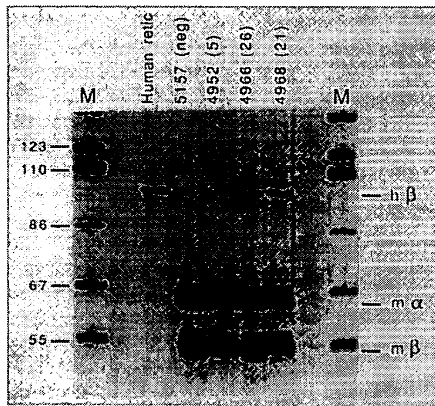

Figure 4. Primer extension analysis of fetal liver RNA from HS I–VI β transgenic mice. Human α-, mouse α-, and mouse β-globin-specific oligonucleotides were end labeled with [α-^{32}P]ATP (3000 Ci/mM) and hybridized together with 5 μg of mouse fetal liver RNA or 0.5 μg of human reticulocyte RNA and then extended with reverse transcriptase to map the 5′ ends of human β-, mouse α-, and mouse β-globin mRNAs. The products were electrophoresed on an 8.0% urea–polyacrylamide gel, and the gel was autoradiographed for 8 hr at −70°C with an intensifying screen. The authentic human β-globin primer extension product is 98 bp, and the correct mouse α- and β-globin products are 65 and 53 bp, respectively. Markers are end-labeled *Hpa*II fragments of the plasmid pSP64. Accurate quantitative values of human β-globin and mouse β-globin mRNAs were determined by solution hybridization with human β-globin and mouse β-globin-specific oligonucleotides as described by Townes et al. (1985b). Levels of human β-globin mRNA expressed as a percentage of endogenous mouse β-globin mRNA are listed in parenthesis after each sample number.

mouse α- and β-globin products are 65 and 53 bp, respectively. All three of the animals that contained the HS I–VI β transgene expressed correctly initiated human β-globin mRNA; and the levels of expression; which are listed in parentheses after each sample number, ranged from 5.0 to 26% of endogenous mouse β-globin mRNA. As there are four copies of the mouse β-globin gene per diploid genome (2β^s and 2β^t alleles in the β single haplotype mouse; Weaver et al. 1981), the levels of human and mouse β-globin mRNAs were divided by their respective gene copy numbers to make a direct comparison of expression. The corrected values for human β-globin mRNA ranged from 20 to 84% of endogenous mouse β-globin mRNA, and the average level of expression was 52% per gene copy (Table 1).

To determine whether the downstream HS VI site was required for high level human β-globin gene expression, a construct containing only the five upstream HS sites [HS I–V (30) β; Fig. 2] was analyzed in transgenic mice. This construct contains the five HS sites on a 30-kb fragment linked upstream of the human β-globin gene. Thirteen animals that contained intact copies of the transgene were obtained, and all 13 expressed human β-globin mRNA in fetal liver. Figure 5 illustrates the primer extension gel of fetal liver RNA from the HS I–V (30) β construct. Levels of human β-globin mRNA ranged from 18 to 316% of endogenous mouse β-globin mRNA. When these values were corrected for transgene copy number, the average level of expression per gene copy was 108% of endogenous mouse β-globin mRNA (Table 1).

A construct that contained all five upstream HS sites on a smaller fragment (22 kb) was also assayed for activity. Nine animals containing intact copies of the HS I–V (22) β transgene (Fig. 2) were obtained, and all nine expressed human β-globin mRNA in fetal liver. Fetal liver RNA from eight of these samples was analyzed by primer extension. The results are illustrated in Figure 6. All eight animals expressed correctly initiated human β-globin mRNA, and the levels of expression ranged from 52 to 380% of endogenous mouse β-globin mRNA. The lowest expressor (4854), which expressed human β-globin mRNA at 1.0% of the level of mouse β-globin mRNA, was not included on the gel. When the level of expression for all nine animals was corrected for transgene copy number, the average level of expression per gene copy was 109% of endogenous mouse β-globin mRNA (Table 1).

To determine whether all five upstream HS sites are required for high level erythroid expression, a construct containing only HS I and HS II on a 13-kb *Mlu*I–*Cla*I fragment was inserted upstream of the human β-globin gene (Fig. 2) and tested for activity. Thirteen animals that contained intact copies of the HS I,II (13) β trans-

Table 1. *Summary of HS β transgene expression*

Transgene	Fraction expressors	Percent endogenous mouse β-globin mRNA[a]	Percent expression per gene copy[b]	
			mean	range
HS I–VI β	3/3	5–26	52	20–84
HS I–V (30) β	13/13	18–316	108	16–200
HS I–V (22) β	9/9	1–380	109	2–208
HS I,II (13) β	13/13	9–347	49	9–92
HS II (5.8) β	6/7	8–108	40	6–84
HS II (1.9) β	4/4	56–194	40	13–63
β	7/23	0.2–23	0.3	0.1–0.6

Human and mouse β-globin mRNA levels were quantitated by solution hybridization with human β- and mouse β-globin-specific oligonucleotides, as described (Townes et al. 1985). The values of percent expression per gene copy were calculated assuming four mouse β-globin genes per cell. Mice used in this study (C57BL/6 × SJL) F2 have the *Hbb*^{s} or single haplotype. The β-globin locus in this haplotype contains two adult β-globin genes (β^s and β^t) per haploid genome (Weaver et al. 1981). These mice also have two α-globin genes (α1 and α2) per haploid genome (Whitney et al. 1981; Erhart et al. 1987). Copies per cell of HS β transgenes were determined by densitometric scanning of the Southern blots illustrated in Fig. 3.

[a] $\left(\frac{\text{h}\ \beta\ \text{mRNA}}{\text{m}\ \beta\ \text{mRNA}} \times 100\right)$.

[b] $\left(\frac{\text{h}\ \beta\ \text{mRNA/h}\ \beta\ \text{gene}}{\text{m}\ \beta\ \text{mRNA/m}\ \beta\ \text{gene}} \times 100\right)$.

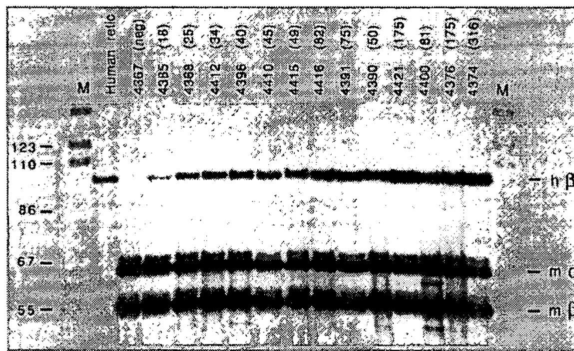

Figure 5. Primer extension analysis of fetal liver RNA from HS I–V (30) β transgenic mice. As described in the legend to Fig. 4, 5 μg of fetal liver RNA was analyzed.

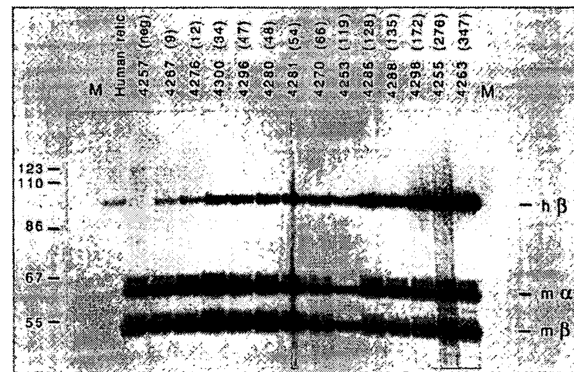

Figure 7. Primer extension analysis of fetal liver RNA from HS I,II (13) β transgenic mice. As described in the legend to Fig. 4, 5 μg of fetal liver RNA was analyzed.

gene were obtained, and all 13 animals expressed correctly initiated human β-globin mRNA in fetal liver (Fig. 7). Levels of expression ranged from 9.0 to 347% of endogenous mouse β-globin mRNA. When these values were corrected for transgene copy number, the average level of human β-globin expression was 49% of endogenous mouse β-globin expression (Table 1).

The 13.0-kb *Mlu*I–*Cla*I fragment containing HS I and HS II was then divided into a 5.8-kb *Mlu*I–*Bst*EII fragment containing HS II and a 7.2-kb *Bst*EII–*Cla*I fragment containing HS I. Each of these fragments was inserted upstream of the human β-globin gene (Fig. 2) and injected into fertilized eggs. Unfortunately, no HS I β transgenic animals were obtained. However, nine animals containing the HS II (5.8) β construct were identified by DNA dot hybridization, and seven of these nine animals contained intact copies of the transgene. Fetal liver RNA from all nine samples was analyzed by solution hybridization and primer extension, and eight of nine animals expressed correctly initiated human β-globin mRNA (Fig. 8). The single animal (5120) that did not express any human β-globin mRNA was the only one of 51 HS β transgenic animals that did not express the transgene. The levels of expression for samples 5140 and 5153 were low but, as described above, both of these samples contained rearranged copies of the transgene. Also, the fetal liver RNA of sample 5127 was somewhat degraded. The levels of human β-globin mRNA for samples 5127, 5118, 5132, 5131, 5148, and 5136 ranged from 8.0 to 108% of endogenous mouse β-globin mRNA. When these levels were corrected for transgene copy number, the values ranged from 6.0 to 84%, and the average level of human β-globin mRNA per gene copy was 40% of endogenous mouse β-globin mRNA (Table 1).

To begin to determine the minimal HS II sequence required for high level expression, a 1.9-kb *Kpn*I–*Pvu*II

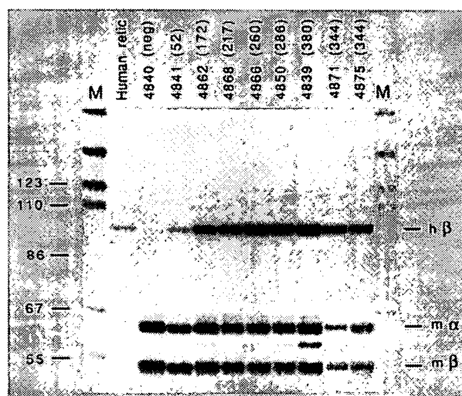

Figure 6. Primer extension analysis of fetal liver RNA from HS I–V (22) β transgenic mice. As described in the legend to Fig. 4, 5 μg of fetal liver RNA was analyzed.

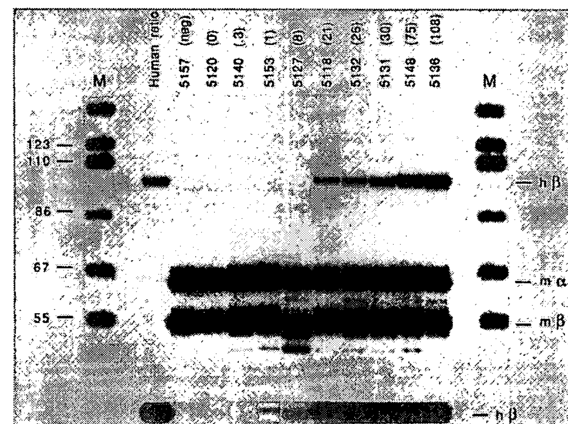

Figure 8. Primer extension analysis of fetal liver RNA from HS II (5.8) β transgenic mice. As described in the legend to Fig. 1, 5 μg of fetal liver RNA was analyzed. (*Bottom*) A 3-day exposure of the human β-globin, 98-bp primer extension product is shown in the insert. Samples 5140 and 5153 contained rearranged copies of the transgene (data not shown), and the RNA from sample 5127 was degraded slightly. Sample 5120 was the only one of 51 transgenic mice that contained an intact copy of the transgene but did not express any human β-globin mRNA.

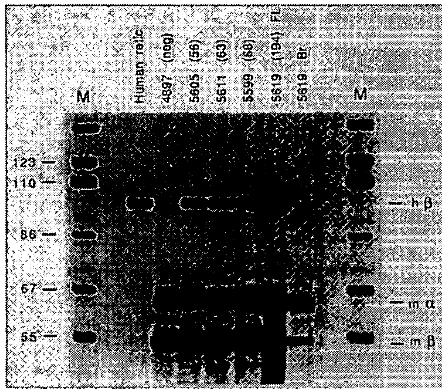

Figure 9. Primer extension analysis of fetal liver RNA from HS II (1.9) β transgenic mice. As described in the legend to Fig. 4, 5 μg of fetal liver RNA was analyzed. Five micrograms of both fetal liver and brain RNA were analyzed for sample 5619.

fragment containing HS II was inserted upstream of the human β-globin gene (Fig. 2) and tested for activity in transgenic mice. Four animals that contained intact copies of the transgene were obtained, and all four expressed correctly initiated human β-globin mRNA in fetal liver (Fig. 9). The levels of human β-globin mRNA ranged from 56 to 194% of endogenous mouse β-globin mRNA. When these values were corrected for transgene copy number, the average level of human β-globin mRNA was 40% of endogenous mouse β-globin mRNA (Table 1).

Finally, the human β-globin gene without HS sites was injected into fertilized eggs and assayed for expression in 16-day fetal liver. In this experiment, only 7 of 23 mice that contained intact copies of the transgene expressed human β-globin mRNA, and the levels of expression ranged from 0.2 to 23% of endogenous mouse β-globin mRNA. When these levels were corrected for transgene copy number, the average level of human β-globin mRNA was 0.3% of endogenous mouse β-globin mRNA (Table 1).

Tissue specificity of HS β-globin transgene expression

Fetal liver and brain RNA from the highest expressor of each set of transgenic animals were analyzed for human β-, mouse α-, and mouse β-globin mRNA by primer extension to assess the tissue specificity of human β-globin gene expression. Data in Figure 10 and in the last two lanes of Figure 9 demonstrate that the human β-globin gene is expressed in fetal liver and not in brain. The small amount of human β-globin mRNA in the brain results from blood contamination because equivalent amounts of mouse α- and β-globin mRNA are also observed in this nonerythroid tissue. Solution hybridization analysis demonstrated that the ratio of human β-globin mRNA to mouse β-globin mRNA was virtually identical in fetal liver and brain in all 50 HS β transgenic mice. These data strongly suggest that the HS sites act specifically in erythroid tissue to stimulate high levels of human β-globin gene expression in transgenic mice.

Discussion

Summary of HS β-globin expression

A summary of the results presented above are listed in Table 1. In this study only 7 of 23 animals without HS sites expressed the transgene. In contrast, 50 of 51 animals that contained HS sites inserted upstream of the human β-globin gene expressed correctly initiated human β-globin mRNA in fetal liver and no expression was detected in fetal brain. These results, like those of Grosveld et al. (1987) with a construct containing HS I–VI β, suggest that the HS sites activate expression regardless of the site of transgene integration. However, expression is not totally position independent. The range of expression varied widely with all of the constructs tested, and levels of human β-globin mRNA were not absolutely correlated with transgene copy number. Nevertheless, the average levels of expression per gene copy were high for all of the HS β-globin constructs tested. The HS I–V (30) β and HS I–V (22) β constructs were expressed at an average level of 108 and 109%, respectively, of endogenous mouse β-globin per gene copy, and all other HS β constructs were expressed

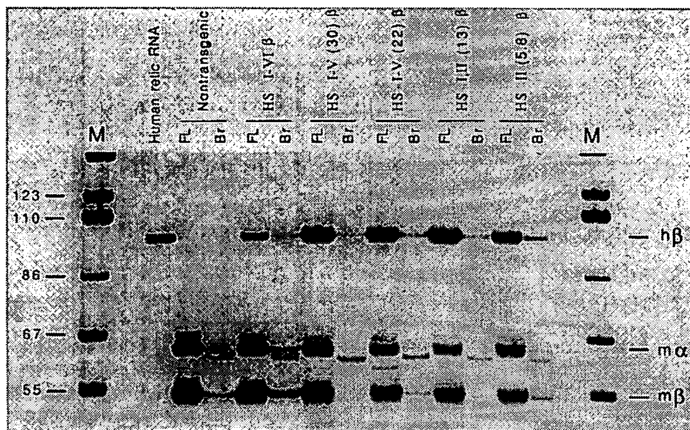

Figure 10. Primer extension analysis of fetal liver and brain RNA of HS β transgenic mice. As described in the legend to Fig. 4, 5 μg of fetal liver and brain RNA from the highest expressor of each set of HS β transgenic mice were analyzed. The low level of human β-globin mRNA observed in the brain is the result of blood contamination because equivalent levels of mouse α- and β-globin mRNAs are also observed in this tissue.

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at 40–49% of endogenous mouse β-globin per gene copy. This high level of expression was obtained even when a 1.9-kb fragment containing only HS II was inserted upstream of the human β-globin gene. The average level of expression per gene copy for a human β-globin construct that did not contain HS sites was only 0.3% of endogenous mouse β-globin. This average level of expression is 133–363 times lower than constructs containing HS sites. Finally, we suspect that the average level of expression for the HS I–VI β construct was lower than 100% per gene copy because only three animals were obtained.

Role of individual HS sites

Southern blots of fetal liver DNA from all 51 of the HS β transgenic mice generated in this study demonstrated head-to-tail tandem arrays of the transgene (data not shown). Therefore, every animal contains at least one copy of the human β-globin gene that is flanked on either side by HS sites. This is true even for animals that contain one or fewer copies per cell of the transgene. These animals must be mosaics (Wilke et al. 1986) with multiple tandemly linked transgenes in only a fraction of their cells. Although the data demonstrate that HS VI is not required for high level expression, a copy of HS II or one of the other upstream HS sites may substitute for HS VI when inserted downstream of the β-globin gene in the tandem array. To determine whether a downstream HS site is required for high level expression, animals containing a single copy of HS I–V β or HS II β will have to be produced.

We have not yet tested the activity of HS III, HS IV, or HS V, inserted individually upstream of the human β-globin gene. However, one or more of these sites may be active because transgenic animals that contain HS I–V consistently express higher levels of human β-globin mRNA than animals that contain HS I and HS II or HS II alone. Individual sites and various combinations of sites are now being tested to determine the minimal sequences required for maximal expression. As individual sites may be functionally redundant, it will also be interesting to test constructs containing multiple copies of HS II inserted upstream of the human β-globin gene to determine whether multimers of an individual site can substitute for HS I–V.

Because HS I β transgenic animals were not obtained, we do not know whether HS I alone can stimulate β-globin gene expression. However, two pieces of data argue strongly that HS I is not sufficient to enhance expression. First, we have demonstrated recently that the human α-globin gene is expressed at high levels in transgenic mice when placed downstream of HS I and HS II (Ryan et al. 1989). Of 12 HSI, HSII, α-globin mice, 11 expressed correctly initiated human α-globin mRNA specifically in erythroid tissue, and the average percent expression per gene copy was 57% of endogenous mouse β-globin mRNA. The single animal that did not express human α-globin mRNA had intact copies of HS I α-globin, but the HS II site had been deleted upon integration. This result suggests that HS I alone cannot enhance expression. Second, a very interesting deletion in a Hispanic γδβ-thalassemic patient has recently been defined by C. Driscoll et al. (pers. comm.). A 30-kb deletion that ends 9.8 kb upstream of the ε-globin gene removes HS V–II but leaves HS I intact (Fig. 1). The patient, who has a β^S gene on this same chromosome, makes no sickle hemoglobin. The data from this patient and the transgenic animal described above strongly suggest that HS I cannot, by itself, stimulate expression of downstream globin genes.

HS site effect on other genes

The effects of erythroid-specific HS sites on other tissue specifically expressed genes has not been tested. However, the experiments of Nandi et al. (1988) strongly suggest that the SV40 promoter can be dramatically influenced by HS sites. Murine erythroleukemia (MEL) cells containing human chromosome 11 were transfected with a construct containing a modified human β-globin gene and an SVneo gene. G418-resistant cells were identified that contained this construct inserted specifically into the human β-globin locus or at nonspecific chromosomal sites. When these cells were induced to differentiate with dimethylsulfoxide (DMSO), SVneo mRNA was induced to high levels in cells with site-specific integrants but not in cells with random integrants. These results strongly suggest that expression from heterologous promoters can be greatly enhanced by the HS sites. We have also demonstrated that SVneo expression is induced to high levels in MEL cells transfected with cosmids containing HS I–V β linked to the SVneo gene (unpubl.).

Human β-globin domain

Several groups have suggested that HS sites mark the boundaries of the human β-globin domain and that these sites are responsible for opening the β-globin domain specifically in erythroid tissue (Tuan et al. 1985; Forrester et al. 1986, 1987; Grosveld et al. 1987). Forrester et al. (1987) have demonstrated recently that these HS sites are formed in human fibroblasts that have been fused with MEL cells. These hybrids synthesize high levels of human β-globin mRNA. Presumably, *trans*-acting factors present in MEL cells interact with the hypersensitive site sequences both upstream and downstream of the human β-globin locus and organize the previously closed chromatin domain into an open domain. Therefore, Forrester et al. (1987) have suggested that the sequences be called 'locus activating regions,' or LARs. Similarly, in the developing human embryo, *trans*-acting factors present in early erythroid cells may interact with hypersensitive site sequences and activate the β-globin locus for expression.

Model for developmental regulation

Choi and Engel (1988) have demonstrated recently that sequences at the immediate 5′ end of the chicken β-

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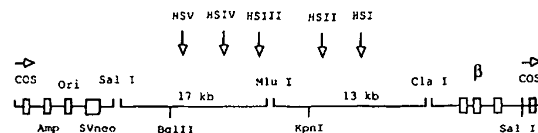
globin gene are involved in temporal specificity in transient expression assays. These sequences apparently bind factors that influence the ability of this promoter to compete with the ε-globin gene promoter for interactions with a single erythroid enhancer (Choi and Engel 1988; Nickol and Felsenfeld 1988) located in the chicken β-globin locus. Although similar mechanisms may be involved in developmental stage-specific expression of human globin genes, the situation is probably more complex. The major determinants of erythroid tissue specificity in humans appear to be the HS sequences. In fact, these sequences carry out two important functions: They organize the entire β-globin locus for expression specifically in erythroid tissue, and they act as an enhancer to direct high level expression. These two separate but related functions are evident in the experiments described above. First, the HS sites increase the fraction of transgenic animals that express the human β-globin gene. Of 51 HS β-globin mice, 50 expressed the transgene specifically in erythroid tissue compared with 7 of 23 animals containing the β-globin gene alone. Apparently, the HS sequences ensure that the transgene will be in an open chromatin domain regardless of the site of integration. Second, HS sites stimulated the average level of β-globin gene expression 133- to 363-fold compared to the average level of the β-globin gene alone. Therefore, these sequences constitute a powerful enhancer that may work in concert with enhancers in and surrounding individual genes.

Although human β-globin genes in transgenic mice are expressed specifically in adult erythroid tissue without HS sites, high levels of correctly regulated expression may require interactions between HS sequences, promoters, and proximal enhancers. A model for globin gene regulation can be envisioned that incorporates the two important functions of HS sites and the concept of competition between various regulatory sequences. HS sequences could be activated in early erythroid cell precursors and organize the entire β-globin locus into an open chromatin domain that is stable throughout development. Within the open domain, promoters and enhancers in and surrounding the ε-, γ-, and β-globin genes could then compete for interactions with the HS master enhancer to determine which of these genes will be expressed. Promoter and proximal enhancer binding factors synthesized in yolk sac, fetal liver, and bone marrow could influence these competitive interactions either positively or negatively and subsequently determine developmental specificity. Transgenic mouse experiments with constructs containing human ε-, γ-, and β-globin genes inserted separately or in various combinations downstream of the HS sites should help define important interactions between regulatory sequences and should, in general, provide meaningful insights into the complex mechanisms that regulate multigene families during development.

Methods

Construction of HS β-globin clones

Lambda clones containing HS sites I–IV (5′εII and 5′εIII; Li et al. 1985) were kindly provided by Oliver Smithies, and a λ clone containing HS VI (λ4) was kindly provided by Don Fleenor and Russell Kaufman. A 1.9-kb *Hin*dIII fragment containing HS III was prepared from 5′εIII and subcloned into pUC19. A 1.3-kb *Bam*HI–*Hin*dIII fragment from this plasmid was then used to screen a human placenta genomic library in EMBL 4 (Stratagene) and several clones that overlapped with 5′εIII were isolated. One clone that contained a 17.5-kb insert extended ~11.0 kb upstream of the *Eco*RI site at the 5′ end of the 5′εIII clone. This new clone, which was designated 5′εIV, contained HS V. Cosmid clone HS I–V (30) β was constructed as follows. A 17-kb *Sal*I–*Mlu*I fragment was prepared from 5′εIV; the *Sal*I site was from the EMBL 4 *Sal*I–*Bam*HI cloning site, and the *Mlu*I site was a natural site in the insert. This 17-kb fragment contained HS V, HS IV, and HS III. A 13-kb *Mlu*I–*Cla*I fragment containing HS II and HS I was prepared from 5′εII. These two fragments were inserted into the cosmid vector pCV001 (Lau and Kan 1983) in a four-way ligation. The left arm was a 9.0-kb *Mlu*I–*Sal*I fragment obtained from pCV001; the *Mlu*I site was destroyed by S1 digestion. This fragment contained a *cos* site, an ampicillin-resistance gene, a ColEI origin, and the SVneo gene. The right arm was a 6.6-kb *Cla*I–*Hin*dIII fragment that contained the human β-globin gene on a 4.1-kb *Hpa*I–*Xba*I fragment and a *cos* site from pCV001 on a 2.5-kb *Sal*I–*Hin*dIII fragment. The *Hpa*I and *Xba*I sites on either side of the β-globin gene were changed to *Cla*I and *Sal*I, respectively, in the right arm plasmid.



These four fragments were ligated in a 2 : 1 : 1 vector arms to inserts and packaged (Gigapack Gold; Stratagene). *Escherichia coli* ED8767 was then infected with the packaged cosmids and plated on ampicillin plates. Large-scale cultures of ampicillin-resistant colonies were grown and cosmids were prepared by standard procedures (Maniatis et al. 1982).

The HS I–V (22) β cosmid was constructed as follows. A 12-kb *Bgl*II fragment containing HS V, HS IV, HS III, and HS II was subcloned from HS I–V (30) β into a modified pUC plasmid, and a 10.7-kb *Sal*I–*Kpn*I fragment containing HS V, HS IV, and HS III was prepared from this plasmid. The *Sal*I site of this fragment was from the pUC polylinker, and the *Kpn*I site was a natural site in the insert. A 10.9-kb *Kpn*I–*Cla*I fragment containing HS II and HS I was isolated from 5′εII and subcloned into a modified pUC plasmid. The 10.7-kb *Sal*I–*Kpn*I fragment containing HS V, HS IV, and HS III was ligated to the 10.9-kb *Kpn*I-*Cla*I fragment containing HS II and HS I and the two cosmid vector arms described above. The ligation mixture was packaged, ED8767 cultures were infected, and cosmids were prepared from ampicillin-resistant colonies.

HS I–VI β was prepared as follows. A 12.0-kb *Hpa*I–*Bam*HI fragment containing HS VI was subcloned from λ4 into a modified pUC19 plasmid and then isolated from this plasmid as a 12.0-kb *Xho*I–*Sal*I fragment. This fragment was cloned into the *Sal*I site downstream of the human β-globin gene in the right-arm plasmid described above. The right-arm plasmid was then linearized with *Cla*I and dephosphorylated with calf intestinal phosphatase (Boehringer–Mannheim). This 21-kb right-arm fragment and the 9.0-kb *Mlu*I–*Sal*I left-arm fragment described above were ligated with the 10.7-kb *Sal*I–*Kpn*I fragment containing HS V, HS IV, and HS III and the 10.9-kb *Kpn*I–*Cla*I frag-

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ment containing HS II and HS I in a 2 : 1 : 1 molar ratio of vector arms to inserts. The ligation mixture was packaged, ED8767 cultures were infected, and cosmids were prepared from ampicillin-resistant colonies.

HS I,II (13) β was derived from HS I–V (22) β after digestion with *Mlu*I and *Sal*I. HS II (5.8) β and HS II (1.9) β were constructed by subcloning the 5.8-kb *Mlu*I–*Bst*EII fragment or the 1.9-kb *Kpn*I–*Pvu*II fragment into modified pUC plasmids containing the human β-globin gene.

Sample preparation and microinjection

All of the constructs were removed from vector sequences by digestion with the appropriate enzymes and isolated on low-gelling temperature agarose (FMC) gels. Gel slices were melted, extracted twice with phenol [buffered with 0.1 M Tris-HCl (pH 8.0), 1.0 mM EDTA], once with phenol/chloroform, and once with chloroform and precipitated with ethanol. After resuspension in TE [10 mM Tris-HCl (pH 8.0), 1.0 mM EDTA], the fragments were again extracted with phenol, phenol/chloroform, and chloroform and precipitated with ethanol. The purified fragments were washed with 70% ethanol, resuspended in sterile TE, and microinjected into the male pronuclei of F2 hybrid eggs from C57BL/6 × SJL parents as described by Brinster et al. (1985).

DNA analysis

Total nucleic acids were prepared from 16-day fetal liver and brain, as described previously (Brinster et al. 1985). Samples that contained the injected constructs were determined by DNA dot hybridization of brain nucleic acids with human β-globin and HS II-specific probes that were labeled by extension of random primers (Feinberg and Vogelstein 1983). The human β-globin probe was a 790-bp *Hin*fI fragment from IVS 2, and the HS II probe was a 1.9-kb *Hin*dIII fragment spanning the HS II site. Hybridizations were performed at 68°C for 16 hr in 5× SSC, 5× Denhardt's solution, 100 μg/ml herring sperm DNA, and 0.1% SDS. Filters were washed three times for 20 min each at 68°C in 2× SSC, and 0.1% SDS and for 20 min at 68°C in 0.2× SSC and 0.1% SDS if necessary to reduce background.

For Southern blots, 10 μg of fetal liver DNA from animals that were positive with HS II and/or β-globin probes were digested with *Bam*HI and *Pst*I, electrophoresed on 1.0% agarose gels, blotted onto nitrocellulose, and hybridized with the β and HS II probes described above. The hybridization conditions for Southern blots were the same as described for DNA dots.

RNA analysis

RNA was prepared from total nucleic acids by digesting the sample with DNase I (Worthington, RNase-free) at 10 μg/ml for 20 min at 37°C in 10 mM Tris-HCl (pH 7.5), 10 mM MgCl, and 50 mM NaCl. The reaction was stopped with EDTA, and the sample was digested with proteinase K (100 μg/ml) for 15 min at 37°C. After digestion, RNA was purified by phenol/chloroform and chloroform extraction, precipitated with ethanol, and resuspended in TE.

Quantitation of human and mouse β-globin mRNA was determined by solution hybridization with oligonucleotide probes as described (Townes et al. 1985b). Primer extensions were performed as described by Townes et al. (1985a,b), except that only 5 μg of fetal liver or brain RNAs were analyzed and three oligonucleotides were used in each reaction. The human β primer 5'-AGACGGCAATGACGGGACACC-3' corresponds to sequences from +78 to +98 of the human β-globin gene. The mouse α primer 5'-CAGGCAGCCTTGATGTTGCTT-3' corresponds to sequences from +45 to +65 of the mouse α1- and α2-globin genes, which are identical in this region. The mouse β primer 5'-TGATGTCTGTTTCTGGGGTTGTG-3' corresponds to sequences +31 to +53 of the mouse β^s-globin gene. Although there are 2-bp differences in the β^s and β^t genes in the region covered by this oligonuceotide, comparison of solution hybridization results (obtained with a different oligonucleotide that is perfectly complimentary to β^s and β^t; see Townes et al. 1985b) with primer extension data suggests that the primer anneals with equal efficiency to β^s- and β^t-globin mRNA under the hybridization conditions used.

Acknowledgments

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The "β-like-globin" gene domain in human erythroid cells

(β-globin gene cluster/DNA sequences/DNase I-hypersensitive sites/domain boundary/enhancers)

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ABSTRACT **We have mapped the distribution of the major and minor DNase I-hypersensitive sites in the human "β-like-globin" gene domain. The minor DNase I-hypersensitive sites map close to the 5′ end of each of the β-like-globin genes. Their presence is specifically associated with the transcription of the immediate downstream β-like-globin genes. The major DNase I-hypersensitive sites map in what appear to be the 5′ and 3′ boundary areas of the human β-like-globin gene domain, a region estimated to span at least 90 kilobases of DNA. These major sites are present in various erythroid cells, which express predominantly either the embryonic, the fetal, or the adult β-like-globin genes, and seem to be involved in defining the active β-like-globin gene domain in cells of erythroid lineage. The four major DNase I-hypersensitive sites in the 5′ boundary area, when correlated with sequencing data, are shown to be located in DNA regions containing enhancer core-like sequences and alternating purine and pyrimidine bases.**

The human "β-like-globin" genes (hemoglobin β-chain gene cluster) encode, respectively, one embryonic (ε), two fetal ($^{G}\gamma$ and $^{A}\gamma$), and two adult (δ and β) globin chains. These genes have been shown to reside within ≈50 kilobases (kb) of chromosomal DNA in the transcriptional order 5′ ε-$^{G}\gamma$-$^{A}\gamma$-δ-β 3′ (ref. 1; see Fig. 1). These structurally related genes are normally expressed exclusively in cells of erythroid lineage. Furthermore, their expression undergoes a developmental stage-related switching mechanism: the embryonic ε-globin gene is expressed in the early embryo; the fetal γ-globin genes are expressed during most of fetal life; and the adult δ- and β-globin genes, in adulthood (2).

In an attempt to locate the regulatory elements important in controlling the differential expression of the human β-like-globin genes during erythroid differentiation and development, we have mapped the DNase I-hypersensitive sites in the flanking DNA of the β-like-globin gene complex in several human cells: a human leukemia cell line (K562) in which the embryonic ε-globin gene is predominantly expressed (3, 4); a human erythroleukemia cell line (HEL), which expresses predominantly the fetal γ-globin genes (5); normal nucleated bone marrow cells of adult humans, in which the β-globin gene is predominantly expressed; and a human promyelocytic leukemia cell line (HL60), which expresses none of the β-like-globin genes. In agreement with others (6), we have found DNase I-hypersensitive sites close to the 5′ end of the transcribed globin genes, which we named minor hypersensitive sites because of the relatively high DNase I concentration required for their detection. In addition, we have found major DNase I-hypersensitive sites in what appear to be the 5′ and 3′ boundary areas of the β-like-globin gene domain, far upstream and far downstream of the expressed globin genes. These major hypersensitive sites are present in all three erythroid cell types, regardless of whether the predominantly expressed globin gene is the embryonic ε-, fetal γ-, or adult β-globin gene, but they are absent in HL60 cells, which do not express the β-like-globin genes. Their presence may thus serve to define and mark the active β-like-globin gene domain in erythroid cells during differentiation and development. Sequencing data of the DNase I-hypersensitive sites in the 5′ boundary area show that they each contain 2 or 3 enhancer core-like sequences and 10–26 consecutive or nonconsecutive pairs of alternating purine and pyrimidine bases.

EXPERIMENTAL PROCEDURES

Cells were grown as described (7). Human bone marrow cells were collected from cancer patients with normal marrow who were to undergo chemotherapy and bone marrow reinfusion. Isolated by dextran column chromatography, ≈25% of the nucleated cells were erythroid.

DNase I-digestion, gel electrophoresis, RNA isolation, blotting, and hybridization were carried out as described (7).

RESULTS

Globin Gene Transcription in K562, HEL, Adult Human Marrow, and HL60 Cells. Nuclear and cytoplasmic RNAs were isolated from cells, and individual globin gene transcription was detected by "dot-blot" hybridization with ε-, γ-, δ-, or β-globin specific cDNA probes (7). The results are not shown but may be summarized briefly. In K562 cells, the embryonic ε-globin gene is transcribed, and the fetal γ-globin genes are also transcribed but at a lower level; transcription of the adult δ- and β-globin genes is not detected. The transcriptional pattern of the β-like-globin genes in K562 cells thus bears resemblance to the embryonic pattern. In HEL cells, the fetal γ-globin genes are transcribed at a higher level than the ε-globin gene; the transcriptional pattern thus resembles that of the β-like-globin genes in the fetus. In adult nucleated human marrow cells, the β-globin gene is predominantly transcribed. In HL60 cells, none of the β-like-globin genes is detectably transcribed.

DNase I-Hypersensitivity Mapping. The locations of the plasmid probes and the restriction fragments chosen to map the β-like-globin area are presented in Fig. 1. We are able to map an area covering ≈100 kb in the human β-like-globin gene cluster, from a *Pvu* II restriction site 25 kb upstream of the ε-globin gene to a *Bam*HI site 25 kb downstream of the β-globin gene (Fig. 1). If not otherwise stated, the data shown are for hemin-treated K562 and HEL cells.

DNase I-Hypersensitive Sites Upstream of the ε-Globin Gene in K562 Cells. In the 13.8-kb *Kpn* I fragment in K562 cells (Fig. 2*a*), there are five degradation bands (8.1, 4.6, 3.9, 2.5, and 1.8 kb). The 8.1-kb band is very dark and well-defined and is clearly discernible at the lowest DNase I concentration

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Abbreviation: kb, kilobase(s).
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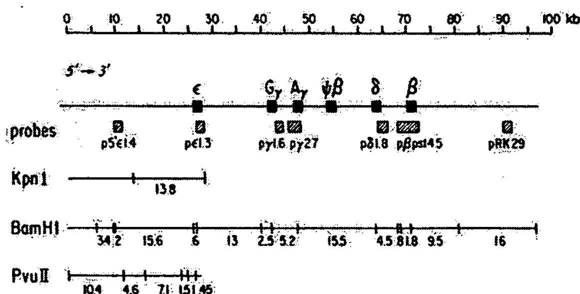


FIG. 1. Restriction map of the human β-like-globin gene cluster. Hatched boxes denote the locations, with respect to the globin genes, of the hybridization probes. ψβ, a pseudo β-globin gene. Restriction sites were determined from sequencing data (8, 27).

(lane 1 in Fig. 2a). It is therefore generated by cleavage at the most sensitive site in the region. This site is marked by a thick vertical arrow in Fig. 2b at about 6 kb upstream of the ε-globin gene. The 4.6-kb degradation band is discernible in lane 2 of Fig. 2a and is generated by cleavage at a less sensitive site, marked by a less thick vertical arrow in Fig. 2b. The other three degradation bands (3.9, 2.5, and 1.8 kb) are rather diffuse (Fig. 2a) and become discernible at the highest DNase I concentration (Fig. 2a, lane 3); they are generated by cleavage at the three least sensitive sites (thin vertical arrows in Fig. 2b). The data shown are for K562 cells without hemin treatment. In K562 cells treated with hemin, these hypersensitive sites are also present; however, there is one additional site at around −3.7 kb (7).

The DNase I-hypersensitive sites 5′ of the ε-globin gene are not found in HL60 cells (7), which do not transcribe the ε-globin gene; the appearance of these sites in K562 cells seems, therefore, to be associated with ε-globin gene transcription.

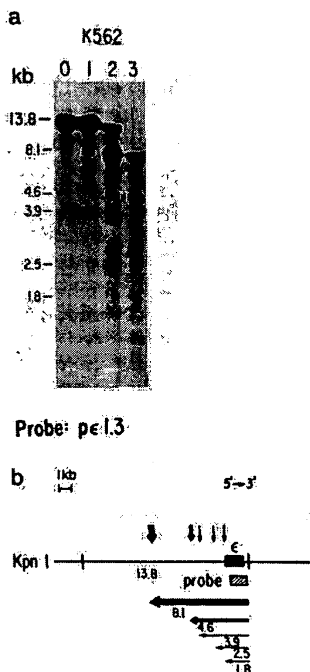


FIG. 2. (a) Southern blot of K562 DNA, from cells not treated with hemin, cleaved with *Kpn* I. DNase I at 0, 10, 15, or 20 μg/ml (lanes 0, 1, 2, and 3, respectively). (b) Distribution of DNase I-hypersensitive sites upstream of the ε-globin gene. Horizontal arrows denote the degradation fragments. Vertical arrows mark the location of the DNase I-hypersensitive sites.

DNase I-Hypersensitive Sites in the γ-, δ-, β-Globin Gene Region of K562 and HL60 Cells. For mapping the DNase I-hypersensitive sites around the γ-globin genes, *Bam*HI enzyme was chosen. There are two DNase I-hypersensitive sites generating respective degradation bands at 4.1 and 1.1 kb (Fig. 3a, lanes 2 and 3). The hypersensitive site generating the 4.1-kb degradation band has been placed at the 5′ end of the Aγ-globin gene (Fig. 3b). The 1.1-kb degradation band is probably generated by a hypersensitive site at the 5′ end of the Gγ-globin gene (Fig. 3b). The placement of hypersensitive sites 5′ of the transcribed γ-globin genes in K562 cells is in agreement with findings of others (9, 10). These hypersensitive sites are less sensitive to DNase I than the two most sensitive sites upstream of the ε-globin gene (Fig. 2) and are marked with thin vertical arrows in Fig. 3b. In HL60 cells, in which the γ-globin genes are not transcribed, no DNase I-hypersensitive site is detected in the $^{G}\gamma$- and $^{A}\gamma$-globin gene region (Fig. 3a). The bands below the 5.2- and 2.5-kb bands in lane 3 of the HL60 blot are nonspecific background contamination.

In both K562 and HL60 cells, where no δ- or β-globin gene transcripts are detected, the four major *Bam*HI fragments in the δ- and β-globin gene region exhibit no DNase I-sensitive degradation band (not shown). A hypersensitive site immediately 5′ of the δ-globin gene in K562 cells, correlating with a very small amount of δ-globin gene transcripts as detected by nuclease S1 mapping (11), has been reported (10). In our hands, however, this site is not well-defined and is much less sensitive to DNase I digestion than the hypersensitive sites 5′ of the ε- or of the γ-globin gene.

Hypersensitive Sites Far Downstream of the β-Globin Gene in K562, HL60, HEL, and Nucleated Adult Human Marrow Cells. In K562 cells, where no β-globin gene transcript is detected, we observe no DNase I-hypersensitive site in the 9.5-kb *Bam*HI fragment spanning about 8.5 kb of DNA immediately downstream of the β-globin gene (Fig. 3b). In the 16-kb *Bam*HI fragment downstream of this 9.5-kb fragment, however, we detect a DNase I-hypersensitive site that

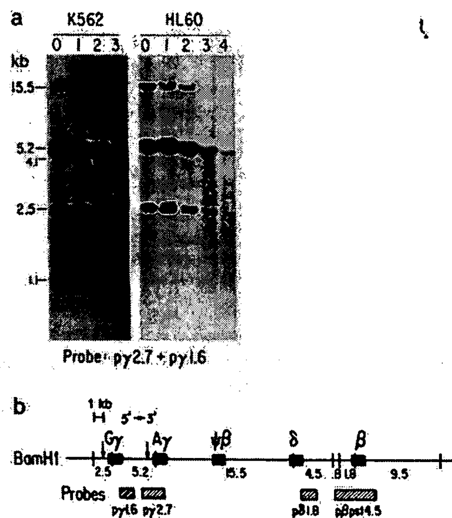


FIG. 3. (a) Southern blots of DNA from K562 or HL60 cells, cleaved with *Bam*HI. DNase I concentrations were 0, 10, 15, or 20 μg/ml for K562 nuclear DNA (lanes 0–3, respectively) and 0, 2.5, 5, 10, or 15 μg/ml for HL60 nuclear DNA (lanes 0–4, respectively). (b) Distribution of DNase I-hypersensitive sites in the γ-,δ-,β-globin gene region.

generates an 11-kb degradation band (Fig. 4*a*). Since the hybridization probe (pRK29) does not hybridize to the 5′ or the 3′-terminal sequences of the 16-kb fragment, we can place the hypersensitive site on either the 3′ side or the 5′ side of the pRK29 probe (these alternative sites are marked by solid and broken vertical arrows, respectively, in Fig. 4*b*). We have placed the hypersensitive site (denoted HS VI in Fig. 4*b*) on the 3′ side of the pRK29 probe, because we also observe a 4.5-kb degradation band produced from a 9-kb *Pst* I fragment (12), which does not contain the 7-kb of DNA spanning the alternative hypersensitive site (not shown); HS VI is situated about 20 kb downstream of the β-globin gene (Fig. 4*b*). In HL60 cells, we detect no DNase I-sensitive degradation bands (Fig. 4*a*) that are derived from the 16-kb parental *Bam*HI fragment. HS VI is therefore not present in HL60 cells, which express none of the β-like-globin genes.

The presence of HS VI in K562 cells does not seem to be associated with nearby non-globin genes that are being actively transcribed. The DNA sequence immediately upstream of HS VI, subcloned in pRK29, does not hybridize to K562 RNAs in RNA dot-blotting experiments (unpublished data) and therefore does not seem to serve as a template for RNA transcription. Further upstream of HS VI is a cluster of repetitive DNA sequences (13), belonging to the *Kpn* I middle-repetitive sequence family (14), which is unlikely to contain structural genes. Downstream of HS VI there are also clusters of repetitive sequences, including, among others, members of the *Kpn* I and *Alu*-repetitive sequence (15) families (R. Kaufman, personal communication), which are also unlikely to contain structural genes. HS VI, located about 20 kb downstream of the β-globin gene, thus seems to be associated with ε- and γ-globin gene transcription in K562 cells. To determine whether HS VI is associated with β-like-globin gene transcription in other cells of erythroid lineage, we have mapped the area downstream of the β-globin gene in a human erythroleukemia cell line (HEL), which expresses predominantly the γ-globin genes (ref. 5 and unpublished data). In HEL cells we find the same 11-kb degradation fragment (Fig. 4*a*); HS VI is therefore also present in HEL cells. Furthermore, in nucleated adult human marrow cells containing erythroid cell precursors, which express predominantly the β-globin gene (unpublished), we also detect the 11-kb degradation band (Fig. 4*a*). This band is relatively faint because the amount of erythroid DNA which can give rise to the 11-kb degradation band is only one-quarter of the total DNA sample. The 11-kb degradation band generated by cleavage at HS VI is clearly discernible in lanes 2 of the K562, HEL, and marrow blots in Fig. 4*a*. HS VI is therefore more sensitive to DNase I than those sites immediately 5′ of the ε- and γ-globin genes but less sensitive than the most sensitive site 6-kb upstream of the ε-globin gene.

In summary (see Fig. 6), HS VI is present in cells that express predominantly the embryonic ε-globin gene (in K562), the fetal γ-globin genes (in HEL), or the adult β-globin gene (in adult marrow). HS VI is, however, not present in HL60 cells, which express none of the β-like-globin genes.

DNase I-Hypersensitive Sites Far Upstream of the ε-Globin Gene. In a 15.6-kb *Bam*HI fragment far upstream of the ε-globin gene (Fig. 5*c*), there are two hypersensitive sites producing degradation bands at 9.2 and 4.7 kb, respectively (Fig. 5*a*, lane 1). The hypersensitive site generating the 9.2-kb

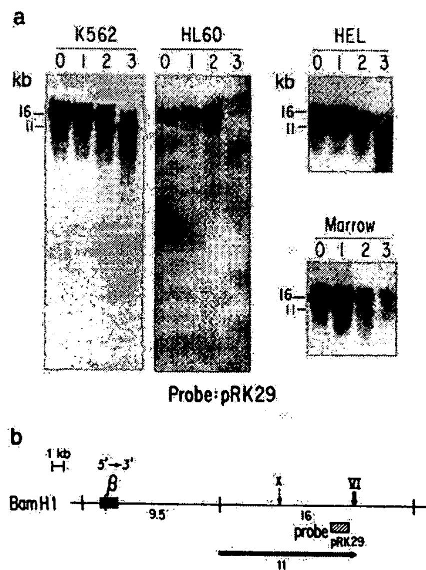
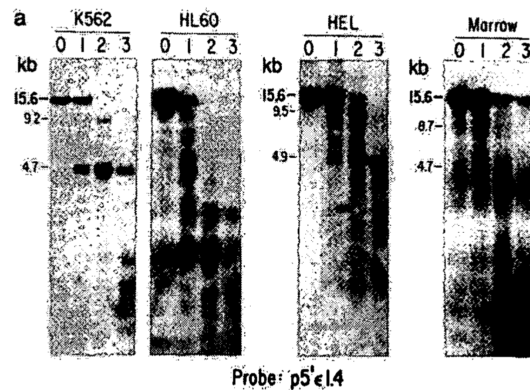
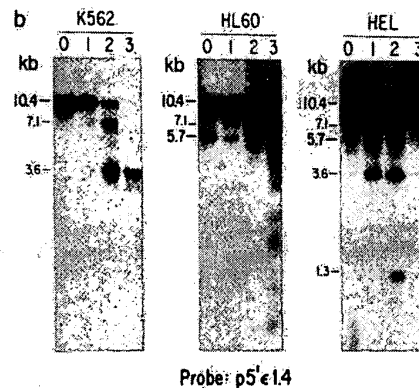


FIG. 4. (*a*) Southern blots of DNA from K562, HL60, HEL, or marrow cells, cleaved with *Bam*HI. DNase I concentrations were 0, 10, 15, or 20 μg/ml for lanes 0–3, respectively. (*b*) Location of the DNase I-hypersensitive site far downstream of the β-globin gene. Vertical arrow VI marks one possible location of the DNase I-hypersensitive site; broken vertical arrow X marks the alternative location of the DNase I-hypersensitive site.





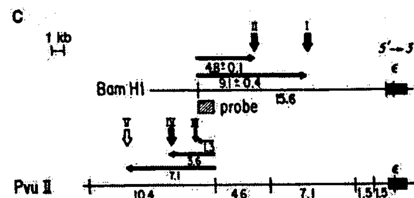


FIG. 5. (*a* and *b*) Southern blots of DNA cleaved with *Bam*HI (*a*) or *Pvu* II (*b*). DNase I concentrations were 0, 10, 15, or 20 μg/ml for the samples in lanes 0–3, respectively. (*c*) Distribution of DNase I-hypersensitive sites (HS I–V) in the area far upstream of the ε-globin gene.

degradation band is located 6.4 kb from the *Bam*HI site bordering the 3′ end of the 15.6-kb *Bam*HI fragment and is also about 6.4-kb from the ε-globin gene, since the 3′ *Bam*HI site abuts the ε-globin gene (Fig. 5*c*). This hypersensitive site is marked by vertical arrow I in Fig. 5*c*. This site, HS I, 6.4-kb upstream of the ε-globin gene, and the most sensitive site 6.1-kb upstream of the ε-gene (Fig. 2) are probably one and the same site. The hypersensitive site whose cleavage generates the 4.7-kb degradation band is marked by vertical arrow II in Fig. 5*c* and is located about 11-kb upstream of the ε-globin gene. In the HL60 blot (Fig. 5*a*), we detect no degradation bands. In the HEL blot, however, we detect degradation bands at 9.5 and 4.9 kb; in the marrow blot, we detect degradation bands at 8.7 and 4.7 kb (Fig. 5*a*). The sizes of the 9.5-kb band in HEL cells, of the 8.7-kb band in marrow cells, and of the 9.2-kb band in K562 cells are within the error of measurement (2–8%) of the technique; therefore, we consider these bands to be generated from cleavage at the same HS I present in each cell line. We think that the 4.7-kb degradation bands in K562 and marrow cells and the 4.9-kb band in HEL cells are generated by cleavage at the same HS II present in each cell line.

In a 10.4-kb *Pvu* II restriction fragment further upstream of the ε-globin gene, we detect major degradation bands at 7.1 and 3.6 kb in K562 (Fig. 5*b*, lanes 1 and 2). The hypersensitive site (HS IV) generating the 3.6-kb degradation band is marked by vertical arrow IV in Fig. 5*c*. It is located about 17.5-kb upstream of the ε-globin gene. The hypersensitive site (HS V) generating the 7.1-kb degradation band is marked by open vertical arrow V and is located about 21.5 kb upstream of the ε-globin gene. In HL60 cells, which express no β-like-globin genes, the 7.1-kb degradation band generated by HS V is present but the 3.6-kb degradation band generated by HS IV is not. The 5.7-kb band present in all four lanes in the HL60 blots (present also in the HEL blot in Fig. 5*b*) is a cross-hybridization band with the p5′ε1.4 probe, observed because both the HL60 and HEL blots were hybridized and rinsed under less stringent conditions than the K562 blot in Fig. 5*b*. In HEL cells, the 7.1-kb degradation band generated by HS V and the 3.6 kb degradation band generated by cleavage at HS IV are both present (Fig. 5*b*, lanes 1 and 2). In addition, there is a 1.3-kb degradation band (Fig. 5*b*) generated by cleavage at HS III (Fig. 5*c*), which is, however, not detected in either K562 or HL60 cells and may be a site peculiar to the HEL cells.

In summary (Fig. 6), HS I and HS II are present in K562, HEL, and nucleated marrow cells expressing at least one β-like-globin gene but are absent in HL60 cells which express none of the β-like-globin genes. Likewise, HS IV is present in both K562 and HEL cells but is absent in HL60 cells. The presence of HS I, HS II, and HS IV thus appears to be

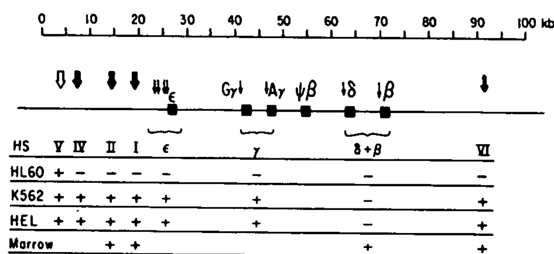


FIG. 6. The distribution of major and minor DNase I-hypersensitive sites in the human β-like-globin gene domain. HS V, located probably outside of the β-like-globin gene domain, is marked with an unfilled vertical arrow. Data on HS ε, HS γ, and HS δ+β in HEL and on HS δ+β in marrow cells are from our unpublished observations. + and − represent the presence or absence of various hypersensitive sites in each cell type.

associated with β-like-globin gene transcription. The most upstream site, HS V at −21.5 kb, is present not only in K562 and HEL cells but also in HL60 cells. The relationship, if any, of HS V to β-like-globin gene transcription is uncertain.

The presence of HS I, HS II, and HS IV in K562, HEL, and nucleated marrow cells does not seem to be associated with other nearby structural genes, because the area of DNA spanned by HS I, II, and IV, with the exception of only two short gaps of nonrepetitive DNA, is comprised of repetitive DNA sequences (unpublished data, and R. Kaufman, personal communication) and therefore does not seem to contain structural genes. The nonrepetitive sequence gaps, however, could code for structural genes. DNA corresponding to the p5′ε1.4 probe, which was subcloned from one of the nonrepetitive sequence gaps, is indeed transcribed in K562 cells, as determined by the RNA dot-blotting technique; the transcripts, found mostly in the nucleus (unpublished data), are unlikely to code for a protein product in K562 cells.

The DNA upstream of the ε-globin gene up to an area 2 kb 5′ of HS IV has been sequenced (27). Correlating the sequence data with the locations of the above hypersensitive sites, we found that HS I, at −6 kb, is in an area that contains, within 800 bases, three enhancer core-like sequences (16), a stretch of 28 consecutive thymidylate residues, and a stretch of 21 consecutive pairs of alternating purine or pyrimidine bases $[(CA)_{15}(TA)_6]$. HS II, at −11 kb, is in an area that contains, within 900 bases, two enhancer core-like sequences and a stretch of 26 consecutive pairs of alternating purine and pyrimidine bases $[(TA)_{10}(CA)_2(TA)_2(CG)(TA)_{11}]$. HS III, at −14.5 kb, is in an area that contains, within 400 bases, three enhancer core-like sequences and a fourth enhancer core-like sequence, which is followed by 10 nonconsecutive pairs of purine and pyrimidine bases embedded in short stretches of purine or pyrimidine bases [GGGAAAGGTGGGGGAGG-$(CA)_2$G(CA)(TA)(GC)(AT)A(GC)A(GC)(AT)TTT-T(CA)TT]. HS IV, at −17.5 kb, is in an area which contains, within 600 bases, two enhancer core-like sequences and 12 nonconsecutive pairs of purine and pyrimidine bases embedded in short stretches of purine or pyrimidine bases [(CA)-(TA)(CA)CTCT$(CA)_5$AA(CA)(TA)A(AC)(TA)AA]. We do not know the sequence features of HS V because it is outside of the area whose sequence has been determined.

DISCUSSION

The distribution of the major and minor DNase I-hypersensitive sites in the entire human β-like-globin gene cluster is presented in Fig. 6. The minor hypersensitive sites include the 4 sites within 4 kb upstream of the ε-globin gene (denoted HS ε in Fig. 6) and the sites immediately 5′ of the Gγ- and Aγ- (HS γ in Fig. 6) and of the δ- and β-globin genes (HS δ+β in Fig. 6). The sites immediately 5′ of the globin genes are situated close to the promoter region of the individual globin genes and appear to be associated with the transcriptional activity of the adjoining globin gene. For example, in K562 and HEL cells, these minor hypersensitive sites are present 5′ of the actively transcribed ε- and γ-globin genes but are absent 5′ of the inactive β-globin gene; conversely, in adult human marrow cells containing erythrocyte precursors, the minor hypersensitive sites are present 5′ of the active β-globin gene (ref. 10 and unpublished data). The major DNase I-hypersensitive sites HS I, HS II, and HS IV, situated upstream of the ε-globin gene, and HS VI, situated downstream of the β-globin gene also seem to be associated with β-like-globin gene expression, since they are present in K562, HEL, and adult nucleated marrow cells, which express the β-like-globin genes, and are absent in HL60 cells, which do not express the β-like-globin genes. However, they differ from the minor DNase I-hypersensitive sites in at least four respects. First, they are much more sensitive to DNase I digestion and seem therefore to be located in a much more

open and accessible chromatin structure. Second, they are located much farther from the globin genes than the minor hypersensitive sites and seem to bracket the β-like-globin genes and the minor hypersensitive sites. Third, the appearance of HS I, II, and IV and of HS VI, which is separated from HS I, II, and IV by 70–85 kb, seems to be coordinately controlled. Both the far upstream sites (HS I, II, and IV) and the far downstream site (HS VI) either are present (in K562, HEL, and nucleated marrow cells) or are absent (in HL60). Fourth, the appearance of these major hypersensitive sites is not closely coupled to any specific globin gene transcription as is that of the minor hypersensitive sites; they are present in specific cell lines irrespective of whether the predominantly expressed globin gene is the embryonic ε-globin gene in the K562 cell, or the fetal γ-globin genes in the HEL cell, or the adult β-globin gene in the adult nucleated marrow cell (Fig. 6). As with the active ovalbumin multigene family in chicken oviduct (17) and the β-like-globin genes in chicken erythroblasts (18), it is possible that the human β-like-globin genes may also be organized in an active chromatin domain in K562, HEL, and adult nucleated marrow cells, with HS I, II, and IV and HS VI possibly marking the locations of the 5′ and 3′ boundaries of the active chromatin domain. The most upstream major hypersensitive site, HS V, which is present not only in cells expressing the β-like-globin genes but also in HL60 cells not expressing these genes, may then represent either the limit of the 5′ boundary of the human β-like-globin gene domain or part of the boundary area of a neighboring domain. If the limit of the 5′ boundary lies somewhere between HS IV and HS V and if the beginning of the 3′ boundary is in an area marked by HS VI, the human β-like-globin gene domain would then span at least 90 kb of DNA. We do not know how far downstream from HS VI the 3′ boundary extends because of the unavailability of a unique-sequence probe in this area (R. Kaufman, personal communication).

Active chromatin domains show overall sensitivity toward DNase I digestion when compared to unexpressed genes or DNA outside of the domain (17, 18). This overall nuclease sensitivity may represent a state of transcriptional preactivation of the structural genes contained within such active domains (19). Thus, in the active β-like-globin gene domain in K562 and HEL cells, the actively transcribed embryonic ε-globin (unpublished data) and fetal γ-globin genes as well as the nontranscribed β-globin gene display the same overall DNase I sensitivity (10). This suggests that the transcribed ε- and γ- as well as the nontranscribed β-globin genes are all in a transcriptionally preactivated state. For actual transcription to take place, the chromatin structure around the preactivated globin genes needs to be further modulated by additional factors. The presence of the minor hypersensitive site 5′ of the transcribed ε- and γ-globin genes and the absence of these minor DNase I-hypersensitive sites 5′ of the nontranscribed β-globin gene in K562 and HEL cells (Fig. 6) may reflect such a requirement of further modulation in chromatin structure. A possible function of major hypersensitive sites I, II, IV, and VI may be to organize and maintain the β-like-globin gene domain in an overall DNase I-sensitive, transcriptionally preactivated state, such that the chromatin structure 5′ of the embryonic ε-, fetal γ-, and adult β-globin genes could be further modulated by cellular signals affecting the transcription of each specific globin gene.

The nucleotide sequence of the DNA upstream of the ε-globin gene up to 2 kb 5′ of HS IV has been determined (unpublished data). Correlating the sequence data with the locations of the major DNase I-hypersensitive sites, we found common sequence features in HS I–IV. These major hypersensitive sites all contain two or three enhancer core-like sequences (16) and 10–26 consecutive or nonconsecutive pairs of alternating purine and pyrimidine bases, found also in many transcriptional enhancers (20). The immunoglobulin enhancer sequences display tissue-specific DNase I hypersensitivity (21, 22) and are recognized by lymphoid-specific cellular factors (23–25). The DNA sequences contained in the above major DNase I-hypersensitive sites, which share common sequence features with these enhancers (23–26), might also possess enhancer function and be recognized by erythroid-specific cellular factors. Whether HS I, II, and IV can serve as transcriptional enhancers for the β-like-globin genes remains to be investigated.

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Nucleotide Sequence of 16-Kilobase Pairs of DNA 5′ to the Human ϵ-Globin Gene*

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We have determined the nucleotide sequence of a 16-kilobase pair (kb) region of DNA on the 5′ side of the human embryonic globin gene (ϵ). This sequence, when combined with previously published sequences, gives an uninterrupted sequence of 21 kb extending from approximately 19.5 kb upstream of the ϵ-globin gene to 0.3 kb 3′ to its poly(A)-addition site. Computer-assisted analysis of this DNA reveals no large regions of self-homology but it shows the presence of seven members of the *Alu* family of repeated DNA, two very short members of the *Kpn* family of repeated DNA, one unusual direct repeat of 39 base pairs, and two potential stem and loop structures. The overall frequencies of mono- and dinucleotides within the 21 kb approximate those found in the total human genome but the distribution of (G + C)-rich regions signal many sequences of interest. The occurrences of some of the polynucleotides and polydinucleotides also differ appreciably from randomness, and the region contains several large stretches of these simple sequences. We found no evidence of strand asymmetry in the region. Homology between the nucleotide sequences of the human ϵ- and $^{G}\gamma$-globin genes extends only 100 base pairs 5′ to the genes. One region, about 10 kb upstream of the gene, shows an apparent clustering of unusual sequence features. We discuss our sequence data in relation to the studies by others of DNAase hypersensitive sites and 5′ transcription endpoints in this part of the β-globin gene cluster.

The human β-globin gene cluster spans approximately 45 kb[1] from the transcriptional start of the message for the 5′ gene, ϵ, through the poly(A)-addition signal of the 3′ gene, β. The nucleotide sequences of the functional globin structural genes, ϵ, $^{G}\gamma$, $^{A}\gamma$, δ, and β, and all of the intergenic DNA from the ϵ-globin gene through the β-globin gene have been determined (compiled by Collins and Weissman, 1984). There has, however, been little systematic effort to search by DNA sequence analysis for structural features around the human β-globin gene cluster that might be related to its overall control.

We describe here the determination of the nucleotide sequence of approximately 16 kb of DNA upstream of the human ϵ-globin gene. These sequences, when combined with the 3.4 kb of DNA sequence from the "$\psi\beta_2$" region (Shen and Smithies, 1982) and with 2 kb from the structural ϵ-globin gene and 5′ flanking DNA (Barelle *et al.*, 1980a; Barelle *et al.*, 1980b; Di Segni *et al.*, 1981), give an uninterrupted sequence of 21 kb of DNA extending from 19.5 kb upstream of the ϵ-globin gene to downstream of its poly(A)-addition signal. We have carried out a computer-assisted analysis of this region and find several interesting structural features. Although these features cannot at this time be absolutely correlated with any functional attributes of the β-globin gene cluster, the clustering of unusual features in some parts of the region suggest that they are likely to have biological function. Our data provide a framework for other investigations, such as those correlating the expression of the human β-globin gene cluster in different cell types with the nuclease sensitivity of the chromatin and should help in selecting promising regions for more intensive study.

EXPERIMENTAL PROCEDURES

DNA Source—The human DNA used for cloning was from an embryonic fibroblast culture (563) prepared from a karyotypically normal first trimester female abortus by Dr. R. M. DeMars at the University of Wisconsin, Madison (Slightom *et al.*, 1980).

Phage Libraries and Cloning Procedures—Two recombinant bacteriophage libraries were screened for overlapping clones spanning the region 5′ to the ϵ-globin gene. One library was constructed by ligating 10–22 kb DNA fragments from a partial *Eco*RI digest of 563 DNA into the vector Charon 4A (Blattner *et al.*, 1978). The other library was constructed by ligating 15–30 kb DNA fragments from a partial *Bam*HI digestion of 563 DNA into the vector Charon 28 (Rimm *et al.*, 1980). The recombinant molecules were packaged *in vitro* (Blattner *et al.*, 1978) and amplified in *Escherichia coli* on agar plates prior to screening. Phage isolations and plasmid subcloning procedures were as described by Slightom *et al.* (1980). Plasmid DNA was isolated as described by Maniatis *et al.* (1982).

Recombinant Phages—The specific recombinant phages and plasmids used in this study are illustrated in Fig. 1 which also shows their nomenclature. Plasmid probe p5′ϵ0.7B, containing a 0.7-kb *Bam*HI fragment including some 5′ flanking DNA and the 5′ portion of the ϵ-globin gene (J. Devereux and P. S. Henthorn, our laboratory), was used in the isolation of phage 5′ϵI. Plasmid probe p5′ϵ3.1HBg, made from a *Hin*dIII-*Bgl*II fragment of phage 5′ϵI, was used in the isolation of phage 5′ϵII. Plasmid probe p5′ϵ1.4BH, made from a *Bam*HI-*Hin*dIII fragment of phage 5′ϵII, was used in the isolation of phage 5′ϵIII.

Plasmid Subclones—Plasmid subclones p5′ϵ1, p5′ϵ2, p5′ϵ3, p5′ϵ4, and p5′ϵ5 were prepared from *Hin*dIII and *Hin*dIII-*Bam*HI fragments from the phage 5′ϵII. Plasmid subclones p5′ϵ6 and p5′ϵ7 were prepared from *Hin*dIII and *Hin*dIII-*Eco*RI fragments from the phage 5′ϵIII.

Overall Sequencing Strategy—The insert from each of the seven plasmids (p5′ϵ1–p5′ϵ7) was isolated. Each insert fragment was first digested on a test scale with restriction enzymes selected for their low exonuclease and nicking activities and because they give overhanging 5′ or flush ends. On the basis of the size and separation of the resulting fragments, one enzyme was then selected for the first preparative end-labeling step using 5–10 μg of the insert. The result-

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[1] The abbreviations used are: kb, kilobase pair; bp, base pair.

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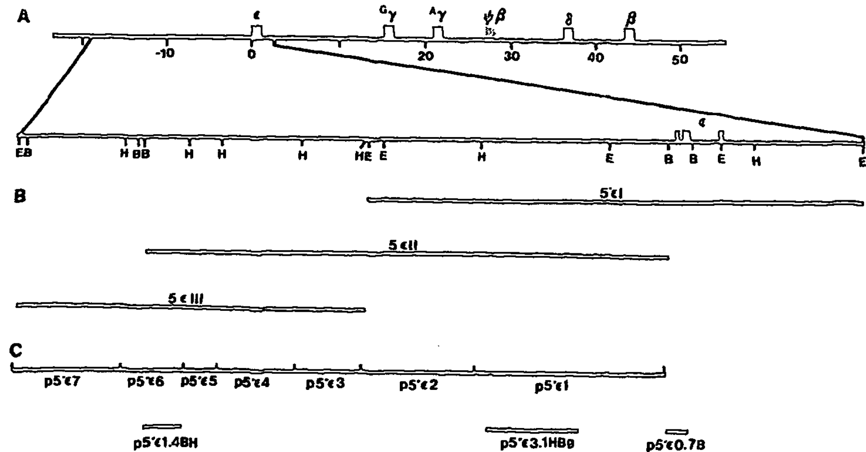

FIG. 1. Map of the region 5′ to the human ε-globin gene and the phage clones and plasmid subclones used in this study. *A*, the human β-globin gene cluster (*upper line*) and a restriction map of the region 5′ to the ε-globin gene (*lower line*) are shown. *H*, *Hin*dIII; *E*, *Eco*RI; *B*, *Bam*HI. The scale is in kb. *B*, overlapping phage clones, 5′εI, 5′εII, and 5′εIII span this region as shown. *C*, plasmids p5′ε7, p5′ε6, p5′ε5, p5′ε4, p5′ε3, p5′ε2, and p5′ε1 were used as DNA sources in the sequencing experiments. Plasmid p5′ε1.4BH, p5′ε3.1HBg, and p5′ε0.7B were used as probes to obtain clones 5′εI, 5′εII, and 5′εIII.

ing fragments were labeled on their 5′ ends with polynucleotide kinase and all were isolated from a polyacrylamide gel, including any that were unresolved. A small amount of each isolated fragment (or mixture of fragments) was then tested for strand separation after denaturation. The fragments were then either preparatively separated into single strands or were cleaved by a second enzyme between the two labeled ends prior to sequencing as described by Maxam and Gilbert (1977).

Second and third end-labeling steps were carried out on each insert fragment using other restriction enzymes. The labeled fragments were again separated, tested for strand separation or for recutting with another enzyme, and were then sequenced. At this stage, in favorable cases, the sequence was close to completion with only some gaps needing to be filled. More often, a fourth end-labeling was required with another enzyme. Gaps were filled by selecting appropriate five or six base recognition enzymes based on the incompletely determined sequence.

This subclone sequencing strategy usually resulted in a complete sequence of the plasmid insert with, in most cases, about 90% of the sequence being determined on both strands and the remainder being sequenced at least twice on the same strand.

A final step in assembling the total sequence was used to establish whether any small DNA sequences were lost. Fragments which overlap the adjacent plasmid subclones were isolated from the parent phage and sequenced. The DNA sequence and sizes of all of the overlapping fragments were as predicted, indicating that no DNA fragments had been lost during the subcloning.

Hybridizations—All hybridizations were done using the conditions described by Vanin *et al.* (1983) with final washes at 68 °C in 3 × SSC plus 0.5% sodium dodecyl sulfate.

Computer Analysis—The DNA sequence was analyzed using software for the VAX computer provided by the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

RESULTS

Nucleotide Sequence—We present in Fig. 2 the nucleotide sequence of 21,381 bp of DNA from about 19.5 kb upstream of the human ε-globin gene to 0.3 kb downstream of its poly(A)-addition signal. This 21 kb of uninterrupted sequence is a composite of three sequences assembled to allow a logical presentation of our computer analysis. *Capital letters* in Fig. 2 indicate 15,965 bp of the new sequence. *Lower case letters* indicate 3,347 bp of the DNA sequence from earlier work of our laboratory (Shen and Smithies, 1982) that is included for continuity. *Lower case letters* are also used for 2,069 bp of DNA sequence from Barelle *et al.* (1980a and 1980b) that was appended so that some known coding sequences could be included in the computer analysis.

The sequence of 2.4 kb of DNA 5′ to the ε-globin gene was previously determined by Barelle *et al.* (1980b) and Di Segni *et al.* (1981). Our sequence differed from theirs by about 2.6%, with the majority of the differences occurring within regions where the sequence was determined on only one strand by Barelle *et al.* (1980b) who considered their sequence within this region to be about 98% accurate. Thus, the differences between our sequence and theirs are probably due in part to errors in determining the DNA sequence and in part to the different sources of the sequenced DNA.

We have used a simple strategy in attempting to identify important regions within this extensive length of DNA. The strategy is to record diagrammatically the nonrandom features within the sequence, on the assumption that any clustering of unusual features will direct our attention to regions that have acquired unique characteristics via selection or other unusual events. Fig. 3 summarizes the unusual features which we describe in the following sections.

Internal Comparisons of the DNA Sequence—We compared the 21 kb of the sequence shown in Fig. 2 directly with itself and with its complementary strand using a dot matrix program. Dots were recorded whenever the two sequences used in the comparisons had 14 or more nucleotides identical out of the 20 nucleotides compared. Relatedness as distant as that between the coding regions of the adult α-globin and fetal γ-globin genes, thought to have separated about 500 million

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FIG. 2. **Nucleotide sequence of the region 5′ to the human ε-globin gene.** The 21,381 bp of the DNA sequence is shown from 5′ to 3′. The *first nucleotide corresponding to the initiation codon* of the ε-globin gene is given the coordinate 0. Nucleotides 5′ to this position are sequentially numbered with negative coordinates, and nucleotides 3′ to this position are sequentially numbered with positive coordinates. The nucleotide sequence from position −9131 through −5771, shown in *lower case letters*, was determined by Shen and Smithies (1982). The nucleotide sequence from position −234 through 1840, also shown in *lower case letters*, was determined by Barelle *et al.* (1980b). *Eco*RI (*E*), *Hin*dIII (*H*), and *Bam*HI (*B*) restriction sites are marked. Features of interest are indicated alongside the body of the figure and by various *underlines* and *arrows*. These features include exons 1–3 of the ε-globin gene, seven *Alu* repeats, *Alu*5ε1-*Alu*5ε7, two *Kpn* family repeats, *Kpn*5ε1 and *Kpn*5ε2, a direct repeat, 5ε39DR, and two potential stem and loop structures, 5εSL1 and 5εSL2 (the "stem" and "loop" positions are marked with *heavy* and *thin broken underlines*). The locations of the tracts of polynucleotides diagrammed in Fig. 3 are shown *above* the *sequence* by suitable symbols; for example, at position −19,300, *A13* signifies a tract of 13 adenines.

E
GAATTCTAATCTCCCTCTCAACCCTACAGTCACCCATTTGGTATATTAAAGATGTGTTGTCTACTGTCTAGTATCCCTCAAGTAGTGTCAGGAATTAGTCATTTAAATAGTCTGCAAGCCAGGAGTGGTGGCTCATGTCTGTAATTCCAG -19391

A13
Alu5ε7 CACTGGAGAGGTAGAAGTGGGAGGACTGCTTGAGCTCAAGAGTTTGATATTATCCTGGACAACATAGCAAGACCTCGTCTCTACTTAAAAAAAAAAAAATTAGCCAGGCATGTGATGTACACCTGTAGTCCCAGCTACTCAGGAGGCCGA -19241

B
AATGGGAGGATCCCTTGAGCTCAGGAGGTCAAGGCTGCAGTGAGACATGATCTTGCCACTGCACTCCAGCCTGGACAGCAGAGTGAAACCTTGCCTCACGAAACAGAATACAAAAACAAACAAACAAAAAACTGCTCCGCAATGCGCTTC -19091

CTTGATGCTCTACCACATAGGTCTGGGTACTTTGTACACATTATCTCATTGCTGTTCGTAATTGTTAGATTAATTTTGTAATATTGATATTATTCCTAGAAAGCTGAGGCCTCAAGATGATAACTTTTATTTTCTGGACTTGTAATAGCT -18941

TTCTCTTGTATTCACCATGTTGTAACTTTCTTAGAGTAGTAACAATATAAAGTTATTGTGAGTTTTTGCAAACACAGCAAACACAACGACCCATATAGACATTGATGTGAAATTGTCTATTGTCAATTTATGGGAAAACAAGTATGTACT -18791

TTTTCTACTAAGCCATTGAAACAGGAATAACAGAACAAGATTGAAAGAATACATTTTCCGAAATTACTTGAGTATTATACAAAGACAAGCACGTGGACCTGGGAGGAGGGTTATTGTCCATGACTGGTGTGTGGAGACAAATGCAGGTTT -18641

ATAATAGATGGGATGGCATCTAGCGCAATGACTTTGCCATCACTTTTAGAGAGCTCTTGGGGACCCCAGTACACAAGAGGGGACGCAGGGTATATGTAGACATCTCATTCTTTTTCTTAGTGTGAGAATAAGAATAGCCATGACCTGAGT -18491

Y16
TTATAGACAATGAGCCCTTTTCTCTCTCCCACTCAGCAGCTATGAGATGGCTTGCCCTGCCTCTCTACTAGGCTGACTCACTCCAAGGCCCAGCAATGGGCAGGGCTCTGTCAGGGCTTTGATAGCACTATCTGCAGAGCCAGGGCCGAG -18341

AAGGGGTGGACTCCAGAGACTCTCCCTCCCATTCCCGAGCAGGGTTTGCTTATTTATGCATTTAAATGATATATTTATTTTAAAAGAAATAACAGGAGACTGCCCAGCCCTGGCTGTGACATGGAAACTATGTAGAATATTTTGGGTTCC -18191

T9 Y18
ATTTTTTTTTCCTTCTTTCAGTTAGAGGAAAAGGGGCTCACTGCACATACACTAGACAGAAAGTCAGGAGCTTTGAATCCAAGCCTGATCATTTCCATGTCATACTGAGAAAGTCCCCACCCTTCTCTGAGCCTCAGTTTCTCTTTTTAT -18041

AAGTAGGAGTCTGGAGTAAATGATTTCCAATGGCTCTCATTTCAATACAAAATTTCCGTTTATTAAATGCATGAGCTTCTGTTACTCCAAGACTGAGAAGGAAATTGAACCTGAGACTCATTGACTGGCAAGATGTCCCCAGAGGCTCTC -17891

ATTCAGCAATAAAATTCTCACCTTCACCCAGGCCCACTGAGTGTCAGATTTGCATGCACTAGTTCACGTGTGTAAAAAGGAGGATGCTTCTTTCCTTTGTATTCTCACATACCTTTAGGAAAGAACTTAGCACCCTTCCCACACAGCCAT -17741

CCCAATAACTCATTTCAGTGACTCAACCCTTGACTTTATAAAAGTCTTGGGCAGTATAGAGCAGAGATTAAGAGTACAGATGCTGGAGCCAGACCACCTGAGTGATTAGTGACTCAGTTTCTCTTAGTAATTGTATGACTCAGTTTCTTC -17591

ATCTGTAAAATGGAGGGTTTTTTAATTAGTTTGTTTTTGAGAAAGGGTCTCACTCTGTCACCCAAATGGGAGTGTAGTGGCAAAATCTCGGCTCACTGCAACTTGCACTTCCCAGGCTCAAGCGGTCCTCCCACCTCAACATCCTGAGTA -17441

Alu5ε6 GCTGGAACCACAGGTACACACCACCATACCTCGCTAATTTTTTGTATTTTTGGTAGAGATGGGGTTTCACATGTTACACAGGATGGTCTCAGACTCCGGAGCTCAAGCAATCTGCCCACCTCAGCCTTCCAAAGTGCTGGGATTATAAGC -17291

ATGATTACAGGAGTTTTAACAGGCTCATAAGATTGTTCTGCAGCCCGAGTGAGTTAATACATGCAAAGAGTTTAAAGCAGTGACTTATAAATGCTAACTACTCTAGAAATGTTTGCTAGTATTTTTTGTTTAACTGCAATCATTCTTGCT -17141

GCAGGTGAAAACTAGTGTTCTGTACTTTATGCCCATTCATCTTTAACTGTAATAATAAAAATAACTGACATTTATTGAAGGCTATCAGAGACTGTAATTAGTGCTTTGCATAATTAATCATATTTAATACTCTTGGATTCTTTCAGGTAG -16991

ATACTATTATTATCCCCATTTTACTACAGTTAAAAAAACTACCTCTCAACTTGCTCAAGCATACACTCTCACACACACAAACATAAACTACTAGCAAATAGTAGAATTGAGATTTGGTCCTAATTATGTCTTTGCTCACTATCCAATAAA -16841

TATTTATTGACATGTACTTCTTGGCAGTCTGTATGCTGGATGCTGGGGATACAAAGATGTTTAAATTTAAGCTCCAGTCTCTGCTTCCAAAGGCCTCCCAGGCCAAGTTATCCATTCAGAAAGCATTTTTTACTCTTTGCATTCCACTGT -16691

TTTTCCTAAGTGACTAAAAAATTACACTTTATTCGTCTGTGTCCTGCTCTGGGATGATAGTCTGACTTTCCTAACCTGAGCCTAACATCCCTGACATCAGGAAAGACTACACCATGTGGAGAAGGGGTGGTGGTTTTGATTGCTGCTGTC -16541

TTCAGTTAGATGGTTAACTTTGTGAAGTTGAAAACTGTGGCTCTCTGGTTGACTGTTAGAGTTCTGGCACTTGTCACTATGCCTATTATTTAACAAATGCATGAATGCTTCAGAATATGGGAATATTATCTTCTGGAATAGGGAATCAAG -16391

H
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GGGATTTCTACCCTGAGCAAAAGGCTGGTCTTGATTAATTCCCAAACTCATATAGCTCTGAGAAAGTCTATGCTGTTAACGTTTTCTTGTCTGCTACCCCATCATATGCACAACAATAAATGCAGGCCTAGGCATGACTGAAGGCTCTCT -16091

CATAATTCTTGGTTGCATGAATCAGATTATCAACAGAAATGTTGAGACAAACTATGGGAAGCAGGGTATGAAAGAGCTCTGAATGAAATGGAAACCGCAATGCTTCCTGCCCATTCAGGGCTCCAGCATGTAGAAATCTGGGGCTTTGT -15941

B
GAAGACTGGCTTAAAATCAGAAGCCCCATTGGATAAGAGTAGGGAAGAACCTAGAGCCTACGCTGAGCAGGTTTCCTTCATGTGACAGGGAGCCTCCTGCCCCGAACTTCCAGGGATCCTCTCTTAAGTGTTTCCTGCTGGAATCTCCTC -15791

B
ACTTCTATCTGGAAATGGTTTCTCCACAGTCCAGCCCCTGGCTAGTTGAAAGAGTTACCCATGCAGAGGCCCTCCTAGCATCCAGAGACTAGTGCTTAGATTCCTACTTTCAGCGTTGGACAACCTGGATCCACTTGCCCAGTGTTCTTC -15641

CTTAGTTCCTACCTTCGACCTTGATCCTCCTTTATCTTCCTGAACCCTGCTGAGATGATCTATGTGGGGAGAATGGCTTCTTTGAGAAACATCTTCTTCGTTAGTGGCCTGCCCCTCATTCCCACTTTAATATCCAGAATCACTATAAGA -15491

R19
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CTATTGTTTTCCATCCTCACGCTAACTCCATAAAAAAACACTATTATTATCTTTATTTTGCCATGACAAGACTGAGCTCAGAAGAGTCAAGCATTTGCCTAAGGTCGGACATGTCAGAGGCAGTGCCAGACCTATGTGAGACTCTGCAGC -15191

TACTGCTCATGGGCCCTGTGCTGCACTGATGAGGAGGATCAGATGGATGGGGCAATGAAGCAAAGGAATCATTCTGTGGATAAAGGAGACAGCCATGAAGAAGTCTATGACTGTAAATTTGGGAGCAGGAGTCTCTAAGGACTTGGATTT -15041

CAAGGAATTTTGACTCAGCAAACACAAGACCCTCACGGTGACTTTGCGAGCTGGTGTGCCAGATGTGTCTATCAGAGGTTCCAGGGAGGGTGGGGTGGGGTCAGGGCTGGCCACCAGCTATCAGGGCCCAGATGGGTTATAGGCTGGCAG -14891

GCTCAGATAGGTGGTTAGGTCAGGTTGGTGGTGCTGGGTGGAGTCCATGACTCCCAGGAGCCAGGAGAGATAGACCATGAGTAGAGGGCAGACATGGGAAAGGTGGGGAGGCACAGCATAGCAGCATTTTTCATTCTACTACTACATGG -14741

GACTGCTCCCCTATACCCCCAGCTAGGGGCAAGTGCCTTGACTCCTATGTTTTCAGGATCATCATCTATAAAGTAAGAGTAATAATTGTGTCTATCTCATAGGGTTATTATGAGGATCAAAGGAGATGCACACTCTCTGGACCAGTGGCC -14591

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H
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AAATCTGACTTCACTGGAAACATGGAAGCCCAAGGTTCTGAACATGAGAAATTTTTAGGAATCTGCACAGGAGTTGAGAGGAAACAAGATGGTGAAGGGACTAGAAACCACATGAGAGACACGAGGAAATAGTGTAGATTTAGGCTGGA -14141

GGTAAATGAAAGAGAAGTGGGAATTAATACTTACTGAAATCTTTCTATATGTCAGGTGCCATTTTATGATATTTAATAATCTCATTACATATGGTAATTCTGTGAGATATGTATTATTGAACATACTATAATTAATACTAATGATAAGTA -13991

ACACCTCTTGAGTACTTAGTATATGCTAGAATCAAATTTAAGTTTATCATATGAGGCCGGGCACGGTGGCTCATATATGGGATTACATGCCTGTAATCCCAGCACTTTGGGAGGCCAAGGCAATTGGATCACCTGAGGTCAGGAGTTCCA -13841

Alu5ε5 GACCAGCCTGGCCAACATGGTGAAACCCCTTCTCTACTAAAAATACAAAAATCAGCCAGGTGTGGTGGCACGCGTCTATAATCCCAGCTACTCAGGAGGCTGAGGCAGGAGAATCACTTGAACCCAGGAGGTGGAGGTTGCAGTGAGC -13691

A13, R17
TAAGATTGCACCACTGCACTCCAGCCTAGGCGACAGAGTGAGACTCCATCTCAAAAAAAAAAAAAGAAGTTTATTATATGAATTAACTTAGTTTTACTCACACCAATACTCAGAAGTAGATTATTACCTCATTTATTGATGAGGAGCCCA -13541

ATGTACTTGTAGTGTAGATCAACTTATTGAAAGCACAAGCTAATAAGTAGACAATTAGTAATTAGAAGTCAGATGGTCTGAGCTCTCCTACTGTCTACATTACATGAGCTCTTATTAACTGGGGACTCGAAAATCAAAGACATGAAATAA -13391

H
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CCTTGCTTCTTACTTTTACTTCTTGGCGATTGGAAGTTGTGGTCCAAGCCACAGTTGCAGACCATACTTCCTCAACCATAATTGCATTTCTTCAGGAAAGTTTGAGGGAGAAAAAGGTAAAGAAAAATTTAGAAACAACTTCAGAATAAA -13091

GAGATTTTCTCTTGGGTTACAGAGATTGTCATATGACAAATTATAAGCAGACACTTGAGAAAACTGAAGGCCCATGCCTGCCCAAATTACCCTTTGACCCCTTGGTCAAGCTGCAACTTTGGTTAAAGGGAGTGTTTATGTGTTATAGTG -12941

RY6
TTCATTTACTCTTCTGGTCTAACCCATTGGCTCCGTCTTCATCCTGCAGTGACCTCAGTGCCTCAGAAACATACATATGTTTGTCTAGTTTAAGTTTGTGTGAAATTCTAACTAGCGTCAAGAACTGAGGGCCCTAAACTATGCTAGGAA -12791

TAGTGCTGTGGTGCTGTGATAGGTACACAAGAAATGAGAAGAAACTGCAGATTCTCTGCATCTCCCTTTGCCGGGTCTGACAACAAAGTTTCCCCAAATTTTACCAATGCAAGCCATTTCTCCATATGCTAACTACTTTAAAATCATTTG -12641

GGGCTTCACATTGTCTTTCTCATCTGTAAAAAGAATGGAAGAACTCATTCCTACAGAACTCCCTATGTCTTCCCTGATGGGCTAGAGTTCCTCTTTCTCAAAAATTAGCCATTATTGTATTTCCTTCTAAGCCAAAGCTCAGAGGTCTTG -12491

TATTGCCCAGTGACATGCACACTGGTCAAAAGTAGGCTAAGTAGAAGGGTACTTTCACAGGAACAGAGAGCAAAAGAGGTGGGTGAATGAGAGGGTAAGTGAGAAAAGACAAATGAGAAGTTACAACATGATGGCTTGTTGTCTAAATAT -12341

FIG. 2

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RY6
CTCCTAGGGAATTATTGTGAGAGGTCTGAATAGTGTTGTAAAATAAGCTGAATCTGCTGCCTAACATTAACAGTCAAGAAATACCTCCGAATAACTGTACCTCCAATTATTCTTTAAGGTAGCATGCAACTGTAATAGTTGCATGTATAT −12191
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R22
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AT10 RY27 AT11
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RY6 RY6
5εSL2 CTCTATACCTAGAAGCGGCAGAATCAGGCTTTATTAATACATGTGTATAGATTTTTAGGATCTATACACATGTATTAATATGAAACAAGGATATGGAAGAGGAAGGCATGAAAACAGGAAAAGAAAACAAACCTTGTTTGCCATTTTAAG −10391
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5ε39DR TCTACTCGGTTTCGCTGTGAATGAATGATGTCTTGTCAGCCTACTTGGTTTCGCTAAGAGCACAGAGAGAAGATTTAGTGATGCTATGTAAAAACTTCCTTTTGGTTCAAGTGTATGTTTGTGATAGAAATGAAGACAGGCTACATGAT −9641
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H E
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T12 Y16
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RY6
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AC12 RY18 AT6 RY7
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H
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FIG. 2.—*continued*

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TTTCTTAGAATTTTAATCATGAAAATAAATGAAGGCATCTTTACTTACTCAAGGTCCCAAAAGGTCAAAGAAACCAGGAAAGTAAAGCTATATTTCAGCGGAAAATGGGATATTTATGAGTTTTCTAAGTTGACAGACTCAAGTTTTAAC −4991

CTTCAGTGCCCATGATGTAGGAAAGTGTGGCATAACTGGCTGATTCTGGCTTTCTACTCCTTTTTCCCATTAAAGATCCCTCCTGCTTAATTAACATTCACAAGTAACTCTGGTTGTACTTTAGGCACAGTGGCTCCCGAGGTCAGTCAC −4841

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R15

Kpn5ε1 CACATGGACTCATAGAGGGAAACAACGCATACTGGGGCCTATCAGAGGGTGGAGGGTGAGAGAAGGAGAGGATCAGGAAAAATCACTAATGGATGCTAAGCGTAATACCTGAGTGATGAGATCATCTATACAACAAACCCCCTTGACATT −4541

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R15

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E

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T19

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A14 R47

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A10 R15

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5εSL1 CAAGAAGAGAGCCCCAGGCAATACTCACAAAGTAGCCAGTGTCCCCTGTGGTCATAGAGAAATGAAAAGAGAGAGGATTCCCTGGAAGCACTGGATGTAATCTTTTCTGTCTGTCCTCTCTAGGGAATCACCCCAAGGTACTGTACTTTG −641

GGATTAAGGCTTTAGTCCCACTGTGGACTACTTGCTATTCTGTTCAGTTTCTAGAAGGAACTATGTACGGTTTTTGTCTCCCTAGAGAAACTAAGGTACAGAAGTTTTGTTTACAATGCACTCCTTAAGAGAGCTAGAACTGGGTGAGAT −491

TCTGTTTTAACAGCTTTATTTCTTTTCCTTGGCCCTGTTTTTGTCAACTGTCACCACCTTTAAGGCAAATGTTAAATGTGCTTTGGCTGAAACTTTTTTTTCCTATTTTGAGATTTGCTCCTTTATATGAGGCTTTCTTGGAAAAGGAGA −341

B

ATGGGAGAGATGGATATCATTTTGGAAGATGATGAAGAGGGTAAAAAAGGGGACAAATGGAAATTTGTGTTGCAGATAGATGAGGAGCCAACAAAAAAGAGCCTCAGgatccagcacacattatcacaaacttagtgtccatccatcact −191

gctgaccctctccggacctgactccacccctgaggacacaggtcagccttgaccaatgacttttaagtaccatggagaacagggggccagaacttcggcagtaaagaataaaaggccagacagagaggcagcagcacatatctgcttccg −41

Exon1 acacagctgcaatcactagcaagctctcaggcctggcatcatggtgcattttactgctgaggagaaggctgccgtcactagcctgtggagcaagatgaatgtggaagaggctggaggtgaagccttgggcaggtaagcattggttctcaa 109

tgcatgggaatgaagggtgaatattaccctagcaagttgattgggaaagtcctcaagatttttgcatctctaattttgtatctgatatggtgtcatttcatagactcctcgttgtttacccctggacccagagatttttgacagcttt 259

Exon 2 ggaacctgtcgtctccctctgccatcctgggcaaccccaaggtcaaggcccatggcaagaaggtgctgacttcctttggagatgctattaaaaacatggacaacctcaagcccgcctttgctaagctgagtgagctgcactgtgacaag 409

B

ctgcatgtggatcctgagaacttcaaggtgagttcaggtgctggtgatgtgattttttggctttatattttgacattaattgaagctcataatcttattggaaagaccaacaaagatctcagaaatcatgggtcgagcttgatgttagaa 559

cagcagacttctagtgagcataaccaaaacttacatgattcagaactagtgacagtaaaggactactaacagcctgaattggcttaacttttcaggaaatcttgccagaacttgatgtgtttatcccagagaattgtattatagaattgt 709

agacttgtgaaagaagaatgaaatttggcttttggtagatgaaagtccatttcaaggaaatagaaatgccttattttatgtgggtcatgataattgaggtttagaagagatttttgcaaaaaaataaaagatttgctcaaagaaaaata 859

agacacatttctaaaatatgttaaatttcccatcagtattgtgaccaagtgaaggcttgtttccgaatttgttggggatttaaactcccgctgagaactcttgcagcactcacattctacatttacaaaattagacaattgcttaaa 1009

gaaaaacagggagagagggaacccaataatactggtaaaatggggaagggggtgagggtgtaggtaggtagaatgttgaatgtagggctcatagaataaaattgaacctaagctcatctgaatttttgggtggcacaaaccttggaac 1159

agtttgaggtcagggttgtctaggaatgtaggtataaagccgtttttgtttgtttgtttgtttttcatcaagttgttttcggaaacttctactcaacatgcctgtgtgttattttgtcttttgcctaacagctcctgggtaacgtgatg 1309

Exon3 gtgattattctggctactcactttggcaaggagttcacccctgaagtgcaggctgcctggcagaagctggtgtctgctgtcgccattgccctggcccataagtaccactgagttctcttccagtttgcaggtcttcctgtgaccctgaca 1459

ccctccttctgcacatggggactgggcttggccttgagagaagccttctgtttaataaagtacattttcttcagtaatcaaaaattgcaattttatcttctccatcttttactcttgtgttaaaaggaaaaagtgttcatgggctgagg 1609

E

gatggagagaaacataggaagaaccaagagcttccttaagaaatgtatggggggcttgtaaaattaatgtggatgttatgggagaattcccaagattcccaaggaggatgatatgatggagaaaaatctttatcggggtgggaaaatggtt 1759

aattaagtggcagagactcctaggcagttttactgcaccggggaaagaaggagctgttgtggtacctgagaaagcagatt 1840

FIG. 2—*continued*

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TABLE I
Characteristics of the seven Alu repeats

	Location	Homology	Orientation	Direct repeats
		%		
Alu5ε7	−19,424 to −19,109	71	5'-3'	No
Alu5ε6	−17,572 to 17,286	76	3'-5'	No
Alu5ε5	−13,935 to −13,622	85	5'-3'	AAGTTTATCATATGA
Alu5ε4	−11,521 to 11,200	81	5'-3'	No
Alu5ε3	−8,929 to −8,609	82	5'-3'	AAACATCAAATTCCTTGA
Alu5ε2	−2,628 to −2,322	84	3'-5'	AAAAATATCTGCAAT
Alu5ε1	−1,597 to −1,308	82	5'-3'	AGAAATGGATGGAGA

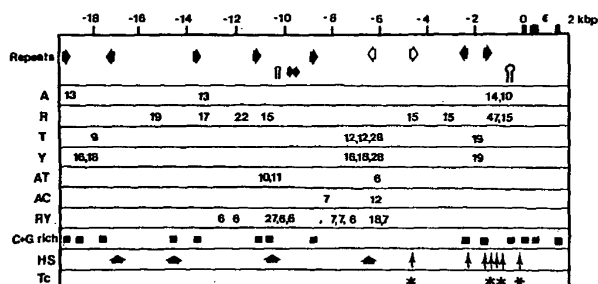

FIG. 3. **Summary of the unusual sequence features in the region 5' to the human ε-globin gene.** The *upper line* shows the extent and coordinates of the DNA sequence under analysis. The locations of the coding regions of the ε-globin gene are shown by *heavy bars. Repeats, solid arrows* represent the locations of the seven *Alu* repeats; *open arrows* mark the locations of *Kpn5ε2* and *Kpn5ε1*; *double arrows* mark the location of 5ε39DR; and *stem* and *loop structures* mark the locations of 5εSL1 and 5εSL2. Polynucleotides and polydinucleotides: *A*, poly(A); *R*, poly(purine); *T*, poly(T); *Y*, poly(pyrimidine); *AT*, poly(AT); *AC*, poly(AC); and *RY*, poly(purine-pyrimidine). For each polynucleotide and polydinucleotide, the *number* shows the number of nucleotides of dinucleotides at the indicated approximate location. The precise locations of these polynucleotides and polydinucleotides are shown in Fig. 2 by appropriate symbols; for example, at position −19,300 A13 signifies a tract of 13 adenines. C + G-rich, *solid blocks* represent regions with 50% or greater cytosine and guanine content. *HS*, the locations of the major and minor nuclease hypersensitive sites are, respectively, marked with *heavy* and *light arrows* (Tuan and London, 1984; Zhu *et al.*, 1984).[2] *Tc*, the locations of the minor transcriptional initiation sites are marked with *asterisks* (Allan *et al.*, 1983).

years ago, are readily detected with this degree of stringency (Shen and Smithies, 1982). In the direct and inverted plots, seven members of the *Alu* family of repeats were easily identified as a string of dots because each *Alu* repeat has homologous sequences in both the same and in opposite orientations. The internally duplicated nature of each of the *Alu* repeats was likewise visible, as were the short direct repeats of four of the *Alu* repeats.

No evidence of any large regions of self-homology, direct or inverted, was observed in the 21 kb of DNA sequence. This confirms the observation of Shen and Smithies (1982) that no globin pseudogene or other structure homologous to globin genes occurs in this region upstream of the ε-globin gene. We conclude that this 21-kb sequence did not evolve by the duplication of any substantial part of itself, although, as will be discussed below, the ε-globin gene has a short 5' flanking sequence related to the equivalent parts of other genes of the β-globin gene cluster.

Several small repeats within this sequence were visible on the dot matrix plot, and are indicated on the sequences shown in Figs. 2 and 3. A pair of contiguous direct repeats of 39 bp, 5ε39DR, containing 36 matching nucleotides is located at position −9813 through −9777. Two long inverted duplications with the potential of forming stem and loop structures were also visible on the dot matrix plot. One potential stem and loop structure, 5εSL2, is located at positions −10509 through −10450 and can form a perfectly paired stem of 21 nucleotide pairs plus one G-T pair (with a total of 47 hydrogen bonds in the stem) and a loop of 5 nucleotides. Since other notable features are located in this neighborhood, this potential stem and loop structure may have some structural significance. A second potential stem and loop structure, 5εSL1, with a total of 37 hydrogen bonds in the 18 nucleotide pair stem and 101 bases in the loop is located at positions −807 through −671 close to the 5' start of the ε-globin gene transcript.

A pair of inverted repeats of about 164 base pairs with 2 length differences and 78% identity between the two sequences occurs at positions −6637 through −6474 and positions −4696 through −4480. The 5' member of the pair is flanked by a pair of short direct repeats with 16 out of 17 identical nucleotides. The 3' member is flanked by short direct repeats with 14 out of 15 identical nucleotides. The presence of these short repeats suggests that both members of this pair of inverted repeats are inserted sequences. We show below that they are short stretches of DNA belonging to the *Kpn* or L1 family of repeated DNA.

Members of the Alu Family of Repetitive DNA—The most prominent landmarks in our DNA sequence are the seven members of the *Alu* family of repeated DNA. We have designated them *Alu5ε1* through *Alu5ε7* consecutively with *Alu5ε1* closest to the ε-globin gene. The DNA sequences of two of the *Alu* family repeats, *Alu5ε1* and *Alu5ε2*, were previously determined by Barelle *et al.* (1980b) and Di Segni *et al.* (1981). Our DNA sequences of these two *Alu* repeats are essentially identical to theirs.

Table I summarizes features of the seven *Alu* repeats. The DNA sequence of the *Alu* repeat was compared to a consensus sequence derived from the 12 *Alu* repeats found near or within the human β-globin gene cluster. The homology of each of the seven *Alu* repeats to the consensus sequence ranged from 71 to 85%. Four of the *Alu* repeats, *Alu5ε1*, *Alu5ε2*, *Alu5ε3*, and *Alu5ε5*, have flanking short direct repeats. The lack of homology between these short direct repeats between the DNA, flanking all seven *Alu* repeats, strongly suggests that each *Alu* repeat was inserted into its present location by an independent event.

Members of the Kpn Family of Repetitive DNA—A portion of the repetitive DNA sequences of the human genome consists of members of the *Kpn* or L1 family of repeated DNA (Thayer and Singer, 1983; diGiovanni *et al.*, 1983; Grimaldi *et al.*, 1984). Comparison of the pair of inverted repeats at about positions −6500 and −4500 with the published sequences of *Kpn* family members showed that they are members of this family. Accordingly, we named them *Kpn5ε1* (at

[2] D. Tuan, W. Solomon, Q. Li, and I. M. London, personal communication.

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Consensus   GCAAACTATC ACAAGAACAG AAAACCAAAC ACCGCATGTT CTCACTCATA  5782
Kpn5ε1      cacctgcaaa cttcgtgaga gatgaggcag aggtacacta cgaaagcaac
Kpn5ε2      gaaaataaat tacattccaa aaatttaact gagactttaa aaaaaaaaa

                 5792
Consensus   GGTGGGAATT G.AACAATGA GAACACATGG ACACAGGAAG GGGAACATCA  5832
Kpn5ε1      agttagaagc taaatgATGA GAACACATGG ACTCATAGAG GGAAACAACG
Kpn5ε2      aaaaaaaaaa aaaaaaccag tgatcCATGG ACACAGGGAG GGGAACATCA

Consensus   CACACTGGGG CCTGTTATGG GGTGGGGGGA GGGGGGAGGG ATAGCATTAG  5882
Kpn5ε1      CATACTGGGG CCTATCA.GA GGGTGGAGGG TGAGAGAAGG AGAGGATCAG
Kpn5ε2      CACACTGGGG CCTGTTG.GG GGTGGGGGGC TAGGGGAAGG ATAGCATTAG

                      5897                   C
Consensus   GAGATATACC TAATG..... .......... ...TAAATGA TGAGTTGATG  5932
Kpn5ε1      GAAAAATCAC TAATGGATGC TAAGCGTAAT ACCTGAGTGA TGAGATCATC
Kpn5ε2      GAGAAATACC TAATG..... .......... ...TAGATGA CGGGTTGATG

Consensus   GGTGCAGCAC ACCAACATGG CACATGTATA CATATGTAAC AAACCTGCAC  5982
Kpn5ε1      TATACAACAA ACCCCCTTGA CATTCATTTA TCTATGTAAC AAACCTGCAC
Kpn5ε2      GGTGCAGCAA ACCACCATG. .......... .......... ..........

Consensus   GTTGTGCACA TGTACCCTAG AACTTAAAGT ATAATAATAA TAAAAA....  6032
Kpn5ε1      ATCCTGTACA CGTACCCCTG AACTTAAAAT AAAAGTTGAA AACAAgaaag
Kpn5ε2      .....GCACA TGTACCCCAG AACTTAAAGC ATATTaaaaa aacagtgatc

Consensus   .......... .......... .......... .......... ..........
Kpn5ε1      caacagtttg aacacttgtt atggtctatt ctctcattct ttacaattac
Kpn5ε2      ataaaagaag ctcaaattta actataagag acggaatggc tcccacaatt
```

FIG. 4. **Comparison of the *Kpn* family members to a consensus sequence.** The sequences of *Kpn5ε1* and *Kpn5ε2* are compared to a consensus sequence derived from 15 sequences from humans and monkeys. The human sequences were: *Kpn5ε1* and *Kpn5ε2* (this paper); pCD-*Kpn*I-8, pCD-*Kpn*I-4, pCD-*Kpn*I-3, Ig-*Kpn*I-7, Ig-*Kpn*I-84, and Ig-*Kpn*I-83 (diGiovanni *et al.*, 1983); pPD16 (Deininger *et al.*, 1981); H*Kpn*E13 and H*Kpn*10.[3] The monkey sequences were: *Kpn*I RET (Thayer and Singer, 1983); LS-1 (Lerman *et al.*, 1983); A11.1;[4] and pa7 (Potter and Jones, 1983). The *numbering system* used is that of Singer.[5] Direct repeats flanking *Kpn5ε1* and *Kpn5ε2* are *boxed*. The "stem" of the potential stem and loop structure discussed in the text is *overlined* in the consensus sequence with *broken lines*. The 9-bp direct repeats at the region of the 36-bp length difference in *Kpn5ε2* are *underlined* in the consensus sequence with *solid lines*.

−4500) and *Kpn5ε2* (at −6500). Several interesting features of these sequences are illustrated in Fig. 4 in which the sequences of *Kpn5ε1* and *Kpn5ε2* are compared with a consensus sequence compiled from the 15 primate *Kpn* family sequences listed in the legend to Fig. 4. *Kpn5ε1* and *Kpn5ε2* differ from the consensus sequence by 19.5 and 7.8%, respectively, and from each other by 22%, suggesting that they are no more related to each other than they are to the other known *Kpn* sequences. The short terminal repeats flanking *Kpn5ε1* and *Kpn5ε2* (*boxed* in Fig. 4) show no relationship to one another, which indicates the *Kpn5ε1* and *Kpn5ε2* were independently introduced into the region upstream of the ε-globin gene.

Kpn5ε1 and *Kpn5ε2* have 3′ ends that extend to the 3′-most boundary of the full length *Kpn* family members. The 5′ ends of *Kpn5ε1* and *Kpn5ε2* are separated by only 9 bp relative to the consensus sequence and lie very near the site in the consensus sequence (position 5792) where the homology between the human *Kpn* consensus sequence and the analogous mouse *Bam*HI family sequence, *Bam* 5, ends (Singer *et al.*, 1983). The clustering of the "endpoints" of these three *Kpn* family sequences (*Bam* 5, *Kpn5ε1* and *Kpn5ε2*) within a region of 17 bp (positions 5792 through 5815) may be due to interruptions in some type of transcriptional process. A potential stem and loop structure is located in the consensus sequence very near the 5′ boundary of each of the truncated *Kpn* sequences (the DNA sequences comprising the stem are *overlined* in the consensus sequence of Fig. 4 by a *broken line*). Formation of a stem and loop structure at this location in the RNA template might block the formation of the DNA copy by reverse transcriptase. Alternatively this site may be the 5′ end of an RNA transcript or lie within a region of the RNA template that is particularly susceptible to nuclease cleavage. Any of these processes would result in the clustering of endpoints of truncated *Kpn* sequences at this location.

A discontinuity between these *Kpn* family sequences occurs at position 5898. *Kpn5ε1* and the African green monkey sequences LS-1 (Lerman *et al.*, 1983) and A11.1[4] have 18 bp in this region while *Kpn5ε2* and most of the other *Kpn* family sequences have only one or no nucleotides. Remarkably, the three 18-bp sequences from *Kpn5ε1*, LS-1, and A11.1 do not appear to have more than random similarity to each other even though they are all of the same length. The extra 18 bp in A11.1 is clearly due to the addition of DNA as it is a nearly perfect duplication of the neighboring consensus sequence from positions 5917 through 5934. The origins of the extra 18 bp in *Kpn5ε1* and LS-1 are less clear, although in both cases a closely similar but shorter sequence occurs in the neighborhood of position 5794.

Kpn5ε2 differs from *Kpn5ε1* and all of the other *Kpn* family sequences in lacking the 36 bp between positions 5951 and 5987. This length difference is likely to be the result of a deletion of DNA. A pair of identical direct repeats of 9 bp (*underlined* in the consensus sequence with a *solid line*) flanking this length difference may have predisposed such a deletion.

Other Families of Repetitive DNA—We compared the 21 kb of DNA sequence 5′ to the ε-globin gene with the published sequences of various other families of repeated DNA. Included in these comparisons were: human (Deininger *et al.*, 1981), monkey (Thayer and Singer, 1983), and bovine (Sano and Sager, 1982) satellite DNA; a repetitive element found in Epstein Barr virus (Jones and Griffin, 1983); several families of repeated DNA found in the rat genome (Parker *et al.*, 1981; Sealy *et al.*, 1981); the human *Hin*fI family of repeated DNA (Shimizu *et al.*, 1983); and the human O and K families (Sun *et al.*, 1984) of repeated DNA. None showed significant homology to the 21-kb sequence, and we conclude that representatives of these repetitive families do not occur in this region.

Base Composition—The base composition of the 21 kb codon-synonomous strand is 30.2% A, 29.4% T, 20.7% G, and 19.7% C. These values closely approximate the nucleotide frequencies found in human genomic DNA (Chargaff, 1950; Shapiro and Chargaff, 1957; Swartz *et al.*, 1962). Nonetheless, the distribution of mononucleotides proves to be a simple statistic that is able to signal many unusual structural features within the 21 kb. The region as a whole contains 40.5% (G + C) and 59.5% (A + T), but several local regions of the sequence have in excess of 50% (G + C). These regions are marked on Fig. 3. This simple statistic picks out all seven *Alu* family repeats, the 5′ flanking region, and the three coding regions of the ε-globin gene. Four other regions within the 21 kb have a (G + C)-content of greater than 50%. Two of these (G + C)-rich regions, at positions −11,000 through −10,700 and positions −740 through −540, are associated with regions containing simple sequences and potential stem and loop structures, but a careful examination of the other two regions revealed no unusual sequence features. Their significances, if any, remains to be determined.

Dinucleotide Distributions—The observed dinucleotide frequencies were compared to those expected from the appropri-

[3] C. Schmidt, personal communication.

[4] G. Grimaldi, J. Skowronski, and M. F. Singer, personal communication.

[5] M. Singer, personal communication.

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ate products of the observed mononucleotide frequencies. We note a large deficit in the dinucleotide CpG (100 × observed/expected = 15.5%) and modest excesses of the dinucleotides TpG (120%), CpA (118%), GpG (121%), ApG (120%), CpC (120%), and CpT (128%). These values are consistent with previous observations that the dinucleotide CpG is underrepresented in the genomes of higher vertebrates (see, for example Ehrlich and Wang, 1981). In some CpG dinucleotides the cytosine residue is methylated as 5-methylcytosine, and it has been suggested that the under-representation of the dinucleotide CpG is the result of deamination of these methylated cytosine residues to uracil (Bird, 1980; McClelland and Ivarie, 1982). We observe an excess of the dinucleotides TpG and CpA (the transition products of CpG) as well as an excess of GpG, ApG, CpC, and CpT dinucleotides (the transversion products of CpG), which suggests that the deficit of CpG may be due to mutational processes other than those exclusively involving the deamination of the 5-methylcytosine residue. In contrast to recent reports describing CpG enrichment in the 5′ gene flanking regions (McClelland and Ivarie, 1982) or clustered within certain genes (Tykocinski and Max, 1984), we find no evidence for any local CpG enrichment in the 21 kg of sequence we have examined.

Simple Sequences—We scanned the codon-synonymous strand of the 21-kb sequence for the occurrence of simple polynucleotides $(X)_n$ with $n \geq 6$. Table II shows the observed number of occurrences of polynucleotides and the expected numbers assuming that the sequence is a random permutation of 21 kb having the observed frequencies of the four mononucleotides. The χ^2 value for each is also given. For each polynucleotide the observed number is not significantly different from the expected number although poly(A) and poly(T) stretches occur in slight excess. However, several of the poly(A) and poly(T) stretches are longer than would be expected to occur by chance in 21 kb of random sequence. We have recorded in Fig. 3 all the occurrences of poly(A) or poly(T) greater than or equal to 9 nucleotides in length (less than 1 of each would be expected by chance). There are four stretches of $A_n \geq 9$, of which three are associated with members of the *Alu* family of repeats, and one is located at position −959. There are five stretches of $T_n \geq 9$; one is associated with *Kpn*5ε2, and the other four are located at positions −18,238, −7,472, −6,786, and −1,911. Possibly the overall slight excess numbers of poly(A) and poly(T) stretches are due to the occurrences of these long stretches. Poly(C) or poly(G) tracts occurred in numbers and in lengths close to those expected in a random sequence of 21 kb.

Table II also shows the observed and expected occurrences

TABLE II

Frequencies of polynucleotides and polydinucleotides

Polynucleotides	Expected[a]	Observed	χ^2	Probability[b]
$(A)_{n\geq6}$	23	27	0.7	
$(G)_{n\geq6}$	2	0	2.0	
$(C)_{n\geq6}$	2	1	0.5	
$(T)_{n\geq6}$	19	26	2.6	
Polydinucleotides	**Expected[c]**	**Observed**	χ^2	**Probability[b]**
$(AG)_n$ and $(GA)_n$	14	23	5.8	<0.02
$(AC)_n$ and $(CA)_n$	13	10	0.7	
$(AT)_n$ and $(TA)_n$	39	19	10.3	<0.002
$(GC)_n$ and $(CG)_n$	4	0	4.0	<0.05
$(GT)_n$ and $(TG)_n$	12	10	0.3	
$(TC)_n$ and $(CT)_n$	10	12	0.4	

[a] Calculated from observed frequencies of mononucleotides.
[b] Probabilities greater than 0.05 are not listed.
[c] Calculated from observed frequencies of dinucleotides.

(calculated from the observed occurrences of dinucleotides) of the polydinucleotides $(XY)_n$ and $(YX)_n$, with $n \geq 3$ and the χ^2 value for each. The polynucleotides $(AT)_n$ and $(TA)_n$ are significantly underrepresented. We expected 4 but found no stretches of $(CG)_n$ or $(GC)_n$ which indicates that they are also underrepresented even after allowing for the low frequency of the CpG dinucleotide. The polynucleotides $(AG)_n$ and $(GA)_n$ are considerably overrepresented in the 21 kb of sequence. A similar analysis of the 11.5 kb of DNA containing the two human fetal globin genes showed that this sequence also had a deficit of $(AT)_n$ and $(TA)_n$ polydinucleotides and an excess of $(AG)_n$ and $(GA)_n$ polydinucleotides (Smithies *et al.*, 1981).

The polydinucleotides of length $n \geq 6$ are recorded in Fig. 3. Some of the observed polydinucleotides are of exceptional length or are clustered. The most striking stretch of polydinucleotides, located at position −10,658 through −10,605, is a perfect run of 28 alternating purines and pyrimidines with only four nucleotides preventing it from being an uninterrupted sequence of $(AT)_{28}$. A computer search of all published human DNA sequences shows that the only other copy of poly$(AT)_n$ with $n \geq 10$, even allowing a mismatch of up to 2 nucleotides, is located about 600 bp upstream of the human β-globin gene (Poncz *et al.*, 1983).

The locations of other tracts of alternating purine and pyrimidine residues, $(RY)_n$ or $(YR)_n$ with $n \geq 6$, are recorded in Fig. 3. Purine-pyrimidine tracts appear to be clustered in two locations, near positions −10,600 and −8,200. Several features of alternating purine pyrimidine stretches suggest that they may have biological significance. Alternating purine pyrimidine tracts are able to form left-handed or Z-DNA *in vitro* and *in vivo* (reviewed by Rich, 1983). In addition (see below), recent experiments indicate that some of the major nuclease hypersensitive sites of the human β-globin gene cluster are located within these regions of alternating purines and pyrimidines (Tuan and London, 1984; Zhu *et al.*, 1984).

Homopurine and homopyrimidine tracts were present in about the expected number, although several tracts were longer than would be expected by chance. Homopurine tracts $(R)_n$ and homopyrimidine tracts $(Y)_n$ of length $n \geq 15$ were recorded on Fig. 3. Three regions near positions −18,000, −7,500, and −1,500 have exceptionally long tracts of homopurines and homopyrimidines.

Strand Asymmetries—In a previous paper (Smithies *et al.*, 1981) we noted significant asymmetries in the base composition and occurrence of simple sequences of the two strands of the DNA of the fetal globin region. We examined the 21 kb of DNA sequence for strand asymmetries and could find no convincing examples with respect to the occurrence in the two strands of polynucleotide and polydinucleotide sequences *versus* their complementary sequences of G *versus* C, of A *versus* T, or of purines *versus* pyrimidines.

Homologies to the Other Prenatal Globin Genes—Comparisons of the coding sequences and short lengths of flanking regions of the five productive β-like globin genes (Barelle *et al.*, 1980a; Slightom *et al.*, 1980; Spritz *et al.*, 1980; Lawn *et al.*, 1980) showed that the human embryonic gene has been diverging for about 120 million years from its closest relatives, the fetal globin genes (Efstradiatis *et al.*, 1980; Hardison, 1985). We therefore compared longer lengths of flanking sequences from the ε- and $^G\gamma$-globin genes using the dot matrix program followed by a program based on this comparison. The first is that, although *Alu* repeats occur in the same orientation and at approximately the same distance upstream of the ε- and $^G\gamma$-globin genes, they are not homologous, except in the sense that all *Alu* repeats have a common ancestor. This is also true of the *Alu* repeat located 5′ to the δ-globin

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gene. Thus, the small direct repeats which flank the *Alu* repeats are not the same in these three examples and there is no homology in the flanking sequences outside these direct repeats. Our second finding is that the detectable homology between the ε- and $^G\gamma$-globin genes extends only about 100 bp upstream of the mRNA cap site.

DISCUSSION

Clustering of Unusual Features—Within the 21 kb of sequence analyzed here, one region (see Figs. 2 and 3) between *Alu*5ε4 and *Alu*5ε3 shows a particularly marked clustering of unusual sequence features. Within less than 2 kb of DNA, there is a potential stem and loop structure (5εSL2), a unique 39-bp direct repeat (5ε39DR), a (G + C)-rich stretch of sequence, a 54-nucleotide tract of repeated alternating purines and pyrimidines $(RY)_{28}$, and a 15-nucleotide long stretch of purines $(R)_{15}$. Furthermore, at least part of this 2-kb region must be conserved during evolution as judged by our finding cross-hybridization in the relevant human and goat DNAs (data not shown) at a level requiring about 70% identity of sequence. Other clusterings of a less pronounced nature can also be found. For example, in the species-conserved region around *Kpn*5ε2, there are three $(T)_n$ tracts, with $n = 12$, 12, and 28, and six $(RY)_n$ tracts, with $n = 7$, 7, 7, 6, 18, and 7, within less than 2 kb of DNA.

Other Studies of the Region 5′ to the ε-Globin Gene—Relatively little is known about nucleotide sequences predisposing the organization of chromosomal DNA into functional units or domains. However, two different types of study suggest that the boundary of the β-type globin gene cluster domain is encompassed in the region we have sequenced 5′ to the ε-globin gene.

Chromatin of the region corresponding to our DNA sequence data has recently been mapped for S1, DNase I, and micrococcal nuclease sensitivity (Tuan and London, 1984; Zhu *et al.*, 1984).[2] Minor nuclease hypersensitive sites, which specifically correlate with the transcription of the ε-globin gene, and major nuclease hypersensitive sites, which are present only in erythroid cells but which do not correlate specifically with the expression of a particular β-type globin gene, have been identified. We record their approximate locations in Fig. 3. In addition, a major nuclease hypersensitive site is located a few kb upstream of the region we have sequenced and is present in cells that do not express the β-type globin genes as well as cells that do express globin genes.[2] This nuclease hypersensitive site may not be specifically associated with the expression of the β-globin gene cluster. Thus, the region corresponding to the 21 kb of the DNA sequence appears to be close to and may include the 5′ end of the β-globin gene cluster domain as judged by the nuclease sensitivity of chromatin in cells that are actively expressing β-type globin genes.

Minor transcriptional initiation sites within the β-globin gene cluster have also been determined (Allan *et al.*, 1983). The approximate locations of these sites are shown in Fig. 3. The 5′-most minor transcriptional initiation site maps near position −4540 (Allan *et al.*, 1983). Thus, as judged by these studies, the sequenced region encompasses the 5′ transcriptional boundary of the β-globin gene cluster.

It was our premise in undertaking this study that regions containing unusual DNA sequence features would eventually be correlated with regions of biological interest. However, at this time the sequence features, such as the clustering of unusual features we noted between *Alu*5ε3 and *Alu*5ε4, can only be loosely correlated with the DNase hypersensitive sites and 5′ transcription boundaries of the β-globin gene cluster, and thus with biological function. More correlative data will be needed before the significance of these features can be established. Meanwhile our nucleotide sequence will provide a solid anatomical framework for carrying out future studies aimed at understanding the regulation of this gene cluster.

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Claim 1

1. (Currently Amended) A recombinant vector comprising:

(a) a region comprising a nucleotide sequence encoding a functional globin; and

(b) a 3.2-kb portion of a human ß-globin locus control region (LCR) which consists essentially of an HS2-spanning nucleotide fragment extending between BstXI and SnaBI restriction sites of said LCR, an HS3-spanning nucleotide fragment extending between BamHI and HindIII restriction sites of said LCR and an HS4-spanning nucleotide fragment extending between BamHI and BanII restriction sites of said LCR, said vector providing expression of globin when introduced into a mammal *in vivo*.

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The β-globin dominant control region: hypersensitive site 2

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The Dominant Control Region (DCR) of the human β-globin gene locus consists of four strong hypersensitive sites (HSS) upstream of the ϵ-globin gene. Addition of these sites confers copy number dependent expression on the human β-globin gene in murine erythroleukaemia cells and transgenic mice, at levels comparable with the endogenous mouse globin genes. We have shown previously that a 1.9 kb fragment comprising HSS 2 accounts for 40–50% of the full effect of the DCR. In this paper we describe a deletional analysis of HSS 2. We show that a 225 bp fragment is sufficient to direct high levels of expression of the human β-globin gene which is copy number dependent and integration site independent. This 225 bp fragment overlaps the major region that is hypersensitive '*in vivo*'. DNase I footprinting shows the presence of four binding sites for the erythroid specific protein NF-E1; the three other footprinted regions display a remarkable redundancy of the sequence GGTGG and bind a number of proteins including Sp1 and the CACC box protein. The significance of these results for the regulation of globin gene expression is discussed.

Key words: Dominant Control Region/erythroid factors/β-globin

Introduction

The human β-globin gene locus consists in the 5′ to 3′ direction of the ϵ-globin gene, which is expressed in embryonic stages, the Gγ and Aγ genes, which are expressed during fetal development and the δ- and β-globin genes which are expressed during adult life. The gene cluster comprises ~60 kb (for review, see Collins and Weissman, 1984) and expression is restricted to cells of the erythroid lineage. To understand the mechanism underlying the stage and tissue specific expression of the genes in the human β-globin gene cluster, DNA fragments containing the individual genes were used to generate transgenic mice (Magram *et al.*, 1985; Chada *et al.*, 1985; Kollias *et al.*, 1987; Behringer *et al.*, 1987). It was shown that the γ-globin genes behaved like the mouse embryonic βH1 gene, while the human β-globin gene followed the expression pattern of the mouse βmajor gene. However, expression levels were very low and dependent on the integration site in the mouse genomic DNA.

Recently, we have shown that the addition of a region containing four very strong hypersensitive sites (HSS) found upstream of the ϵ-gene (Tuan *et al.*, 1985; Forrester *et al.*, 1987) to a β-globin gene fragment containing all its known local regulatory elements overcomes the dominant action of position effects (Grosveld *et al.*, 1987). Moreover, expression of the transgene was found to match that of the endogenous globin genes and was dependent on the copy number of the integrated construct. For this reason, the construct was termed the 'minilocus' and the HSS were called the Dominant Control Region (DCR) of the human β-globin gene cluster. The four 5′ HSS were contained in a 20 kb fragment in the original minilocus; we subsequently linked the HSS together as a 6.5 kb fragment and showed that this configuration, designated the microlocus, was comparable with the minilocus as defined in murine erythroleukaemia (MEL) cells and transgenic mice (Blom van Assendelft *et al.*, 1989; Talbot *et al.*, 1989). The analysis of a similar construction has recently been reported by Forrester *et al.* (1989).

The discovery of DCR sequences in the human β-globin gene locus has opened the way for a realistic approach towards gene therapy and to the development of animal models for human haemoglobinopathies. The feasibility of the latter has been demonstrated in our laboratory by the development of transgenic mice that carry the human allele for sickle haemoglobin under the control of the DCR (Greaves *et al.*, 1990). The erythrocytes of a mouse producing more β^s than endogenous mouse β-globin sickled both *in vivo* and *in vitro*, providing a mouse model to study anti-sickling drugs and gene therapy protocols.

For somatic gene therapy, introduction of the transgene via retroviral insertion in stem cells appears to be the most realistic approach at the moment. For this, the development of retroviral constructs passaging with high titres is essential to obtain a high frequency of infection in the targeted cells. When single HSS of the DCR were tested in MEL cells, it was shown that HSS 1 and 4 gave a level of ~10% of the microlocus, while both HSS 2 and 3 conferred ~50% to a linked β-globin gene (Collis *et al.*, 1990). These results have been confirmed in transgenic mice (Fraser *et al.*, 1990).

In this paper we describe a detailed analysis of HSS 2. Fine mapping shows that the HSS 2 is, in fact, a small hypersensitive region rather than a defined site and functional analysis of MEL cells and transgenic mice shows that a core 225 bp fragment, coinciding with the HSS, allows high level of position independent expression.

Results

Fine mapping of HSS 2 in MEL cells

The locations of the HSS in the β-globin DCR were previously mapped on large restriction fragments (Tuan *et al.*, 1985; Forrester *et al.*, 1987; Grosveld *et al.*, 1987). This allowed us to construct a smaller fully functional DCR containing the individual HSS on 1–2 kb fragments (Talbot

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et al., 1989; Figure 1C). In this microlocus construct, HSS 2 is present on a 1.9 kb *Hind*III fragment. To determine the position of HSS 2 more accurately, we took advantage of a MEL cell line containing four copies of the minilocus construct (clone C, Blom van Assendelft et al., 1989). Nuclei were isolated (Gorski et al., 1986) and treated with different amounts of DNase I for 5 min on ice. As a non-erythroid control, mouse L-cells containing the same construct were used (Blom van Assendelft et al., 1989). Southern blots of *Hind*III digested DNA were probed for HSS 2 via indirect end-labelling. With a probe specific for the 5′ end of the 1.9 kb *Hind*III fragment, we reproducibly found two hypersensitive regions in MEL cells, the 5′ region being the weakest (Figure 2, panel B). With the 3′ probe, only the stronger 3′ region is seen (Figure 2, panel A). We were unable to detect hypersensitivity in L-cells (Figure 2, panels A and B). From these results we infer that the 5′ hypersensitive site is located between nucleotides 950 and 1150, and the 3′ site between 1250 and 1550 (Figure 3, top line).

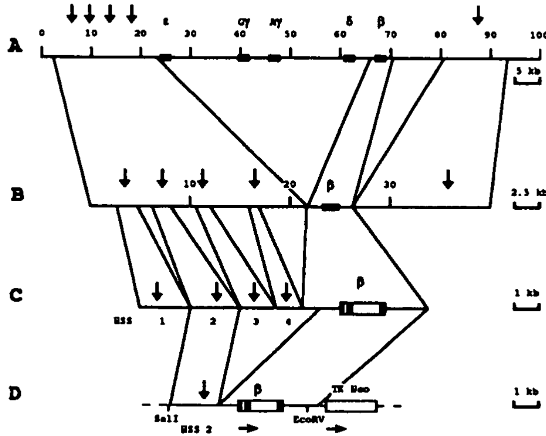

Fig. 1. The Dominant Control Region of the human β-globin gene cluster. (A) The human β-globin gene cluster on the short arm of chromosome 11. The DCR, characterized by four hypersensitive sites 5′ to the ε-globin, and one hypersensitive site 3′ to the β-globin gene is indicated by vertical arrows: (B) The minilocus described by Grosveld et al. (1987), combining the DCR and the 3′ hypersensitive site with the human β-globin gene. (C) The microlocus constructed by Talbot et al. (1989), containing the DCR region as four restriction fragments of 1−2 kb. (D) The plasmid constructs used in this study. All the fragments tested are cloned in the *Hpa*I site at 800 bp in front of the cap site of the human β-globin gene. The *Sal*I and *Eco*RV sites used to isolate the DNA containing the gene plus HSS 2 for transgenic mice are indicated.

Functional analysis of HSS 2 deletions

A series of HSS 2 deletions was made (Figure 3) and cloned in the *Hpa*I site at position −800 in front of the human β-globin gene as shown in Figure 1D. Except when indicated otherwise, the natural sense orientation was used. Plasmids were linearized with *Pvu*I and transfected into MEL cells by electroporation. After selection in G418 containing medium, populations were induced by the presence of 2% DMSO for 4 days and expression of the construct was measured by quantitative S1 analysis using expression of the endogenous mouse α-globin genes as a control (Figure 4).

Construct 1 is the 1.9 kb *Hind*III fragment, which serves as a reference for the full activity of HSS 2 on its own. Construct 2 is a doublet of this fragment. Interestingly, expression of the test gene is remarkably higher with this doublet, indicating that this tandem array allows co-operativity between the two HSS 2 fragments (Table I). Constructs 3−8 are various 5′ and 3′ fragments of HSS 2; analysis of their expression patterns allows us to draw the conclusion that only the fragments containing the major 3′ HSS (Figure 3, Table I) give the full expression observed with the original HSS 2 fragment. Constructs 6 and 7 are particularly instructive, since construct 6 contains the 5′ part of the 1.9 kb *Hind*III fragment, including the weak 5′ HSS, while construct 7 contains the remaining 3′ part comprising the 3′ HSS. The 5′ border of construct 7 is just inside the 5′ border of the strong HSS as mapped in MEL cells (Figures 2 and 3). Only construct 7 gives a level of

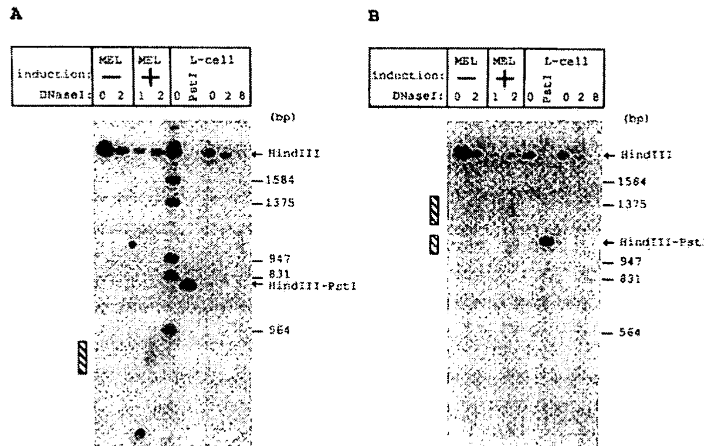

Fig. 2. Fine mapping of HSS 2 in MEL and L-cells. Nuclei were isolated and treated with DNase I (see Materials and methods). DNA was purified and restricted with *Hind*III and Southern blotted. MEL DNA was from uninduced (−) cells or from cells induced for 2 days with 2% DMSO (+). The amount of DNase I (μg/mg DNA) is indicated. The major and minor hypersensitive regions are indicated by dashed blocks. (A) Hybridized with the 3′ *Bst*NI−*Hind*III fragment. (B) Hybridized with the 5′ *Hind*III−*Bam*HI fragment. The position of the major and the minor hypersensitive regions within the 1.9 kb fragment are indicated in Figure 3.

expression comparable with the full site; the level of construct 6 is essentially the same as that of the vector without any DCR fragment (Table I).

These results prompted us to test a 225 bp *HphI–Fnu*4HI fragment which is slightly smaller than the major HSS. This fragment was tested in both the sense (construct 9) and the antisense (construct 10) orientation. The results are shown in Figure 4, panels A and B and Table I. Even this small restriction fragment provides nearly full expression when compared with constructs 1,3,5,7 and 8, independent of orientation. This predicts that this small fragment is capable of providing position independent high levels of expression. However, analyses using MEL cell populations are rather limiting in assaying position independent expression. First of all, cell transformants would have to be cloned and analysed for expression and transgene integrity. Second, the test construct must be integrated into active chromatin regions since the transfected cells are selected in G418 for expression of the linked marker tk-*neo* gene, thus biasing the result. Finally, only a limited copy number range can be achieved in these MEL cells and transcription levels per gene are decreased with increasing copy numbers (>4–5) with similar type small constructs (Talbot *et al.*, 1990).

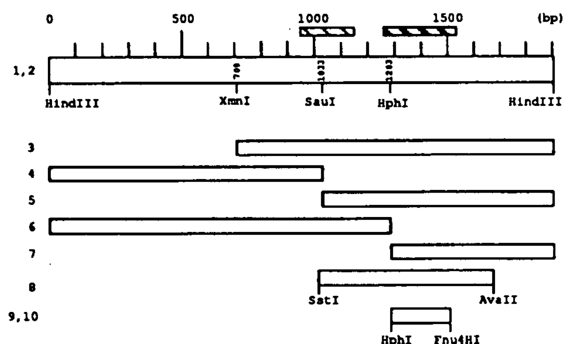

Fig. 3. Deletions of HSS 2 tested in stably transformed MEL cells. All fragments are cloned in the *HpaI* site of the vector GSE 1273 in the sense orientation, with the exception of construct 10, which is the antisense orientation of construct 9. Construct 2 contains the 1.9 kb *HindIII* fragment duplicated in a tandem array. Hatched boxes indicate hypersensitive sites.

Transgenic mice

To test for position independent levels of expression we used the *SalI–Eco*RV fragment of construct 9 (Figures 1 and 3) for microinjection into fertilized mouse eggs. Transgenic 13.5 day fetuses were collected and expression was compared with 13.5 day fetuses containing the 1.9 kb *HindIII* fragment driving the human β-globin gene (Fraser *et al.*, 1990). To determine copy numbers of seven transgenic fetuses, the initial blots of placenta DNA were probed with a human β-globin probe and an endogenous mouse Thy-1 probe as a loading control. To screen for mosaicism of the transgene, DNA from body, head and yolk sac was then also analysed, using the same probes. This showed that mice 31 and 32 were mosaic, since different tissues had a different ratio of human β-globin to Thy-1 signal on the blots (not shown).

Figure 4B and Table I show the results of a quantitative RNA analysis in fetal livers of the seven transgenic fetuses. As expected for mosaic mice, 31 and 32 show low levels of expression per copy of the gene. The other mice show expression levels per gene of 40–50% when compared with the construct containing the entire HSS 2 region (nos. 49 and 59). The exceptions are the mice with extremely high copy numbers (nos. 30 and 354), showing lower levels of

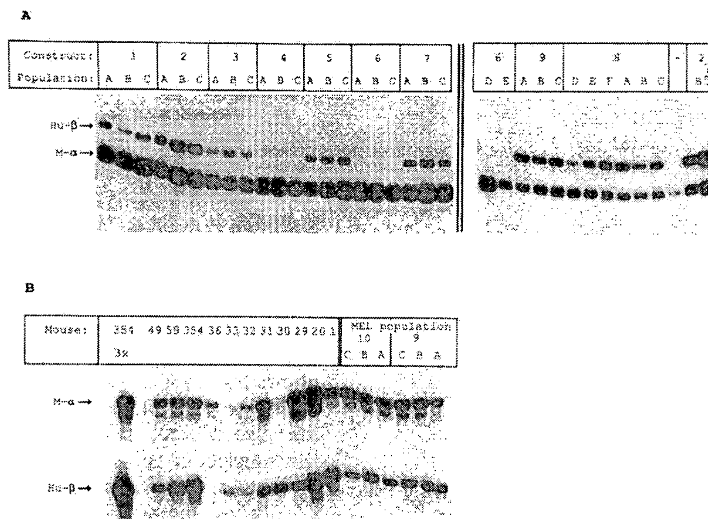

Fig. 4. S1 analysis of the hypersensitive site 2 deletions. In **panel A** a probe for the 3′ end of the human β-globin mRNA was used, in **panel B** a probe for the 5′ end. The probe for the endogenous mouse α-globin mRNA was for the 5′ end in all cases. The combination of a human 3′ β-globin probe and the mouse 5′ α-globin probe results in a background β-signal which was subtracted from all β signals to obtain the values in Table I. Constructs are numbered as in Figure 3. Transgenic mice 1, 20 and 29–33 were made with the *SalI–Eco*RV fragment of construct 9 (Figures 1 and 3). Mouse 36 is the non-transgenic control. Transgenic mice 49, 59 and 354 contain the 1.9 kb *HindIII* fragment 3′ to the 4.8 kb *BglII* fragment of the human β-globin gene (Fraser *et al.*, 1990). Specific activities: panel A, left: β:α = 1:5.3; panel A, right: β:α = 2.7:1; panel B, β:α = 1.5:1.

Table I. Expression of hypersensitive site 2 constructs in MEL cells and transgenic mice

MEL populations

Construct	Copy no.	Mouse α	Human β	% Hβ/Mα	% exp/copy	Av.
1A	>8	13282	3509	24	–	
1B	1	14785	1968	13	52	44
1C	2	17882	3127	18	36	
2A	2	8687	4636	53	106	
2B	2.5	17599	10770	61	98	106
2C	2	13672	7767	57	114	
3A	2	8733	2177	25	50	
3B	3	7075	3345	47	63	59
3C	2	7174	2266	32	64	
4A	1	11304	182	2	8	
4B	1	15832	134	1	4	5
4C	1	9121	47	0.5	2	
5A	3	6597	2822	43	57	
5B	2.5	10904	3711	34	54	52
5C	2	17914	4113	23	46	
6A	1	17603	59	0.3	1	
6B	0.5	16339	81	0.5	2	
6C	0.5	10372	44	0.4	3	2
6D	3	32389	122	0.4	0.53	
6E	dl	nd	nd	–	–	
7A	3	10866	3411	31	42	
7B	2	20820	4171	20	40	41
7C	2	15761	3145	20	40	
8A	4.5	8769	5046	58	52	
8B	3	7134	2285	32	43	
8C	4	8234	4060	49	49	50
8D	dl	11367	1537	13	–	
8E	3	10617	3257	30	40	
8F	3	9190	4555	50	67	
9A	5	9222	4634	50	40	
9B	2	11403	3631	32	64	48
9C	3.5	12922	4543	35	40	
10A	1.5	9025	2369	26	69	
10B	7.5	9688	5436	56	30	52
10C	2.0	10754	3052	29	58	
1273A no DCR	1	13951	117	1	4	
1273B no DCR	1.5	20703	314	1.5	6	5
1273C no DCR	2	17178	346	2	4	
1401A µl	3.5	13210	12716	96	110	
1401B µl	4.5	12606	14182	112	100	97
1401C µl	5	12999	13088	101	80	

Transgenic mice

Mouse no.	Copy no.	Mouse α	Human β	% Hβ/Mα	% exp/copy
1	11	8101	11060	136	49
20	5	27494	13696	49	39
29	1	24666	3084	12	48
30	50	1814	4696	259	21
31 mosaic	30	11017	4171	38	5
32 mosaic	13	2874	1201	42	13
33	19	715	1388	194	41
354	50	7538	14504	192	15
59	5	8103	8536	105	84
49	2	6655	3492	53	106
36 non transgenic	0	3228	43	1	–

Bands were cut out of the gel and Cerenkov counted. A similar sized gel fragment just above the band of interest was also counted for background correction. The data given are corrected for the relative specific activities of the probes used. Copy numbers were determined from Southern blots as described (Talbot *et al.*, 1990). The microlocus controls were those used by Talbot *et al.*, (1989). dl, deletion; µl, microlocus; nd, not determined.

expression, a phenomenon previously observed for both HSS 2 and 3 (Fraser *et al.*, 1990). This indicates that although the small *Hph*I–*Fnu*4HI fragment shows a reduced activity when compared with the large *Hin*dIII fragment in fetal liver versus MEL cells, this core fragment has retained the capacity to provide copy number dependent, integration site independent expression on the β-globin gene.

DNase I footprinting

Based on the expression data in transgenic mice and MEL cells we analysed protein–DNA interactions in the 225 bp *Hph*I–*Fnu*4HI fragment by *in vitro* DNase I footprinting. Figure 5 shows the results obtained with nuclear extracts (Gorski *et al.*, 1986) from uninduced MEL cells, representing the non-expressing adult erythroid stage and anaemic adult spleen expressing the globin genes at high levels. Apart from one hypersensitive site which is stronger with the anaemic spleen extract (indicated by the top arrow in footprint 2 in Figure 5A), the same patterns are observed with both extracts, i.e. six footprints, numbered 1–6 in Figure 5 and summarized in Figure 6. Footprinting with extracts from fetal liver, induced MEL cells, K562 cells, HeLa cells and adult liver showed that the non-erythroid tissues lacked footprints 1, 3 and 5. No differences were observed between the footprints obtained with the different erythroid cell extracts.

The three erythroid specific footprints 1, 3 and 5, represent binding sites for the major erythroid specific factor NF-E1 (Wall *et al.*, 1988; Evans *et al.*, 1988; Tsai *et al.*, 1989). As a hallmark of NF-E1 binding very strong hypersensitive sites are observed immediately upstream of the binding site (indicated by horizontal arrows in Figure 5 and vertical arrows in Figure 6). Competition experiments with NF-E1 specific oligonucleotides of the human β-globin gene 3′ enhancer (Wall *et al.*, 1988) also confirm that footprints 1, 3 and 5 are NF-E1 sites (not shown). Another consensus NF-E1 site is present in footprint 4, but the presence of NF-E1 is obscured by protein binding to the neighbouring remarkable sequence motif that is repeated in footprints 2, 4 and 6.

This motif 5′-GnnnGGTGG-3′ occurs in the same orientation twice in footprint 2, three times in footprint 4 and once in footprint 6 (Figure 6). The presence of additional Gs in the sequence predicts that at least part of these footprints is generated by the general transcription factor Sp1 (Kadonaga *et al.*, 1987) and the CACC binding protein TEF2 (Xiao *et al.*, 1987). We therefore carried out bandshift/competition experiments to determine which complexes are formed with these regions and compared these with genuine Sp1 or CACC box binding sites. Figure 7 shows that each of the oligonucleotides forms a number of complexes in MEL extracts including Sp1 and TEF2, which are also present in HeLa cells (not shown). The Sp1 oligonucleotide (Figure 7, panel Sp1) contains a dimer Sp1 binding site (Gidoni *et al.*, 1985) which forms four complexes, the doublet due to binding of the 95 kd and 105 kd forms of Sp1 (Jackson and Tjian, 1988) and a slower mobility complex [labelled (2×)] which is the result of two Sp1 binding sites on the same oligonucleotide. The nature of the faster mobility complex (also labelled Sp1) which is observed in all of our extracts is, at present, not clear. It could be a degradation product of Sp1 (Gustafson and Kedes, 1989), but could also be a different protein (Xiao *et al.*,

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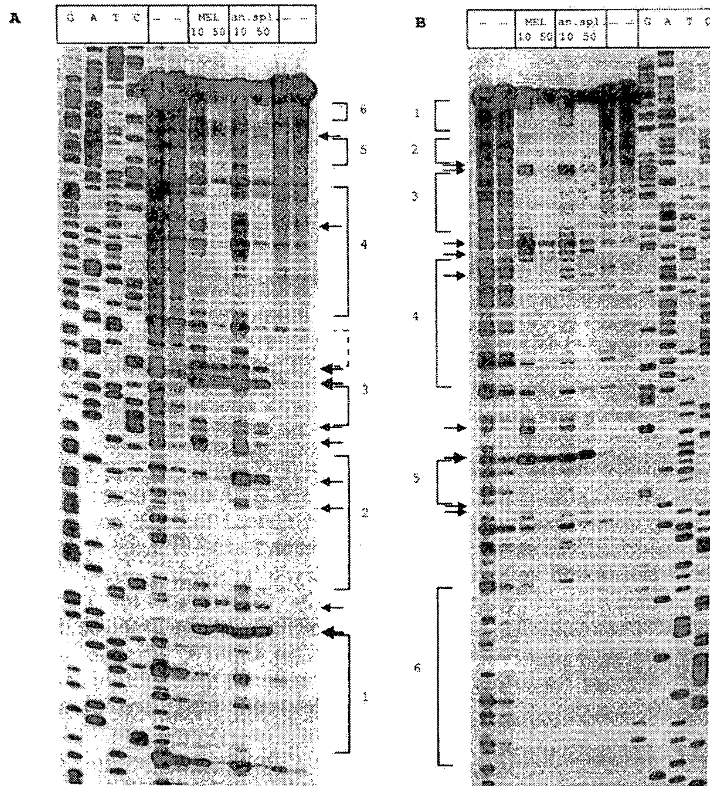

Fig. 5. DNase I footprinting of the 225 bp *HphI–Fnu4HI* fragment. The DNA in the 'no protein' (−) lanes was treated with 1, 0.5, 0.25 and 0.125 μg DNase I from left to right in each panel. DNA pre-incubated with 10 μg nuclear extract from MEL cells or anaemic spleens (an.spl.) was treated with 1 μg DNase I, DNA pre-incubated with 50 μg nuclear extract was treated with 2 μg DNase I. **Panel A** shows the sense strand, **panel B** the antisense strand. Footprinted regions are indicated by numbered brackets, a weak footprint by a dotted bracket. Arrows indicate hypersensitive sites.

1987). In support of the latter possibility, is the fact that an antibody specific for Sp1 (gift of S.Jackson) does not affect the mobility of this band (E.Spanopoulou and F.Grosveld, unpublished). The CACC box oligonucleotide (derived from the β-globin gene promoter, Figure 7, lanes CACC) binds the CACC box protein (labelled CACC) in addition to those proteins bound by the Sp1 oligonucleotide. It also binds a number of fast mobility complexes. The site 2 probes 2, 4 and 6 (see Figure 6) specifically form a number of additional complexes (labelled 1−10). Of the additional complexes, only number 1 can be competed efficiently by the CACC box oligonucleotide, while none are competed by the Sp1 oligonucleotide. It is at present not clear which of these proteins is functionally important.

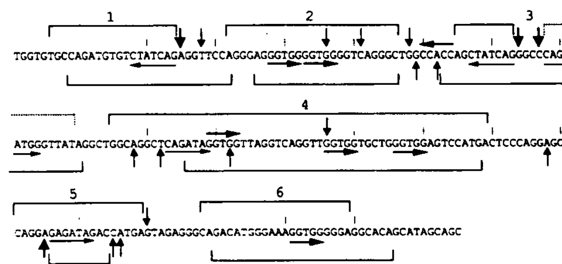

Fig. 6. Summary of the footprinted regions in the 225 bp *HphI–Fnu4HI* fragment. Protected regions on the sense and antisense strand are indicated by brackets, a dotted bracket indicates weak protection; vertical arrows indicate hypersensitive sites. Thin horizontal arrows indicate NF-E1 consensus binding sites; thick horizontal arrows indicate the GGTGG motif.

Discussion

The DCR of the human β-globin gene cluster has been defined to the DNA region between 5 and 25 kb upstream of the ε-globin gene (Grosveld *et al.*, 1987). This region contains four erythroid specific 'super' hypersensitive sites for DNase I (Tuan *et al.*, 1985; Forrester *et al.*, 1987), which were shown to be the functional elements of the DCR (Talbot *et al.*, 1989; Forrester *et al.*, 1989). Deletional analysis of the microlocus construct demonstrated that HSS 2 and 3, as single sites, conferred high levels of expression on the human β-globin test gene in a stable transfection assay (Collis *et al.*, 1990). Interestingly, when tested in transient expression assays in K562 and MEL cells, only HSS 3 was found to stimulate CAT activity (Tuan *et al.*, 1989 and C.H.Chang and P.Dierks, submitted, respectively). This suggests that integration into chromatin is an important prerequisite for proper functioning of the DCR. It clearly distinguishes 'DCR' type elements from classical enhancers, which were originally defined in transient transcription assays (for review see Serfling *et al.*, 1985; Maniatis *et al.*, 1987; Dynan, 1989) and do not necessarily provide integration

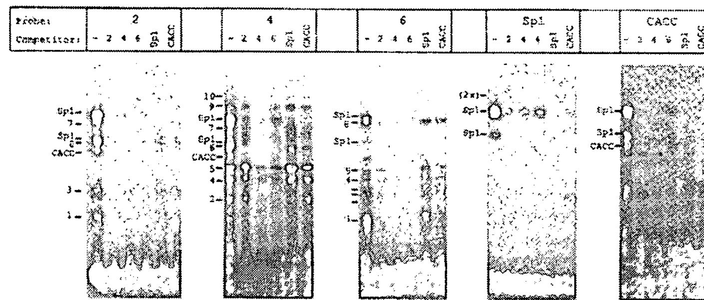

Fig. 7. Gel mobility shift and competition assays of the footprinted regions 2, 4 and 6. Probes covering footprints 2, 4 and 6 (Figure 6) and probes for the SV40 GC-box (Sp1) and the CACC box of the human β-globin gene (CACC) (see Materials and methods) were used in a gel shift assay with nuclear extracts from MEL cells. Competitors were added in 100-fold molar excess as indicated. The position of bands specific for the Sp1 and CACC proteins are indicated by Sp1 and CACC labels. Other specific complexes are numbered relative to their mobility.

position independent expression. HSS 3 appears to be a combination of a DCR element (Forrester *et al.*, 1989; Collis *et al.*, 1990; Fraser *et al.*, 1990; Talbot *et al.*, 1990) and a powerful enhancer (Curtin *et al.*, 1989; Tuan *et al.*, 1989; Ryan *et al.*, 1989) stimulating transcription in both stable and transient assays and in transgenic mice. To understand the mechanism by which 'DCR' type elements act, it is therefore important to define the minimal requirements for this activity, as we describe in this paper for HSS 2 of the human β-globin gene cluster.

Precise mapping of HSS 2 reveals that there is a major hypersensitive region of ~250 bp in the 3' end of the 1.9 kb *Hin*dIII fragment, and a minor site of 150 bp 5' to the major site. The minor site is found only when probing from the 5' end of the *Hin*dIII fragment and footprinting analysis (not shown) shows very few, if any, protein binding sites. Since deletional analysis in MEL cells shows that all the constructs containing the major site (1, 3, 5, 7, 8, 9 and 10) retain the full activity of HSS 2, we concentrated our analysis on this part of HSS 2. HSS 1 and 3 have also been mapped in detail in our laboratory (O.Hanscombe, unpublished; Talbot *et al.*, 1990) and a remarkable common feature is that all the sites have core fragments of between 200 and 300 bp in length. This may indicate that there is a size requirement for DCR elements, roughly coinciding with the length of the nucleosomal repeat unit.

Based on the mapping results in MEL cells (Figure 3 and Table I), we tested the smallest fragment for HSS 2 in transgenic mice. This fragment has retained the most important properties of the entire 1.9 kb HSS 2, i.e. orientation and integration position independent expression of the β-globin gene, albeit at a reduced level. The latter could be due to the fact that the smallest fragment has lost some enhancing sequences, e.g. a potential NF-E2 binding site (Mignotte *et al.*, 1989a,b), which is located just upstream of this fragment and could act as an enhanson (Talbot *et al.*, 1990). It also highlights the occurrence of differences between MEL cell assays and transgenic mice analysis, something we have previously observed with HSS 1 (Collis *et al.*, 1990; Fraser *et al.*, 1990). This could be due to stage specific differences between the two systems or some inherent limitation of the MEL cells.

DNase I footprinting with erythroid nuclear extracts shows that there are six protected regions in the 225 bp fragment. These comprise at least three NF-E1 binding sites (Wall *et al.*, 1988; Tsai *et al.*, 1989) and three regions which have the motif GnnnGGTGG in common (Figure 6). The NF-E1 and GGTGG motifs occur in an alternating order and we have noticed that the combination of these motifs is a common feature of many promoter and regulatory elements of erythroid specific genes. The spacing between the two motifs is often ~30 bp. Table II lists a number of such combinations to substantiate this observation. We propose that the GGTGG array is a key determinant for gene expression in the erythroid lineage and it will be important to determine which protein interacts functionally with the G-motif in erythroid cells. Sp1 (Gidoni *et al.*, 1985) and the CACC box binding protein, TEF2 (Xiao *et al.*, 1987) are both abundant in erythroid cells and both bind to this sequence *in vitro* (Figure 7), but these proteins are not erythroid specific. It has been shown that both of these can interact with other distal transcription elements and their factors to mediate their effect to the transcriptional machinery (Schüle *et al.*, 1988). However, the GGTGG motif is different from the Sp1 and the CACC consensus and it is clear that a number of other ubiquitous proteins are bound to this region which may provide the main activating function *in vivo*; a clue to this is provided by the conserved G residues 5' and 3' to the GGTGG repeats in HSS 2 (Table II). Point mutations at all the positions should resolve which of the proteins is active in a functional analysis. It is conceivable that the combination of such a factor and a tissue specific factor (NF-E1) is sufficient to activate and direct high levels of erythroid specific gene expression. Although the NF-E1 protein has the ability to *trans*-activate (Tsai *et al.*, 1989; Evans and Felsenfeld, 1989), the presence of NF-E1 sites alone is not sufficient for activation *in vivo*, since each of the globin genes (without a DCR) contains multiple NF-E1 binding sites, but is only expressed at low levels and in a highly position dependent manner. Therefore, NF-E1 could be a major activator only in combination with a second factor, where one protein could be the first to bind, without activation of the genes, but enabling the second to bind, thus mediating a transcriptional effect.

Finally, the work described here contributes to the construction of a fully functional DCR of the human β-globin gene cluster containing at least three of the HSS on an ~1 kb fragment. This should greatly facilitate the construction of retroviral vectors which passage with titres high enough to transduce cultured bone marrow stem cells efficiently. It would be a significant step forward if β-thalassaemia could be cured by grafting of autologous 'repaired' bone marrow

Table II. Comparison of different GGTGG arrays in erythroid promoters and regulatory elements

AACCTCTGATAGACAC	a 19 s	GGGGAGGGTGGGGTGGG	s 30 a	GGGCCCTGATAGCTGG	HSS 2, 1324
AACCTCTGATAGACAC	a 24 s	GGGTGGGGTGGGGTCAG	s 25 a	GGGCCCTGATAGCTGG	HSS 2, 1329
GGGCCCAGATGGGTTA	s 30 s	CAGATAGGTGGTTAGGT			HSS 2, 1395
GCTCTCAGATAGGTGG	s 22 s	CAGGTTGGTGGTGCTGG	s 41 s	CAGGAGAGATAGACCA	HSS 2, 1412
GCTCTCAGATAGGTGG	s 32 s	GTGCTGGGTGGAGTCCA	s 31 s	CAGGAGAGATAGACCA	HSS 2, 1422
CAGGAGAGATAGACCA	s 34 s	GGGAAAGGTGGGGGAGG			HSS 2, 1487
ATCGTGAGATAGACGT	a 27 s	AGAAGGGGTGGACTCCA			HSS 1, 899
CAGGGCAGATGGCAAA	s 26 a	TAGTCAGGTGGTCAGCT			HSS 3, 1019
CAGGGCAGATGGCAAA	s 42 a	GTTTGAGGTGGAGTTTT			HSS 3, 1035
		TGCCATGGTGGTTTGCT	s 27 a	TAATGTAGATGACGGG	HSS 4, 247
		GTTGGGGGTGGGGGGCT	a 24 s	AGTGTGTGATGTTCCC	HSS 4, 247
CAGCAGTGATGGATGG	a 30 a	CACAGGGGTGGAGTCAG			H. ε, -165
GCATTGAGATAGTGTG	a 44 a	CCCATGGGTGGAGTTTA			H. Gγ, -143
GGCCTATGATAGGGTA	a 10 a	ATTTGGGGTGGGGCCTA	a 40 s	TGTTTAAGATTAGCAT	H. β-gl.enh., +231
		TTGTGGGGTGGCGCGTG	a 30 a	GGCTCCAGATTCAGAG	H. α-gl., -713
CGAGCGGGATGGGCGG	s 23 s	GTGGCGGGTGGAGGGTG			H. α-gl., -202
CGAGCGGGATGGGCGG	s 30 s	GTGGAGGGTGGAGACGT			H. α-gl., -195
		GGAAGGTGGGCCTGG	s 12 s	GGCCTGGGATAACAGC	H. Gph. A, -51
		AGGAAGGGTGGGGCCTG	a 31 a	GTAAAGAGATAAGGCC	H. PBGD, -100
		GCTGGGTGTGCCC	s 32 s	CCTCAGATAAGACC	Rat PK, -51
		CAGCTGGGTGGGGGCAG	s 16 s	GGTTGCAGATAAACAT	Ch. β^A enh., +1882
		CAGCTGGGTGGGGGCAG	s 31 a	AAGTCTTGATAGCAAA	Ch. β^A enh., +1882

								GGTGG									
G	8	6	14	6	7	14	21	21	0	21	20	9	15	4	7	6	10
A	3	5	1	6	6	5	0	0	0	0	0	7	0	3	1	3	4
T	3	7	3	5	6	2	0	0	21	0	1	4	2	8	4	6	6
C	5	1	3	4	2	0	0	0	0	0	0	1	4	6	9	5	0
Consensus:	-	-	G	-	-	G	G	G	T	G	G	r	G	y	^{C}g	-	r

The GGTGG and NF-E1 motifs are underlined. Frequencies of nucleotides in each position are given. Note that all the sequences are given in the orientation that allows alignment, as indicated by s (sense) and a (antisense). Distances are calculated from the central base in the GGTGG motif to the central base in the NF-E1 motif RNGATNR. Numbering given for the HSS of the DCR is from the 5′ restriction sites used to construct the microlocus (Talbot *et al.*, 1989); others are relative to the cap site. H., human; gl., globin; enh., enhancer; Gph., glycophorin; PBGD, phosphobilinogen deaminase; PK, pyruvate kinase; Ch., chicken.

stem cells, opening the road to the treatment of human haematopoietic diseases by somatic gene therapy protocols.

Materials and methods

Plasmid constructions

Plasmid GSE1273 contains the human β-globin gene as a 4.8 kb *Bgl*II fragment linked to a tk-*neo*^{r} gene (Talbot *et al.*, 1989). All the fragments tested were blunted and cloned in *Hpa*I digested GSE1273, replacing the 650 bp *Hpa*I fragment in the 5′ flanking region of the human β-globin gene (see Figure 1).

DNase I sensitivity

Nuclei were isolated according to Gorski *et al.* (1986) and resuspended at a DNA concentration of 1 mg/ml in 15 mM Tris pH 7.5, 60 mM KCl, 15 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine. DNase I (Worthington) was added to concentrations

of 1–8 μg/ml, and the reaction was started by the addition of $MgCl_2$ to 10 mM and $CaCl_2$ to 1 mM final concentration. The reaction was allowed to proceed for 5 min on ice, and stopped by the addition of 1 vol 50 mM EDTA, 1% SDS. DNA was isolated, digested with appropriate restriction enzymes, and analysed by Southern blotting.

Tissue culture and cell transfections

The MEL cell clone β-C and the L-cell population 4, each containing 4–5 copies of the human β-globin minilocus construct, have been described previously (Blom van Assendelft *et al.*, 1989).

The MEL cell line C88 was maintained in standard αMEM plus 10% fetal calf serum. Plasmid constructs were linearized at the *PvuI* site and transfected by electroporation as follows: log phase MEL cells (2×10^7 cells per transfection) were washed and resuspended in 1.5 ml HEPES buffered saline containing 50 μg of the linearized plasmid. After incubation on ice for 10 min the cells were electroporated with three pulses from a Hoefer 'Pro-Genitor' apparatus set to deliver 250 V for 10 ms. After 5 min at room temperature, they were divided to generate three independent transfected populations (Antoniou *et al.*, 1988). MEL cells were induced to undergo erythroid differentiation by incubation in the presence of 2% (v/v) DMSO for 4 days.

Transgenic mice

Plasmids were cut with *EcoRV* and *SalI* and the fragment containing the human β-globin gene was separated from plasmid sequences by agarose gel electrophoresis and recovered from the gel via isotachophoresis (Öfverstedt *et al.*, 1984). DNA was dissolved at 1–2 μg/ml in microinjection buffer and oocytes were injected into the male pronucleus, as described (Kollias *et al.*, 1987). Fetuses were collected 13.5 days after transfer to pseudopregnant foster mothers and analysed as described (Grosveld *et al.*, 1987).

RNA analysis

RNA was isolated from transfected cell populations and 13.5 day fetal mouse liver by the method of Auffray and Rougeon (1980).

Quantitative S1 nuclease analysis using human β-globin and mouse α-globin DNA probes was performed as described (Kollias *et al.*, 1987; Antoniou *et al.*, 1988; Talbot *et al.*, 1989).

DNA analysis

Southern blotting was performed essentially as described by Southern (1975) using nylon membranes (Nytran, Schleicher and Schüll) and the hybridization conditions described by Church and Gilbert (1985). Copy numbers of the human β-globin gene were determined by laser densitometry using the signal of the endogenous mouse Thy-1 gene as an internal loading control. 13.5 day transgenic fetuses were screened for mosaicism of the transgene by comparing the human β-globin/mouse Thy-1 ratio in DNA from placenta, body, head and yolk sac.

DNase I footprinting

The 225 bp *HphI–Fnu*4HI fragment of HSS 2 was blunted and cloned in both orientations in the *SmaI* site of M13mp8. 1 μg of single-stranded template DNA was annealed to 5 pmol of kinased Universal 17 mer sequencing primer and extended using Klenow polymerase and all four cold dNTPs. The reaction products were phenol extracted and passed over a Sephadex G50 column. After overnight digestion with 10 U *EcoRI*, the DNA was phenol extracted, ethanol precipitated and dissolved in 200 μl of TE buffer. Each footprinting reaction contained 1 μl of the probe mentioned above (3000 c.p.m.), 2 μg poly(dI-dC) and 10–50 μg nuclear protein as described by Wall *et al.*, 1988, but was pre-incubated for 10 min on ice. The samples were analysed on 6% sequencing gels, using dideoxy sequencing reactions as markers.

Nuclear extracts were prepared according to Gorski *et al.* (1986), with modifications as described in Wall *et al.* (1988). HeLa nuclear extracts were prepared according to Dignam *et al.* (1983).

Gel mobility shift assays and competition studies

Gel mobility shift assays were performed as described previously (deBoer *et al.*, 1988), using 5 μg MEL nuclear extract per reaction. Competitions were done by adding 100-fold molar excess of the indicated double-stranded oligonucleotides before addition of the extract.

Nucleotide sequences of the oligonucleotides used in these studies were:

oligonucleotide 2 sense strand:
ATCACAGGTTCCAGGGAGGGTGGGGTGGGGTCAGGGCTGGCC-AC
oligonucleotide 4 sense strand:
GCTCAGATAGGTGGTTAGGTCAGGTTGGTGGTGCTGGGTGGAG-TCCATGACTCCCAG
oligonucleotide 6 sense strand:
TAGAGGGGAGACATGGGAAAGGTGGGGGAGGCACAGCATAG
Sp1 oligonucleotide late strand:
CGATGGGCGGAGTTAGGGGCGGGACTAT
CACC oligonucleotide sense strand:
CGATCCGTAGAGCCACACCCTAGGTAT

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The minimal requirements for activity in transgenic mice of hypersensitive site 3 of the β globin locus control region

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Proper expression of the human β-like globin genes is completely dependent on the presence of the locus control region or LCR, a region containing four DNase hypersensitive sites (HS1–4) situated 5′ to the structural genes. Linkage of the LCR to a transgene results in copy number-dependent transcription, independent of the site of integration in the host genome. We have analysed a small region of the LCR (HS3) in transgenic animals to determine the minimal interactions that are required for this property. The results show that a specific combination of a G-rich sequence flanked on each side by one binding site for the transcription factor GATA1 is essential to obtain position-independent expression of a linked β globin gene in erythroid cells. The overall transcriptional activity of HS3 is achieved through synergy with other combinations of similar binding sites.

Key words: β-globin/GATA1/Hypersensitive 3/locus control region/Sp1/transgenic mice

Introduction

The proper expression of the human β globin locus is completely dependent on the presence of the locus control region (LCR; Figure 1A), which is located to the 5′ side of the β-like globin genes. It is characterized by four developmentally stable erythroid-specific hypersensitive sites, HS1-4 (Tuan *et al.*, 1985; Forrester *et al.*, 1987; Grosveld *et al.* 1987) that control chromatin structure over a distance of at least 150 kb. Deletion of this region leads to an inactive chromatin structure and silencing of the genes (Kioussis *et al.* 1983; Forrester *et al.*, 1990). In hindsight it is therefore not surprising that an isolated β globin gene is expressed at very low levels, if at all, in the red cells of transgenic mice (Magram *et al.*, 1985; Townes *et al.*, 1985; Kollias *et al.*, 1986). Perhaps more importantly, this low level is dependent on the integration site in the mouse genome and therefore virtually unrelated to the number of integrated transgenes (see Perez-Stable and Costantini, 1990 and references therein). This phenomenon, known as 'position effect', has been observed for many genes and is thought to be dependent on the combination of the regulatory sequences of the transgene and the regulatory elements which lie *in cis* to the integration site. In contrast, addition of the LCR leads to full expression of each copy of a β globin gene in erythroid cells of transgenic mice, independent of the site of integration of the transgene (Grosveld *et al.*, 1987). The formation of complexes between the LCR and the gene is probably highly preferred in erythroid cells, excluding interactions between the gene and other regulatory regions present at the site of integration. An alternative explanation for the absence of position effects would be the presence of insulating sequences on the construct, preventing the interaction with neighbouring regulatory sequences. This has been demonstrated in the case of the Drosophila *scs* elements (Kellum and Schedl, 1991). Three arguments favour the first possibility. First, copy number-dependent, integration position-independent expression is still observed when only small fragments of the LCR are linked to the transgene (Fraser *et al.*, 1990). This argues against the second possibility unless insulators are colocalized with each of the activators. Secondly, low levels of non-tissue-specific expression can be observed both in the presence and the absence of the complete LCR (Blom van Assendelft *et al.*, 1989). Lastly, the LCR-dependent DNase I sensitivity (Forrester *et al.*, 1990; S.Pruzina, unpublished) in the human genome extends both to the 5′ and 3′ sides, well beyond any globin sequences used in transgenic experiments.

The LCR activity of each of the hypersensitive sites has been localized to 200–300 bp fragments containing binding sites for two erythroid-specific proteins GATA1 and NF-E2 and a number of general transcription factors, such as Sp1, TEF2, AP1 and USF (Forrester *et al.*, 1989; Philipsen *et al.*, 1990; Talbot *et al.*, 1990; Caterina *et al.*, 1991; Pruzina *et al.*, 1991; Liu *et al.*, 1992; Lowrey *et al.*, 1992). A recurring motif (Mignotte *et al.* 1989) in each of the sites is the presence of a G-rich sequence flanked by binding sites for the factor GATA1 (Philipsen *et al.*, 1990). The latter has been shown to be largely specific for the erythroid lineage (deBoer *et al.*, 1988; Evans *et al.*, 1988; Wall *et al.*, 1988; Martin *et al.*, 1990; Romeo *et al.*, 1990).

GATA1 is necessary for erythroid development and absence of GATA1 appears to arrest erythroid differentiation at an early stage (Pevny *et al.*, 1991). It has been implied that GATA1 is directly involved in the positive regulation of the globin genes on the basis of the frequent occurrence of GATA1 binding sites in the globin loci and its ability to stimulate transcription in transient cotransfection assays (Evans *et al.*, 1990; Martin and Orkin, 1990). In recent transgenic mice experiments, it has also been implicated in negative regulation of the γ globin genes (Berry *et al.*, 1992).

The most striking arrangement of GATA1 binding sites and the G-rich element occurs at HS3. This site can drive expression of both the human γ and β globin genes and it is the most active site of the LCR in the embryonic yolk sac and foetal liver of transgenic mice (Fraser *et al.*, 1990, 1993). The core fragment contains a triple repeat of a combination of GATA1 binding sites and G-rich sequences that are spaced ~30 bp apart (Philipsen *et al.*, 1990). It directs copy number-dependent expression of the β globin gene at a level of 40% of that observed with the full LCR.

In this paper we describe a detailed mutational analysis of HS3 in transgenic mice and show that a specific combina-

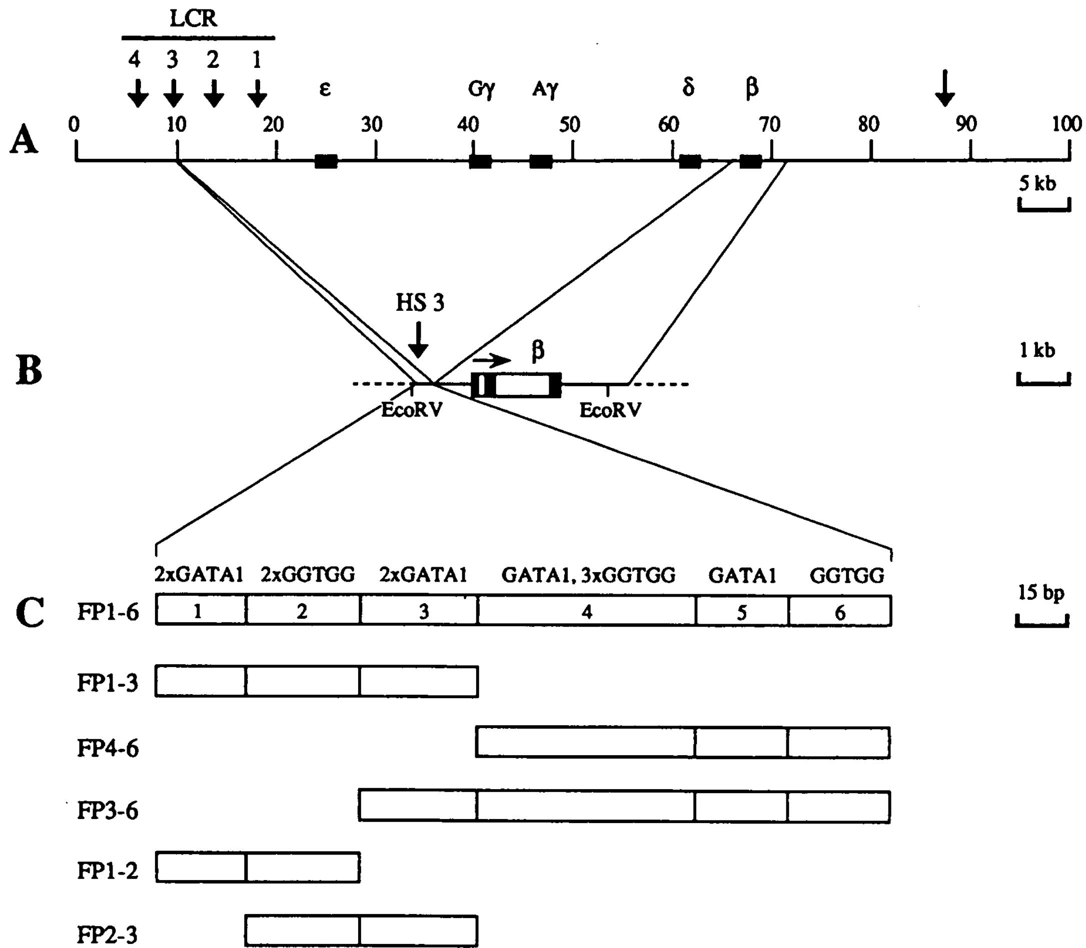

Fig. 1. The human β globin locus and hypersensitive site 3. **A.** The human β globin locus on the short arm of chromosome 11. The four hypersensitive sites (HS) of the LCR are indicated by vertical arrows; one non LCR HS 3' of the β globin gene is also shown. **B.** The human β globin test gene used in this study. The position of the HS3 inserts at 815 bp upstream from the cap site is indicated. The *Eco*RV sites were used to excise microinjection fragments from the plasmids. C. The core element of HS3 is schematically divided into six footprinted regions, numbered 1–6 (Philipsen *et al.*, 1990). 'GATA1' denotes the presence of consensus motifs (deBoer *et al.*, 1988; Evans *et al.*, 1988; Wall *et al.*, 1988) for the erythroid-specific transcription factor GATA1. 'GGTGG' indicates the occurrence of this G-rich sequence capable of binding ubiquitous factors like Sp1 (Gidoni *et al.*, 1985; Philipsen *et al.*, 1990; R.Li *et al.*, 1991) and TEF2 (Xiao *et al.*, 1987; see also Philipsen *et al.* 1990). Deletions of HS3 analysed in this paper are shown below.

tion of a G-rich sequence flanked on each side by one binding site for the transcription factor GATA1 is essential to obtain position-independent expression of a linked β globin gene in erythroid cells.

Results

Our previous analyses of HS3 in transgenic mice showed that a 225 bp fragment was sufficient to provide copy number-dependent expression of a β globin gene in transgenic mice (Fraser *et al.*, 1990; Philipsen *et al.*, 1990). This fragment, which is normally located 15 kb upstream of the embryonic ϵ gene, contains six repeats of the motif GGTGG and six potential binding sites for the transcription factor GATA1, as shown schematically in Figure 1C (Philipsen *et al.*, 1990). Each of these blocks of binding sites gives a 30–50 bp footprint, as shown by *in vitro* factor binding experiments. In order to determine which of these footprints (labelled 1–6 in Figure 1C) are required for position-independent expression, we carried out a deletion experiment by first dividing the fragment into two halves containing either footprints 1–3 or 4–6 (FP1–3 or FP4–6). These were cloned 815 bp upstream of the β globin gene (Figure 1B) and the resulting fragments were injected into fertilized mouse eggs. Embryos were collected at day 13.5 of gestation, DNA was prepared from placenta, head and yolk sac, and RNA was prepared from the foetal liver. The head DNA was Southern blotted and hybridized with a β globin probe to determine which of the embryos were transgenic. In order to exclude mosaic animals (Costantini and Lacey, 1981), the three DNA preparations of each of the transgenics were subsequently hybridized to a probe for the transgene and the resulting signal compared with that obtained from a hybridization with the single copy endogenous *Thy1* gene by analysis on a phosphorimager. This determines both the copy number of the transgene and the degree of mosaicism in each of the embryos. Only non mosaic embryos, i.e. those that contained the same signal for the transgene in the

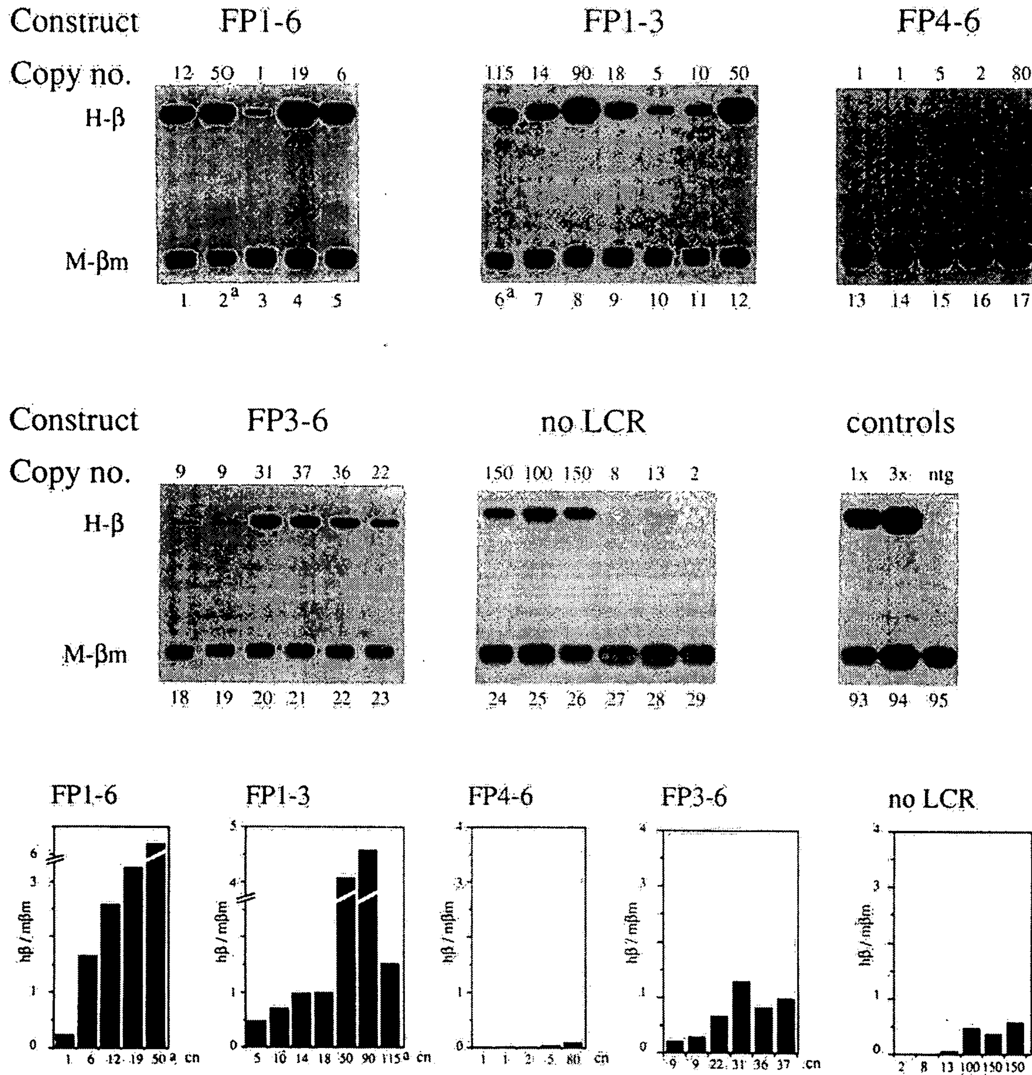

Fig. 2. Functional analysis of HS3 deletions in transgenic mice. Approximately 1 μg of 13.5 day foetal liver or 1/4 of total body RNA (fetus #6) was used per sample and hybridized to probes specific for the 5' end of mouse β major (endogenous erythroid-specific reference, M-βm) and human β globin (test gene, H-β) mRNA. After digestion with S1 nuclease, protected fragments (95 bp for mouse β major and 160 bp for human β globin mRNA) were separated on 6% polyacrylamide–7 M urea sequencing gels. The constructs analysed are as indicated in Figure 1. Letters at the bottom of the lane indicate status of the animal at the time of dissection, [a], foetus was anaemic and had died before the time of dissection (see legend to Table I). Copy numbers are indicated on top of each lane. The protected bands were quantified with the phosphorimager. Only human β globin transgene signals above 0.2% of the mouse β major signal were used for quantification. Very low expression levels were confirmed by a separate SI assay using different specific activity probes which resulted in a 10-fold amplification of the human signal (not shown). Graphs at the bottom show the expression level versus the copy number of each mouse.

different tissues, were analysed further. It should be pointed out that this Southern blot analysis does not guarantee that all of the embryos are fully transgenic.

The level of transgene RNA relative to the endogenous mouse β major RNA was determined by S1 nuclease protection analysis. Figure 2 shows that the activity of the HS3 fragment was associated with FP1-3 rather than FP4-6, which was indistinguishable from the no LCR control. The expression level of FP1-3 was lower than that observed for FP1-6, but it was still copy number-dependent (Figure 2, graph), resulting in a low standard deviation of the average expression level per copy of the transgene (see Table I and Discussion). We therefore conclude that sequences within FP4-6 synergize with FP1-3. The major difference in binding sites between FP1-3 and FP4-6 is the fact that FP1-3 contains two double GATA1 motifs. We therefore decided to add FP3 (a double GATA1 motif) to FP4-6. The analysis of this construct (FP3-6, Figure 2) showed that although the activity has increased considerably over that obtained with FP4-6 and was copy number-dependent, it was well below that obtained with FP1-3 (Table I). The combination of these data indicate that FP3 is very important for the activity of HS3.

This was confirmed when the construct containing only FP1-2 was tested, because the activity of this fragment had been reduced to a very low level when compared with FP1-3 (Figures 2 and 3). However, FP3 is not the only GATA1 binding site required for activity, because the deletion of FP1 from FP1-3 had the same effect as deletion of FP3 (construct

FP2-3; Figure 3). This indicates that GATA1 binding sites are required in at least two physically distinct positions to allow HS3 to be active.

These results therefore leave three footprints as the smallest fragment to confer efficient and copy number-dependent expression to a β globin gene. FP1 and FP3 each contain two potential GATA1 binding sites, while FP2 is characterized by the G-rich sequence (Figure 4A). In order to determine whether the complexity of FP1-3 can be reduced

Table I. Expression of HS3 constructs in transgenic mice

Construct	Average expression	Standard deviation
FP1−6	100	14
FP1−3	30	6
FP4−6	2.9	1.6
FP3−6	12	2.4
FP1−2	3.6	2.7
FP2−3	2.7	2.4
mutant 1	0.7	0.5
mutant 2	1.7	1.4
mutant 3	15	6
mutant 4	15	3
mutant 5	1.5	2.1
mutant 6	1.0	0.9
no LCR	1.0	0.8

The expression level for each mouse was corrected for copy number and standardized to the expression level of FP1-6 (100% per gene copy). The values for each construct were averaged and the standard deviation was calculated. Note that the FP1-6, mouse 2 (Figure 2) and FP1-3, mouse 6 (Figure 2) were not included in the table because these mice were severely anaemic and had died prior to dissection. This indicates that (at least part of) the red cells were expressing high levels of the transgene (Grosveld *et al.*, 1987; Talbot *et al.*, 1989). These mice could not be tested for mosaicism, because three separate tissues could not be obtained. Inclusion of these mice leads to values of FP1-6, 91 ± 23 and FP1-3, 27 ± 10.

still further, we decided to use point mutagenesis to disable each of the binding sites. We therefore determined the contact sites of each of the proteins that bind to these footprints *in vitro* (summarized in Figure 4B). Both GATA1 sites in FP1 are bound by this protein with the proximal site providing the stronger contacts. FP1 also binds an unknown ubiquitous protein X. FP2 binds ubiquitous proteins (Philipsen *et al.*, 1990), including Sp1 (Gidoni *et al.*, 1985), TEF2 (Xiao *et al.*, 1987) and a number of unknown proteins (Spanopoulou *et al.*, 1991). The proximal GATA1 site in FP3 has strong GATA1 contacts, while the distal motif does not appear to interact with GATA1.

Mutations were first introduced into the GATA1 motifs and their effect was analysed by *in vitro* binding experiments using MEL cell nuclear protein extracts (Figure 5). Based on the deletion experiments above, which show that the presence of both FP1 and FP3 is required to obtain efficient expression, three mutants were designed to determine whether the GATA1 sites are essential (Figure 4A). Gel retardation experiments (Figure 5A and B) showed that mutant 1 had lost almost all GATA1 and protein X binding. Mutant 2 had lost all GATA1 binding in FP3 and showed a severely reduced binding of protein X and GATA1 in FP1. Mutant 3 showed normal binding of X and a small reduction of GATA1 binding to FP1, while GATA1 binding to FP3 was hardly affected. Mutant 4 was designed to test the role of protein X. It had a similar reduction in GATA1 binding as mutant 3 in FP1, but had a greatly reduced capacity to bind X. Analysis of the expression of each of the mutants (Figure 6) showed that mutants 1 and 2 are inactive. Together with the data obtained for FP1-2 and FP2-3, this result indicated that the proximal GATA1 binding sites (Figure 4A) in FP1 and FP3 are essential for activity. This was confirmed by the results obtained with mutants 3 and 4; they have the same activity and from this we conclude that the binding site for factor X and the distal GATA motif

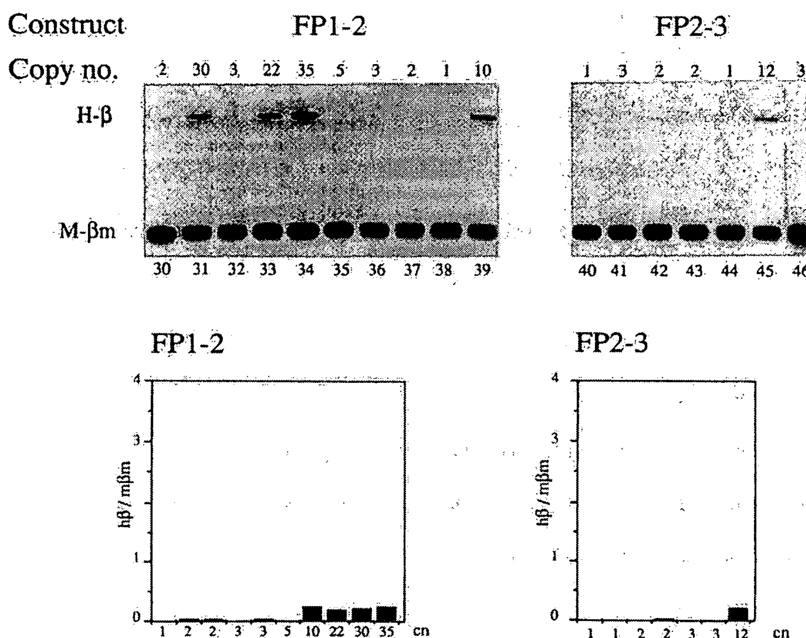

Fig. 3. Functional analysis of deletion mutants FP1-2 and FP2-3. For details see legend to Figure 2.

in footprint 3 play no role in the activity of FP1-3 (Table I). When taken together with the data from FP1-2, this confirms that the proximal GATA1 site in FP3 is essential for activity. However the activity of mutants 3 and 4 was about half of that observed for FP1-3. This leads us to conclude that the distal GATA1 site in FP1 is not essential, but contributes to overall activity. The results observed with mutants 3 and 4 in conjunction with the data for FP2-3 also confirm that the proximal GATA1 site in FP1 is essential for activity.

We then determined whether the central G-rich motif is required for activity or whether the presence of the GATA1 sites alone is sufficient. To this end we constructed two new mutants, 5 and 6. In mutant 5, each triple G or quadruple G motif is interrupted by changing the central Gs to Ts (Figure 4A). These mutations led to the inhibition of all protein binding activity as shown in Figure 5C. In mutant 6 we substituted the central four Gs only, which should affect the binding of all the proteins that require the type of binding site used by Sp1 (Spanopoulou *et al.*, 1991). The result in Figure 5C shows that indeed the binding of all the proteins with the exception of one (band 6 in Philipsen *et al.*, 1990) was severely reduced. The analysis of mutants 5 and 6 in transgenic mice showed that both of the mutations reduced the activity of HS3 to that observed in the absence of any LCR elements. From this we conclude that the central core of four G residues in FP2 is absolutely essential for activity.

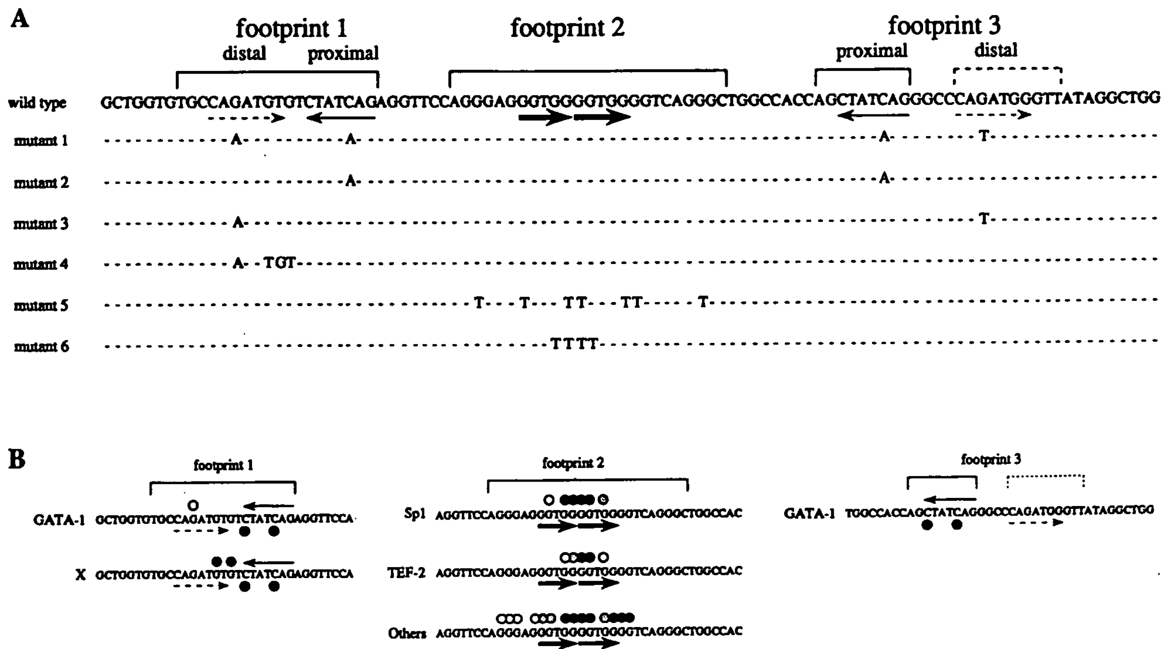

Fig. 4. Mutations and contact sites in HS3 FP1-3. **A.** The sequence of FP1-3 is shown. The bases changed in the mutants are indicated below the sequence. **B.** Oligonucleotide probes covering FP1, FP2 or FP3 were used in a methylation-interference assay (Materials and methods). Strong interference is indicated by a closed circle; moderate interference by a shaded circle; and weak interference by an open circle. Consensus motifs for GATA-1 are denoted by arrows; imperfect matches by stippled arrows. Two GGTGG repeats in FP2 are underlined with bold arrows. Note that for FP2 a summary of the interference patterns of as yet unidentified factors is given in the third line (see Figure 5C).

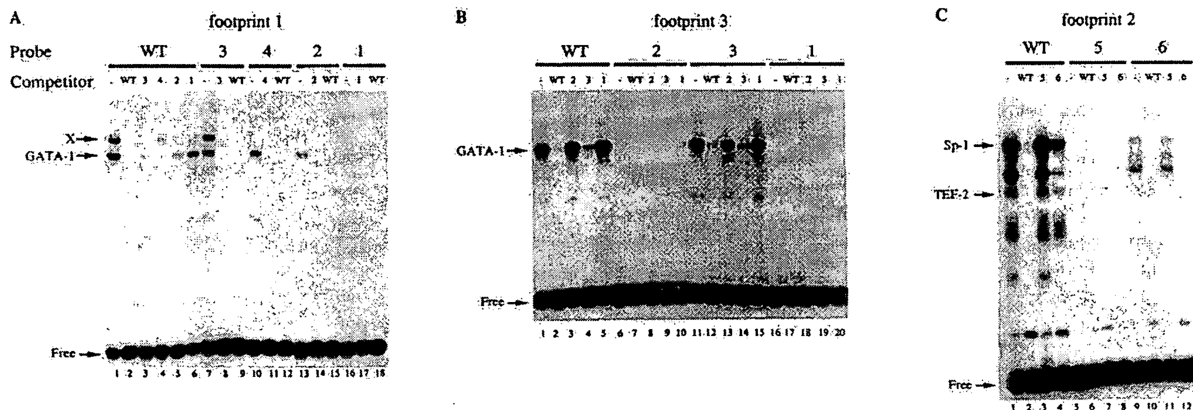

Fig. 5. Gel retardation analysis of mutations in FP1-3 of HS3. Probes and competitors are as shown above the lanes (see Figure 4A). Protein–DNA complexes and free probe are indicated, X indicates a ubiquitous unknown complex.

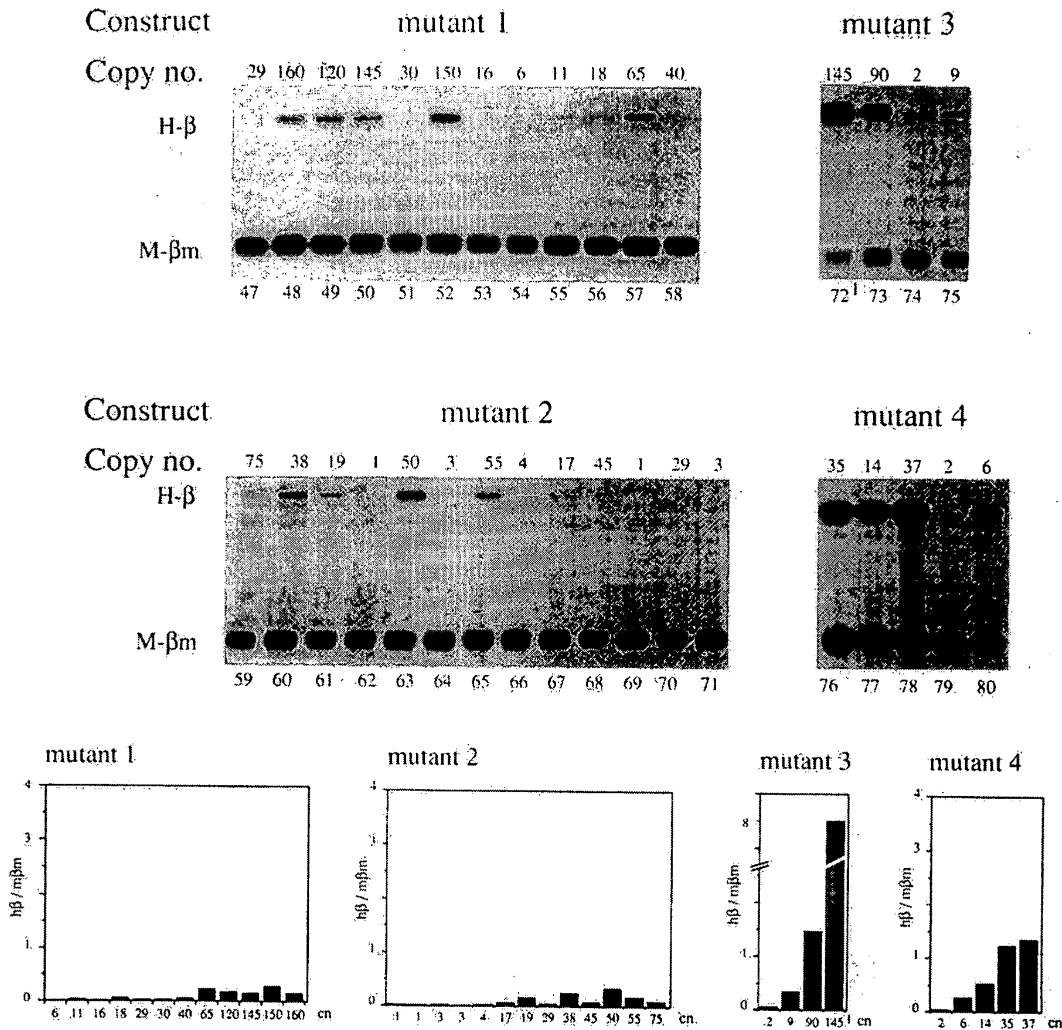

Fig. 6. Functional analysis of mutants 1–4 in transgenic mice. The mutations in the constructs are shown in Figure 4. See Figure 2 for other details of the S1 nuclease analysis. 1: fetus 72 was an anaemic foetus.

Discussion

In this paper we have analysed the minimal interactions required for position-independent expression of the β globin gene in transgenic mice, using hypersensitive site 3 from the globin LCR. This is the first report that ascribes the functional properties of the globin LCR to individual factor binding sites. Importantly, it also shows that this function can be abrogated by the introduction of specific point mutations in these binding sites. Position independence was measured by determining the expression level per integrated copy of the transgene. We used transgenic mice rather than cell transfection assays because the latter are dependent on the expression of a linked marker gene. This results in selection for positive position effects, which as shown in a parallel series of experiments (not shown), gives rise to a high background level of expression. It therefore becomes impossible to obtain meaningful data from constructs with low levels of expression. In transgenic mice, integration of the transgene occurs well before the generation of hematopoietic cells and does not depend on selection for expression. Position effects are therefore expected to result in considerable differences in the average expression level of a particular construct. We observe a large standard deviation for the following constructs: no LCR, FP1-2, FP2-3, FP4-6, mutants 1, 2, 5 and 6 (Table I). The large standard deviation does not appear to be related to the number of animals per construct, because even when at least 10 animals are analysed (e.g. mutants 1 and 2, Table I), it does not decrease. On this basis we interpret a large standard deviation (at least 50%) in the average level of expression as an indicator of position effects. We conclude that constructs FP1-6, FP1-3, FP3-6 and mutants 3 and 4 confer position-independent expression to the linked β globin gene.

The minimal construct that provides position independent expression is mutant 4, which contains the G-rich motif and the two proximal GATA1 binding sites. The transcriptional activity of this construct is doubled to the level observed for FP1-3 by a synergistic effect with the distal GATA1 site in FP1. A further 3-fold increase is obtained by addition of FP4-6. Since FP3-6 can also provide position-independent expression, it is clear that HS3 contains a number of functionally redundant elements capable of synergizing with each other.

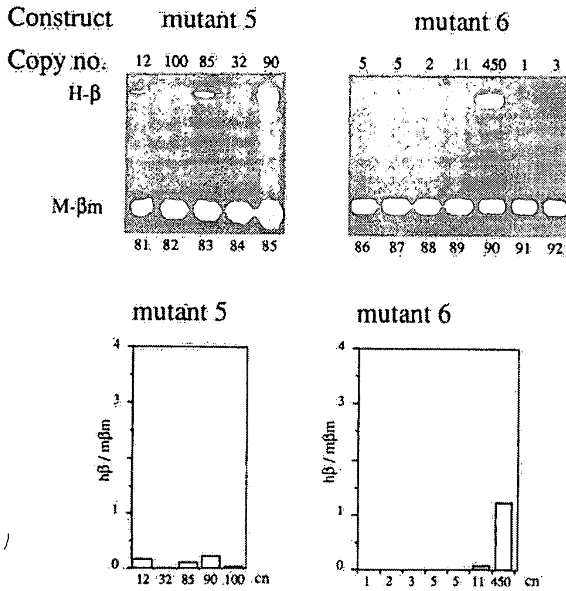

Fig. 7. Functional analysis of mutants 5 and 6 in transgenic mice. The mutations in the constructs are shown in Figure 4. See Figure 2 for other details of the S1 nuclease analysis.

The synergy described above is measured in terms of levels of transcription, but one of the most striking properties of the LCR is that the DNase hypersensitive sites are formed before transcription of the globin genes takes place (Forrester *et al.*, 1987; Blom van Assendelft *et al.*, 1989). Even very small LCR fragments are capable of creating a DNase hypersensitive site larger than the fragment itself (Lowrey *et al.*, 1992). Together with its long range effects, this suggests that an important part of the function of the LCR is the setting up of a domain that is poised for transcription and that this forms the basis for position-independent expression. Our data show that the cooperation of at least three proteins is required to achieve position independence and that the binding sites for these proteins have to occur in a particular arrangement. For example, FP1-2 contains two GATA1 sites followed by the G-rich motif, but it is not active. However, a construct containing the G-rich sequence between two GATA1 sites (mutants 3 and 4), does confer position independence. The difference between FP1-2 and mutant 3 is not due to an inactive distal GATA1 site in FP1-2, because this site can synergize with the core at the level of transcriptional efficiency (compare mutants 3 and 4 with FP1-3). Interestingly, a similar architecture of GATA1 and G-rich elements in FP3-6 also confers position independence. This indicates a central role for the proximal GATA1 site in FP3.

These data provide the first functional evidence that GATA1 is directly involved in the activation of the globin locus *in vivo*. These results agree very well with the recent *in vivo* footprinting data, which show that FP1-5 are occupied by factors in erythroid cells (Strauss and Orkin, 1992). The only possible exception is FP6, which appears not to be occupied *in vivo* by footprinting data. We have not addressed the role of FP6 in this study and we therefore cannot make any direct conclusion about its function.

Interestingly, the two proximal, but not the distal, GATA1 binding sites in FP1-3 are completely conserved between the human HS3 and the homologous sequence in the goat (Q.Li *et al.*, 1991), which supports our observation on the role of these sites. The G-rich motif in FP2 appears to be less well conserved. However, *in vitro* binding experiments demonstrate that the two slowest migrating complexes observed with the human sequence (Figure 5C) are also formed efficiently with the goat sequence (not shown). Although the goat sequence has yet to be tested functionally, it suggests that Sp1 plays a key role in erythroid-specific transcriptional activation. Sp1 would be an attractive candidate because it has been shown to be able to loop DNA (R.Li *et al.*, 1991; Mastrangelo *et al.*, 1991; Su *et al.*, 1991), a process thought to be central to gene activation in general (for review see Ptashne, 1988) and to the interactions between the LCR and the globin genes in particular (Hanscombe *et al.*, 1991). It should, however, be noted that neither the human nor goat sequence contains the optimal Sp1 binding site (Letovsky and Dynan, 1989) and although Sp1 can function through such sites (R.Li *et al.*, 1991), it cannot be excluded that one of the other complexes (Figure 5C) or an as yet to be detected factor is important.

In conclusion we have shown that a small fragment of HS3 containing a minimum number of three binding sites, gives rise to position-independent expression in transgenic mice. HS3 has minimal activity in transient transfection assays (Tuan *et al.*, 1989; P.Dierks and T.Ley, personal communications), but it is capable of activating globin gene expression to a high level when present in chromatin. The fact that this activity can be retained by a specific combination of a very limited number of binding sites, suggests that it will be possible to analyse globin gene activation at the molecular level.

Materials and methods

Plasmid constructions

All the oligonucleotides used for cloning purposes were phosphorylated with polynucleotide kinase. Plasmid GSE 1758 was made by cutting the plasmid GSE 1273 (Philipsen *et al.*, 1990), containing the human β-globin gene as a 5 kb *Bgl*II fragment, with *Not*I and *Hpa*I, blunting and ligating to a polylinker containing *Eco*RV, *Not*I, *Cla*I, *Hin*dIII, *Xho*I, *Spe*I, *Asp*718 and *Sal*I sites, recreating the *Hpa*I site at −815. HS3 deletions were made by polymerase chain reaction (PCR), using the following primers. Construct FP1-3: oligo fp 1, sense strand plus oligo fp 3, antisense strand. Construct FP1-2: oligo fp 1, sense strand plus oligo fp 2, antisense strand. Construct FP3-6: oligo fp 3, sense strand plus oligo fp 6, antisense strand. Construct FP4-6: oligo fp 4, sense strand plus oligo fp 6, antisense strand. PCR was performed as recommended by the suppliers. PCR products were gel purified, blunted and ligated to *Hin*dIII linkers (constructs FP1-3 and 1-2) or *Asp*718 linkers (constructs FP3-6 and 4-6). After digestion with the appropriate restriction enzymes they were cloned in their natural orientation relative to the human β-globin gene in GSE 1758.

The construct containing FP2 and FP3 (construct FP2-3 in Figure 1) was made by direct cloning of two double-stranded oligonucleotides. The 5′ oligonucleotides provided a 5′ *Cla*I end and a 3′ 8 bp single stranded overlap with the 3′ oligonucleotides, which also had a 3′ *Hin*dIII end. This allows efficient three fragment ligation into *Cla*I/*Hin*dIII cut GSE 1758.

The point mutations in constructs containing FP1-3 were generated via a similar strategy.

All the constructs were checked by sequencing using a Sequenase kit (USB). The following oligonucleotides were used for PCR: oligo fp 1, sense GCTGGTGTGCCAGATGTGTCTATCAGAGGTTCCAGGGAGG; oligo fp 1, antisense CCTCCCTGGAACCTCTGATAGACACATCTG-GCACACCAGC; oligo fp 2, antisense TGGCCAGCCCTGACCCCA-CCCCACCCTCCCTGGAACCTCTGAT; oligo fp 3, sense TGGCCACCAGCTATCAGGGCCCAGATGGGTTATAGGCTGG; oligo fp 3, antisense CAGCCTATAACCCATCTGGGCCCTGATAGCTGGTG-GCCA; oligo fp 4, sense GCTCAGATAGGTGGTTAGGTCAGGTTG-GTGGTGCTGGGTGGAGTCCATGACTCCCAGGAGCCAG; oligo fp 6, antisense CTATGCTGTGCCTCCCCCACCTTTCCCATGTCTCC-CCTCTA.

The following oligonucleotides were used for direct cloning: oligo 1, *Cla*I, sense cgatGCTGGTGTGCCAGATGTGTCTATCAG; oligo 1, *Cla*I, antisense TGGAACCTCTGATAGACACATCTGGCACACCAGCat; oligo 1, *Cla*I, mutant 1, sense cgatGCTGGTGTGCCA**A**ATGTGTCTAT**A**AG; oligo 1, *Cla*I, mutant 2, sense cgatGCTGGTGTGCCAGATGTGTCTAT**A**AG; oligo 1, *Cla*I, mutant 3, sense cgatGCTGGTGTGCCA**A**ATGTGTCTATCAG; oligo 1, *Cla*I, mutant 4, sense cgatGCTGGTGTGCCA**A**AT**TGT**TCTATCAG; oligo 2, *Cla*I, sense cgatGAGGTTCCAGGGAGGGTGGGGTGGGGTCAGGGCTGGCCAC; oligo 2, *Cla*I, antisense CCCTGACCCCACCCCACCCTCCCTGGAACCTCat; oligo 2, sense AGGTTCCAGGGAGGGTGGGGTGGGGTCAGGGCTGGCCAC; oligo 2, antisense CCCTGACCCCACCCCACCCTCCC; oligo 2, mutant 5, sense AGGTTCCAG**T**GAG**T**GTG**TT**GTG**TT**GTCAG**T**GCTGGCCAC; oligo 2, mutant 6, sense AGGTTCCAGGGAGGGT**TTTT**TGGGGTCAGGGCTGGCCAC; oligo 3, *Hin*dIII, sense CAGCTATCAGGGCCCAGATGGGTTATAGGCTGGa; oligo 3, *Hin*dIII, antisense agcttCCAGCCTATAACCCATCTGGGCCCTGATAGCTGGTGGCCA; oligo 3, *Hin*dIII, mutant 1, sense CAGCTAT**A**AGGGCCCA**T**ATGGGTTATAGGCTGGa; oligo 3, *Hin*dIII, mutant 2, sense CAGCTAT**A**AGGGCCCAGATGGGTTATAGGCTGGa; oligo 3, *Hin*dIII, mutant 3, sense CAGCTATCAGGGCCCA**T**ATGGGTTATAGGCTGGa.

Bases added to create restriction sites are shown in lower case letters; mutated bases are underlined and in bold type.

Transgenic mice

Plasmids were digested with *Eco*RV (see Figure 1B) and fragments with the human β globin gene were isolated from agarose gels using a Gene Clean kit. After further purification on an Elutip column (Schleicher and Schuell), fragments were dissolved at a concentration of 2 μg/ml in microinjection buffer (10 mM Tris–Cl, pH 7.5 and 0.1 mM EDTA) and fertilized eggs were injected into the male pronucleus (Kollias *et al.*, 1986). Foetuses were collected 13.5 days post transfer as described (Grosveld *et al.*, 1987).

DNA analysis

Genomic mouse DNA was cut with *Eco*RI, Southern blotted and probed for the human β globin transgene, with the mouse *Thy1* gene as a loading control (Grosveld *et al.*, 1987; Philipsen *et al.*, 1990). To assess mosaicism the ratio was compared in DNA from head, yolk sac and placenta.

RNA analysis

Total RNA from either fetal liver or the whole body was used for S1 analysis. Approximately 1 μg of fetal liver RNA or 1/4 of total body RNA (in case of anaemic foetuses that had died prior to dissection) was used for quantification by mixing DNA probes specific for the 5′ ends of human β-globin and mouse β major mRNA (Antoniou *et al.*, 1988). Probe excess was demonstrated by using three times the amount of RNA of one particular sample.

Quantification

Copy numbers were determined from Southern blots and expression data from S1 sequencing gels, using a Phosphor Imager (Molecular Dynamics). Each value was obtained from at least two independent experiments.

Gel mobility shift assays and methylation interference

Gel mobility shift assays were done essentially as described previously (deBoer *et al.*, 1988; Talbot *et al.*, 1990), using 5–10 μg of nuclear extract (Gorski *et al.*, 1986) from MEL cells per reaction. The oligonucleotides shown above were used as probes and competitors; they were blunted with Klenow DNA polymerase after annealing. Competitions were done by adding 100-fold molar excess of the indicated double-stranded oligonucleotide before addition of the extract.

Methylation interference was performed as described previously (deBoer *et al.*, 1988; Talbot and Grosveld, 1991), with the following modifications: 50 ng of DMS-treated DNA probe was used with 100 μg nuclear extract in a 100 μl reaction. After electrophoresis, the bands were dry-blotted onto DE 81 paper (Whatman), cut out and eluted in 2 M NaCl. Following piperidine cleavage, the final products were analysed on 20% sequencing gels.

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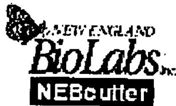

Custom Digest

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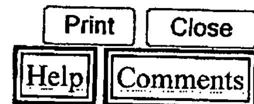

DNA Type: Unmethylated

#	Ends	Coordinates	Length (bp)
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3	BstXI-BstXI	44825-51028	6204
4	BstXI-BstXI	33659-38596	4938
5	BstXI-BstXI	16379-20940	4562
6	SnaBI-BstXI	21836-26309	4474
7	BstXI-BstXI	12674-16378	3705
8	BstXI-BstXI	2237-5656	3420
9	BstXI-BstXI	5657-8054	2398
10	BstXI-BstXI	26310-28567	2258
11	(LeftEnd)-BstXI	1-2236	2236
12	BstXI-BstXI	63522-65734	2213
13	BstXI-SnaBI	59848-61871	2024
14	BstXI-BstXI	30435-32445	2011
15	BstXI-BstXI	10780-12673	1894
16	BstXI-BstXI	28568-30434	1867
17	BstXI-BstXI	69795-71583	1789
18	BstXI-(RightEnd)	71584-73308	1725
19	SnaBI-BstXI	61872-63521	1650
20	BstXI-BstXI	9470-10779	1310
21	BstXI-BstXI	68096-69368	1273
22	BstXI-BstXI	65735-66932	1198
23	BstXI-BstXI	66933-68095	1163
24	BstXI-BstXI	51029-52016	988
25	BstXI-SnaBI	20941-21835	895
26	BstXI-SnaBI	8055-8911	857
27	BstXI-BstXI	32580-33271	692
28	SnaBI-BstXI	8912-9469	558
29	BstXI-BstXI	69369-69794	426
30	BstXI-BstXI	33272-33658	387
31	BstXI-BstXI	52017-52398	382
32	BstXI-BstXI	32446-32579	134

#	Ends	Coordinates	Length (bp)
1	(LeftEnd)-BstXI	1-2236	2236
2	BstXI-BstXI	2237-5656	3420
3	BstXI-BstXI	5657-8054	2398
4	BstXI-SnaBI	8055-8911	857
5	SnaBI-BstXI	8912-9469	558
6	BstXI-BstXI	9470-10779	1310
7	BstXI-BstXI	10780-12673	1894
8	BstXI-BstXI	12674-16378	3705
9	BstXI-BstXI	16379-20940	4562
10	BstXI-SnaBI	20941-21835	895
11	SnaBI-BstXI	21836-26309	4474
12	BstXI-BstXI	26310-28567	2258
13	BstXI-BstXI	28568-30434	1867
14	BstXI-BstXI	30435-32445	2011
15	BstXI-BstXI	32446-32579	134
16	BstXI-BstXI	32580-33271	692
17	BstXI-BstXI	33272-33658	387
18	BstXI-BstXI	33659-38596	4938
19	BstXI-BstXI	38597-44824	6228
20	BstXI-BstXI	44825-51028	6204
21	BstXI-BstXI	51029-52016	988
22	BstXI-BstXI	52017-52398	382
23	BstXI-BstXI	52399-59847	7449
24	BstXI-SnaBI	59848-61871	2024
25	SnaBI-BstXI	61872-63521	1650
26	BstXI-BstXI	63522-65734	2213
27	BstXI-BstXI	65735-66932	1198
28	BstXI-BstXI	66933-68095	1163
29	BstXI-BstXI	68096-69368	1273
30	BstXI-BstXI	69369-69794	426
31	BstXI-BstXI	69795-71583	1789
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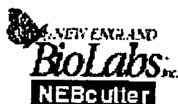

HS2 core

Custom Digest

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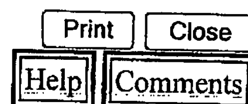

DNA Type: Unmethylated

#	Ends	Coordinates	Length (bp)
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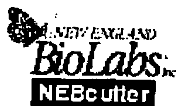
2	HindIII-SnaBI	13770-21835	8066
3	HindIII-HindIII	21887-28975	7089
4	HindIII-HindIII	28976-35767	6792
5	SnaBI-HindIII	61872-67377	5506
6	HindIII-HindIII	10411-13769	3359
7	HindIII-HindIII	38067-41364	3298
8	(LeftEnd)-HindIII	1-3266	3266
9	HindIII-(RightEnd)	70839-73308	2470
10	HindIII-HindIII	6159-8486	2328
11	HindIII-SnaBI	59590-61871	2282
12	HindIII-HindIII	3267-5172	1906
13	HindIII-HindIII	68953-70838	1886
14	HindIII-HindIII	67378-68952	1575
15	SnaBI-HindIII	8912-10410	1499
16	HindIII-HindIII	5173-6158	986
17	HindIII-HindIII	41365-42117	753
18	HindIII-HindIII	37340-38066	727
19	HindIII-HindIII	35768-36464	697
20	HindIII-HindIII	36765-37339	575
21	HindIII-SnaBI	8487-8911	425
22	HindIII-HindIII	36465-36764	300
23	SnaBI-HindIII	21836-21886	51

#	Ends	Coordinates	Length (bp)
1	(LeftEnd)-HindIII	1-3266	3266
2	HindIII-HindIII	3267-5172	1906
3	HindIII-HindIII	5173-6158	986
4	HindIII-HindIII	6159-8486	2328
5	HindIII-SnaBI	8487-8911	425
6	SnaBI-HindIII	8912-10410	1499
7	HindIII-HindIII	10411-13769	3359
8	HindIII-SnaBI	13770-21835	8066
9	SnaBI-HindIII	21836-21886	51
10	HindIII-HindIII	21887-28975	7089
11	HindIII-HindIII	28976-35767	6792
12	HindIII-HindIII	35768-36464	697
13	HindIII-HindIII	36465-36764	300
14	HindIII-HindIII	36765-37339	575
15	HindIII-HindIII	37340-38066	727
16	HindIII-HindIII	38067-41364	3298
17	HindIII-HindIII	41365-42117	753
18	HindIII-HindIII	42118-59589	17472
19	HindIII-SnaBI	59590-61871	2282
20	SnaBI-HindIII	61872-67377	5506
21	HindIII-HindIII	67378-68952	1575
22	HindIII-HindIII	68953-70838	1886
23	HindIII-(RightEnd)	70839-73308	2470



HS 2-Core

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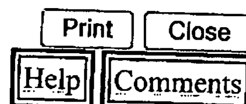

ng 000007.1 - digested with: HindIII, XbaI

DNA Type: Unmethylated

#	Ends	Coordinates	Length (bp)
1	HindIII-XbaI	59590-65421	5832
2	HindIII-XbaI	42118-47465	5348
3	XbaI-HindIII	54336-59589	5254
4	XbaI-XbaI	14236-18950	4715
5	XbaI-XbaI	31994-35159	3166
6	XbaI-XbaI	51359-54335	2977
7	XbaI-HindIII	18951-21886	2936
8	XbaI-HindIII	26433-28975	2543
9	XbaI-XbaI	23914-26432	2519
10	(LeftEnd)-XbaI	1-2352	2352
11	HindIII-HindIII	6159-8486	2328
12	HindIII-XbaI	38067-40095	2029
13	XbaI-XbaI	47466-49459	1994
14	HindIII-XbaI	10411-12399	1989
15	XbaI-HindIII	65422-67377	1956
16	HindIII-HindIII	3267-5172	1906
17	HindIII-HindIII	67378-68952	1575
18	HindIII-XbaI	68953-70444	1492
19	XbaI-HindIII	12400-13769	1370
20	XbaI-HindIII	40096-41364	1269
21	XbaI-XbaI	8861-10082	1222
22	XbaI-XbaI	22709-23913	1205
23	XbaI-XbaI	50208-51358	1151
24	HindIII-HindIII	5173-6158	986
25	XbaI-HindIII	2353-3266	914
26	HindIII-XbaI	21887-22708	822
27	XbaI-XbaI	71854-72656	803
28	HindIII-HindIII	41365-42117	753
29	XbaI-XbaI	49460-50207	748
30	XbaI-XbaI	30220-30959	740
31	HindIII-HindIII	37340-38066	727
32	XbaI-XbaI	29517-30219	703
33	HindIII-HindIII	35768-36464	697
34	XbaI-(RightEnd)	72657-73308	652

#	Ends	Coordinates	Length (bp)
1	(LeftEnd)-XbaI	1-2352	2352
2	XbaI-HindIII	2353-3266	914
3	HindIII-HindIII	3267-5172	1906
4	HindIII-HindIII	5173-6158	986
5	HindIII-HindIII	6159-8486	2328
6	HindIII-XbaI	8487-8860	374
7	XbaI-XbaI	8861-10082	1222
8	XbaI-HindIII	10083-10410	328
9	HindIII-XbaI	10411-12399	1989
10	XbaI-HindIII	12400-13769	1370
11	HindIII-XbaI	13770-14235	466
12	XbaI-XbaI	14236-18950	4715
13	XbaI-HindIII	18951-21886	2936
14	HindIII-XbaI	21887-22708	822
15	XbaI-XbaI	22709-23913	1205
16	XbaI-XbaI	23914-26432	2519
17	XbaI-HindIII	26433-28975	2543
18	HindIII-XbaI	28976-29516	541
19	XbaI-XbaI	29517-30219	703
20	XbaI-XbaI	30220-30959	740
21	XbaI-XbaI	30960-31397	438
22	XbaI-XbaI	31398-31993	596
23	XbaI-XbaI	31994-35159	3166
24	XbaI-HindIII	35160-35767	608
25	HindIII-HindIII	35768-36464	697
26	HindIII-HindIII	36465-36764	300
27	HindIII-HindIII	36765-37339	575
28	HindIII-HindIII	37340-38066	727
29	HindIII-XbaI	38067-40095	2029
30	XbaI-HindIII	40096-41364	1269
31	HindIII-HindIII	41365-42117	753
32	HindIII-XbaI	42118-47465	5348
33	XbaI-XbaI	47466-49459	1994
34	XbaI-XbaI	49460-50207	748

35	XbaI-HindIII	35160-35767	608
36	XbaI-XbaI	31398-31993	596
37	HindIII-HindIII	36765-37339	575
38	XbaI-XbaI	71291-71853	563
39	HindIII-XbaI	28976-29516	541
40	HindIII-XbaI	13770-14235	466
41	HindIII-XbaI	70839-71290	452
42	XbaI-XbaI	30960-31397	438
43	XbaI-HindIII	70445-70838	394
44	HindIII-XbaI	8487-8860	374
45	XbaI-HindIII	10083-10410	328
46	HindIII-HindIII	36465-36764	300

35	XbaI-XbaI	50208-51358	1151
36	XbaI-XbaI	51359-54335	2977
37	XbaI-HindIII	54336-59589	5254
38	HindIII-XbaI	59590-65421	5832
39	XbaI-HindIII	65422-67377	1956
40	HindIII-HindIII	67378-68952	1575
41	HindIII-XbaI	68953-70444	1492
42	XbaI-HindIII	70445-70838	394
43	HindIII-XbaI	70839-71290	452
44	XbaI-XbaI	71291-71853	563
45	XbaI-XbaI	71854-72656	803
46	XbaI-(RightEnd)	72657-73308	652

HS3

Custom Digest

ng 000007.1 - digested with: BamHI, HindIII

DNA Type: Unmethylated

#	Ends	Coordinates	Length (bp)
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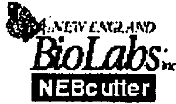
2	HindIII-HindIII	21887-28975	7089
3	HindIII-BamHI	13770-19307	5538
4	BamHI-HindIII	62614-67377	4764
5	BamHI-HindIII	55215-59589	4375
6	HindIII-BamHI	28976-32353	3378
7	HindIII-HindIII	10411-13769	3359
8	BamHI-HindIII	309-3266	2958
9	BamHI-BamHI	32354-34949	2596
10	HindIII-HindIII	6159-8486	2328
11	BamHI-BamHI	60677-62613	1937
12	BamHI-HindIII	19960-21886	1927
13	HindIII-HindIII	8487-10410	1924
14	HindIII-HindIII	68953-70838	1886
15	HindIII-BamHI	38067-39885	1819
16	HindIII-HindIII	67378-68952	1575
17	BamHI-HindIII	39886-41364	1479
18	BamHI-HindIII	3878-5172	1295
19	BamHI-(RightEnd)	72016-73308	1293
20	HindIII-BamHI	70839-72015	1177
21	HindIII-HindIII	5173-6158	986
22	BamHI-HindIII	34950-35767	818
23	BamHI-BamHI	59882-60676	795
24	HindIII-HindIII	41365-42117	753
25	HindIII-HindIII	37340-38066	727
26	HindIII-HindIII	35768-36464	697
27	BamHI-BamHI	19308-19959	652
28	HindIII-HindIII	36765-37339	575
29	HindIII-BamHI	3267-3714	448
30	(LeftEnd)-BamHI	1-308	308
31	HindIII-HindIII	36465-36764	300
32	HindIII-BamHI	59590-59881	292
33	BamHI-BamHI	3715-3877	163

#	Ends	Coordinates	Length (bp)
1	(LeftEnd)-BamHI	1-308	308
2	BamHI-HindIII	309-3266	2958
3	HindIII-BamHI	3267-3714	448
4	BamHI-BamHI	3715-3877	163
5	BamHI-HindIII	3878-5172	1295
6	HindIII-HindIII	5173-6158	986
7	HindIII-HindIII	6159-8486	2328
8	HindIII-HindIII	8487-10410	1924
9	HindIII-HindIII	10411-13769	3359
10	HindIII-BamHI	13770-19307	5538
11	BamHI-BamHI	19308-19959	652
12	BamHI-HindIII	19960-21886	1927
13	HindIII-HindIII	21887-28975	7089
14	HindIII-BamHI	28976-32353	3378
15	BamHI-BamHI	32354-34949	2596
16	BamHI-HindIII	34950-35767	818
17	HindIII-HindIII	35768-36464	697
18	HindIII-HindIII	36465-36764	300
19	HindIII-HindIII	36765-37339	575
20	HindIII-HindIII	37340-38066	727
21	HindIII-BamHI	38067-39885	1819
22	BamHI-HindIII	39886-41364	1479
23	HindIII-HindIII	41365-42117	753
24	HindIII-BamHI	42118-55214	13097
25	BamHI-HindIII	55215-59589	4375
26	HindIII-BamHI	59590-59881	292
27	BamHI-BamHI	59882-60676	795
28	BamHI-BamHI	60677-62613	1937
29	BamHI-HindIII	62614-67377	4764
30	HindIII-HindIII	67378-68952	1575
31	HindIII-HindIII	68953-70838	1886
32	HindIII-BamHI	70839-72015	1177
33	BamHI-(RightEnd)	72016-73308	1293

HS3-core



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ng 000007.1 - digested with: HindIII

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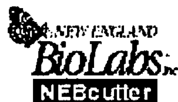
DNA Type: Unmethylated

#	Ends	Coordinates	Length (bp)
1	HindIII-HindIII	42118-59589	17472
2	HindIII-HindIII	13770-21886	8117
3	HindIII-HindIII	59590-67377	7788
4	HindIII-HindIII	21887-28975	7089
5	HindIII-HindIII	28976-35767	6792
6	HindIII-HindIII	10411-13769	3359
7	HindIII-HindIII	38067-41364	3298
8	(LeftEnd)-HindIII	1-3266	3266
9	HindIII-(RightEnd)	70839-73308	2470
10	HindIII-HindIII	6159-8486	2328
11	HindIII-HindIII	8487-10410	1924
12	HindIII-HindIII	3267-5172	1906
13	HindIII-HindIII	68953-70838	1886
14	HindIII-HindIII	67378-68952	1575
15	HindIII-HindIII	5173-6158	986
16	HindIII-HindIII	41365-42117	753
17	HindIII-HindIII	37340-38066	727
18	HindIII-HindIII	35768-36464	697
19	HindIII-HindIII	36765-37339	575
20	HindIII-HindIII	36465-36764	300

#	Ends	Coordinates	Length (bp)
1	(LeftEnd)-HindIII	1-3266	3266
2	HindIII-HindIII	3267-5172	1906
3	HindIII-HindIII	5173-6158	986
4	HindIII-HindIII	6159-8486	2328
5	HindIII-HindIII	8487-10410	1924
6	HindIII-HindIII	10411-13769	3359
7	HindIII-HindIII	13770-21886	8117
8	HindIII-HindIII	21887-28975	7089
9	HindIII-HindIII	28976-35767	6792
10	HindIII-HindIII	35768-36464	697
11	HindIII-HindIII	36465-36764	300
12	HindIII-HindIII	36765-37339	575
13	HindIII-HindIII	37340-38066	727
14	HindIII-HindIII	38067-41364	3298
15	HindIII-HindIII	41365-42117	753
16	HindIII-HindIII	42118-59589	17472
17	HindIII-HindIII	59590-67377	7788
18	HindIII-HindIII	67378-68952	1575
19	HindIII-HindIII	68953-70838	1886
20	HindIII-(RightEnd)	70839-73308	2470



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ng 000007.1 - digested with: Fnu4HI, HphI

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DNA Type: Unmethylated

#	Ends	Coordinates	Length (bp)	#	Ends	Coordinates	Length (bp)
1	HphI-Fnu4HI	65063-66792	1730	1	(LeftEnd)-HphI	1-22	22
2	Fnu4HI-Fnu4HI	26329-27641	1313	2	HphI-Fnu4HI	23-335	313
3	HphI-HphI	31227-32460	1234	3	Fnu4HI-HphI	336-604	269
4	Fnu4HI-HphI	20904-22120	1217	4	HphI-Fnu4HI	605-1087	483
5	HphI-HphI	22818-23993	1176	5	Fnu4HI-HphI	1088-1660	573
6	HphI-HphI	69196-70360	1165	6	HphI-HphI	1661-1666	6
7	HphI-Fnu4HI	57990-59092	1103	7	HphI-HphI	1667-2000	334
8	HphI-Fnu4HI	3247-4347	1101	8	HphI-Fnu4HI	2001-2291	291
9	HphI-Fnu4HI	38094-39191	1098	9	Fnu4HI-Fnu4HI	2292-2399	108
10	HphI-Fnu4HI	56544-57584	1041	10	Fnu4HI-HphI	2400-2416	17
11	HphI-HphI	60992-62023	1032	11	HphI-Fnu4HI	2417-2993	577
12	Fnu4HI-HphI	33105-34131	1027	12	Fnu4HI-HphI	2994-3246	253
13	HphI-HphI	59986-60991	1006	13	HphI-Fnu4HI	3247-4347	1101
14	HphI-HphI	49536-50536	1001	14	Fnu4HI-Fnu4HI	4348-4371	24
15	HphI-Fnu4HI	12024-12954	931	15	Fnu4HI-HphI	4372-4549	178
16	HphI-HphI	63892-64798	907	16	HphI-Fnu4HI	4550-4773	224
17	HphI-Fnu4HI	8131-9016	886	17	Fnu4HI-Fnu4HI	4774-5125	352
18	HphI-Fnu4HI	27654-28537	884	18	Fnu4HI-Fnu4HI	5126-5128	3
19	HphI-HphI	16753-17630	878	19	Fnu4HI-Fnu4HI	5129-5147	19
20	Fnu4HI-Fnu4HI	71894-72728	835	20	Fnu4HI-HphI	5148-5354	207
21	HphI-HphI	47636-48468	833	21	HphI-HphI	5355-5671	317
22	HphI-Fnu4HI	5732-6561	830	22	HphI-HphI	5672-5731	60
23	HphI-Fnu4HI	53518-54284	767	23	HphI-Fnu4HI	5732-6561	830
24	HphI-HphI	46886-47635	750	24	Fnu4HI-HphI	6562-7144	583
25	HphI-Fnu4HI	62644-63362	719	25	HphI-Fnu4HI	7145-7257	113
26	Fnu4HI-HphI	43002-43702	701	26	Fnu4HI-Fnu4HI	7258-7576	319
27	HphI-Fnu4HI	46157-46836	680	27	Fnu4HI-HphI	7577-8130	554
28	Fnu4HI-HphI	15329-15999	671	28	HphI-Fnu4HI	8131-9016	886
29	HphI-Fnu4HI	25317-25976	660	29	Fnu4HI-HphI	9017-9345	329
30	Fnu4HI-HphI	41321-41959	639	30	HphI-Fnu4HI	9346-9651	306
31	HphI-Fnu4HI	41960-42589	630	31	Fnu4HI-HphI	9652-10140	489
32	HphI-Fnu4HI	55503-56128	626	32	HphI-HphI	10141-10765	625
33	HphI-HphI	10141-10765	625	33	HphI-HphI	10766-11384	619
34	HphI-Fnu4HI	37262-37886	625	34	HphI-HphI	11385-11420	36

35	HphI-HphI	10766-11384	619	35	HphI-HphI	11421-11544	124
36	HphI-HphI	14153-14769	617	36	HphI-HphI	11545-11807	263
37	Fnu4HI-HphI	54285-54877	593	37	HphI-HphI	11808-12023	216
38	HphI-HphI	52213-52799	587	38	HphI-Fnu4HI	12024-12954	931
39	Fnu4HI-HphI	6562-7144	583	39	Fnu4HI-Fnu4HI	12955-13504	550
40	HphI-Fnu4HI	2417-2993	577	40	Fnu4HI-HphI	13505-13507	3
41	HphI-HphI	18297-18870	574	41	HphI-HphI	13508-13676	169
42	Fnu4HI-HphI	1088-1660	573	42	HphI-Fnu4HI	13677-13713	37
43	HphI-HphI	29775-30329	555	43	Fnu4HI-HphI	13714-13864	151
44	Fnu4HI-HphI	7577-8130	554	44	HphI-HphI	13865-14152	288
45	Fnu4HI-Fnu4HI	16043-16595	553	45	HphI-HphI	14153-14769	617
46	HphI-HphI	50537-51087	551	46	HphI-HphI	14770-14917	148
47	Fnu4HI-Fnu4HI	12955-13504	550	47	HphI-Fnu4HI	14918-15157	240
48	HphI-HphI	29123-29655	533	48	Fnu4HI-Fnu4HI	15158-15328	171
49	HphI-HphI	51303-51806	504	49	Fnu4HI-HphI	15329-15999	671
50	Fnu4HI-Fnu4HI	68168-68669	502	50	HphI-Fnu4HI	16000-16042	43
51	HphI-Fnu4HI	20006-20506	501	51	Fnu4HI-Fnu4HI	16043-16595	553
52	Fnu4HI-HphI	9652-10140	489	52	Fnu4HI-Fnu4HI	16596-16603	8
53	HphI-Fnu4HI	605-1087	483	53	Fnu4HI-HphI	16604-16752	149
54	Fnu4HI-Fnu4HI	59260-59735	476	54	HphI-HphI	16753-17630	878
55	HphI-HphI	43703-44175	473	55	HphI-HphI	17631-18013	383
56	HphI-Fnu4HI	35953-36420	468	56	HphI-Fnu4HI	18014-18070	57
57	HphI-Fnu4HI	70361-70826	466	57	Fnu4HI-HphI	18071-18296	226
58	HphI-HphI	71264-71727	464	58	HphI-HphI	18297-18870	574
59	HphI-Fnu4HI	40869-41320	452	59	HphI-HphI	18871-19055	185
60	HphI-HphI	24876-25316	441	60	HphI-HphI	19056-19094	39
61	Fnu4HI-HphI	57585-57989	405	61	HphI-Fnu4HI	19095-19479	385
62	Fnu4HI-Fnu4HI	42602-43001	400	62	Fnu4HI-Fnu4HI	19480-19482	3
63	HphI-Fnu4HI	19095-19479	385	63	Fnu4HI-Fnu4HI	19483-19507	25
64	HphI-HphI	48469-48852	384	64	Fnu4HI-Fnu4HI	19508-19569	62
65	HphI-HphI	17631-18013	383	65	Fnu4HI-HphI	19570-19628	59
66	HphI-HphI	28755-29122	368	66	HphI-HphI	19629-19678	50
67	Fnu4HI-HphI	44530-44897	368	67	HphI-Fnu4HI	19679-19936	258
68	HphI-HphI	40436-40799	364	68	Fnu4HI-Fnu4HI	19937-19951	15
69	Fnu4HI-Fnu4HI	4774-5125	352	69	Fnu4HI-HphI	19952-19989	38
70	HphI-HphI	22241-22591	351	70	HphI-HphI	19990-20005	16
71	HphI-HphI	52800-53144	345	71	HphI-Fnu4HI	20006-20506	501
72	Fnu4HI-HphI	56202-56543	342	72	Fnu4HI-HphI	20507-20613	107
73	HphI-HphI	48858-49196	339	73	HphI-HphI	20614-20862	249
74	HphI-HphI	49197-49535	339	74	HphI-HphI	20863-20877	15
75	HphI-HphI	53145-53483	339	75	HphI-Fnu4HI	20878-20903	26

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HS3/HS4

BamHI's cuts

ng 000007.1

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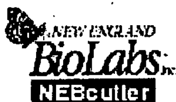
#	Cut position (blunt - 5' ext. - 3' ext.)	5'... Site with flanks ...3'
1	308/312	298 CGAAATGGGA G^GATC_C CTTGAGCTCA
2	3714/3718	3704 GAACTTCCAG G^GATC_C TCTCTTAAGT
3	3877/3881	3867 TGGACAACCT G^GATC_C ACTTGCCCAG
4	19307/19311	19297 AAGAGCCTCA G^GATC_C AGCACACATT
5	19959/19963	19949 AGCTGCATGT G^GATC_C TGAGAACTTC
6	32353/32357	32343 AAAACAGGAG G^GATC_C TAGATATTCC
7	34949/34953	34939 AGCTGCATGT G^GATC_C TGAGAACTTC
8	39885/39889	39875 AGCTGCATGT G^GATC_C TGAGAACTTC
9	55214/55218	55204 AGCTGCACGT G^GATC_C TGAGAACTTC
10	59881/59885	59871 CACAAGAGAA G^GATC_C ATAGTTCATC
11	60676/60680	60666 TAGAAATAGA G^GATC_C AGTTTCTTTT
12	62613/62617	62603 AGCTGCACGT G^GATC_C TGAGAACTTC
13	72015/72019	72005 AGCTGAAACT G^GATC_C TTCCTTACAC

HS-3 | HS-4



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ng 000007.1 - digested with: BamHI

DNA Type: Unmethylated

#	Ends	Coordinates	Length (bp)
1	BamHI-BamHI	3878-19307	15430
2	BamHI-BamHI	39886-55214	15329
3	BamHI-BamHI	19960-32353	12394
4	BamHI-BamHI	62614-72015	9402
5	BamHI-BamHI	34950-39885	4936
6	BamHI-BamHI	55215-59881	4667
7	BamHI-BamHI	309-3714	3406
8	BamHI-BamHI	32354-34949	2596
9	BamHI-BamHI	60677-62613	1937
10	BamHI-(RightEnd)	72016-73308	1293
11	BamHI-BamHI	59882-60676	795
12	BamHI-BamHI	19308-19959	652
13	(LeftEnd)-BamHI	1-308	308
14	BamHI-BamHI	3715-3877	163

#	Ends	Coordinates	Length (bp)
1	(LeftEnd)-BamHI	1-308	308
2	BamHI-BamHI	309-3714	3406
3	BamHI-BamHI	3715-3877	163
4	BamHI-BamHI	3878-19307	15430
5	BamHI-BamHI	19308-19959	652
6	BamHI-BamHI	19960-32353	12394
7	BamHI-BamHI	32354-34949	2596
8	BamHI-BamHI	34950-39885	4936
9	BamHI-BamHI	39886-55214	15329
10	BamHI-BamHI	55215-59881	4667
11	BamHI-BamHI	59882-60676	795
12	BamHI-BamHI	60677-62613	1937
13	BamHI-BamHI	62614-72015	9402
14	BamHI-(RightEnd)	72016-73308	1293

HS4

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BanII's cuts

ng 000007.1

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#	Cut position (blunt - 5' ext. - 3' ext.)	5'... Site with flanks ...3'
1	186/182	172 AGGACTGCTT G▴AGCT▾C AAGAGTTTGA
2	321/317	307 AGGATCCCTT G▴AGCT▾C AGGAGGTCAA
3	955/951	941 CACTTTTAGA G▴AGCT▾C TTGGGGACCC
4	1066/1062	1052 TATAGACAAT G▴AGCC▾C TTTTCTCTCT
5	1157/1153	1143 GCAATGGGCA G▴GGCT▾C TGTCAGGGCT
6	1388/1384	1374 AGAGGAAAAG G▴GGCT▾C ACTGCACATA
7	2203/2199	2189 TCAGACTCCG G▴AGCT▾C AAGCAATCTG
8	3251/3247	3237 GAAAATCTGT G▴AGCT▾C CTCACCATAT
9	3529/3525	3515 GGGTATGAAA G▴AGCT▾C TGAATGAAAT
10	3573/3569	3559 TGCCCATTCA G▴GGCT▾C CAGCATGTAG
11	4278/4274	4264 TGACAAGACT G▴AGCT▾C AGAAGAGTCA
12	4365/4361	4351 TACTGCTCAT G▴GGCC▾C TGTGCTGCAC
13	4628/4624	4614 CCAGCTATCA G▴GGCC▾C AGATGGGTTA
14	5998/5994	5984 TATTGATGAG G▴AGCC▾C AATGTACTTG
15	6084/6080	6070 CAGATGGTCT G▴AGCT▾C TCCTACTGTC
16	6110/6106	6096 TACATTACAT G▴AGCT▾C TTATTAACTG
17	6733/6729	6719 CAAGAACTGA G▴GGCC▾C TAAACTATGC
18	7540/7536	7526 TGGTATTTCT G▴GGCT▾C ATTTGGCCCC
19	7617/7613	7603 CATCAGGGAT G▴GGCT▾C ATACTCACTG
20	8088/8084	8074 GGACTGCTTG G▴AGCT▾C AGGAGTTCAA
21	12751/12747	12737 TAAGGCAACA G▴AGCT▾C CTTTTTTTTT
22	13473/13469	13459 AGCATAGTCC G▴AGCT▾C TTATCTATAT
23	16236/16232	16222 AAGAAAATGT G▴GGCT▾C TGCAACTGGC
24	18763/18759	18749 AGCAAGAAGA G▴AGCC▾C CAGGCAATAC
25	20639/20635	20625 GTTGAATGTA G▴GGCT▾C ATAGAATAAA
26	24286/24282	24272 CTTGTTTATT G▴GGCT▾C TTTTTTGGTT
27	27360/27356	27346 TTGGCTATAT G▴GGCT▾C TTTTTTGATT
28	28882/28878	28868 TCAAGATTTA G▴AGCT▾C CTTTTATCAT
29	30368/30364	30354 AGTCGTGTCT G▴AGCT▾C AGAGTCTCCT
30	30863/30859	30849 GGGACCCAGA G▴AGCC▾C ACAGGGCTTT
31	33217/33213	33203 GGGGAAGGTA G▴AGCT▾C TCCTCCAATA
32	34276/34272	34262 ATCCTCTTGG G▴GGCC▾C CTTCCCCACA

33	35805/35801	35791 GGCCAGGCTG G▲AGCT▼C TCAGCTCACT
34	35985/35981	35971 AGATACCACT G▲AGCT▼C ACTGCCCATG
35	36443/36439	36429 AGCGAGGAAG G▲GGCT▼C AACGAAGAAA
36	37098/37094	37084 AGGTTTAGAG G▲AGCT▼C ATGAGAGCAG
37	37221/37217	37207 CCCAGGAAGA G▲AGCC▼C TGACCAGGAA
38	39212/39208	39198 ATCCTCTTGG G▲GGCC▼C CTTCCCCACA
39	40721/40717	40707 GGCCAGGCTG G▲AGCT▼C TCAGCTCACT
40	41871/41867	41857 AGGTTTAGCT G▲AGCT▼C ATAAGAGCAG
41	42959/42955	42945 TGCTGAGCAG G▲AGCT▼C TTTAGTTTAA
42	45980/45976	45966 TTGGATATCT G▲GGCT▼C TGACTGTGCA
43	48860/48856	48846 CCACCCCAAA G▲AGCT▼C ACCTCACCAT
44	49670/49666	49656 CTACAATTAT G▲GGCT▼C TTTCTTATAA
45	50303/50299	50289 TCCAGAACCA G▲AGCC▼C ATAACCAGAG
46	52668/52664	52654 TATTCTTTCT G▲AGCT▼C CAGATCCACA
47	58848/58844	58834 ATGAAAATCT G▲AGCC▼C AGTGGAGGAA
48	60886/60882	60872 ATGACTGACA G▲GGCC▼C TTAGGGAACA
49	64258/64254	64244 ACTGTCCTGT G▲AGCC▼C TTCTTCCCTG
50	67578/67574	67564 GAACTGGGTG G▲AGCC▼C ACCACAGCTC
51	69041/69037	69027 TGCCCTACAA G▲AGCT▼C CTGAAGGAAG
52	71730/71726	71716 AATCAAAAAA G▲AGCC▼C ACATCACCAA
53	71879/71875	71865 GAACAGAACA G▲AGCC▼C TCAGAAATAA

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ng 000007.1 - digested with: BamHI, BanII

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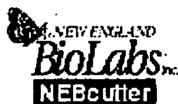
DNA Type: Unmethylated

#	Ends	Coordinates	Length (bp)	#	Ends	Coordinates	Length (bp)
1	BanII-BanII	8089-12751	4663	1	(LeftEnd)-BanII	1-186	186
2	BanII-BanII	20640-24286	3647	2	BanII-BamHI	187-308	122
3	BamHI-BanII	55215-58848	3634	3	BamHI-BanII	309-321	13
4	BanII-BanII	64259-67578	3320	4	BanII-BanII	322-955	634
5	BanII-BanII	24287-27360	3074	5	BanII-BanII	956-1066	111
6	BanII-BanII	42960-45980	3021	6	BanII-BanII	1067-1157	91
7	BanII-BanII	45981-48860	2880	7	BanII-BanII	1158-1388	231
8	BanII-BanII	13474-16236	2763	8	BanII-BanII	1389-2203	815
9	BanII-BanII	69042-71730	2689	9	BanII-BanII	2204-3251	1048
10	BanII-BamHI	52669-55214	2546	10	BanII-BanII	3252-3529	278
11	BanII-BanII	16237-18763	2527	11	BanII-BanII	3530-3573	44
12	BanII-BanII	50304-52668	2365	12	BanII-BamHI	3574-3714	141
13	BanII-BanII	37222-39212	1991	13	BamHI-BamHI	3715-3877	163
14	BanII-BamHI	60887-62613	1727	14	BamHI-BanII	3878-4278	401
15	BamHI-BanII	62614-64258	1645	15	BanII-BanII	4279-4365	87
16	BanII-BanII	27361-28882	1522	16	BanII-BanII	4366-4628	263
17	BanII-BamHI	30864-32353	1490	17	BanII-BanII	4629-5998	1370
18	BanII-BanII	28883-30368	1486	18	BanII-BanII	5999-6084	86
19	BanII-BanII	67579-69041	1463	19	BanII-BanII	6085-6110	26
20	BanII-BanII	4629-5998	1370	20	BanII-BanII	6111-6733	623
21	BamHI-(RightEnd)	72016-73308	1293	21	BanII-BanII	6734-7540	807
22	BanII-BanII	40722-41871	1150	22	BanII-BanII	7541-7617	77
23	BanII-BanII	41872-42959	1088	23	BanII-BanII	7618-8088	471
24	BanII-BanII	33218-34276	1059	24	BanII-BanII	8089-12751	4663
25	BanII-BanII	2204-3251	1048	25	BanII-BanII	12752-13473	722
26	BanII-BamHI	58849-59881	1033	26	BanII-BanII	13474-16236	2763
27	BamHI-BanII	32354-33217	864	27	BanII-BanII	16237-18763	2527
28	BamHI-BanII	34950-35805	856	28	BanII-BamHI	18764-19307	544
29	BamHI-BanII	39886-40721	836	29	BamHI-BamHI	19308-19959	652
30	BanII-BanII	1389-2203	815	30	BamHI-BanII	19960-20639	680
31	BanII-BanII	48861-49670	810	31	BanII-BanII	20640-24286	3647
32	BanII-BanII	6734-7540	807	32	BanII-BanII	24287-27360	3074
33	BamHI-BamHI	59882-60676	795	33	BanII-BanII	27361-28882	1522
34	BanII-BanII	12752-13473	722	34	BanII-BanII	28883-30368	1486

35	BamHI-BanII	19960-20639	680	35	BanII-BanII	30369-30863	495
36	BanII-BamHI	34277-34949	673	36	BanII-BamHI	30864-32353	1490
37	BanII-BamHI	39213-39885	673	37	BamHI-BanII	32354-33217	864
38	BanII-BanII	36444-37098	655	38	BanII-BanII	33218-34276	1059
39	BamHI-BamHI	19308-19959	652	39	BanII-BamHI	34277-34949	673
40	BanII-BanII	322-955	634	40	BamHI-BanII	34950-35805	856
41	BanII-BanII	49671-50303	633	41	BanII-BanII	35806-35985	180
42	BanII-BanII	6111-6733	623	42	BanII-BanII	35986-36443	458
43	BanII-BamHI	18764-19307	544	43	BanII-BanII	36444-37098	655
44	BanII-BanII	30369-30863	495	44	BanII-BanII	37099-37221	123
45	BanII-BanII	7618-8088	471	45	BanII-BanII	37222-39212	1991
46	BanII-BanII	35986-36443	458	46	BanII-BamHI	39213-39885	673
47	BamHI-BanII	3878-4278	401	47	BamHI-BanII	39886-40721	836
48	BanII-BanII	3252-3529	278	48	BanII-BanII	40722-41871	1150
49	BanII-BanII	4366-4628	263	49	BanII-BanII	41872-42959	1088
50	BanII-BanII	1158-1388	231	50	BanII-BanII	42960-45980	3021
51	BamHI-BanII	60677-60886	210	51	BanII-BanII	45981-48860	2880
52	(LeftEnd)-BanII	1-186	186	52	BanII-BanII	48861-49670	810
53	BanII-BanII	35806-35985	180	53	BanII-BanII	49671-50303	633
54	BamHI-BamHI	3715-3877	163	54	BanII-BanII	50304-52668	2365
55	BanII-BanII	71731-71879	149	55	BanII-BamHI	52669-55214	2546
56	BanII-BamHI	3574-3714	141	56	BamHI-BanII	55215-58848	3634
57	BanII-BamHI	71880-72015	136	57	BanII-BamHI	58849-59881	1033
58	BanII-BanII	37099-37221	123	58	BamHI-BamHI	59882-60676	795
59	BanII-BamHI	187-308	122	59	BamHI-BanII	60677-60886	210
60	BanII-BanII	956-1066	111	60	BanII-BamHI	60887-62613	1727
61	BanII-BanII	1067-1157	91	61	BamHI-BanII	62614-64258	1645
62	BanII-BanII	4279-4365	87	62	BanII-BanII	64259-67578	3320
63	BanII-BanII	5999-6084	86	63	BanII-BanII	67579-69041	1463
64	BanII-BanII	7541-7617	77	64	BanII-BanII	69042-71730	2689
65	BanII-BanII	3530-3573	44	65	BanII-BanII	71731-71879	149
66	BanII-BanII	6085-6110	26	66	BanII-BamHI	71880-72015	136
67	BamHI-BanII	309-321	13	67	BamHI-(RightEnd)	72016-73308	1293

HS4 core



Custom Digest

ng 000007.1 - digested with: AvaI, SacI

Print Close Help Comments

DNA Type: Unmethylated

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2	AvaI-SacI	18074-28882	10809	2	SacI-SacI	187-321	135
3	AvaI-SacI	8223-12751	4529	3	SacI-SacI	322-955	634
4	SacI-(RightEnd)	69042-73308	4267	4	SacI-AvaI	956-1234	279
5	AvaI-SacI	44959-48860	3902	5	AvaI-SacI	1235-2203	969
6	SacI-SacI	48861-52668	3808	6	SacI-AvaI	2204-2294	91
7	AvaI-AvaI	14687-18073	3387	7	AvaI-SacI	2295-3251	957
8	SacI-AvaI	37099-39942	2844	8	SacI-SacI	3252-3529	278
9	AvaI-SacI	31059-33217	2159	9	SacI-SacI	3530-4278	749
10	SacI-AvaI	42960-44958	1999	10	SacI-SacI	4279-6084	1806
11	SacI-SacI	6111-8088	1978	11	SacI-SacI	6085-6110	26
12	SacI-SacI	4279-6084	1806	12	SacI-SacI	6111-8088	1978
13	SacI-AvaI	33218-35006	1789	13	SacI-AvaI	8089-8222	134
14	AvaI-AvaI	65619-67267	1649	14	AvaI-SacI	8223-12751	4529
15	SacI-SacI	28883-30368	1486	15	SacI-SacI	12752-13473	722
16	AvaI-SacI	67777-69041	1265	16	SacI-AvaI	13474-13842	369
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18	SacI-SacI	35986-37098	1113	18	AvaI-AvaI	14687-18073	3387
19	AvaI-SacI	1235-2203	969	19	AvaI-SacI	18074-28882	10809
20	AvaI-SacI	2295-3251	957	20	SacI-SacI	28883-30368	1486
21	AvaI-AvaI	13843-14686	844	21	SacI-AvaI	30369-31058	690
22	AvaI-SacI	35007-35805	799	22	AvaI-SacI	31059-33217	2159
23	AvaI-SacI	39943-40721	779	23	SacI-AvaI	33218-35006	1789
24	SacI-SacI	3530-4278	749	24	AvaI-SacI	35007-35805	799
25	SacI-SacI	12752-13473	722	25	SacI-SacI	35806-35985	180
26	SacI-AvaI	41872-42593	722	26	SacI-SacI	35986-37098	1113
27	SacI-AvaI	30369-31058	690	27	SacI-AvaI	37099-39942	2844
28	SacI-SacI	322-955	634	28	AvaI-SacI	39943-40721	779
29	AvaI-AvaI	67268-67776	509	29	SacI-SacI	40722-41871	1150
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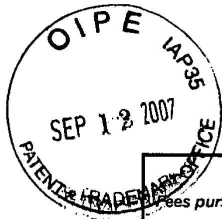
31	AvaI-SacI	42594-42959	366	31	AvaI-SacI	42594-42959	366
32	SacI-AvaI	956-1234	279	32	SacI-AvaI	42960-44958	1999
33	SacI-SacI	3252-3529	278	33	AvaI-SacI	44959-48860	3902
34	(LeftEnd)-SacI	1-186	186	34	SacI-SacI	48861-52668	3808

35	SacI-SacI	35806-35985	180	35	SacI-AvaI	52669-65618	12950
36	SacI-SacI	187-321	135	36	AvaI-AvaI	65619-67267	1649
37	SacI-AvaI	8089-8222	134	37	AvaI-AvaI	67268-67776	509
38	SacI-AvaI	2204-2294	91	38	AvaI-SacI	67777-69041	1265
39	SacI-SacI	6085-6110	26	39	SacI-(RightEnd)	69042-73308	4267



PTO/SB/17 (06-07)
Approved for use through 06/30/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL
For FY 2007

[X] Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 1,155.00

Complete if Known	
Application Number	10/188,221-Conf. #9026
Filing Date	July 1, 2002
First Named Inventor	Michel Sadelain
Examiner Name	M. Marvich
Art Unit	1633
Attorney Docket No.	64836(51590)

METHOD OF PAYMENT (check all that apply)

[ ] Check [ ] Credit Card [ ] Money Order [ ] None [ ] Other (please identify): ____

[X] Deposit Account Deposit Account Number: 04-1105 Deposit Account Name: Memorial Sloan-Kettering Cancer Center

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

[X] Charge fee(s) indicated below
[ ] Charge fee(s) indicated below, **except for the filing fee**
[X] Charge any additional fee(s) or underpayments of fee(s) under 37 CFR 1.16 and 1.17
[X] Credit any overpayments

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

Application Type	FILING FEES Fee ($)	FILING FEES Small Entity Fee ($)	SEARCH FEES Fee ($)	SEARCH FEES Small Entity Fee ($)	EXAMINATION FEES Fee ($)	EXAMINATION FEES Small Entity Fee ($)	Fees Paid ($)
Utility	300	150	500	250	200	100	
Design	200	100	100	50	130	65	
Plant	200	100	300	150	160	80	
Reissue	300	150	500	250	600	300	
Provisional	200	100	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims		Extra Claims		Fee ($)		Fee Paid ($)
22	- 47 =		x		=	

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims		Extra Claims		Fee ($)		Fee Paid ($)
1	- 3 =		x		=	

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims

Fee ($)	Fee Paid ($)

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets		Extra Sheets		Number of each additional 50 or fraction thereof		Fee ($)		Fee Paid ($)
	- 100 =		/50 =		(round up to a whole number) x		=	

4. OTHER FEE(S)

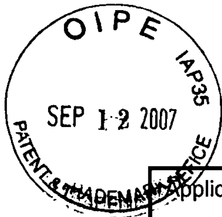
	Fees Paid ($)
Non-English Specification, $130 fee (no small entity discount)	
Other (e.g., late filing surcharge): 2253 Extension for response within third month	510.00
2801 Request for continued examination (RCE) (see 37 ...	395.00
2401 Notice of appeal	250.00

SUBMITTED BY

Signature	[signature]	Registration No. (Attorney/Agent)	32,360	Telephone	(617) 517-5509
Name (Print/Type)	Peter C. Lauro			Date	September 12, 2007

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service as Express Mail, Label No. EM 053203019 US, on the date shown below in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: September 12, 2007 Signature: [signature] (Peter C. Lauro)



Certificate of Express Mailing Under 37 CFR 1.10

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P.O. Box 1450
Alexandria, VA 22313-1450

on September 12, 2007
Date

Signature

Peter C. Lauro
Typed or printed name of person signing Certificate

32,360
Registration Number, if applicable

(617) 517-5509
Telephone Number

Note: Each paper must have its own certificate of mailing, or this certificate must identify each submitted paper.

Fee Transmittal (1 page)
Three Month Request for Extension of Time Under 37 CFR 1.136(a) (1 page)
Request for Continued Examination Transmittal (1 page)
Amendment and Response with Enclosures (20 pages)
Amendment Transmittal (1 page)
Declaration of Jason W. Plotkin with Exhibits 1-11 (76 pages)
Notice of Appeal (1 page)
Charge $1,155.00 to deposit account 04-1105

PTO/SB/06 (12-04)
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PATENT APPLICATION FEE DETERMINATION RECORD
Substitute for Form PTO-875

Application or Docket Number: 10/188221

APPLICATION AS FILED – PART I

FOR	NUMBER FILED (Column 1)		NUMBER EXTRA (Column 2)	SMALL ENTITY RATE ($)	SMALL ENTITY FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))								
SEARCH FEE (37 CFR 1.16(k), (i), or (m))								
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))								
TOTAL CLAIMS (37 CFR 1.16(i))	41	minus 20 =	*	x =		OR	x =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	4	minus 3 =	*	x =			x =	
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).							
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))								
				TOTAL			TOTAL	

* If the difference in column 1 is less than zero, enter "0" in column 2.

APPLICATION AS AMENDED – PART II

11/30/06

AMENDMENT A	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 23	Minus	** 41	= 0	x =		OR	x =	
Independent (37 CFR 1.16(h))	* 2	Minus	*** 5	= 0	x =		OR	x =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0

9/1/07

AMENDMENT B	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 22	Minus	** 23	= 4	x =		OR	x =	
Independent (37 CFR 1.16(h))	* 1	Minus	*** 5	=	x =		OR	x =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

EAST Search History

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L20	10	mouse adj pgk adj promoter and dhfr	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 12:04
L19	68	mouse adj pgk adj promoter	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 12:04
L18	5	human adj dhfr same amino adj acid adj "22"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 12:03
L16	1	human adj dhfr and mouse adj pgk adj promoter	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:47
L17	0	09/247054 and pgk	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:46
L15	1	dhfr same mouse adj pgk adj promoter	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:46
L11	1	09/247054 and (hsv or retrovirus or lentivirus)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:46
L14	29	dhfr same pgk adj promoter	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:45
L13	203139	dhfr same pgk promoter	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:44

EAST Search History

L12	15	globin and (lcr or locus adj control adj region) same dhfr	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:44
L10	1	globin same (lcr or locus adj control adj region) same dhfr	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:41
L8	1	09/247054 and dhfr	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:37
L9	84	globin same (lcr or locus adj control adj region) and dhfr	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:36
L7	4	globin same (lcr or locus adj control adj region) and (heat adj sensitive or hs2 adj10 hs3 adj10 hs4) and dhfr	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:36
L6	0	globin same (lcr or locus adj control adj region) and (heat adj sensitive or hs2 adj10 hs3 adj10 hs4) same dhfr	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:31
L5	1	globin same (lcr or locus adj control adj region) and (heat adj sensitive or hs or hs2 adj10 hs3 adj10 hs4) same dhfr	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:31
L4	75	globin same (lcr or locus adj control adj region) and (heat adj sensitive or hs or hs2 adj10 hs3 adj10 hs4) and dhfr	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:31
L2	290	globin same (lcr or locus adj control adj region) and (heat adj sensitive or hs or hs2 adj10 hs3 adj10 hs4)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:31
S59	4	globin and (lcr or locus adj control adj region) adj20 (hs2 adj10 hs3 adj10 hs4)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:28

EAST Search History

S3	8840	Sadelin.in. or Rivella.in. or May.in. or Bertino.in.	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2007/10/27 11:28
L3	11117	Sadelin.in. or Rivella.in. or May.in. or Bertino.in.	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:28
S39	198	(DCR or LAR or LCR) same globin and sequence	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:27
L1	42	(DCR or LAR or LCR) adj20 globin and dhfr	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2007/10/27 11:27

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www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/188,221	07/01/2002	Michel Sadelain	64836(51590)	9026

21874 7590 10/30/2007
EDWARDS ANGELL PALMER & DODGE LLP
P.O. BOX 55874
BOSTON, MA 02205

EXAMINER	
MARVICH, MARIA	

ART UNIT	PAPER NUMBER
1633	

MAIL DATE	DELIVERY MODE
10/30/2007	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

PTOL-90A (Rev. 04/07)

Office Action Summary	Application No.	Applicant(s)
	10/188,221	SADELAIN ET AL.
	Examiner	Art Unit
	Maria B. Marvich, PhD	1633

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *12 September 2007*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-18,43,44,46 and 47* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-18,44 and 46* is/are rejected.

7)☒ Claim(s) *43 and 47* is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *8/2/07*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 9/12/07 has been entered.

Claim Objections

Applicant is advised that should claim 44 be found allowable, claim 45 will be objected to under 37 CFR 1.75 as being a substantial duplicate thereof. When two claims in an application are duplicates or else are so close in content that they both cover the same thing, despite a slight difference in wording, it is proper after allowing one claim to object to the other as being a substantial duplicate of the allowed claim. See MPEP § 706.03(k).

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.
>
> (e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this

subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

Claim 1 are rejected under 35 U.S.C. 102(b) as being anticipated by Ryan et al (Genes and Development, 1989, Vol 3, pages 314-323; see entire document).

Ryan et al teach recombinant vectors encompassing the 3.2 kb portion of the human b-globin LCR that consists essentially of HS2, HS3 and HS4 as recited in claim 1 (see e.g. page 321, col 2 and figure 2). By inclusion of the term "consisting essentially of" in the amended claim language, it appears that applicants have attempted to limit the invention to the four recited steps. However, the specification does not define the use of the term "consisting essentially of". Absent a clear indication in the specification or claims as to what is considered a material change in the basic and novel characteristics of "consisting essentially of", it will be construed as equivalent to "comprising" (see MPEP 2111.03).

Claims 1, 10, 44 and 46 are rejected under 35 U.S.C. 102(e) as being anticipated by Antoniou et al (US patent 6, 797,494; see entire document).

Antoniou et al teach recombinant retroviral vectors encompassing the 3.2 kb portion of the human b-globin LCR that consists essentially of HS2, HS3 and HS4 as recited in claim 1 (see e.g. figure 3, col 1, lines 17-21). By inclusion of the term "consisting essentially of" in the amended claim language, it appears that applicants have attempted to limit the invention to the four recited steps. However, the specification does not define the use of the term "consisting essentially of". Absent a clear indication in the specification or claims as to what is considered a material change in the basic and novel characteristics of "consisting essentially of", it will be construed as equivalent to "comprising" (see MPEP 2111.03).

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 1-18, 44 and 46 are rejected under 35 U.S.C. 103(a) as being unpatentable over Ryan et al (Genes and Development, 1989, Vol 3, pages 314-323; see entire document) or Antoniou et al (US patent 6, 797,494; see entire document) in view of Bertino et al (US patent 6,642,043; see entire documents) and Melton et al (NAR, 1997, pages 3937-3943; see entire document).

Applicants claim a recombinant vector comprising a 3.2 portion of a human b-globin LCR further comprising a region encoding DHFR.

The teachings of are described above and are applied as before except;

Neither Antoniou et al or Ryan et al teach use of a mutant DHFR.

Bertino et al teach use of a mutant DHFR with a mutation in amino acid 22 and 31 of the human DHFR sequences that has been shown to have superior properties to reduce sensitivity of the enzyme to antifolate inhibition i.e. amino acid 22 mutants(see e.g. abstract).

Melton et al teach that mouse pgk promoter is capable of expressing marker genes stably at multiple locations in the genome (see abstract).

It would have been obvious to one of ordinary skill in the art at the time the invention was made to substitute the markers taught by Ryan and Antoniou with the DHFR sequences as

taught by Bertino et al under control of the mouse PGK promoter as taught by Melton et al because Ryan and Antoniou et al teach that the LCR regions are capable of driving expression of globin and because Bertino et al teaches that DHFR is an excellent marker that is resistant to antifolates and because Melton et al teach that it is within the ordinary skill of the art to express marker genes from mouse pgk promoters. Bertino et al and Melton et al demonstrates uses of known elements in recombinant vectors for which it would have been obvious to use to improve similar vectors using skill that was available at the time of filing with well-established methods. Based upon the teachings of the cited references, the high skill of one of ordinary skill in the art, and absent evidence to the contrary, there would have been a reasonable expectation of success to result in the claimed invention.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Maria B. Marvich, PhD whose telephone number is (571)-272-0774. The examiner can normally be reached on M-F (7:00-4:00).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Joseph Woitach, PhD can be reached on (571)-272-0739. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Maria B Marvich, PhD
Examiner
Art Unit 1633

Used In Lieu of PTO/SB/08A/B
(Based on PTO 04-07 version)

Substitute for form 1449/PTO				Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	10/188,221-Conf. #9026
				Filing Date	July 1, 2002
				First Named Inventor	Michel Sadelain
				Art Unit	1633
(Use as many sheets as necessary)				Examiner Name	M. Marvich
Sheet	1	of	1	Attorney Docket Number	64836(51590)

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number: Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document: Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
MM	CA	ZUFFEREY et al., "Self-Inactivating Lentivirus Vector for Safe and Efficient In Vivo Gene Delivery", Journal of Virology, Dec. 1998, Vol. 72, No. 12, pp. 9873-9880.	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

Examiner Signature	M. Marvich	Date Considered	10/25/07

233076

Notice of References Cited	Application/Control No. 10/188,221	Applicant(s)/Patent Under Reexamination SADELAIN ET AL.	
	Examiner Maria B. Marvich, PhD	Art Unit 1633	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-6,642,043	11-2003	Bertino et al.	435/252.3
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

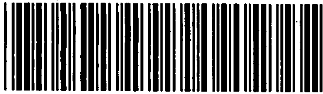
*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Ryan et al, A single erythroid-specific DNAse I super-hypersensitive site activates high levels of human b-globin gene expression in transgenic mice, Genes and Development, Vol 3, pages 314-323; see entire document)
	V	Melton et al, Stability of HPRT marker gene expression at different gene-targeted loci: observing and overcoming a position effect, Nucleic Acids Research, 1997, Vol. 25, No. 19 3937–3943.
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes



Application/Control No.	Applicant(s)/Patent under Reexamination
10/188,221	SADELAIN ET AL.
Examiner	**Art Unit**
Maria B. Marvich, PhD	1633

SEARCHED			
Class	Subclass	Date	Examiner

INTERFERENCE SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES (INCLUDING SEARCH STRATEGY)	DATE	EXMR
East, PALM inventor search	9/30/2005	MM
East databases- USPAT, PGPUB, EPO, JPO, Derwent, IBM-IDB search notes attached	9/30/2005	MM
STN databases- Caplus, Scisearch, Medline search notes attached	9/30/2005	MM
East, STN search history updated, search ntoes attached	7/17/2006	MM
East, STN search history updated, search notes attached	2/25/2007	MM
East, STN search history updated, search notes attached	10/24/2007	MM

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with § 1.6(a)(4).

Dated: February 29, 2008 Signature: _______/Peter C. Lauro/_________
Peter C. Lauro, Esq. , Reg. No. 32,360

Docket No.: 64836(51590)
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Sadelain, *et al.*

Serial No.: 10/188,221

Filed: July 1, 2002

For: *VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES*

Confirmation No.: 9026

Group Art Unit: 1633

Examiner: Maria Marvich

Mail Stop: Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AND RESPONSE UNDER 37 C.F.R. § 1.111

Dear Sir:

Applicants submit this paper in response to the non-final Office Action mailed on October 30, 2007 in the above-referenced patent application. Applicants submit concurrently herewith a Request for a One-Month Extension of Time and the required fee based on small entity status. The Commissioner is authorized to charge any additional fees occasioned by this paper, or credit any overpayment of such fees, to Deposit Account No. 04-1105, under Order No. 64836(51590).

Amendments to the claims are reflected in the listing of claims beginning on page 2.

Remarks/Arguments begin on page 6.

AMENDMENTS TO THE CLAIMS

Please amend claim 1 and please cancel claim 46 without prejudice, without admission, without surrender of subject matter, and without any intention of creating any estoppel as to equivalents and please add claims 48-50. This following listing of claims will replace all prior versions, and listings, of claims in the application.

1. (Currently amended) A recombinant vector comprising:

(a) a region comprising a nucleotide sequence encoding a functional globin; and

(b) a 3.2-kb nucleotide fragment ~~portion of a human ß-globin locus control region (LCR)~~ which consists essentially of an HS2-spanning nucleotide fragment extending between BstXI and SnaBI restriction sites of a human ß-globin locus control region (LCR)~~of said LCR~~, an HS3-spanning nucleotide fragment extending between BamHI and HindIII restriction sites of said LCR and an HS4-spanning nucleotide fragment extending between BamHI and BanII restriction sites of said LCR, said vector providing expression of globin when introduced into a mammal *in vivo*.

2. (Previously presented) The vector of claim 1, further comprising a region encoding a dihydrofolate reductase.

3. (Previously presented) The vector of claim 2, further comprising a mouse PGK promoter, to control the expression of the dihydroflate reductase.

4. (Previously presented) The vector of claim 3, wherein the dihydrofolate reductase is a human dihydrofolate reductase.

5. (Previously presented) The vector of claim 4, wherein the human dihydrofolate reductase is a mutant form having increased resistance to antifolates as compared to wild-type human dihydrofolate reductase, said mutant form differing in amino acid sequence from wild-type human dihydrofolate reductase as a result of a set of mutations.

6. (Previously presented) The vector of claim 5, wherein the set of mutations comprises a mutation at an amino acid corresponding to amino acid 22 of the wild-type sequence and a mutation at an amino acid corresponding to amino acid 31 of the wild type sequence.

7. (Previously presented) The vector of claim 2, wherein the dihydrofolate reductase is a human dihydrofolate reductase.

8. (Previously presented) The vector of claim 7, wherein the human dihydrofolate reductase is a mutant form having increased resistance to antifolates as compared to wild-type human dihydrofolate reductase, said mutant form differing in amino acid sequence from wild-type human dihydrofolate reductase as a result of a set of mutations.

9. (Previously presented) The vector of claim 8, wherein the set of mutations comprises a mutation at an amino acid corresponding to amino acid 22 of the wild-type sequence and a mutation at an amino acid corresponding to amino acid 31 of the wild type sequence.

10. (Previously presented) The vector of claim 1, wherein the functional globin is human ß-globin.

11. (Previously presented) The vector of claim 10, further comprising a region encoding a dihydrofolate reductase.

12. (Previously presented) The vector of claim 11, further comprising a mouse PGK promoter, wherein the mouse PGK promoter controls the expression of the region encoding a dihydrofolate reductase.

13. (Previously presented) The vector of claim 12, wherein the dihydrofolate reductase

is a human dihydrofolate reductase.

14. (Previously presented) The vector of claim 13, wherein the human dihydrofolate reductase is a mutant form having increased resistance to antifolates as compared to wild-type human dihydrofolate reductase, said mutant form differing in amino acid sequence from wild-type human dihydrofolate reductase as a result of a set of mutations.

15. (Previously presented) The vector of claim 14, wherein the set of mutations comprises a mutation at an amino acid corresponding to amino acid 22 of the wild-type sequence and a mutation at an amino acid corresponding to amino acid 31 of the wild type sequence.

16. (Previously presented) The vector of claim 11, wherein the dihydrofolate reductase is a human dihydrofolate reductase.

17. (Previously presented) The vector of claim 16, wherein the human dihydrofolate reductase is a mutant form having increased resistance to antifolates as compared to wild-type human dihydrofolate reductase, said mutant form differing in amino acid sequence from wild-type human dihydrofolate reductase as a result of a set of mutations.

18. (Previously presented) The vector of claim 17, wherein the set of mutations comprises a mutation at an amino acid corresponding to amino acid 22 of the wild-type sequence and a mutation at an amino acid corresponding to amino acid 31 of the wild type sequence.

19-42. (Canceled)

43. (Previously presented) The vector of claim 1, further comprising 2 GATA-1 binding sites at the junction between the HS3 and HS4 fragments.

44. (Previously presented) The vector of claim 1, wherein the vector is a lentiviral vector.

45-46. (Canceled)

47. (Previously presented) The vector of claim 1, wherein the vector is pTNS9.

48. (New) The vector of claim 1, wherein the functional globin is a β-globin.

49. (New) The vector of claim 1, wherein the functional globin is a γ-globin.

50. (New) The vector of claim 1, wherein the functional globin is an α-globin.

REMARKS

I. Status of the Claims and Formal Matters

Claims 1-18, 43, 44, 46 and 47 are pending in the application and have been examined. Claim 46 has been canceled, claim 1 has been amended and claims 48-50 have been added. Accordingly, claims 1-18, 43, 44 and 47-50 will pending in the application upon entry of the amendments presented herein.

Claims 48-50 now further indicate that the functional globin is a β-globin, a γ-globin or an α-globin. Support for the addition of claims 48-50 can be found in the specification, at least, for example, in the paragraph spanning pages 3 and 4. No new matter is introduced by this amendment.

Applicants wish to thank Examiner Marvich for her assistance during the telephonic interview conducted on February 25, 2008.

II. Objections to the Claims

In the Office Action Summary, under the Disposition of Claims (p. 1), claims 43 and 47 are described as objected to. As no description of the objection to claims 43 and 47 appears in the Office Action, it is assumed that the objection was made to indicate that claims 43 and 47 would be allowable if re-written in independent form to include the elements of Claim 1. In view of the amendments to claim 1 presented herein, it is believed that all claims are allowable, including claims 43 and 47.

Under Claims Objections (p. 2), the Office Action indicates that claims 44 and 45 are objected to as substantial duplicates. Upon review of the claims, claims 44 and 46 were found to be identical. Consequently, Applicants believe the Examiner intended to indicate that claims 44 and 46 were objected to as substantial duplicates (which arose inadvertently when changing claim dependencies). Accordingly, Applicants have canceled claim 46 and believe that this was the original intent of the Examiner's objection to these claims.

Applicants would appreciate the Examiner's concurrence that the foregoing is correct.

III. The Rejections under 35 U.S.C. § 102 Are Overcome

Claim 1 is rejected under 35 U.S.C. § 102(b) as being anticipated by Ryan, *et al.* (Genes and Development, 1989, Vol. 3, pages 314-323). Claims 1, 10, 44 and 46 are rejected under 35 U.S.C. § 102(e) as being anticipated by Antoniou, *et al.* (USSN 6,797,494). It is alleged that the claims read upon the vectors described by Ryan and Antoniou. Applicants respectfully traverse the rejection.

Applicants have amended claim 1 to clarify the vector contents relating to the HS-spanning nucleotide fragments of a human ß-globin locus control region (LCR). In particular, claim 1 now recites that the claimed recombinant vector has a 3.2-kb nucleotide fragment which consists essentially of an HS2-spanning nucleotide fragment extending between BstXI and SnaBI restriction sites of a human ß-globin locus control region (LCR), an HS3-spanning nucleotide fragment extending between BamHI and HindIII restriction sites of said LCR and an HS4-spanning nucleotide fragment extending between BamHI and BanII restriction sites of said LCR.

Applicants respectfully submit that Ryan, *et al.* and Antoniou, *et al.* fail to teach or suggest the 3.2-kb nucleotide fragment of claim 1. The HS-containing fragments of Ryan are depicted in Fig. 2 (p. 315). Of this group, Ryan shows only a 30-kb and a 22-kb recombinant nucleotide fragment that contain at least HS2, HS3 and HS4 (these fragments also contain HS1 and HS5 as well as other sequences). The HS-containing fragments of Antoniou, *et al.* are depicted in Fig. 3. Of this group, Antoniou, *et al.* show only a single 5.5-kb nucleotide fragment that contains HS2, HS3 and HS4. Based on size, the Ryan, *et al.* and Antoniou, *et al.* fragments clearly differ from the 3.2 kb fragment of Claim 1. Further, based on composition, neither Ryan, *et al.* nor Antoniou, *et al.* show a single fragment of 3.2 kb that combines the recited HS2-, HS3- and HS4-spanning fragments as in present claim 1. Hence, neither reference anticipates claim 1 presented herein. Moreover, because claims 10, 44 and 46 incorporate the elements of claim 1, these claims are also novel in view of Ryan, *et al.* and Antoniou, *et al.*. Therefore, Applicants respectfully request reconsideration and withdrawal of the rejections to claims 1, 10, 44 and 46 under 35 U.S.C. §§ 102(b) and 102(e).

IV. The Rejections under 35 U.S.C. § 103 Are Overcome

Claims 1 – 18, 44 and 46 are rejected under 35 U.S.C. § 103 as being unpatentable over Ryan, *et al.* (Genes and Development, 1989, Vol. 3, pages 314-323) or Antoniou et al. (USSN 6,797494) in view of Bertino, *et al.* (USSN 6,642,043) and Melton, *et al.* (NAR 1997 3937-3943). Applicants respectfully traverse the rejection.

There is no teaching or suggestion of the 3.2-kb nucleotide fragment of claim 1 in either Ryan, *et al.* or Antoniou, *et al.* Likewise, Bertino, *et al.* and Melton, *et al.* are silent regarding the 3.2-kb nucleotide fragment of claim 1 and therefore, fail to cure the defects of Ryan, *et al.* and Antoniou, *et al.* Therefore, Applicants respectfully request reconsideration and withdrawal of the rejections to claims 1 – 18, 44 and 46 under 35 U.S.C. § 103.

CONCLUSION

In view of the foregoing amendments and remarks presented herein, Applicants respectfully request reconsideration and withdrawal of all rejections and allowance of the application with all pending claims. If a telephone conversation with Applicants' attorney(s) would help to expedite the prosecution of the above-identified application, the Examiner is invited to contact the undersigned.

Dated: February 29, 2008

Respectfully submitted,

By: /Peter C. Lauro/
Peter C. Lauro, Esq.
Registration No.: 32,360
EDWARDS ANGELL PALMER & DODGE LLP
P.O. Box 55874
Boston, Massachusetts 02205
(617) 517-5509

PTO/SB/21 (10-07)
Approved for use through 10/31/2007. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Application Number	10/188,221-Conf. #9026
Filing Date	July 1, 2002
First Named Inventor	M. Sadelain
Art Unit	1633
Examiner Name	M. Marvich
Attorney Docket Number	64836(51590)
Total Number of Pages in This Submission	11

ENCLOSURES (*Check all that apply*)

- [x] Fee Transmittal Form
  - [ ] Fee Attached
- [x] Amendment/Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [x] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
  - [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) ______
  - [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [ ] Other Enclosure(s) (please Identify below):

Return Receipt Postcard
Declarations with Exhibits

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	EDWARDS ANGELL PALMER & DODGE LLP		
Signature	/Peter C. Lauro/		
Printed name	Peter C. Lauro, Esq.		
Date	February 29, 2008	Reg. No.	32,360

PTO/SB/17 (10-07)
Approved for use through 06/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no person are required to respond to a collection of information unless it displays a valid OMB control number.

Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL
For FY 2008

[x] Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 60.00

Complete if Known	
Application Number	10/188,221 - Conf. # 9026
Filing Date	July 1, 2002
First Named Inventor	M. Sadelain
Examiner Name	M. Marvich
Art Unit	1633
Attorney Docket No.	64836(51590)

METHOD OF PAYMENT (check all that apply)

[ ] Check [ ] Credit Card [ ] Money Order [ ] None [ ] Other (please identify): ____________

[x] Deposit Account Deposit Account Number: 04-1105 Deposit Account Name: Edwards Angell Palmer & Dodge

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

[x] Charge fee(s) indicated below
[ ] Charge fee(s) indicated below, **except for the filing fee**
[x] Charge any additional fee(s) or underpayments of fee(s) under 37 CFR 1.16 and 1.17
[x] Credit any overpayments

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
Application Type	**Fee ($)**	**Small Entity Fee ($)**	**Fee ($)**	**Small Entity Fee ($)**	**Fee ($)**	**Small Entity Fee ($)**	**Fees Paid ($)**
Utility	310	155	510	255	210	105	
Design	210	105	100	50	130	65	
Plant	210	105	310	155	160	80	
Reissue	310	155	510	255	620	310	
Provisional	210	105	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	**Fee ($)**	**Small Entity Fee ($)**
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	210	105
Multiple dependent claims	370	185

Total Claims		**Extra Claims**		**Fee ($)**		**Fee Paid ($)**
24	-47 =	0	x	0	=	

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims		**Extra Claims**		**Fee ($)**		**Fee Paid ($)**
1	- 3 =	0	x		=	

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims

Fee ($)	**Fee Paid ($)**

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $260 ($130 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets		**Extra Sheets**		**Number of each additional 50 or fraction thereof**		**Fee ($)**		**Fee Paid ($)**
	- 100 =		/50 =		(round **up** to a whole number) x		=	

4. OTHER FEE(S) **Fees Paid ($)**

Non-English Specification, $130 fee (no small entity discount)

Other (e.g., late filing surcharge): 2251 Extension for response within first month 60.00

SUBMITTED BY					
Signature	/Peter C. Lauro/	Registration No. (Attorney/Agent)	32,360	Telephone	(617) 517-5509
Name (Print/Type)	Peter C. Lauro, Esq.			Date	February 29, 2008

Electronic Patent Application Fee Transmittal

Application Number:	10188221
Filing Date:	01-Jul-2002
Title of Invention:	Vector encoding human globin gene and use thereof in treatment of hemoglobinopathies
First Named Inventor/Applicant Name:	Michel Sadelain
Filer:	Peter C. Lauro
Attorney Docket Number:	64836(51590)

Filed as Small Entity

Utility Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 1 month with $0 paid	2251	1	60	60

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
		Total in USD ($)		**60**

Electronic Acknowledgement Receipt

EFS ID:	2937289
Application Number:	10188221
International Application Number:	
Confirmation Number:	9026
Title of Invention:	Vector encoding human globin gene and use thereof in treatment of hemoglobinopathies
First Named Inventor/Applicant Name:	Michel Sadelain
Customer Number:	21874
Filer:	Peter C. Lauro
Filer Authorized By:	
Attorney Docket Number:	64836(51590)
Receipt Date:	29-FEB-2008
Filing Date:	01-JUL-2002
Time Stamp:	21:25:54
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$60
RAM confirmation Number	4776
Deposit Account	041105
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes) /Message Digest	Multi Part /.zip	Pages (if appl.)
1	Extension of Time	EOT660856_1.pdf	23325 a79c782a99acdcbbeca21ab54743c718a06de137	no	1
Warnings:					
Information:					
2	Amendment - After Non-Final Rejection	AmendmentandResponse660849_1.pdf	51959 0f093c5b3b0c6c4020a8f2b46b6cae0466bdad12	no	8
Warnings:					
Information:					
3	Miscellaneous Incoming Letter	Transmittal660857_1.pdf	27808 cbeba040a21e11ae364adaff465d2e1720669bbc	no	1
Warnings:					
Information:					
4	Fee Worksheet (PTO-06)	FeeTransmittal660855_1.pdf	44654 09b01983a770ac53ad7ac323634ed5fe817d88ba	no	1
Warnings:					
Information:					
5	Fee Worksheet (PTO-06)	fee-info.pdf	8175 3a4f2cada14c4d0c869fce6134ef74aa8703b571	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	155921		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/22 (10-07)
Approved for use through 10/31/2007. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless if displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a) **FY 2006** **(Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).)**	Docket Number (Optional) 64836(51590)
Application Number 11/016,196-Conf. #6852	Filed July 1, 2002
For VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES	
Art Unit 1633	Examiner M. Marvich

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
X	One month (37 CFR 1.17(a)(1))	$120	$60	$ 60.00
	Two months (37 CFR 1.17(a)(2))	$460	$230	$
	Three months (37 CFR 1.17(a)(3))	$1050	$525	$
	Four months (37 CFR 1.17(a)(4))	$1640	$820	$
	Five months (37 CFR 1.17(a)(5))	$2230	$1115	$

[X] Applicant claims small entity status. See 37 CFR 1.27.

[ ] A check in the amount of the fee is enclosed.

[ ] Payment by credit card. Form PTO-2038 is attached.

[X] The Director has already been authorized to charge fees in this application to a Deposit Account.

[X] The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 04-1105 . I have enclosed a duplicate copy of this sheet.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

[ ] applicant/inventor.

[ ] assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

[X] attorney or agent of record. Registration Number 32,360

[ ] attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 ______

/Peter C. Lauro/	February 29, 2008
Signature	Date
Peter C. Lauro, Esq.	(617) 517-5509
Typed or printed name	Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

[ ] Total of 1 forms are submitted.

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 10/188,221	Filing Date 07/01/2002	☐ To be Mailed

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY ☒ RATE ($)	FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 02/29/2008	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 24	Minus	** 47	= 0	X $25 =	0	OR	X $ =	
Independent (37 CFR 1.16(h))	* 1	Minus	***5	= 0	X $105 =	0	OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE	**0**	OR	TOTAL ADD'L FEE	

AMENDMENT	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/AMANDA FORD/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/188,221	07/01/2002	Michel Sadelain	64836(51590)	9026

21874 7590 03/17/2008
EDWARDS ANGELL PALMER & DODGE LLP
P.O. BOX 55874
BOSTON, MA 02205

EXAMINER	
MARVICH, MARIA	

ART UNIT	PAPER NUMBER
1633	

MAIL DATE	DELIVERY MODE
03/17/2008	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

PTOL-90A (Rev. 04/07)

Interview Summary	**Application No.** 10/188,221	**Applicant(s)** SADELAIN ET AL.	
	Examiner MARIA B. MARVICH	**Art Unit** 1633	

All participants (applicant, applicant's representative, PTO personnel):

(1) *MARIA B. MARVICH*.

(2) *Amy Leahy*.

(3) *Lisa Wilson*.

(4) _____.

Date of Interview: *26 February 2008*.

Type: a)☒ Telephonic b)☐ Video Conference
c)☐ Personal [copy given to: 1)☐ applicant 2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☐ Yes e)☐ No.
If Yes, brief description: _____.

Claim(s) discussed: _____.

Identification of prior art discussed: *Ryan et al and Antoniou et al* .

Agreement with respect to the claims f)☐ was reached. g)☐ was not reached. h)☒ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: *Applicants described amendments to overcome the art rejections under Ryan et al and Antoniou et al that would comprise indicating that the 3.2 kb portion is a fragment comprising components of the LCR. Such an amendment appears to overcome the art rejections however a closer look at the art is required* .

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN A NON-EXTENDABLE PERIOD OF THE LONGER OF ONE MONTH OR THIRTY DAYS FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

/Maria B Marvich, PhD/
Examiner, Art Unit 1633
Examiner's signature, if required

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/188,221	07/01/2002	Michel Sadelain	64836(51590)	9026

21874 7590 06/03/2008

EDWARDS ANGELL PALMER & DODGE LLP
P.O. BOX 55874
BOSTON, MA 02205

EXAMINER	
MARVICH, MARIA	

ART UNIT	PAPER NUMBER
1633	

MAIL DATE	DELIVERY MODE
06/03/2008	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

PTOL-90A (Rev. 04/07)

Office Action Summary	**Application No.** 10/188,221	**Applicant(s)** SADELAIN ET AL.	
	Examiner MARIA B. MARVICH	**Art Unit** 1633	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE _3_ MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on _29 February 2008_.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) _1-18,43,44 and 47-50_ is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) _1-18,44 and 48-50_ is/are rejected.

7)☒ Claim(s) _43 and 47_ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on _07 January 2001_ is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)

3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _12/8/06_.

4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

5) ☐ Notice of Informal Patent Application

6) ☐ Other: _____.

DETAILED ACTION

Claims 1-18, 43, 44 and 47-50 are pending in this application. This office action is in response to an amendment filed 2/29/08.

Claim Objections

Claim 1 is objected to because of the following informalities: Claim 1 recites "which consists essentially of an HS-2 spanning nucleotide fragment extending between BstXI and SnaB1 restriction sites of a human b-globin locus control region (LCR), an HS3-spann"ng nucleotide fragment extending between BanHI and HindIII restriction sites of said LCR and an HS-4 spanning nucleotide fragment extending between BamHI and BanII restriction sites of said LCR". It is recommended that the claim be amended to recite the following, --which consists essentially of an HS-2 spanning BstXI and SnaB1 restriction fragment from a human b-globin locus control region (LCR), an HS3-spanning BanHI and HindIII restriction fragment from said LCR and an HS-4 spanning BamHI and BanII restriction fragment from said LCR.

Claims 5, 8, 14 and 18 are objected to for recitation "mutant form", which is preferably recommended to be amended to recite "mutant dihydrofolate reductase". And subsequently, the recitation in line 3, of "form" should be deleted. As well "as a result of a set of mutations" is inherent and need not be included. This phrase can be deleted.

The recitation in claims 6, 9, 15 and 18 "set of mutations" is more accurately written as --mutant dihydrofolate reductase--.

Claim 43 for clarity should be amended to recite –wherein HS3 and HS4 are operably linked and comprise 2 GATA-1 binding sites at the junction between the HS3 and HS4 fragments--. There is no requirement that the two have a junction in claim 1.

Appropriate correction is required.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

(e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

Claim 1 are rejected under 35 U.S.C. 102(b) as being anticipated by Ryan et al (Genes and Development, 1989, Vol 3, pages 314-323; see entire document). **This rejection is maintained for reasons of record in the office action mailed 10/30/07 and restated below.**

Ryan et al teach recombinant vectors encompassing the 3.2 kb portion of the human b-globin LCR that consists essentially of HS2, HS3 and HS4 as recited in claim 1 (see e.g. page 321, col 2 and figure 2). By inclusion of the term "consisting essentially of" in the amended claim language, it appears that applicants have attempted to limit the invention to the four recited steps. However, the specification does not define the use of the term "consisting essentially of". Absent a clear indication in the specification or claims as to what is considered a material change

in the basic and novel characteristics of "consisting essentially of", it will be construed as equivalent to "comprising" (see MPEP 2111.03).

Claims 1, 10, 44 and 46 are rejected under 35 U.S.C. 102(e) as being anticipated by Antoniou et al (US patent 6, 797,494; see entire document). **This rejection is maintained for reasons of record in the office action mailed 10/30/07 and restated below.**

Antoniou et al teach recombinant retroviral vectors encompassing the 3.2 kb portion of the human b-globin LCR that consists essentially of HS2, HS3 and HS4 as recited in claim 1 (see e.g. figure 3, col 1, lines 17-21). By inclusion of the term "consisting essentially of" in the amended claim language, it appears that applicants have attempted to limit the invention to the four recited steps. However, the specification does not define the use of the term "consisting essentially of". Absent a clear indication in the specification or claims as to what is considered a material change in the basic and novel characteristics of "consisting essentially of", it will be construed as equivalent to "comprising" (see MPEP 2111.03).

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 1-18, 44 and 46 are rejected under 35 U.S.C. 103(a) as being unpatentable over Ryan et al (Genes and Development, 1989, Vol 3, pages 314-323; see entire document) or

Antoniou et al (US patent 6, 797,494; see entire document) in view of Bertino et al (US patent 6,642,043; see entire documents) and Melton et al (NAR, 1997, pages 3937-3943; see entire document). **This rejection is maintained for reasons of record in the office action mailed 10/30/07 and restated below.**

Applicants claim a recombinant vector comprising a 3.2 portion of a human b-globin LCR further comprising a region encoding DHFR.

The teachings of are described above and are applied as before except;

Neither Antoniou et al or Ryan et al teach use of a mutant DHFR.

Bertino et al teach use of a mutant DHFR with a mutation in amino acid 22 and 31 of the human DHFR sequences that has been shown to have superior properties to reduce sensitivity of the enzyme to antifolate inhibition i.e. amino acid 22 mutants(see e.g. abstract).

Melton et al teach that mouse pgk promoter is capable of expressing marker genes stably at multiple locations in the genome (see abstract).

It would have been obvious to one of ordinary skill in the art at the time the invention was made to substitute the markers taught by Ryan and Antoniou with the DHFR sequences as taught by Bertino et al under control of the mouse PGK promoter as taught by Melton et al because Ryan and Antoniou et al teach that the LCR regions are capable of driving expression of globin and because Bertino et al teaches that DHFR is an excellent marker that is resistant to antifolates and because Melton et al teach that it is within the ordinary skill of the art to express marker genes from mouse pgk promoters. Bertino et al and Melton et al demonstrates uses of known elements in recombinant vectors for which it would have been obvious to use to improve

similar vectors using skill that was available at the time of filing with well-established methods. Based upon the teachings of the cited references, the high skill of one of ordinary skill in the art, and absent evidence to the contrary, there would have been a reasonable expectation of success to result in the claimed invention.

Response to Argument

Applicants traverse the claim rejections under 35 U.S.C. 102 and 103 on pages 7-8 of the amendment filed 2/29/08. Applicants' arguments filed 2/29/08 have been fully considered but they are not persuasive. By recitation of a 3.2 kb nucleotide fragment, the claim is not limited to just these sequences but encompasses any number of additional sequences such that even full length LCR sequences are encompassed by the claims. The vector must comprise at the least the restriction fragments but is not limited to these pieces.

Conclusion

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37

CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to MARIA B. MARVICH whose telephone number is (571)272-0774. The examiner can normally be reached on M-F (7:00-4:00).

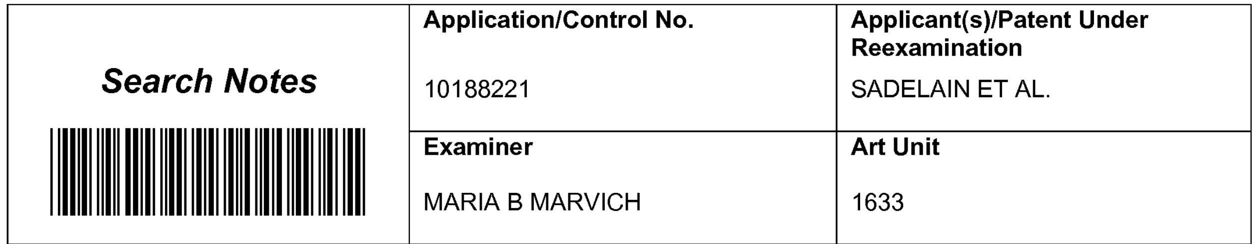
If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Joseph Woitach, PhD can be reached on (571)-272-0739. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Maria B Marvich, PhD
Primary Examiner
Art Unit 1633

/Maria B Marvich, PhD/

Primary Examiner, Art Unit 1633



Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	10188221	SADELAIN ET AL.
	Examiner	**Art Unit**
	MARIA B MARVICH	1633

SEARCHED

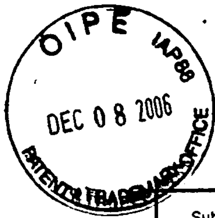
Class	Subclass	Date	Examiner

SEARCH NOTES

Search Notes	Date	Examiner
EAST, STN search updated, search notes updated	5/27/08	MM

INTERFERENCE SEARCH

Class	Subclass	Date	Examiner



PTO/SB/08A/B (09-06)
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Substitute for form 1449/PTO				Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	10/188,221-Conf. #9026
				Filing Date	July 1, 2002
				First Named Inventor	Michel Sadelain
				Art Unit	1633
(Use as many sheets as necessary)				Examiner Name	Maria Marvich
Sheet	1	of	1	Attorney Docket Number	64836(51590)

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
/M.M./	CA	GATLIN et al. "In Vitro Selection of Lentivirus Vector-Transduced Human CD34+ Cells." Human Gene Therapy 11: 1949-1957 (2000)	
/M.M./	CB	SADELAIN "Genetic Treatment of the Haemogloinopathies Recombinations and New Combinations." British Journal of Haematology 98: 247-253 (1997)	
/M.M./	CC	TISDALE et al. "Towards Gene Therapy for Disorders of Golbin Synthesis." Seminars in Hematology 38:4 382-392 (2001)	
/M.M./	CD	RIVELLA et al. "Globin Gene Transfer: a Paradigm for Transgenic Regulation and Vector Safety." Gene Therapy and Regulation 00:0; 1-27 (2003)	
/M.M./	CE	SADELAIN et al. Issues in the Manufacture and Transplantation of Genetically Modified Hematopoietic Stem Cells." Current Opinion in Hematology 7: 364-377 (2000)	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

Examiner Signature	/Maria Marvich/	Date Considered	05/03/2008

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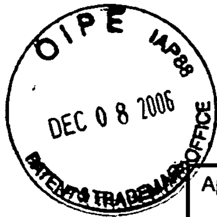

Application No. (if known): 10/188,221 Attorney Docket No.: 64836(51590)

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Information Disclosure Statement (IDS)
IDS Form PTO SB-08 (5 References)
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EAST Search History

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	87	globin same (lcr or locus adj control adj region) and dhfr	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2008/05/27 15:48
L2	4	globin same (lcr or locus adj control adj region) adj20 (hs2 adj10 hs3 adj10 hs4)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2008/05/27 15:48
L3	6	"5610053".pn. or "6090608".pn. or "5631162".pn.	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2008/05/27 15:48
L4	1	L3 and globin and (LCR or locus adj control adj region) and HS2 same hs3 same hs4	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2008/05/27 15:48
L5	100	vector same globin and hemoglobinopathy	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2008/05/27 15:48
L6	4	(lentivirus or lentiviral) same globin same lcr	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2008/05/27 15:48
L7	0	(lcr or locus adj control adj region) adj20 (hs2 and hs3 and hs4) same restriction adj map	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2008/05/27 15:49
L8	9	globin same (lcr or locus adj control adj region) adj20 (hs2 and hs3 and hs4)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2008/05/27 15:49
L9	4	globin and (lcr or locus adj control adj region) adj20 (hs2 adj10 hs3 adj10 hs4)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2008/05/27 15:49

L10	6	"5610053".pn. or "6090608".pn. or "5631162".pn.	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2008/05/27 15:50

5/27/2008 3:52:57 PM
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Docket No.: 64836(51590)
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Michel Sadelain, *et al.*

Application No.: 10/188,221 Confirmation No.: 9026

Filed: July 1, 2002 Art Unit: 1633

For: *VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES* Examiner: M. Marvich

AMENDMENT AND RESPONSE AFTER FINAL ACTION UNDER 37 C.F.R. §1.116

MS AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Madam:

INTRODUCTORY COMMENTS

Applicants submit this paper in response to the Final Office Action mailed on June 3, 2008 in the above-referenced patent application. Applicants also file concurrently herewith a Petition for a three-month Extension of Time and a Notice of Appeal. The Commissioner is authorized to charge any additional fees occasioned by this paper, or credit any overpayment of such fees, to Deposit Account No. 04-1105, under order no. 64836(51590). Please amend the application without prejudice, without admission, without surrender of subject matter, and without any intention of creating any estoppel as to equivalents as follows.

Amendments to the Claims are reflected in the listing of claims, which begins on page 2 of this paper.

Remarks/Arguments begin on page 6 of this paper.

AMENDMENTS TO THE CLAIMS

Please amend claims 1, 5, 6, 8, 9, 14, 15, 17, 18 and 43. The following listing of claims will replace all prior versions, and listings, of claims in the application.

1. (Currently amended) A recombinant vector comprising:
a region comprising a nucleotide sequence encoding a functional globin; and
a 3.2-kb nucleotide fragment which consists essentially of three contiguous nucleotide fragments, said fragments being an HS2-spanning nucleotide fragment extending between BstXI and SnaBI restriction sites of a human ß-globin locus control region (LCR), an HS3-spanning nucleotide fragment extending between BamHI and HindIII restriction sites of said LCR and an HS4-spanning nucleotide fragment extending between BamHI and BanII restriction sites of said LCR, said vector providing expression of globin when introduced into a mammal *in vivo*.

2. (Previously presented) The vector of claim 1, further comprising a region encoding a dihydrofolate reductase.

3. (Previously presented) The vector of claim 2, further comprising a mouse PGK promoter, to control the expression of the dihydrofolate reductase.

4. (Previously presented) The vector of claim 3, wherein the dihydrofolate reductase is a human dihydrofolate reductase.

5. (Currently amended) The vector of claim 4, wherein the human dihydrofolate reductase is a mutant dihydrofolate reductase ~~form~~ having increased resistance to antifolates as compared to wild-type human dihydrofolate reductase~~, said mutant form~~ and differing in amino acid sequence from wild-type human dihydrofolate reductase ~~as a result of a set of mutations~~.

6. (Currently amended) The vector of claim 5, wherein the mutant dihydrofolate reductase ~~set of mutations~~ comprises a mutation at an amino acid corresponding to amino acid 22 of the wild-type sequence and a mutation at an amino acid corresponding to amino acid 31 of the wild type sequence.

7. (Previously presented) The vector of claim 2, wherein the dihydrofolate reductase is a human dihydrofolate reductase.

8. (Currently amended) The vector of claim 7, wherein the human dihydrofolate reductase is a mutant dihydrofolate reductase ~~form~~ having increased resistance to antifolates as compared to wild-type human dihydrofolate reductase~~, said mutant form~~ and differing in amino acid sequence from wild-type human dihydrofolate reductase~~ as a result of a set of mutations~~.

9. (Currently amended) The vector of claim 8, wherein the mutant dihydrofolate reductase ~~set of mutations~~ comprises a mutation at an amino acid corresponding to amino acid 22 of the wild-type sequence and a mutation at an amino acid corresponding to amino acid 31 of the wild type sequence.

10. (Previously presented) The vector of claim 1, wherein the functional globin is human ß-globin.

11. (Previously presented) The vector of claim 10, further comprising a region encoding a dihydrofolate reductase.

12. (Previously presented) The vector of claim 11, further comprising a mouse PGK promoter, wherein the mouse PGK promoter controls the expression of the region encoding a dihydrofolate reductase.

13. (Previously presented) The vector of claim 12, wherein the dihydrofolate reductase is a human dihydrofolate reductase.

14. (Currently amended) The vector of claim 13, wherein the human dihydrofolate reductase is a mutant dihydrofolate reductase ~~form~~ having increased resistance to antifolates as compared to wild-type human dihydrofolate reductase~~, said mutant form~~ and differing in amino acid sequence from wild-type human dihydrofolate reductase~~ as a result of a set of mutations~~.

15. (Currently amended) The vector of claim 14, wherein the mutant dihydrofolate reductase ~~set of mutations~~ comprises a mutation at an amino acid corresponding to amino acid 22 of the wild-type sequence and a mutation at an amino acid corresponding to amino acid 31 of the wild type sequence.

16. (Previously presented) The vector of claim 11, wherein the dihydrofolate reductase is a human dihydrofolate reductase.

17. (Currently amended) The vector of claim 16, wherein the human dihydrofolate reductase is a mutant dihydrofolate reductase ~~form~~ having increased resistance to antifolates as compared to wild-type human dihydrofolate reductase~~, said mutant form~~ and differing in amino acid sequence from wild-type human dihydrofolate reductase~~ as a result of a set of mutations~~.

18. (Currently amended) The vector of claim 17, wherein the mutant dihydrofolate reductase ~~set of mutations~~ comprises a mutation at an amino acid corresponding to amino acid 22 of the wild-type sequence and a mutation at an amino acid corresponding to amino acid 31 of the wild type sequence.

19-42. (Canceled)

43. (Currently Amended) The vector of claim 1, wherein the HS3 fragment and the HS4 fragment are adjacent to each other and have ~~further comprising~~ 2 GATA-1 binding sites at the junction between the HS3 and HS4 fragments.

44. (Previously presented) The vector of claim 1, wherein the vector is a lentiviral vector.

45-46. (Canceled)

47. (Previously presented) The vector of claim 1, wherein the vector is pTNS9.

48. (Previously presented) The vector of claim 1, wherein the functional globin is a β-globin.

49. (Previously presented) The vector of claim 1, wherein the functional globin is a γ-globin.

50. (Previously presented) The vector of claim 1, wherein the functional globin is an α-globin.

REMARKS

I. Status of the Claims and Formal Matters

Claims 1-18, 43, 44 and 47-50 have been examined and are the pending claims. Claims 1, 5, 6, 8, 9, 14, 15, 17, 18 and 43 have been amended. Favorable reconsideration and allowance are respectfully requested.

Claim 1 recites that the three fragments are contiguous. Support for this aspect of the claimed subject matter is provided in Figs. 1 and 2. The remaining amendments are supported by the original claims corresponding to the amended claims and incorporate the changes suggested by the Examiner. No new matter is introduced by this amendment.

Amendment of the claims herein is not to be construed as acquiescence to any objections/rejections in the pending Office Action or any previous Office Actions and was done solely to expedite prosecution of the application. Applicants hereby reserve the right to pursue the subject matter of the claims as originally filed, or similar claims, in this or one or more subsequent patent applications.

II. Objections to the Claims

In the present Office Action, under the Disposition of Claims (p. 1), Claims 43 and 47 remain under objection. As neither a description of the objection nor an explicit rejection of Claims 43 and 47 appears in this or the previous Office Action, Applicants assume that the objection was made to indicate that Claims 43 and 47 would be allowable if re-written in independent form to include the elements of Claim 1. If this is not the case, the Examiner's clarification on this point is again respectfully requested. Applicants believe the present amendment obviates the need for rewriting these claims in independent form.

Under the heading "Claims Objections" beginning at p. 2 of the Office Action, the Examiner has objected to the form of Claim 1. As currently presented, Claim 1 recites that the elements of the 3.2-kb nucleotide fragment are HS-spanning nucleotide fragments that extend between particular restriction sites. As Applicants understand this objection, the Examiner recommends that the elements be recited as particular HS-spanning restriction fragments. Applicants believe that characterization of these

elements as nucleotide fragments more accurately reflects the nature of these elements. As the Examiner will appreciate, those of skill in the art recognize that the ends of a restriction fragment can be sticky, blunt, filled in or made blunt. All of these forms may colloquially be referred to as restriction fragments but are more fully described as a subset of nucleotide fragments that extend between particular restriction sites. Consequently, to ensure that there is no ambiguity, Applicants believe that the current claim language more accurately embraces the subject matter of the present invention. Moreover, for the reasons discussed under the anticipation rejection, Applicants believe this language is preferable. Accordingly, Applicants respectfully decline to incorporate the Examiner's recommendation.

Claims 5, 8, 14 and 17[1] have been objected to for reciting "mutant form." The Examiner has recommended that this phrase be rewritten to recite "mutant dihydrofolate reductase" and to make a few other related changes in these claims as well as in Claims 6, 9, 15 and 18. Applicants have followed the Examiner's helpful suggestions for amending these claims and believe the claims more fully embody Applicants' invention.

Finally, Claim 43 has been objected to as lacking a clear relationship between the HS3- and HS4-spanning fragments and the two GATA-1 binding sites. The claim has been amended as recommended to indicate that the HS3- and HS4-spanning fragments are adjacent to each other and that the two GATA-1 binding sites lie between and at the junction of the two fragments.

III. The § 102 rejections

Claim 1 has been rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Ryan, *et al.* (Genes and Development, 1989, Vol. 3, pages 314-323; hereafter "Ryan"). Further, Claims 1, 10, 44 and 46[2] have been rejected under 35 U.S.C. § 102(e) as allegedly being anticipated by Antoniou, *et al.* (U.S. Patent No. 6,797,494; hereafter "Antoniou"). The Examiner is maintaining these rejections for the reasons of record, namely because the vectors described by Ryan and Antoniou allegedly "encompass the 3.2 kb portion of the human b-globin LCR that consists essentially of HS2, HS3 and

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[1] In the Office Action, the Examiner listed claim 18 as objectionable. It appears clear that the listing of Claim 18 was a typographical error and that Claim 17 was intended to be the objected claim.
[2] Claim 46 was cancelled by the response filed February 29, 2008, rendering moot this aspect of the rejection.

HS4 as recited in claim 1" (Office Action, p. 3, last paragraph and p. 4, second full paragraph). Applicants respectfully disagree and traverse the rejection.

The transition phrase "consisting essentially of" represents a middle ground in claim construction that defines the elements to be excluded from the scope of a claim as those which do not materially affect the basic and novel characteristics of the claimed subject matter. *PPG Industries v. Guardian Industries*, 156 F.3d 1351, 1354 (Fed. Cir. 1998). This transition phrase neither fully opens the claim nor fully closes it. Although MPEP §2111.03 may guide the Examiner to treating the phrase as open for prior art search purposes, that action alone does not open the claim language to unlimited elements. Moreover, the limitations that define the basic and novel properties of the invention can be in the claim, in the specification or implicit in the amendments and arguments made to distinguish the claimed subject matter from the prior art. *Id., University of Cal. v. Eli Lilly & Co.*, 1995 U.S. Dist. LEXIS 19003, 39 U.S.P.Q.2d (BNA) 1225 (S.D. Ind. Dec. 11, 1995).

The relevant basic and novel properties of the claimed 3.2 kb nucleotide fragment are defined in Claim 1 as previously presented (on Feb. 29, 2008) and as currently amended.[3] According to the invention, the basic and novel properties of the claimed subject matter are achieved by the choice and combination of the three specific nucleotide fragments from a human β-globin LCR that produce a vector capable of expressing a functional globin *in vivo* when the vector is introduced into a mammal. The choice of the three nucleotide fragments recited in Claim 1 is specifically defined by (1) the HS spanned by each fragment, (2) the restriction sites defining the ends of each fragment, (3) the size of each fragment, and importantly, (4) the overall combined size of the three fragments to provide a single nucleotide fragment of 3.2 kb. Hence, recombinant vectors comprising this specific assembly of three recited LCR fragments (*i.e.*, a 3.2-kb nucleotide fragment consisting essentially of the three specified fragments—as well as encoding a functional globin) define the scope of the invention,

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[3] Claim 1 as currently amended recites that the three HS fragments are contiguous. According to the Random House Dictionary, contiguous is defined as: 1. touching; in contact, or 2. in close proximity without actually touching; near. *The Random House Dictionary of the English Language* (Stein, Ed.) Random House, New York, 1973, p. 316. For the first meaning, the definition is synonymous with bordering, adjoining and abutting; for the second, the definition is synonymous with adjacent. That the three fragments are contiguous is clear from Figs. 1 and 2. Amending the claim in this manner makes explicit a relationship that was already implicit in the subject matter as previously claimed. As discussed in the text, here and in previous responses, the size sum of the three HS fragments rounds to 3.2 kb, meaning that the fragments are at least adjacent and could easily be adjoined.

whereas any vector lacking the claimed 3.2-kb fragment with the three LCR fragments assembled in a contiguous manner is excluded from the scope of the claimed subject matter.

The simple fact that the combination of the three HS-spanning fragments is 3.2 kb partially (and significantly) closes this aspect of the present claim, qualifies its size and thus provides the boundaries for ascertaining the elements excluded by use of "consisting essentially of" as the transitional phrase. For example, any additional nucleotides added to the 3.2 kb fragment that cause the fragment to exceed 3.2 kb, would alter a basic and novel property of the invention. As Applicants have exhaustively established on the record, the combined size of the three HS-spanning fragments so closely approximates 3.2 kb, that the number of additional nucleotides that could be added to (or removed from) this fragment is relatively few and non-material. For example, the types of non-material nucleotide changes that can be accommodated are those associated with filling in or blunting the sticky end of a restriction fragment, adding a small linker to provide or change a restriction site, or making any other minor change to the sequence that does not alter the functionality of the fragment in driving globin expression, including changes at the ends of or at the junction points of the fragments All such changes are well known in the art and would be readily contemplated, accomplished and analyzed by skilled artisans. However, none of these non-material changes rises to the level of the fragments taught by Ryan or Antoniou.

The HS-containing fragments of Ryan are depicted in Fig. 2 (p. 315). Of this group, Ryan shows only a 30-kb and a 22-kb recombinant nucleotide fragment that contain at least HS2, HS3 and HS4 (these fragments also contain HS1 and HS5 as well as other sequences). The HS-containing fragments of Antoniou are depicted in Fig. 3. Of this group, Antoniou show only a single 5.5-kb nucleotide fragment that contains HS2, HS3 and HS4. Based on size, the Ryan and Antoniou fragments clearly differ from the 3.2 kb fragment of Claim 1.

Further, based on nucleotide composition and arrangement of the HS fragments (*i.e.*, which pieces of the LCR are present), neither Ryan nor Antoniou shows any fragment that combines the recited HS2-, HS3- and HS4-spanning fragments in

contiguity into a single ***3.2-kb*** fragment as claimed in present Claim 1. Ryan's fragments are single, large restriction fragments from the LCR encompassing all 5 HS sites in their natural order and sequence context. Antoniou fragments combine various restriction fragments which are larger and distinct from those claimed by Applicants. Merely because the three HS fragments that Applicants have identified are within the sequence of the Ryan and Antoniou fragments does not mean that those references "encompass" the claimed 3.2-kb fragment and thereby anticipate the present invention. The actual combination must be demonstrated in these references and it is not, as evidenced by Applicants' use of "consisting essentially of" as the transitional phrase, along with bounding this operable LCR fragment at 3.2 kb, which, therefore, serve to distinguish the claimed invention from Ryan and Antoniou as well as establish the basic and novel properties of this nucleotide fragment.

Hence, Applicants respectfully submit that both Ryan and Antoniou lack the 3.2-kb nucleotide fragment of Claim 1, or any suggestion thereof, so that neither reference anticipates present Claim 1. Moreover, because Claims 10 and 44 (specifying, respectively, that the globin is β-globin and the vector is a lentiviral vector) incorporate the elements of Claim 1, these claims are also novel in view of Ryan and Antoniou.

Moreover, to close a claim directed to a nucleotide fragment to the degree discussed above or to overcome the prior art is not unprecedented. In *University of Cal. v. Eli Lilly & Co.*[4] , UCal amended certain claims during prosecution by changing "comprising" (or "having") to "consisting essentially of" to distinguish over the prior art. The UCal claim at issue was directed to an expression vector for human proinsulin and the prior art showed an expression vector encoding a human proinsulin fusion protein (in which the fused portion was removed after expression to produce the same human proinsulin). The former was considered a "tailored" direct expression system and the latter an indirect expression system. By amending the claim to recite that the expression vector consisted essentially of the nucleotides encoding human proinsulin, the Examiner allowed a direct expression system claim over the prior art and the district court concurred in this claim construction. *Id*. At 42. The CAFC later confirmed this construction as well. *Regents of Univ. of Cal. v. Eli Lilly & Co.*, 119 F.3d 1559, 1573-1574 (Fed. Cir. 1997).

[4] See, FN15 and surrounding text at pp. 28-42.

Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejections of Claims 1, 10, and 44 under 35 U.S.C. §§ 102(b) and 102(e).

IV. The § 103 rejection

Claims 1 – 18, 44 and 46[5] are rejected under 35 U.S.C. § 103 as being unpatentable over Ryan or Antoniou in view of Bertino *et al.* (U.S. Patent No. 6,642,043: hereafter "Bertino") and Melton *et al.* (1997) Nucleic Acids Res. Vol? 3937-3943; hereafter "Melton"). Applicants respectfully traverse the rejection.

There is no teaching or suggestion of the 3.2-kb nucleotide fragment of Claim 1 by Ryan or Antoniou. Bertino and Melton are likewise silent regarding the 3.2-kb nucleotide fragment of Claim 1 and, therefore, fail to cure the defects of Ryan and Antoniou. Reconsideration and withdrawal of the rejections to Claims 1 – 18 and 44 under 35 U.S.C. § 103 are respectfully requested.

CONCLUSION

In view of the foregoing amendments and remarks presented herein, reconsideration and withdrawal of all rejections and allowance of the instant application with all pending claims are respectfully solicited. If a telephone conversation with Applicants' attorney(s) would help to expedite the prosecution of the above-identified application, the Examiner is invited to contact the undersigned.

Dated: December 3, 2008

Respectfully submitted,

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[5] See the explanation at footnote 2.

AMENDMENT TRANSMITTAL LETTER			Docket No. 64836(51590)
Application No. 10/188,221-Conf. #9026	Filing Date July 1, 2002	Examiner M. Marvich	Art Unit 1633

Applicant(s): Michel Sadelain et al.

Invention: VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES

TO THE COMMISSIONER FOR PATENTS

Transmitted herewith is an amendment in the above-identified application.

The fee has been calculated and is transmitted as shown below.

CLAIMS AS AMENDED					
	Claims Remaining After Amendment	**Highest Number Previously Paid**	**Number Extra Claims Present**	**Rate**	
Total Claims	24	- 50 =		x	
Independent Claims	1	- 3 =		x	
Multiple Dependent Claims (check if applicable) ☐					
Other fee (please specify): Extension for response within third month					555.00
TOTAL ADDITIONAL FEE FOR THIS AMENDMENT:					555.00

☐ Large Entity ☒ Small Entity

☐ No additional fee is required for this amendment.

☒ Please charge Deposit Account No. 04-1105 in the amount of $ 555.00 .
A duplicate copy of this sheet is enclosed.

☐ A check in the amount of $ ______________ to cover the filing fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☒ The Director is hereby authorized to charge and credit Deposit Account No. 04-1105 as described below. A duplicate copy of this sheet is enclosed.

- ☒ Credit any overpayment.
- ☒ Charge any additional filing or application processing fees required under 37 CFR 1.16 and 1.17.

/Peter C. Lauro/ Dated: December 3, 2008

Peter C. Lauro, Esq.
Attorney/Agent Reg. No.: 32,360

EDWARDS ANGELL PALMER & DODGE LLP
P.O. Box 55874
Boston, Massachusetts 02205
(617) 517-5509

BOS2 709019.1

PTO/SB/31 (11-08)
Approved for use through 12/31/2008. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

NOTICE OF APPEAL FROM THE EXAMINER TO THE BOARD OF PATENT APPEALS AND INTERFERENCES	Docket Number (Optional) 64836(51590)	
	In re Application of Michel Sadelain et al.	
	Application Number 10/188,221-Conf. #9026	Filed July 1, 2002
	For VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES	
	Art Unit 1633	Examiner M. Marvich

Applicant hereby **appeals** to the Board of Patent Appeals and Interferences from the last decision of the examiner.

The fee for this Notice of Appeal is (37 CFR 41.20(b)(1)) $ 540.00

[x] Applicant claims small entity status. See 37 CFR 1.27. Therefore, the fee shown above is reduced by half, and the resulting fee is: $ 270.00

[ ] A check in the amount of the fee is enclosed.

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[x] The Director has already been authorized to charge fees in this application to a Deposit Account.

[x] The Director is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 04-1105 .

[ ] A petition for an extension of time under 37 CFR 1.136(a) (PTO/SB/22) is enclosed.

WARNING: INFORMATION ON THIS FORM MAY BECOME PUBLIC. CREDIT CARD INFORMATION SHOULD NOT BE INCLUDED ON THIS FORM. PROVIDE CREDIT CARD INFORMATION AND AUTHORIZATION ON PTO-2038.

I am the

[ ] applicant /inventor.

[ ] assignee of record of the entire interest. See 37 CFR 3.71. Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96)

[x] attorney or agent of record.
Registration number 32,360

[ ] attorney or agent acting under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34. ______

/Peter C. Lauro/
Signature

Peter C. Lauro, Esq.
Typed or printed name

(617) 517-5509
Telephone number

December 3, 2008
Date

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

[ ] *Total of 1 forms are submitted.

Electronic Patent Application Fee Transmittal

Application Number:	10188221
Filing Date:	01-Jul-2002
Title of Invention:	Vector encoding human globin gene and use thereof in treatment of hemoglobinopathies
First Named Inventor/Applicant Name:	Michel Sadelain
Filer:	Peter C. Lauro/Teresa Lauro
Attorney Docket Number:	64836(51590)

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Notice of appeal	2401	1	270	270
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 3 months with $0 paid	2253	1	555	555
Miscellaneous:				
		Total in USD ($)		**825**

Electronic Acknowledgement Receipt

EFS ID:	4388709
Application Number:	10188221
International Application Number:	
Confirmation Number:	9026
Title of Invention:	Vector encoding human globin gene and use thereof in treatment of hemoglobinopathies
First Named Inventor/Applicant Name:	Michel Sadelain
Customer Number:	21874
Filer:	Peter C. Lauro
Filer Authorized By:	
Attorney Docket Number:	64836(51590)
Receipt Date:	03-DEC-2008
Filing Date:	01-JUL-2002
Time Stamp:	17:06:39
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$825
RAM confirmation Number	2781
Deposit Account	041105
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

File Listing:					
Document Number	**Document Description**	**File Name**	**File Size(Bytes)/ Message Digest**	**Multi Part /.zip**	**Pages (if appl.)**
1	Miscellaneous Incoming Letter	64836CertificateofElectronicFiling120308.pdf	14010 9fbc2abce5803a30955cfa2bb0f86484fef80ce5	no	1
Warnings:					
Information:					
2	Extension of Time	64836ExtensionofTime120308.pdf	31094 96cb12038f6ac77d90f2a074e423d71888fdc0bc	no	1
Warnings:					
Information:					
3	Amendment After Final	64836AmendmentandRespponse120308.pdf	78729 23a60611461f0dea993175bfb498a78d639b3836	no	11
Warnings:					
Information:					
4	Miscellaneous Incoming Letter	64836AmendmentTransmittal.pdf	23460 769edae13502fcc4b1d443ee695b967cb60d0515	no	1
Warnings:					
Information:					
5	Notice of Appeal Filed	64836NoticeofAppeal.pdf	21374 b306815448c0fb5c5702735f11df016c76410dea	no	1
Warnings:					
Information:					
6	Fee Worksheet (PTO-06)	fee-info.pdf	32019 102062effca145714b2806f36f0a47afb84d0c16	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	200686		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Application No. (if known): 10/188,221 Attorney Docket No.: 64836(51590)

Certificate of Electronic Filing Under 37 CFR 1.8

I hereby certify that this correspondence is being transmitted via the Office electronic filing system in accordance with 37 CFR 1.6(a)(4):

MS AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

on December 3, 2008 .
Date

/Peter C. Lauro/
Signature

Peter C. Lauro, Esq.
Typed or printed name of person signing Certificate

32,360	(617) 517-5509
Registration Number, if applicable	Telephone Number

Note: Each paper must have its own certificate of mailing, or this certificate must identify each submitted paper.

Three Month Request for Extension of Time Under 37 CFR 1.136(a) (1 page)
Amendment and Response After Final Action Under 37 C.F.R. 1.116 (11 pages)
Amendment Transmittal (1 page)
Notice of Appeal (1 page)
Charge $825.00 to deposit account 04-1105

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PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a) FY 2009 *(Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).)*	Docket Number (Optional) 64836(51590)
Application Number 10/188,221-Conf. #9026	Filed July 1, 2002
For VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES	
Art Unit 1633	Examiner M. Marvich

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
[ ]	One month (37 CFR 1.17(a)(1))	$130	$65	$
[ ]	Two months (37 CFR 1.17(a)(2))	$490	$245	$
[x]	Three months (37 CFR 1.17(a)(3))	$1110	$555	$ 555.00
[ ]	Four months (37 CFR 1.17(a)(4))	$1730	$865	$
[ ]	Five months (37 CFR 1.17(a)(5))	$2350	$1175	$

[x] Applicant claims small entity status. See 37 CFR 1.27.

[ ] A check in the amount of the fee is enclosed.

[ ] Payment by credit card. Form PTO-2038 is attached.

[x] The Director has already been authorized to charge fees in this application to a Deposit Account.

[x] The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 04-1105 .

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

[ ] applicant/inventor.

[ ] assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

[x] attorney or agent of record. Registration Number 32,360

[ ] attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 ______

/Peter C. Lauro/ — Signature
December 3, 2008 — Date

Peter C. Lauro, Esq. — Typed or printed name
(617) 517-5509 — Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

[ ] Total of 1 forms are submitted.

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 10/188,221	Filing Date 07/01/2002	☐ To be Mailed

APPLICATION AS FILED – PART I

FOR	NUMBER FILED (Column 1)	NUMBER EXTRA (Column 2)	SMALL ENTITY ☒ RATE ($)	SMALL ENTITY FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
			TOTAL			TOTAL	

* If the difference in column 1 is less than zero, enter "0" in column 2.

APPLICATION AS AMENDED – PART II

AMENDMENT	12/03/2008	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 24	Minus	** 47	= 0	X $26 =	0	OR	X $ =	
	Independent (37 CFR 1.16(h))	* 1	Minus	***5	= 0	X $110 =	0	OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE	**0**	OR	TOTAL ADD'L FEE	

AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/DESHONNE T. MARTINO/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Docket No.: 64836(51590)
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Michel Sadelain, *et al.*

Application No.: 10/188,221 | Confirmation No.: 9026

Filed: July 1, 2002 | Art Unit: 1633

For: *VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES* | Examiner: M. Marvich

OK to enter
MM
1/9/09

AMENDMENT AND RESPONSE AFTER FINAL ACTION UNDER 37 C.F.R. §1.116

MS AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

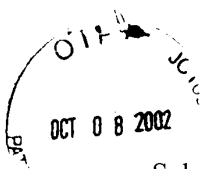
Dear Madam:

INTRODUCTORY COMMENTS

Applicants submit this paper in response to the Final Office Action mailed on June 3, 2008 in the above-referenced patent application. Applicants also file concurrently herewith a Petition for a three-month Extension of Time and a Notice of Appeal. The Commissioner is authorized to charge any additional fees occasioned by this paper, or credit any overpayment of such fees, to Deposit Account No. 04-1105, under order no. 64836(51590). Please amend the application without prejudice, without admission, without surrender of subject matter, and without any intention of creating any estoppel as to equivalents as follows.

Amendments to the Claims are reflected in the listing of claims, which begins on page 2 of this paper.

Remarks/Arguments begin on page 6 of this paper.



Replacement 1449 10/8/02

Substitute for form 1449

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

Application No.: 10/188,221
Applicant: Sadelain
Filing Date: July 1, 2002
Conf. No.: 9026
Title: Vector encoding human globin gene and use thereof in treatment of hemoglobinopathies
Attorney Docket No.: MSK.P-050

Page 1 of 1

U.S. PATENT DOCUMENTS

Examiners Initials	U S Patent No.	Name of Persons or applicant	Date of Publication of Cited Document

FOREIGN PATENT DOCUMENTS

Examiners Initials	Patent No.	Name of Persons or applicant	Date of Publication of Cited Document
/M.M./	WO 97/33988	Sloan-Kettering Institute for Cancer Research	09/18/1997

OTHER PRIOR ART - NON PATENT LITERATURE DOCUMENTS

Examiner Initials	
/M.M./	Huang. et al., "Nonadditivity of Mutational Effects at the Folate Binding Site of *Escherichia* coli Dihydrofolate Reductase", *Biochemistry,* Vol. 33, No. 38, pp. 11576 - 11585, 1994
/M.M./	Ercikan et al., "Effect of codon 22 mutations on substrate and inhibitor binding for human dihydrofolate reductase", *Chemistry and Biology of Pteridines and Folates,* pp 515 - 519, 1993
/M.M./	May, et al., "Therapeutic haemoglobin synthesis in β-thalassaemic mice expressing lentivirus-encoded human β-globin", *Nature,* Vol. 406, pp. 82 - 86, July 6, 2000
/M.M./	D. Bodine, "Globin Gene Therapy: One (Seemingly) Small Vector Change, One Giant Leap in Optimism", *Molecular Therapy,* Vol. 2, No. 2, pp. 101 -102, August 2000

This Information Disclosure Citation List is being submitted as a substitute for Form PTO-1449. The Examiner is requested to place his or her initials on the lines adjacent to the citations to indicate that the reference has been considered. The Examiner is further requested to fill in his or her name and the date the information was considered in blocks at the bottom of this substitute for Form PTO-1449.

/Maria Marvich/ 05/03/2008

Examiner Signature Date Considered

EAST Search History

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S114	5	S106 and (S111 or S112)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 17:08
S115	15	S106 and (S110 or S112)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 17:08
S110	150	hs2 same hs3 same hs4	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 17:07
S111	31721	hs adj "2" same hs adj "3" or hs adj "4"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 17:07
S112	6890	hs adj "2" same hs adj "3" same hs adj "4"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 17:07
S113	3	S106 and vector and (S111 or S112)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 17:07
S109	1	mouse adj pgk adj promoter same dhfr	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 16:52
S107	66	S106 and vector	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 16:51
S108	3	S94 and S107	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 16:51
S103	60	(hs2 or hs3 or hs4) same (lcr or locus adj control adj region)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 16:50

S104	2	heat adj sensitive same (lcr or locus adj control adj region)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 16:50
S105	82890	S101 or S102 or S104	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 16:50
S106	76	S103 or S102 or S104	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 16:50
S102	20	(hs adj "2" or hs adj "3" or hs adj "4") same (lcr or locus adj control adj region)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 16:49
S99	17	S97 and S98	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 16:48
S100	1620	hs2 or hs3 or hs4	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 16:48
S101	82888	hs adj "2" or hs adj "3" or hs adj "4"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 16:48
S94	12190	Sadelin.in. or Rivella.in. or May.in. or Bertino.in.	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 16:47
S95	358	globin same (lcr or locus adj control adj region)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 16:47
S96	0	betaglobin same (lcr or locus adj control adj region)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 16:47

S97	2509	globin and (lcr or locus adj control adj region)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 16:47
S98	58110	heat adj sensitive	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2009/01/09 16:47

1/10/2009 7:43:45 AM
C:\Documents and Settings\mmarvich.USPTO\My Documents\EAST\Workspaces\10188221.wsp

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(FILE 'HOME' ENTERED AT 08:12:44 ON 10 JAN 2009)

     FILE 'MEDLINE, CAPLUS, SCISEARCH' ENTERED AT 08:13:51 ON 10 JAN 2009
L1          1469 S GLOBIN AND LCR
L2           358 S L1 AND HS2
L3           105 S L1 AND HS (A) 2
L4           444 S L2 OR L3
L5           198 DUP REM L4 (246 DUPLICATES REMOVED)
L6           144 S L5 AND PY<=2001
L7             0 S HS2 SAME  HS3 SAME HS4
L8             0 S HS (A) 2 SAME  HS(A) 3 SAME HS (A) 4
L9           216 S LCR AND HS3
L10          166 S LCR AND HS4
L11            2 S L3 AND L9 AND L10
L12           13 S HS (A) 2 AND  HS(A) 3 AND  HS (A) 4
L13           15 S L11 OR L12
L14           12 DUP REM L13 (3 DUPLICATES REMOVED)
L15           11 S L14 AND PY<=2001
L16            4 S L15 AND GLOBIN
L17          164 S TI 1-4
L18        18269 S BETA-GLOBIN
L19           89 S L18 AND HS2 AND HS3 AND HS4
L20            4 S L18 AND HS(A) 2 AND HS (A) 3 AND HS(A) 4
L21            4 S L18 AND HS (A) 2 AND HS (A) 3 AND HS(A) 4
L22           93 S L19 OR L21
L23           43 DUP REM L22 (50 DUPLICATES REMOVED)
L24           32 S L23 AND PY<=2001
L25            9 S L24 AND VECTOR
L26            7 S L25 AND LCR
L27            8 S L25 AND LOCUS
L28            8 S L26 OR L27

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NOTICE OF ALLOWANCE AND FEE(S) DUE

21874 7590 01/26/2009

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P.O. BOX 55874
BOSTON, MA 02205

EXAMINER	
MARVICH, MARIA	
ART UNIT	PAPER NUMBER
1633	

DATE MAILED: 01/26/2009

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/188,221	07/01/2002	Michel Sadelain	64836(51590)	9026

TITLE OF INVENTION: VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$755	$300	$0	$1055	04/27/2009

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

B. If the status above is to be removed, check box 5b on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above, or

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check box 5a on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and 1/2 the ISSUE FEE shown above.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PTOL-85 (Rev. 08/07) Approved for use through 08/31/2010.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
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Alexandria, Virginia 22313-1450
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CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

21874 7590 01/26/2009

EDWARDS ANGELL PALMER & DODGE LLP
P.O. BOX 55874
BOSTON, MA 02205

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/188,221	07/01/2002	Michel Sadelain	64836(51590)	9026

TITLE OF INVENTION: VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$755	$300	$0	$1055	04/27/2009

EXAMINER	ART UNIT	CLASS-SUBCLASS
MARVICH, MARIA	1633	435-320100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 ________________________________

2 ________________________________

3 ________________________________

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. The following fee(s) are submitted:

❑ Issue Fee

❑ Publication Fee (No small entity discount permitted)

❑ Advance Order - # of Copies ______________________

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

❑ A check is enclosed.

❑ Payment by credit card. Form PTO-2038 is attached.

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number _____________ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

❑ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

❑ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature ____ Date ____

Typed or printed name ____ Registration No. ______________________________________

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

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United States Patent and Trademark Office
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www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/188,221	07/01/2002	Michel Sadelain	64836(51590)	9026

21874 7590 01/26/2009

EDWARDS ANGELL PALMER & DODGE LLP
P.O. BOX 55874
BOSTON, MA 02205

EXAMINER	
MARVICH, MARIA	
ART UNIT	PAPER NUMBER
1633	

DATE MAILED: 01/26/2009

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 0 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 0 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

PTOL-85 (Rev. 08/07) Approved for use through 08/31/2010.

Notice of Allowability	**Application No.** 10/188,221	**Applicant(s)** SADELAIN ET AL	
	Examiner MARIA B. MARVICH	**Art Unit** 1633	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *after final amendment 12/3/08*.
2. ☒ The allowed claim(s) is/are *1-18, 43, 44 and 47-50*.
3. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) ☐ All b) ☐ Some* c) ☐ None of the:
   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

4. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.
5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
   1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 10/8/02
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application
6. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
7. ☒ Examiner's Amendment/Comment
8. ☐ Examiner's Statement of Reasons for Allowance
9. ☐ Other _____.

DETAILED ACTION

This office action is in response to an after final amendment filed 12/3/08. The amendment has been entered. Claims 1-18, 43, 44 and 47-50 are pending in this application.

Information Disclosure Statement

The 1449 for the IDS filed 10/8/02 was incorrectly processed and therefore, a replacement 1449 accompanies this office action in which the IDS is properly signed.

EXAMINER'S AMENDMENT

An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in a telephone interview with Amy Leahy and Lisa Wilson on January 8, 2009.

The application has been amended as follows:

IN THE CLAIMS:

Claim 1. (Currently amended) A recombinant vector comprising~~: a region comprising a nucleotide sequence~~ a nucleic acid encoding a functional globin operably linked to [[:and]] a 3.2-kb nucleotide fragment which consists essentially of three contiguous nucleotide fragments

obtainable from a human β-globin locus control region (LCR), ~~said~~ the three fragments being [[an]] a BstXI and SnaBI HS2-spanning nucleotide fragment of said LCR ~~extending between BstXI and SnaBI restriction sites of a human B-globin locus control region (LCR)~~, [[an]] a BamHI and HindIII HS3-spanning nucleotide fragment ~~extending between BamHI and Hindlll restriction sites~~ of said LCR and [[an]] a BamHI and BanII HS4-spanning nucleotide fragment ~~extending between BamHI and Banll restriction sites~~ of said LCR, said vector providing expression of the globin ~~when introduced into~~ in a mammal *in vivo*.

Claim 2. (Currently amended) The vector of claim 1, further comprising a nucleic acid ~~region~~ encoding a dihydrofolate reductase.

Claim 3. (Currently amended) The vector of claim 2, further comprising a mouse PGK promoter [[,]] to control the expression of the dihydrofolate reductase.

Claim 11. (Currently amended) The vector of claim 10, further comprising a nucleic acid ~~region~~ encoding a dihydrofolate reductase.

Claim 12. (Currently amended) The vector of claim 11, further comprising a mouse PGK promoter[[,]] to control ~~wherein the mouse PGK promoter controls~~ the expression of the ~~region encoding a~~ dihydrofolate reductase.

Claim 43. (Currently amended) ~~The vector of claim 1,~~ A recombinant vector comprising a nucleic acid encoding a functional globin operably linked to a 3.2-kb nucleotide fragment which consists essentially of three nucleotide fragments obtainable from a human ß-globin LCR, the three fragments being a BstXI and SnaBI, HS2-spanning nucleotide fragment of said LCR, a BamHI and HindIII, HS3-spanning nucleotide fragment of said LCR, and a BamHI and BanII, HS4-spanning nucleotide fragment of said LCR, wherein the HS3-spanning nucleotide fragment and the HS4-spanning nucleotide fragment are adjacent to each other and ~~have~~ the vector further comprises 2 GATA-1 binding sites at the junction between the HS3-spanning and HS4-spanning nucleotide fragments, said vector providing expression of the globin in a mammal *in vivo*.

Claim 47. (Currently amended) The vector of claim [[1]] 43, wherein the vector is pTNS9.

Conclusion

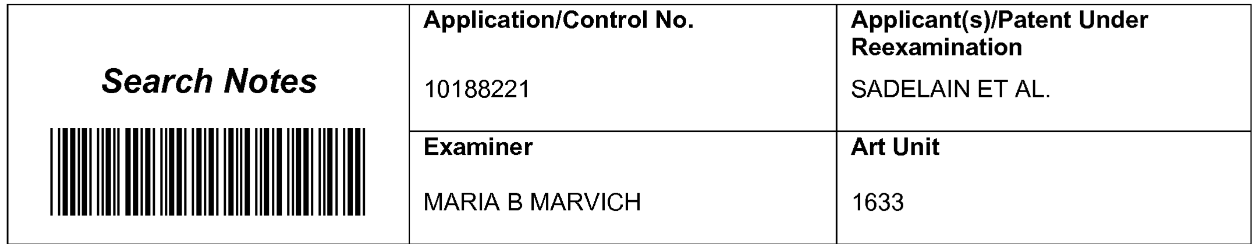
Any inquiry concerning this communication or earlier communications from the examiner should be directed to MARIA B. MARVICH whose telephone number is (571)272-0774. The examiner can normally be reached on M-F (7:00-4:00).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Joseph Woitach, PhD can be reached on (571)-272-0739. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Maria B Marvich, PhD
Primary Examiner
Art Unit 1633

/Maria B Marvich/
Primary Examiner, Art Unit 1633



Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	10188221	SADELAIN ET AL.
	Examiner	**Art Unit**
	MARIA B MARVICH	1633

SEARCHED

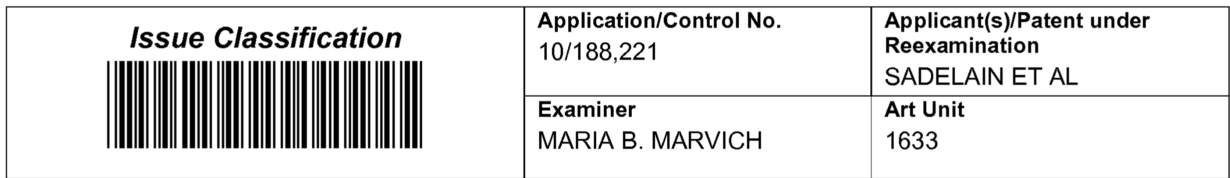
Class	Subclass	Date	Examiner

SEARCH NOTES

Search Notes	Date	Examiner
EAST, STN search updated, search notes updated	5/27/08	MM
East, STN search updated, search notes attached	12/31/08	MM
Consultation with Joe Woitach	12/31/08	MM

INTERFERENCE SEARCH

Class	Subclass	Date	Examiner



Issue Classification	Application/Control No.	Applicant(s)/Patent under Reexamination
	10/188,221	SADELAIN ET AL
	Examiner	Art Unit
	MARIA B. MARVICH	1633

ISSUE CLASSIFICATION

ORIGINAL		INTERNATIONAL CLASSIFICATION									
CLASS	SUBCLASS	CLAIMED					NON-CLAIMED				
435	320.1	C	12	N	15	/00					/

CROSS REFERENCES					INTERNATIONAL CLASSIFICATION (CLAIMED)					NON-CLAIMED				
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)				C	12	N	15	/10					/
435	69.1				C	12	N	15	/67					/
536	24.1	24.2			C	12	N	7	/01					/
424	93.2								/					/
									/					/
									/					/

(Assistant Examiner) (Date)	/Maria Marvich/ 1/8/09	Total Claims Allowed: **24**	
(Legal Instruments Examiner) (Date)	(Primary Examiner) (Date)	O.G. Print Claim(s)	O.G. Print Fig.
		1	No

☐ **Claims renumbered in the same order as presented by applicant** ☐ CPA ☐ T.D. ☐ R.1.47

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
1	1		31		61		91		121		151		181
2	2		32		62		92		122		152		182
3	3		33		63		93		123		153		183
4	4		34		64		94		124		154		184
5	5		35		65		95		125		155		185
6	6		36		66		96		126		156		186
7	7		37		67		97		127		157		187
8	8		38		68		98		128		158		188
9	9		39		69		99		129		159		189
10	10		40		70		100		130		160		190
11	11		41		71		101		131		161		191
12	12		42		72		102		132		162		192
13	13	23	43		73		103		133		163		193
14	14	22	44		74		104		134		164		194
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	26		56		86		116		146		176		206
	27		57		87		117		147		177		207
	28		58		88		118		148		178		208
	29		59		89		119		149		179		209
	30		60		90		120		150		180		210

AMENDMENT

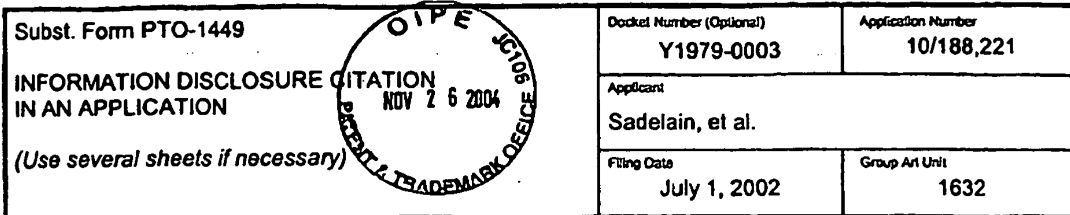
It is respectfully requested that the claims and specification be amended without prejudice, without admission, without surrender of subject matter, and without any intention of creating any estoppel as to equivalents, as follows.

In the Specification:

11 Kindly replace the paragraph beginning at page 13, line 24 and ending at page 14, line ~~12~~ with the following paragraph:

gm 3/2/09

--Donor bone marrow was flushed from the ~~temurs~~ tumors of 8- to 16-week old male c57/BL6 or Hbb$^{th3/+}$ mice[23] obtain from Jackson Laboratories (Bar Harbor, Me.) that had been injected intravenously (IV) 6 days earlier with 5-flurouracil (5-FU) 150 mg/kg body weight obtained from Pharmacia (Piscataway, N.J.). Bone marrow cells were resuspended in X-VIVO-15 serum-free medium and supplemented with 10 ng/mL interleukin-1α (IL-1 α 100 U/mL IL-3, 150 U/mL IL-6, 10 ng/mL Kit ligand obtained from Genzyme (Cambridge, Mass.), 0.5 mM β-mercaptoethanol obtained from Sigma (St. Louis, Mo.), 200-mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Bone marrow cells were ~~ten~~ then pelleted and resuspended in ~~serrum~~ serum-free medium containing concentrated lentiviral supernatant and supplemented with 8 mg/mL polybrene (Sigma), 200 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and cytokines as above, and ~~cultureed~~ cultured for 8 hours. Transduced bone marrow cells (5 X 10^5) were ~~ten~~ then injected IV into each of the irradiated female recipients to establish bone marrow chimeras. Recipients mice (11- to 14-week-old C57/BL6 or Hbb$^{th3/+}$ mice) were irradiated with 10.5 Gy (Split dose 2 X 5.25 Gy) on the day of transplantation.--



Subst. Form PTO-1449 INFORMATION DISCLOSURE CITATION IN AN APPLICATION *(Use several sheets if necessary)*	Docket Number (Optional): Y1979-0003	Application Number: 10/188,221
	Applicant: Sadelain, et al.	
	Filing Date: July 1, 2002	Group Art Unit: 1632

OIPE JC106 NOV 2 6 2004 PATENT & TRADEMARK OFFICE

U. S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
[initials]	5,126,260	6/30/92	Tuan et al.			
	5,631,162	5/20/97	LeBoulch et al.			
	5,834,256	11/10/98	Finer, et al.			
	5,858,740	1/12/99	Finer et al.			
	5,981,276	11/9/99	Sodroski et al.			
	5,994,136	11/30/99	Naldini et al.			
	~~6,103,516~~	1/11/00	Verma et al.			6013516
	6,218,187	4/17/01	Finer et al.			
	6,294,165	9/25/01	Lever et al.			
	6,312,682	11/6/01	Kingsman et al.			
	6,428,953	8/6/02	Naldini et al.			
	6,524,851	2/25/03	Ellis			
	6,544,771	4/8/03	Rivière et al.			

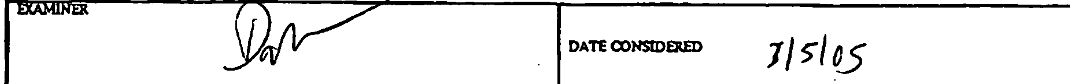
CQ 3/5/09

FOREIGN PATENT DOCUMENTS

	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	Translation YES	Translation NO

OTHER DOCUMENTS *(Including Author, Title, Date, Pertinent Pages, Etc.)*

[initials]	Dull *et al.* (1998) J. Virol. 72:8463-8471, "A Third-Generation Lentivirus Vector with a Conditional Packaging System"
	Naldini et al. (1996) Science 272:263-267, "In Vivo Gene Delivery and Stable Transduction of Nondividing Cells by a Lentiviral Vector"
	Sadelain et al. (1995) Proc. Natl. Acad. Sci. 92:6728-6732, "Generation of a high-titer retroviral vector capable of expressing high levels of the human ß-globin gene"



EXAMINER [signature]	DATE CONSIDERED 3/5/05

EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP § 609; Draw line through citation if not in conformance and not considered. Include copy with next communication to applicant.

NY\264242.1

11/29/2004 EAREGAY1 00000016 10188221
02 FC:1806 180.00 OP

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Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
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or Fax (571) 273-2885

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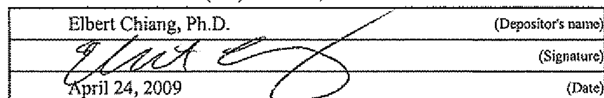
Elbert Chiang, Ph.D. (Depositor's name)


(Signature)

April 24, 2009 (Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/188,221	07/01/2002	Michel Sadelain	64836(51590)	9026

TITLE OF INVENTION: VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES

APPLN. TYPE	SMALL ENTITY	ISSUE FEE	PUBLICATION FEE	TOTAL FEE(S) DUE	DATE DUE
Non-Provisional	yes	$755.00	$300.00	$1,055.00	04/27/2009

EXAMINER	ART UNIT	CLASS-SUBCLASS
M. Marvich	1633	435-320100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
   - [ ] Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
   - [ ] "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list (1) the names of up to 3 registered patent attorneys or agents OR, alternatively, (2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.
   1 Edwards Angell Palmer & Dodge LLP
   2 Peter C. Lauro, Esq.
   3

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(A) NAME OF ASSIGNEE: Memorial Sloan-Kettering Cancer Center

(B) RESIDENCE: (CITY and STATE OR COUNTRY): 1275 York Avenue, New York, NY 10021

Please check the appropriate assignee category or categories (will not be printed on the patent): [ ] Individual [x] Corporation or other private group entity [ ] Government

4a. The following fee(s) are enclosed:
- [x] Issue Fee
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- [ ] Payment by credit card. Form PTO-2038 is attached.
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5. **Change in Entity Status** (from status indicated above)
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Authorized Signature ______ Date April 24, 2009

Typed or printed name Elbert Chiang, Ph.D. Registration No. 60,325

Electronic Patent Application Fee Transmittal

Application Number:	10188221
Filing Date:	01-Jul-2002
Title of Invention:	VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES
First Named Inventor/Applicant Name:	Michel Sadelain
Filer:	Elbert C. Chiang/Alyson Lucas
Attorney Docket Number:	64836(51590)

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Publ. Fee- early, voluntary, or normal	1504	1	300	300
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
		Total in USD ($)		**300**

Electronic Acknowledgement Receipt

EFS ID:	5216700
Application Number:	10188221
International Application Number:	
Confirmation Number:	9026
Title of Invention:	VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES
First Named Inventor/Applicant Name:	Michel Sadelain
Customer Number:	21874
Filer:	Elbert C. Chiang/Alyson Lucas
Filer Authorized By:	Elbert C. Chiang
Attorney Docket Number:	64836(51590)
Receipt Date:	24-APR-2009
Filing Date:	01-JUL-2002
Time Stamp:	16:14:38
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$300
RAM confirmation Number	2335
Deposit Account	041105
Authorized User	

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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	6483651590_-_64836_51590_certificate_of_efiling_001_001PDF.PDF	22778 cf6c5f79f094655e09d84090e862f63e826af58d	no	1
Warnings:					
Information:					
2	Issue Fee Payment (PTO-85B)	6483651590_-_64836_51590_PartB_fee_transmittal_001_001PDF.PDF	91093 2f38cedaf924e503013adc6b00dcebde57a0f4ec	no	1
Warnings:					
Information:					
3	Fee Worksheet (PTO-875)	fee-info.pdf	30473 ce41781c003a6eed35e03aa7f5b38b90ad8d19ad	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	144344		

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<u>New Applications Under 35 U.S.C. 111</u>
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<u>National Stage of an International Application under 35 U.S.C. 371</u>
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

<u>New International Application Filed with the USPTO as a Receiving Office</u>
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Certificate of Electronic Filing Under 37 CFR 1.8

I hereby certify that this correspondence is being transmitted via the Office electronic filing system in accordance with 37 CFR 1.6(a)(4):

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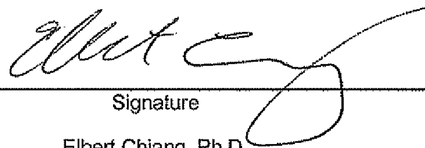
on April 24, 2009 .
Date



Signature

Elbert Chiang, Ph.D.
Typed or printed name of person signing Certificate

60,325
Registration Number, if applicable

(617) 517-5502
Telephone Number

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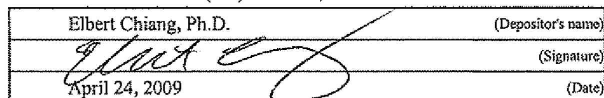
Elbert Chiang, Ph.D. (Depositor's name)



(Signature)

April 24, 2009 (Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/188,221	07/01/2002	Michel Sadelain	64836(51590)	9026

TITLE OF INVENTION: VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES

APPLN. TYPE	SMALL ENTITY	ISSUE FEE	PUBLICATION FEE	TOTAL FEE(S) DUE	DATE DUE
Non-Provisional	yes	$755.00	$300.00	$1,055.00	04/27/2009

EXAMINER	ART UNIT	CLASS-SUBCLASS
M. Marvich	1633	435-320100

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   - ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
   - ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list (1) the names of up to 3 registered patent attorneys or agents OR, alternatively, (2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.
   1. Edwards Angell Palmer & Dodge LLP
   2. Peter C. Lauro, Esq.
   3.

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE	(B) RESIDENCE: (CITY and STATE OR COUNTRY)
Memorial Sloan-Kettering Cancer Center	1275 York Avenue, New York, NY 10021

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

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- ☒ Issue Fee
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   - ☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.
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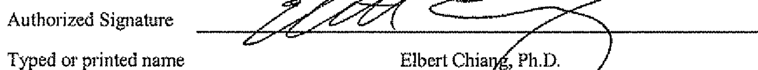
Authorized Signature  Date April 24, 2009

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PTOL-85 (Rev. 08/08) Approved for use through 08/31/2010. OMB 0651-0033 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Electronic Patent Application Fee Transmittal

Application Number:	10188221
Filing Date:	01-Jul-2002
Title of Invention:	VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES
First Named Inventor/Applicant Name:	Michel Sadelain
Filer:	Elbert C. Chiang/Alyson Lucas
Attorney Docket Number:	64836(51590)

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Utility Appl issue fee	2501	1	755	755
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Printed copy of patent - no color	8001	10	3	30
		Total in USD ($)		**785**

Electronic Acknowledgement Receipt

EFS ID:	5217237
Application Number:	10188221
International Application Number:	
Confirmation Number:	9026
Title of Invention:	VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES
First Named Inventor/Applicant Name:	Michel Sadelain
Customer Number:	21874
Filer:	Elbert C. Chiang/Alyson Lucas
Filer Authorized By:	Elbert C. Chiang
Attorney Docket Number:	64836(51590)
Receipt Date:	24-APR-2009
Filing Date:	01-JUL-2002
Time Stamp:	16:44:42
Application Type:	Utility under 35 USC 111(a)

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Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$785
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Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance fees)

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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	PartBfeetransmittal001001.PDF	91093 bbbae26776ad38a9aca79b62ab39754c39f839ce	no	1
Warnings:					
Information:					
2	Fee Worksheet (PTO-875)	fee-info.pdf	32268 8cd4d8f06524a563645e5904bc858454096d5823	no	2
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Information:					
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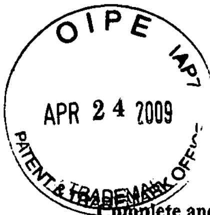

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Elbert Chiang, Ph.D.	(Depositor's name)
	(Signature)
April 24, 2009	(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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Authorized Signature ________ Date April 24, 2009
Typed or printed name Elbert Chiang, Ph.D. Registration No. 60,325

04/27/2009 INTEFSW 00002335 10188221
01 FC:1504 300.00 DA

PTOL-85 (Rev. 08/08) Approved for use through 08/31/2010. OMB 0651-0033 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

04/27/2009 INTEFSW 00002763 10188221
01 FC:2501 755.00 DA
02 FC:8001 30.00 DA

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/188,221	06/02/2009	7541179	64836(51590)	9026

21874 7590 05/13/2009

EDWARDS ANGELL PALMER & DODGE LLP
P.O. BOX 55874
BOSTON, MA 02205

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 431 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Michel Sadelain, New York, NY;
Stefano Rivella, New York, NY;
Chad May, New York, NY;
Joseph Bertino, New York, NY;

IR103 (Rev. 11/05)

Docket No.: 64836(51590)
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re U.S. Patent No. 7,541,179 of:
Sadelain, *et al.*

Issued: June 2, 2009

Application No.: 10/188,221 | Confirmation No.: 9026

Filed: July 1, 2002 | Art Unit: 1633

For: *VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES* | Examiner: Maria Marvich

REQUEST FOR RECONSIDERATION OF PATENT TERM ADJUSTMENT UNDER 37 C.F.R. §1.705(d)

MS Petition
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

This is a Request for Reconsideration of Patent Term Adjustment (PTA) under 37 C.F.R. § 1.705(d) (or, alternatively, a Petition for Review of Patent Term Adjustment under 37 C.F.R. §§ 1.181 & 1.705(d), if appropriate) to correct the Patent Term Adjustment for the above-referenced patent application.

It is respectfully submitted that the Patent Term Adjustment of four hundred thirty-one (431) days accorded by the United States Patent and Trademark Office ("Office") to the above-referenced application in the Issue Notification and the "Determination of Patent Term Adjustment" mailed May 13, 2009, and as shown on the face of the issued patent, is not correct, or at least is not complete. Applicants respectfully request the grant of a ***minimum*** Patent Term Adjustment of six hundred eighty-three (683) days.

STATEMENT OF FACTS

1. Applicants filed the instant application pursuant to 35 U.S.C. § 111(a) on July 1, 2002.

2. A Notice to File Missing Parts of Non-provisional Application was mailed from the Office on September 13, 2002. Applicants filed a response on October 8, 2002 (within three months of the mailing date of the Office Action).

3. The Office mailed a Restriction Requirement on May 5, 2004. This was the first action under 35 U.S.C. § 132 in the application. As this first action was mailed more than 14 months after the date of filing of the application, the application is entitled to 247 days of PTA pursuant to 37 C.F.R. § 1.703(a)(1), as shown on the Patent Term Adjustments page obtained from the PAIR record for the above-referenced patent, a copy of which is attached hereto as Appendix A. Applicants filed a response on June 1, 2004 (within three months of the mailing date of the Restriction Requirement).

4. An Office Action was mailed from the Office on August 25, 2004. Applicants filed a response on November 26, 2004. The Response filed on November 26, 2004, and Applicants were charged with 1 day of delay, as shown in Appendix A.

5. The Office mailed an Office Action on March 31, 2005. As the Office Action of March 31, 2005 was mailed more than 4 months after the date a reply under 37 C.F.R. § 1.111 was filed, the application is entitled to 5 days of PTA pursuant to 37 C.F.R. § 1.703(a)(2), as shown in Appendix A. Applicants filed a response on June 30, 2005 (within three months of the mailing date of the Office Action).

6. An Office Action was mailed from the Office on October 4, 2005. Applicants filed a response on February 6, 2006. The Response filed on February 6, 2006, and Applicants were charged with 33 days of delay, as shown in Appendix A.

7. The Office mailed a Notice on April 4, 2006, stating that the Office regarded the Response filed on February 6, 2006, as informal and non-responsive to the Office Action mailed October 4, 2005. Applicants filed a response on May 4, 2006. The Response filed on May 4, 2006, and Applicants were charged with 87 days of delay, as shown in Appendix A.

8. An Office Action was mailed from the Office on July 31, 2006. Applicants filed a response on November 30, 2006. The Response filed on November 30, 2006, and Applicants were charged with 30 days of delay, as shown in Appendix A.

9. Applicants filed an Information Disclosure Statement (IDS) on December 8, 2006. The IDS was filed as an IDS after an Office Action on the merits pursuant to 37 C.F.R. 1.97(c)(2). The IDS filed on December 8, 2006, and Applicants were charged with 8 days of delay, as shown in Appendix A.

10. A Final Office Action was mailed from the Office on March 12, 2007. On September 12, 2007, Applicants filed a Request for Continued Examination (RCE) and a Response to the Final Office Action. Applicants were charged with 92 days of delay, as shown in Appendix A.

11. On October 30, 2007, a new, non-final Office Action was mailed from the Office. Applicants filed a response on February 29, 2008. The Response filed on February 29, 2008, and Applicants were charged with 30 days of delay, as shown in Appendix A.

12. A Final Office Action was mailed from the Office on June 3, 2008. On December 3, 2008, Applicants filed a response and a Notice of Appeal. The Response and Notice of Appeal filed on December 3, 2008, and Applicants were charged with 91 days of delay, as shown in Appendix A.

13. A Notice of Allowance (together with related papers) was mailed on January 26, 2009. The Notice of Allowance included a "Determination of Patent Term Adjustment Under 34 U.S.C. 154(b)" that indicated that the application would be entitled to 0 days of PTA under 35 U.S.C. § 154(b), "[i]f the issue fee is paid on the date that is three

months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice". Applicants timely paid the Issue Fee on April 24, 2009.

14. An Issue Notification was mailed on May 13, 2009. The Issue Notification included a "Determination of Patent Term Adjustment Under 34 U.S.C. 154(b)" that indicated that the application would be entitled to 431 days of PTA under 35 U.S.C. § 154(b) (apparently calculated as 803 days of the Office delay under 35 U.S.C. § 154(b)(1)(B), as discussed in paragraph 10, above, less 372 days of Applicant delay, as discussed in paragraphs 4, 6, 7, 8, 9, 10, 11, and 12, above).

15. The application issued as U.S. Patent 7,541,179 on June 2, 2009 (more than three years after the date that the application was filed).

16. A review of Appendix A reveals that the Office accorded, as of June 2, 2009 (the issue date), eight hundred three (803) days of Patent Term Adjustment attributable to the Office delay under 35 U.S.C. § 154(b)(1)(B) (albeit reduced by 372 days of Applicant delay). Thus, the total Patent Term Adjustment accorded by the Office, and shown on the face-page of the patent, is four hundred thirty-one (431) days.

17. During prosecution, there was no request for continued examination, no interference proceeding, no imposition of a secrecy order, and no review by the Board of Patent Appeals and Interferences or a Federal Court, other than the Request for Continued Examination filed on September 12, 2007 as discussed at Paragraph 10 herein. The instant patent is not subject to a terminal disclaimer.

18. Except as set forth above, Applicants submit that there were no circumstances constituting a failure by Applicants to engage in reasonable efforts to conclude processing or examination of the patent application.

19. As the instant patent issued on June 2, 2009, and this Request/Petition is filed no later than two months after the issue date, as required by 37 C.F.R. § 1.705(d), this paper is timely filed.

20. The Director is authorized to charge the $200.00 fee for this Request, pursuant to 37 C.F.R. § 1.18(e), to our Deposit Account No. 04-1105 under Order No. 64836(51590).

APPLICANTS ARE ENTITLED TO ADDITIONAL PATENT TERM ADJUSTMENT UNDER 35 U.S.C. § 154(b)(1)(A)

21. From an inspection of Appendix A, Applicants calculate 252 days of delay attributable to the Office under 35 U.S.C. § 154(b)(1)(A) ("The A Delay") as follows: the sum of 247 days pursuant to 37 C.F.R. § 1.703(a)(1) as set forth in paragraph 3 and 5 days pursuant to 37 C.F.R. § 1.703(a)(1) as set forth in paragraph 5.

22. As discussed above in paragraph 10, on September 12, 2007, Applicants filed a Request for Continued Examination (RCE) and a Response to the Final Office Action dated March 12, 2007. The date of September 12, 2007 is 803 days after the date (July 1, 2005) that is three years from the fling date of the application. From an inspection of Appendix A, Applicants calculate 803 days of delay attributable to the Office under 35 U.S.C. § 154(b)(1)(B) ("The B Delay").

23. As discussed above in paragraph 16, an inspection of Appendix A reveals that the Office accorded, as of June 2, 2009 (the issue date), eight hundred three (803) days of Patent Term Adjustment attributable to the Office delay under 35 U.S.C. § 154(b)(1)(B) (albeit reduced by 372 days of Applicant delay). Thus total Patent Term Adjustment accorded by the Office is four hundred thirty-one (431) days.

24. A further inspection of Appendix A reveals that the Office did not accord any days of Patent Term Adjustment under 35 U.S.C. § 154(b)(1)(A); *i.e.*, The A Delay. Because the Office did not accord any days of Patent Term Adjustment under 35 U.S.C. § 154(b)(1)(A), it appears that the Office either (i) has not yet calculated any Patent Term Adjustment due under 35 U.S.C. § 154(b)(1)(A), or (ii) has incorrectly applied the "double-counting" provision of 35 U.S.C. § 154(b)(2)(A).

25. 35 U.S.C. § 154(b)(2)(A) provides:

> To the extent that periods of delay attributable to grounds specified in paragraph (1) overlap, the period of any adjustment granted under this subsection shall not exceed the actual number of days the issuance of the patent was delayed.

26. In a Memorandum Opinion issued on September 30, 2008 in *Wyeth v. Dudas*, the U.S. District Court for the District of Columbia held that Office's interpretation of § 154(b)(2)(A), as published at 69 Fed. Reg. 34238 and (apparently or potentially) applied to the present patent application/patent, is incorrect. *Wyeth v. Dudas*, 580 F. Supp. 2d. 138, 88 U.S.P.Q.2d 1538 (D.D.C. 2008), *appeal docketed* No. 08-5502 (Fed. Cir. Dec. 24, 2008).

27. The *Wyeth* court held that "[t]he only way that periods of time can 'overlap' is if they occur on the same day." *Id.* Accordingly if a delay under § 154(b)(1)(A) occurs on one calendar day, and a delay under § 154(b)(1)(B) occurs on another day, "they do not overlap, and § 154(b)(2)(A) does not limit the extension to one day." *Id.*

28. Applicants respectfully submit that the two hundred fifty two (252) days of Office delay under § 154(b)(1)(A) (The A Delay) do not overlap with the delay under § 154(b)(1)(B), which is eight hundred three (803) days (The B Delay - the time period between the day after the date that is three years after the filing date of the application (*i.e.*, July 1, 2005) and the date a Request for Continued Examination was filed (*i.e.*, September 12, 2007)).

29. The non-overlapping nature of the 252 days of Office delay under § 154(b)(1)(A) (The A Delay) and the 803 day delay under § 154(b)(1)(B) (The B Delay) in this application is illustrated in Figure 1 below.

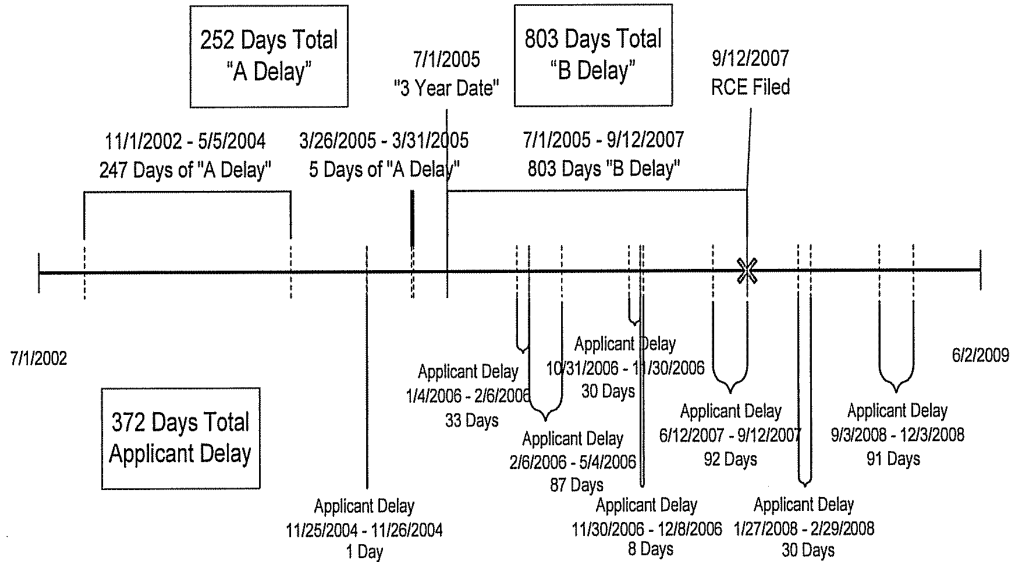


Figure 1

30. Therefore, Applicants contend that the present patent is entitled to an additional two hundred fifty-two (252) days of patent term adjustment under § 154(b)(1)(A).

RELIEF REQUESTED

31. Applicants respectfully request that the Office (i) properly calculate, pursuant to the *Wyeth* decision, the Patent Term Adjustment under 35 U.S.C. § 154(b)(1)(A-B), 35 U.S.C. § 154(b)(2), and 37 C.F.R. § 1.702(a-c) to which the instant patent is entitled, and (ii) grant Patent Term Adjustment equal to the sum of **six hundred eighty-three (683) days** (the sum of the delays under § 154(b)(1)(A) (252 days) and § 154(b)(1)(B) (803 days), less 372 days of Applicant delay, as set forth above).

32. Applicants believe that there are no further fees due in connection with this Petition other than the fee under 37 C.F.R. § 1.18(e). However, if additional fees are due, the Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 04-1105 under Order No. 64836 (51590).

Dated: July 31, 2009

Respectfully submitted,

Electronic signature: /Peter C. Lauro/
Peter C. Lauro, Esq.
Registration No.: 32,360
EDWARDS ANGELL PALMER & DODGE LLP
P.O. Box 55874
Boston, Massachusetts 02205
(617) 517-5509
Attorneys/Agents For Applicants

Attachment (Appendix A)

APPENDIX A

10/188,221	VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES	07-30-2009::10:25:56

Patent Term Adjustments

Patent Term Adjustment (PTA) for Application Number: 10/188,221

Filing or 371(c) Date:	07-01-2002	USPTO Delay (PTO) Delay (days):	803
Issue Date of Patent:	06-02-2009	Three Years:	-
Pre-Issue Petitions (days):	+0	Applicant Delay (APPL) Delay (days):	372
Post-Issue Petitions (days):	+0	Total PTA (days):	431
USPTO Adjustment(days):	+0	Explanation Of Calculations	

Patent Term Adjustment History

Date	Contents Description	PTO(Days)	APPL(Days)
05-13-2009	PTA 36 Months	551	
06-02-2009	Patent Issue Date Used in PTA Calculation		
04-27-2009	Dispatch to FDC	⇑	
04-27-2009	Application Is Considered Ready for Issue	⇑	
04-24-2009	Issue Fee Payment Verified	⇑	
04-24-2009	Issue Fee Payment Received	⇑	
02-09-2009	Sequence Forwarded to Pubs on Tape	⇑	
01-26-2009	Mail Notice of Allowance	⇑	
01-16-2009	Document Verification	⇑	
01-16-2009	Notice of Allowance Data Verification Completed	⇑	
01-16-2009	Case Docketed to Examiner in GAU	⇑	
01-15-2009	Examiner's Amendment Communication	⇑	
12-10-2008	Date Forwarded to Examiner	⇑	
12-03-2008	Amendment/Argument after Notice of Appeal	⇑	
12-03-2008	Notice of Appeal Filed		91
12-03-2008	Request for Extension of Time - Granted		⇑
06-03-2008	Mail Final Rejection (PTOL - 326)		⇑
05-28-2008	Final Rejection		
03-18-2008	Date Forwarded to Examiner		
02-29-2008	Response after Non-Final Action		30
02-29-2008	Request for Extension of Time - Granted		⇑
03-17-2008	Mail Examiner Interview Summary (PTOL - 413)		⇑
02-26-2008	Examiner Interview Summary Record (PTOL - 413)		⇑
10-30-2007	Mail Non-Final Rejection		⇑
10-27-2007	Non-Final Rejection		
08-02-2007	Information Disclosure Statement considered		
09-12-2007	Affidavit(s) (Rule 131 or 132) or Exhibit(s) Received		
09-15-2007	Date Forwarded to Examiner		
09-15-2007	Date Forwarded to Examiner		
09-12-2007	Request for Continued Examination (RCE)		
09-15-2007	DISPOSAL FOR A RCE/CPA/129 (express abandonment if		

APPENDIX A

Date	Description	Pages
	CPA)	
09-12-2007	Notice of Appeal Filed	92
09-12-2007	Request for Extension of Time - Granted	⇑
09-12-2007	Workflow - Request for RCE - Begin	⇑
08-10-2007	Mail Examiner Interview Summary (PTOL - 413)	⇑
08-02-2007	Miscellaneous Incoming Letter	⇑
08-02-2007	Information Disclosure Statement (IDS) Filed	⇑
08-05-2007	Examiner Interview Summary Record (PTOL - 413)	⇑
08-02-2007	Information Disclosure Statement (IDS) Filed	⇑
08-25-2004	Information Disclosure Statement considered	⇑
08-25-2004	Information Disclosure Statement (IDS) Filed	⇑
03-12-2007	Mail Final Rejection (PTOL - 326)	⇑
03-05-2007	Final Rejection	
12-08-2006	Information Disclosure Statement considered	
12-08-2006	Information Disclosure Statement (IDS) Filed	8
12-08-2006	Information Disclosure Statement (IDS) Filed	
12-18-2006	Date Forwarded to Examiner	⇑
11-30-2006	Response after Non-Final Action	30
11-30-2006	Request for Extension of Time - Granted	⇑
10-23-2006	Mail Examiner Interview Summary (PTOL - 413)	⇑
10-11-2006	Examiner Interview Summary Record (PTOL - 413)	⇑
08-03-2006	Correspondence Address Change	⇑
07-31-2006	Mail Non-Final Rejection	⇑
07-24-2006	Non-Final Rejection	
10-08-2002	Information Disclosure Statement considered	
10-08-2002	Information Disclosure Statement (IDS) Filed	
10-08-2002	Information Disclosure Statement (IDS) Filed	
05-15-2006	Date Forwarded to Examiner	
05-04-2006	Response after Non-Final Action	87
04-04-2006	Mail Notice of Informal or Non-Responsive Amendment	⇑
04-04-2006	Correspondence Address Change	⇑
04-04-2006	Change in Power of Attorney (May Include Associate POA)	⇑
03-13-2006	Receipt of all Acknowledgement Letters	⇑
02-08-2006	Date Forwarded to Examiner	⇑
02-06-2006	Informal or Non-Responsive Amendment after Examiner Action	⇑
02-06-2006	Response after Non-Final Action	33
02-06-2006	Request for Extension of Time - Granted	⇑
10-12-2005	Correspondence Address Change	⇑
10-04-2005	Mail Non-Final Rejection	⇑
10-03-2005	Non-Final Rejection	
07-20-2005	Request for Refund	

APPENDIX A

07-25-2005	Date Forwarded to Examiner		
06-30-2005	Supplemental Response		
07-18-2005	Case Docketed to Examiner in GAU		
06-30-2005	Electronic Information Disclosure Statement		
06-30-2005	Information Disclosure Statement (IDS) Filed		
07-07-2005	Date Forwarded to Examiner		
06-30-2005	Response after Non-Final Action		
07-05-2005	CRF Is Good Technically / Entered into Database		
06-28-2005	Case Docketed to Examiner in GAU		
03-31-2005	Mail Non-Final Rejection	5	
03-07-2005	Non-Final Rejection	⇑	
01-11-2005	Case Docketed to Examiner in GAU	⇑	
12-29-2004	Date Forwarded to Examiner	⇑	
11-26-2004	Response after Non-Final Action		1
11-26-2004	Reference capture on IDS		⇑
11-26-2004	Information Disclosure Statement (IDS) Filed		⇑
11-26-2004	Information Disclosure Statement (IDS) Filed		⇑
11-26-2004	Affidavit(s) (Rule 131 or 132) or Exhibit(s) Received		⇑
11-26-2004	Workflow incoming amendment IFW		⇑
08-25-2004	Mail Non-Final Rejection		⇑
08-23-2004	Non-Final Rejection		
06-21-2004	Date Forwarded to Examiner		
06-01-2004	Response to Election / Restriction Filed		
06-01-2004	Workflow incoming amendment IFW		
05-05-2004	Mail Restriction Requirement	247	
05-03-2004	Requirement for Restriction / Election	⇑	
11-17-2003	Information Disclosure Statement (IDS) Filed	⇑	
11-17-2003	Information Disclosure Statement (IDS) Filed	⇑	
09-08-2003	IFW TSS Processing by Tech Center Complete	⇑	
07-15-2003	Case Docketed to Examiner in GAU	⇑	
10-28-2002	Application Dispatched from OIPE	⇑	
10-24-2002	Application Is Now Complete	⇑	
10-08-2002	Additional Application Filing Fees	⇑	
10-08-2002	A statement by one or more inventors satisfying the requirement under 35 USC 115, Oath of the Applic	⇑	
09-24-2002	Letter to Applicant - No government Interest / Patent to Issue	⇑	
09-13-2002	Notice Mailed--Application Incomplete--Filing Date Assigned	⇑	
09-06-2002	Referred by L&R for Third-Level Security Review. Agency Referral Letter Generated	⇑	
09-06-2002	Cleared by L&R (LARS)	⇑	
09-05-2002	IFW Scan & PACR Auto Security Review	⇑	

APPENDIX A

09-04-2002	IFW Scan & PACR Auto Security Review	⇑
07-01-2002	Initial Exam Team nn	⇑

Close Window

Electronic Patent Application Fee Transmittal

Application Number:	10188221
Filing Date:	01-Jul-2002
Title of Invention:	VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES
First Named Inventor/Applicant Name:	Michel Sadelain
Filer:	Peter C. Lauro
Attorney Docket Number:	64836(51590)

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Application for patent term adjustment	1455	1	200	200
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
		Total in USD ($)		**200**

Electronic Acknowledgement Receipt

EFS ID:	5808039
Application Number:	10188221
International Application Number:	
Confirmation Number:	9026
Title of Invention:	VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES
First Named Inventor/Applicant Name:	Michel Sadelain
Customer Number:	21874
Filer:	Peter C. Lauro
Filer Authorized By:	
Attorney Docket Number:	64836(51590)
Receipt Date:	31-JUL-2009
Filing Date:	01-JUL-2002
Time Stamp:	14:17:18
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$200
RAM confirmation Number	435
Deposit Account	041105
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	64836CertificateOfElectronicFiling.pdf	39416 7602108f094a421f4e27787724e4087a8ed0fab9	no	1
Warnings:					
Information:					
2	Patent Term Adjustment Petition	64836RequestForReconsiderationOfPatentTermAdjustment.pdf	629510 23dbf513eae8522a20bf82b497354609f3c30fb5	no	12
Warnings:					
Information:					
3	Fee Worksheet (PTO-875)	fee-info.pdf	30426 809fd21a921776cb6d95445247d2e7bac5ede3a7	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	699352	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Application No. (if known): 10/188,221
U.S. Patent 7,541,179

Attorney Docket No.: 64836(51590)

Certificate of Electronic Filing Under 37 CFR 1.8

I hereby certify that this correspondence is being transmitted via the Office electronic filing system in accordance with 37 CFR 1.6(a)(4):

MS Petition
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

on July 31, 2009 .
Date

/Peter C. Lauro/
Signature

Peter C. Lauro, Esq.
Typed or printed name of person signing Certificate

32,360
Registration Number, if applicable

(617) 517-5509
Telephone Number

Note: Each paper must have its own certificate of mailing.

Request for Reconsideration of Patent Term Adjustment (12 pages)
Charge $200.00 to Deposit Acount No. 04-1105

IN THE UNITED STATES DISTRICT COURT FOR THE

DISTRICT OF COLUMBIA

MEMORIAL SLOAN-KETTERING CANCER CENTER 1275 York Avenue New York, NY 10021 Plaintiff, v. HON. DAVID KAPPOS Under Secretary of Commerce for Intellectual Property and Director of the United States Patent and Trademark Office Office of the General Counsel United States Patent and Trademark Office 600 Dulaney Street Arlington, VA 22314 Defendant.	Civil Action No. ______________

COMPLAINT

Plaintiff Memorial Sloan-Kettering Cancer Center, for its Complaint against the Honorable David Kappos, states as follows:

1. This is an action by the owner of U.S. Patent No. 7,541,179 ("the '179 patent") seeking review of inaccurate and erroneous patent term adjustment calculations made by the United States Patent and Trademark Office ("USPTO"). Specifically, this is an action by Plaintiff under 35 U.S.C. § 154(b)(4)(A) seeking a judgment that the patent term adjustment of 431 days calculated by the USPTO for the '179 patent should be corrected to 683 days.

2. This action arises under 35 U.S.C. § 154 and the Administrative Procedure Act, 5 U.S.C. §§ 701-706.

THE PARTIES

3. Plaintiff Memorial Sloan-Kettering Cancer Center is a corporation organized under the laws of the state of New York and having a principal place of business at 1275 York Avenue, New York, NY 10021.

4. Defendant David Kappos is the Under Secretary of Commerce for Intellectual Property and Director of the USPTO, acting in his official capacity. The Director of the USPTO ("Director") is responsible for superintending or performing all duties required by law with respect to the granting and issuing of patents, and is designated by 35 U.S.C. § 154(b)(3)(B) as the official responsible for determining the period of patent term adjustment.

JURISDICTION AND VENUE

5. This Court has jurisdiction over this action and is authorized to issue the requested relief to Plaintiff pursuant to 28 U.S.C. §§ 1331, 1338(a) and 1361, 35 U.S.C. § 154(b)(4)(A) and 5 U.S.C. §§ 701-706.

6. Venue is proper in this district by virtue of 35 U.S.C. § 154(b)(4)(A).

7. This Complaint is timely filed in accordance with 35 U.S.C. § 154(b)(4)(A).

FACTS

8. On June 2, 2009, the USPTO issued the '179 patent to Plaintiff based on U.S. Patent Application Serial No. 10/188,221, entitled "Vector Encoding Human Globin Gene And Use Thereof In Treatment Of Hemoglobinopathies," and filed on July 1, 2002 by Michel Sadelain, Stefano Rivella, Chad May and Joseph Bertino. A true copy of the '179 patent is attached hereto as Exhibit 1.

9. Plaintiff Memorial Sloan-Kettering Cancer Center is the assignee and owner of the '179 patent as evidenced by an assignment executed by Michel Sadelain, Stefano Rivella, Chad May

and Joseph Bertino and recorded with the USPTO at Reel 013256, Frame 0983 of the USPTO assignment records. Plaintiff Memorial Sloan-Kettering Cancer Center is the real party in interest in this case.

10. Pursuant to 35 U.S.C. § 154, the Director is required to grant a patent term adjustment in accordance with the provisions of 35 U.S.C. § 154(b). Specifically, 35 U.S.C. § 154(b)(3)(D) states that "[t]he Director shall proceed to grant the patent after completion of the Director's determination of a patent term adjustment under the procedures established under this subsection, notwithstanding any appeal taken by the applicant of such determination."

11. In calculating the patent term adjustment, the Director is required to consider USPTO delays under 35 U.S.C. § 154(b)(1)(A) and (B), any overlapping periods in the USPTO delays under 35 U.S.C. § 154(b)(2)(A), and any applicant delays under 35 U.S.C. § 154(b)(2)(C).

12. The patent term adjustment for the '179 patent, as determined by the Defendant under 35 U.S.C. § 154(b) and listed on the face of the '179 patent, is 431 days. *See* Exhibit 1 at 1.

13. Had the USPTO calculated the patent term adjustment properly, the '179 patent would be entitled to 683 days of patent term adjustment.

14. The errors in the USPTO's patent term adjustment calculation are detailed in a recent decision from this Court in *Wyeth v. Dudas*, 580 F. Supp. 2d 138, 88 U.S.P.Q.2d 1538 (BNA) (D.D.C. 2008), *argued*, No. 2009-1120 (Fed. Cir. Oct. 7, 2009), where this Court granted summary judgment against the USPTO holding that the USPTO's patent term adjustment calculation methodology was erroneous as a matter of law and inconsistent with the 35 U.S.C. § 154. A true copy of the *Wyeth v. Dudas* opinion is attached hereto as Exhibit 2.

15. The correct patent term adjustment methodology identified in the *Wyeth v. Dudas* decision governs the USPTO's calculation of patent term adjustment for the Plaintiff's '179 patent.

CLAIM FOR RELIEF

16. The allegations of paragraphs 1-15 are incorporated in this claim for relief as if fully set forth herein.

17. During prosecution of the '179 patent, Plaintiff accrued 252 days of patent term adjustment under 35 U.S.C. § 154(b)(1)(A), and accrued 803 days of patent term adjustment under 35 U.S.C. § 154(b)(1)(B).

18. Under the USPTO's interpretation of 35 U.S.C. § 154, all patent term adjustment accrued under 35 U.S.C. § 154(b)(1)(A) and 35 U.S.C. § 154(b)(1)(B) inherently overlaps and, thus, it has been the USPTO's position that a patent holder is only eligible for the larger of these two amounts of patent term adjustment, that is, 803 days for the '179 patent (less any deductions for applicant delay).

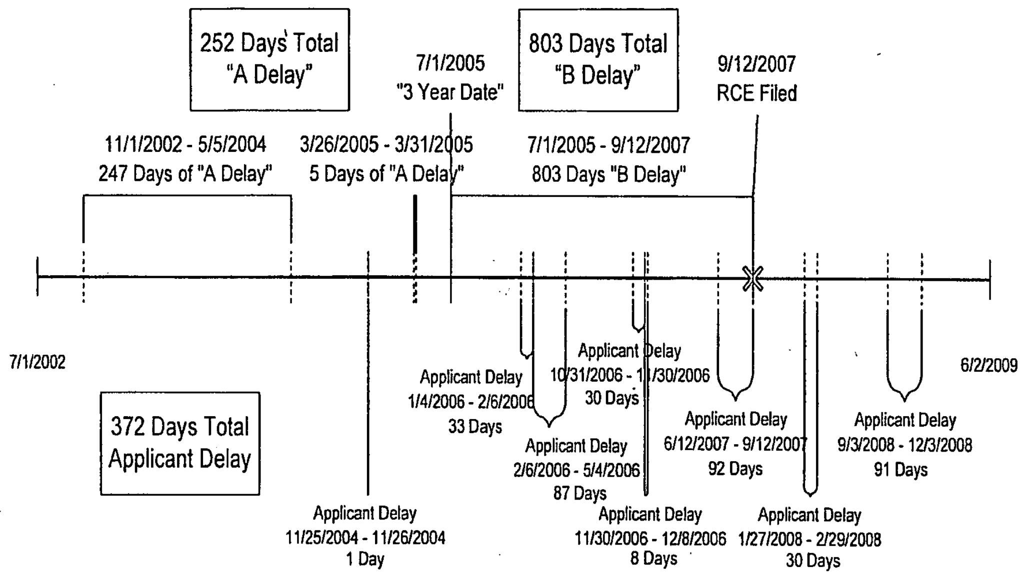
19. In view of the recent decision from this Court in *Wyeth v. Dudas*, the USPTO is obligated to award patent term adjustment under both 35 U.S.C. § 154(b)(1)(A) and 35 U.S.C. § 154(b)(1)(B) except where both delays occur on the same day, in which case the applicant is awarded a single day of patent term adjustment.

20. Plaintiff agrees with the USPTO's calculation of 252 days of "A Delay" under 35 U.S.C. § 154(b)(1)(A).

21. Plaintiff respectfully asserts that USPTO erred in not awarding the full 803 days "B Delay" under 35 U.S.C. § 154(b)(1)(B) for the failure of the USPTO to issue the '179 patent on or before July 1, 2005 (three years after the filing date). Specifically, Plaintiff is entitled to all of

the 803 days of patent term adjustment for the period between July 2, 2005 (three years and one day after the application filing date) and September 12, 2007 (the date on which a Request for Continued Examination ("RCE") was filed).

22. The 252 days of "A Delay" and the 803 days of "B Delay" do not overlap as depicted below:



23. Under the proper analysis set forth in *Wyeth v. Dudas* and as reflected in the above-diagram, Plaintiff is entitled to 683 days of patent term adjustment calculated as follows:

a. 252 days of "A Delay,"

b. plus 803 days of "B Delay,"

c. less 0 days of overlap between the periods of "A Delay" and "B Delay," and

d. less 372 days of applicant delay.

PRAYERS FOR RELIEF

WHEREFORE, Plaintiff requests that this Court:

(a) issue an Order changing the period of patent term adjustment for the '179 patent from 431 days to 683 days and requiring Defendant to issue a Certificate of Correction to alter the terms of '179 patent to reflect the corrected 683 days of patent term adjustment; and

(b) grant such other and further relief as the nature of the case may admit or require and as may be just and equitable.

Date: December 2, 2009

Respectfully submitted,

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EXHIBIT 1

(12) **United States Patent**
Sadelain et al.

(10) **Patent No.: US 7,541,179 B2**
(45) **Date of Patent: Jun. 2, 2009**

(54) **VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES**

(75) Inventors: **Michel Sadelain**, New York, NY (US); **Stefano Rivella**, New York, NY (US); **Chad May**, New York, NY (US); **Joseph Bertino**, New York, NY (US)

(73) Assignee: **Memorial Sloan-Kettering Cancer Center**, New York, NY (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 431 days.

(21) Appl. No.: **10/188,221**

(22) Filed: **Jul. 1, 2002**

(65) **Prior Publication Data**

US 2003/0022303 A1 Jan. 30, 2003

Related U.S. Application Data

(60) Provisional application No. 60/301,861, filed on Jun. 29, 2001, provisional application No. 60/302,852, filed on Jul. 2, 2001.

(51) **Int. Cl.**

C12N 15/00 (2006.01)
C12N 15/10 (2006.01)
C12N 15/67 (2006.01)
C12N 7/01 (2006.01)

(52) **U.S. Cl.** 435/320.1; 435/69.1; 536/24.1; 536/24.2; 424/93.2

(58) **Field of Classification Search** 435/320.1
See application file for complete search history.

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Primary Examiner—Maria Marvich
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(57) **ABSTRACT**

Recombinant lentiviral vectors having a region encoding a functional β-globin gene; and large portions of the β-globin locus control regions which include DNase I hypersensitive sites HS2, HS3 and HS4 provides expression of β-globin when introduced into a mammal, for example a human, in vivo. Optionally, the vector further includes a region encoding a dihydrofolate reductase. The vector may be used in treatment of hemoglobinopathies, including β-thalessemia and sickle-cell disease. For example, hematopoietic progenitor or stem cells may be transformed ex vivo and then restored to the patient. Selection processes may be used to increase the percentage of transformed cells in the returned population. For example, a selection marker which makes transformed cells more drug resistant than un-transformed cells allows selection by treatment of the cells with the corresponding drug.

24 Claims, 4 Drawing Sheets

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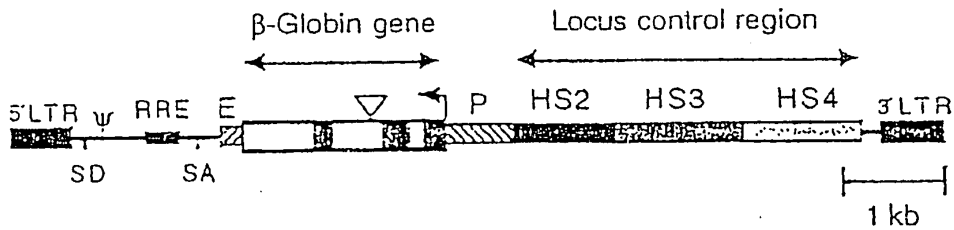


Fig. 1

Clinical Use of Drug Resistance

In Vivo Selection of Genetically Modified Stem Cells

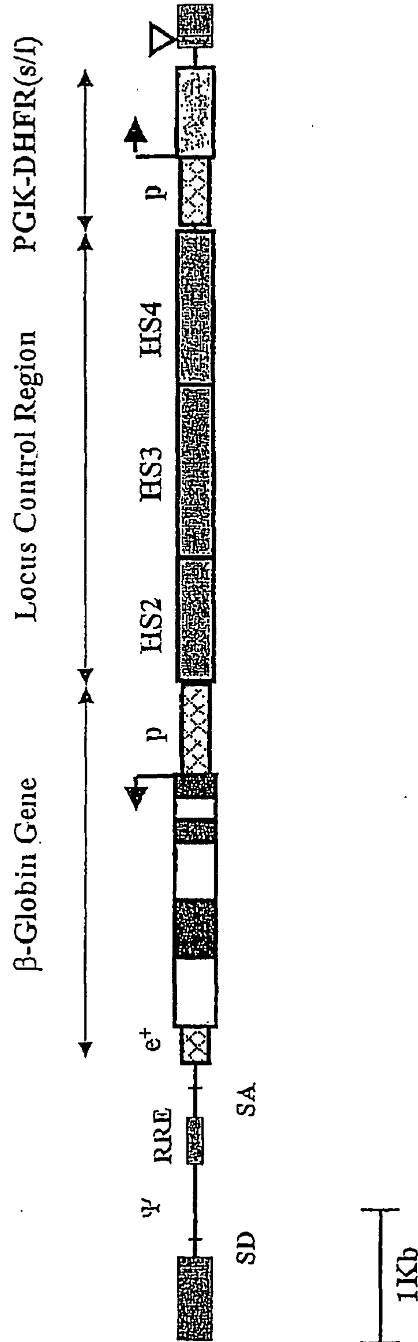


Fig. 2

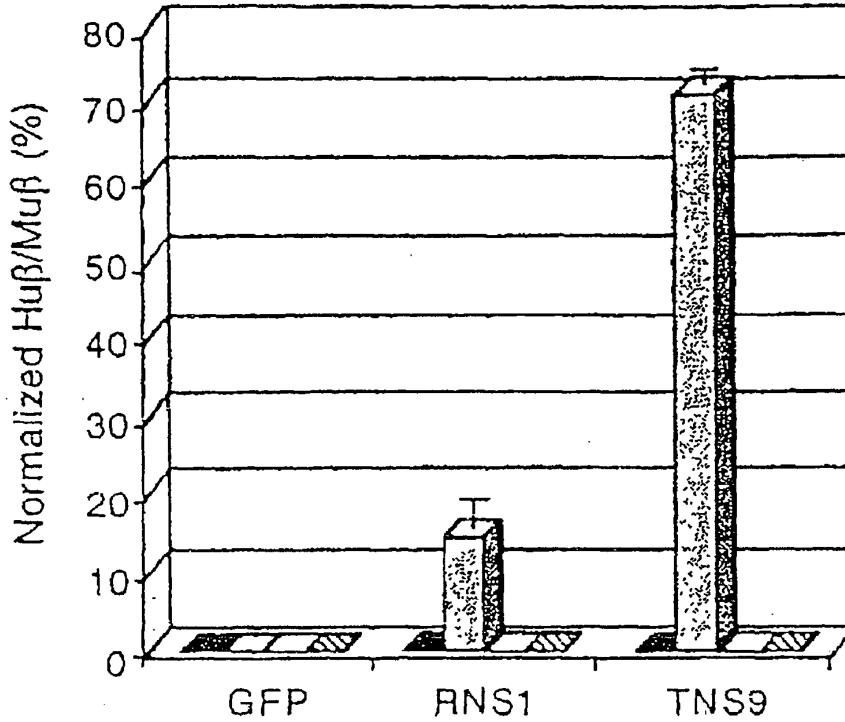


Fig. 3

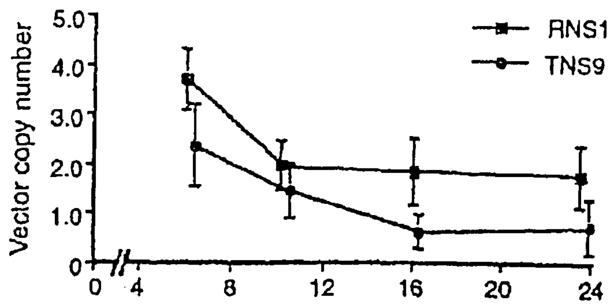


Fig. 4

Fig. 5 A

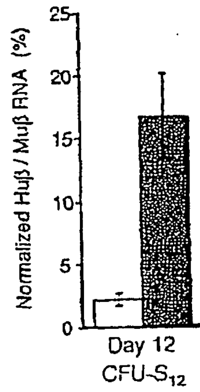
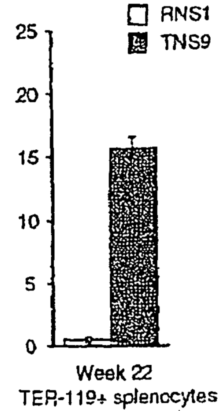


Fig. 5 B



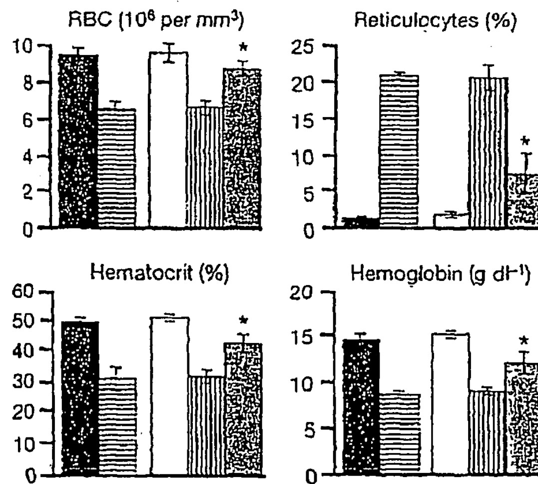


Fig. 6

VECTOR ENCODING HUMAN GLOBIN GENE AND USE THEREOF IN TREATMENT OF HEMOGLOBINOPATHIES

STATEMENT CONCERNING RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/301,861 filed Jun. 29, 2001 and U.S. Provisional Application No. 60/302,852 filed Jul. 2, 2001, both of which are incorporated herein by reference.

STATEMENT CONCERNING GOVERNMENT FUNDING

This application was supported by funds provided under NHLBI grant No. HL57612. The United States government may have certain rights in the invention.

BACKGROUND OF THE INVENTION

This application relates to a vector comprising a mammalian, and particularly a human globin gene and to the use thereof in treatment of hemoglobinopathies, including α- and β-thalessemia and sickle-cell disease.

Current treatment modalities for β-thalassemias consist of either red blood cell transfusion plus iron chelation (which extends survival but is cumbersome, expensive and an imperfect therapy), or allogeneic bone marrow transplant (which carries a lethal risk and is not available to the majority of patients). Thus, there is a substantial need for improved therapeutic approaches. The present invention provides a genetic correction in autologous hematopoietic stem cells, thus using gene therapy to provide a less-risky and more effective long-term treatment.

While gene therapy has been proposed for many years, a significant challenge facing efforts to develop gene therapy vectors is the ability to produce therapeutically useful levels of a desired protein or peptide. The present invention provides a vector which is capable of providing therapeutically meaningful levels of human globin for sustained periods of time. This ability arises from the ability to transmit large genomic regulatory sequences that control expression of the therapeutic gene.

SUMMARY OF THE INVENTION

In accordance with the invention, a recombinant lentiviral vector is provided comprising:

(a) a region comprising a functional globin gene; and

(b) large portions of the β-globin locus control regions which include large portions of DNase I hypersensitive sites HS2, HS3 and HS4. The regions may be the complete site or some lesser site which provides the same functionality as the specific sequences set forth below. This vector provides expression of β-globin when introduced into a mammal, for example a human, in vivo. Optionally, the vector further comprises a region encoding a dihydrofolate reductase.

By incorporation of different globin genes, the vector of the invention may be used in treatment of hemoglobinopathies, including α- and β-thalessemia and sickle-cell disease. For example, hematopoietic progenitor or stem cells may be transformed ex vivo and then restored to the patient. Selection processes may be used to increase the percentage of transformed cells in the returned population. For example, a selection marker which makes transformed cells more drug resistant than un-transformed cells allows selection by treatment of the cells with the corresponding drug. Selection and/or enrichment may also be carried out in vivo, for example using methotrexate or similar antifolates to select for cells rendered resistant by the expression from the vector of a dihydrofolate reductase (DHFR).

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the genomic structure of a recombinant oncoretroviral vector in accordance with the invention.

FIG. 2 shows the genomic structure of recombinant oncoretroviral vector within the scope of the invention.

FIG. 3 shows experimental results demonstrating increased mean β-globin expression in transduced MEL cells.

FIG. 4 shows the average vector copy number in peripheral blood cells, measured periodically for 24 weeks, which confirms highly efficient gene transfer in cells transduced with the vector of the invention.

FIGS. 5A and B show human β-globin expression per endogenous allele 12 days and 22 weeks after introduction of cells transduced with the vector of the invention.

FIG. 6 shows haematocrit level, red blood cell count, reticulocyte count and haemoglobin level fifteen weeks after transplantation with unselected TNS9-transduced $Hbb^{th3/+}$ bone marrow.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect of the present invention, a recombinant lentirviral vector is provided comprising:

(a) a region comprising a functional globin gene; and

(b) large portions of the β-globin locus control regions, which include DNase I hypersensitive sites HS2, HS3 and HS4.

As used in the specification and claims hereof, the term "recombinant lentiviral vector" refers to an artificially created polynucleotide vector assembled from a lentiviral-vector and a plurality of additional segments as a result of human intervention and manipulation.

The term "functional globin gene" refers to a nucleotide sequence the expression of which leads to a globin that does not produce a hemoglobinopathy phenotype, and which is effective to provide therapeutic benefits to an individual with a defective globin gene. The functional globin gene may encode a wild-type globin appropriate for a mammalian individual to be treated, or it may be a mutant form of globin, preferably one which provides for superior properties, for example superior oxygen transport properties. The functional globin gene includes both exons and introns, as well as globin promoters and splice doners/acceptors. Suitably, the globin gene may encode α-globin, β-globin, or γ-globin. β-globin promoters may be sued with each of the globin genes.

The recombinant vectors of the invention also include large portions of the locus control region (LCR) which include DNase I hypersensitive sites HS2, HS3 and HS4. In prior studies, smaller nucleotide fragments spanning the core portions of HS2, HS3 and HS4 have been utilized. Sadelain et al. *Proc. Nat'l Acad. Sci.* (*USA*) 92: 6728-6732 (1995); Lebouich et al., *EMBO J.* 13: 3065-3076 (1994). The term "large portions" refers to portions of the locus control region which encompass larger portions of the hypersensitive sites as opposed to previously tested fragments including only the core elements. The regions may be the complete site or some lesser site which provides the same functionality as the specific sequences set forth below. In preferred embodiments of the invention, the large portions of the locus control regions

are assembled from multiple fragments, each spanning one of the DNase I hypersensitive sites. In addition, the locus control region has two introduced GATA-1 binding sites at the junction between HS3 and HS4. While not intending to be bound by any specific mechanism, it is believed that the incorporation of these transcription factor binding sites enhances the effectiveness of the vector.

The genomic structure of one embodiment of the vector of the invention (TNS9) is shown in FIG. 1. TNS9 incorporates human β-globin gene (from position −618 to +2484) that includes an extended promoter sequence and a 3'-enhancer element. Optionally, a portion of 3'U3 region of the lentiviral backbone can be deleted for increased safety. In FIG. 1, the exons and introns of the human β-globin gene are represented by filled and open boxes, The locations are indicated for the splice donor (SD), splice acceptor (SA), packaging region (Ψ), rev-response element (RRE), human β-globin promoter (P) and 3'-β-globin enhancer (E). Thus, in the vector TNS9, a functional β-globin gene, which includes both the exons and introns of the gene and the relevant control sequences from the human β-globin locus. These are combined with the large fragments of the locus control region. The 3.2 kb LCR assembled into dTNS9 consists of an 840 bp HS2 fragment (SnaBI-BstXI), a 1308 bp HS3 fragment (HindIII-BamHI) and a 1069 bp HS4 fragment (BamHI-BanII).

In a further aspect of the invention, the β-globin gene coding sequence can be exchanged and replaced with either the gamma globin gene (for sickle cell disease) or the alpha globin gene (for alpha-thalassemias). In one strategy, a NcoI-PstI fragment of the β-globin gene is replaced with the corresponding NcoI-HindIII fragment of the gamma globin gene or the NcoI-PstI fragment of the human alpha globin gene. These fragments start at the translational start of each globin gene (spanning the NcoI site) and end past their respective polyadenylation signals. In the second strategy, chimeric genes can be generated by only swapping the coding sequence of each one of the three exons of these genes. Thus, for the gamma globin gene, the result is a vector that comprises the beta globin promoter, the beta globin 5' untranslated region, the gamma exon 1 coding region, the gamma intron 1 the gamma exon 2, the beta intron 2, the gamma exon 3, and the beta 3' untranslated region. Thus all the elements of the TNS9 vector remain in place (promoter, enhancers, 5' and 3' untranslated regions, the LCR elements, the 2 additional GATA-1 binding sites and the introns of the beta globin gene (at least intron 2, which is most important). In a third strategy, the codon usage within exon 3 of the gamma globin gene can be modified so that its sequence will resemble as much as possible that of the beta globin gene. The reason for testing this is that the beta globin gene is always the best expressed.

Additional elements may be included in the vectors of the invention to facilitate utilization of the vector in therapy. For example, the vector may include selectable markers, to confirm the expression of the vector or to provide a basis for selection of transformed cells over untransformed cells, or control markers which allow targeted disruption of transformed cells, and thus the selective removal of such cells should termination of therapy become necessary.

In a further specific embodiment, the vector of the invention includes the mouse PGK promotor and human dihydrofolate reductase (DHFR) cDNA as a transcriptional unit. Mutant forms of DHFR which increase the capacity of the DHFR to confer resistance to drugs such as methotrexate are suitably used. For example, single and double mutants of DHFR with mutations at amino acids 22 and 31 as described in commonly assigned PCT Publication No. WO 97/33988, which is incorporated herein by reference, may be advantageously utilized.

FIG. 2 shows the genomic structure of specific vector within the scope of the invention. The vector includes a deleted LTR, from −456 to −9 of HIV LTR and the PGK promoter (530 bp) from the murine phosphoglycerate kinase 1 gene. It also includes a DHFR-encoding region encoding human DHFR with s/f mutation at amino acid 22. The locus control region and the β-globin region are the same as in TSN9. This vector is designated dTNS9-PD. This incorporation of DHFR into this vector provides transformed cells with a methotrexate-resistant phenotype. As a result, methotrexate, and other antifolates can be used, both in vitro and in vivo as a selection tool to enhance levels of the functional hemoglobin. When hematopoietic stem cells were transformed using dTNS9-PD and reintroduced to mice that were then treated with NMBPR-P (0.5 mg/dose) and TMTX (0.5 mg dose) for five days, observed levels of expressed human β-globin were much higher in mice transduced with dTNS9-PD vectors after treatment with TMTX and NMBPR-P for selection of transduced cells.

The vectors of the invention are used in therapy for treatment of individuals suffering from hemoglobinopathies. In one embodiment of the invention, hematopoietic progenitor or stem cells are transformed ex vivo and then restored to the patient. As used in the specification and claims hereof, the term "hematopoietic progenitor sand stem cells" encompasses hematopoietic cells and non-hematopoietic stem cells, e.g., embryonic stem cells, hematopoietic stem cell precursors, or any of the latter generated by nuclear transfer from a somatic cell. It is know in the art that efficient gene transfer into human embryonic stem cells can be achieved using lentiviral vectors.

Selection processes may be used to increase the percentage of transformed cells in the returned population. For example, a selection marker which makes transformed cells more drug resistant than un-transformed cells allows selection by treatment of the cells with the corresponding drug. When DHFR is used as a selection marker, it can be used for enrichment of transduced cells in vitro, or for in vivo selection to maintain the effectiveness of the vector.

The invention will now be further described with reference to the following non-limiting examples.

EXAMPLE 1

To produce vector TNS9, the human β-globin gene was subcloned from Mβ6L (Sadelain et al. *Proc. Nat'l Acad. Sci.* (*USA*)92: 6728-6732 (1995)) into lentiviral vector pHR'LacZ (Zuffery et al., *Nature* 15: 871-875 (1997)) replacing the CMV-LacZ sequence. pHR'eGFP was constructed by replacing LacZ with the eGFP sequence (Clontech). Viral stocks were generated by triple transfection of the recombinant vectors pCMVΔR8.9 (Zuffrey et al.) and pMD.G in 293T cells as previously described in Dull, et al., *J. Virol.* 72: 8463-8471 (1998). The pseudotyped virions were concentrated by ultracentrifugation resuspended and titrated as described in Gallardo et al., *Blood* 90: 952-957 (1997). For comparison, RSN1 was used which has a similar structure, except that the LCR contains only the core portion of HS2, HS3 and HS4. Northern blot analysis showed full length RNA transcripts, indicating that the recombinant lentiviral genomes are stable. Southern blot analysis on genomic DNA from transduced cells, digested once in each long terminal repeat (LTR) results in a single band corresponding to the expected size for the vector, indicating that the proviral structure is not rearranged.

EXAMPLE 2

To investigate the tissue specificity, stage specificity and expression level of the vector-encoded human B-globin gene, we transduced RNS1 and TNS9 into MEL cells, lymphoid Jurkat cells and myeloid HL-60 cells. Cell-free viral supernatant was used to infect C88 MEL cells in the presence of polybrene (8 μg ml^{-1}). Transduced MEL cells were subcloned by limiting dilution, and screened by PCR for transduction[30] using primers that anneal in the human β-globin promoter sequence (βPS, 5'-GTCTAAGTGATGACAGC-CGTACCTG-3', Seq ID No.: 1) and in HS2 (C2A, 5'-TCAGCCTAGAGT GATGACTCC TATCTG-3', Seq ID No.: 2). Vector copy number and integration site analysis was determined by Southern blot analysis[9]. Transduced MEL cells were induced to maturation by 5-day culture in 5 mM N,N'-hexamethylene bisacetamide (HMBA, Sigma).

To induce β-globin transcription, transduced MEL cell pools were differentiated using hexamethylene bisacetamide HMBA). Human β-globin (β^A) and mouse β-globin transcripts were measured by quantitative primer extension. After normalization to vector copy number and to endogenous β-globin expression per allele, human β-globin levels were 14.2±4.7% for RNS1 and 71.3±2.3% for TNS9 in pooled MEL cells (FIG. 2*a*). MEL, Jurkat and HL-60 cells were transduced with RNS1, TNS9 or control GFP recombinant lentivirus. Human β-globin RNA expression in HMBA induced MEL cells (grey bars) was measured by quantitative primer extension and normalized to mouse β-globin RNA expression per locus. Expression was then normalized to the vector copy number determined by Southern blot. No human β-globin RNA expression was detected in non-induced MEL (black bars), Jurkat (white bars) or HL-60 cells (hatched bars), indicating that globin expression was erythroid- and differentiation-specific. No human β-globin expression was detected in non-induced MEL, Jurkat and HL-60 cells (FIG. 3), indicating that human β-globin expression was appropriately regulated in terms of tissue specificity and state of differentiation. We generated a panel of MEL cell clones that carried a single copy of either vector to distinguish whether the increased expression obtained in HMBA-treated Mel cells transduced with TNS9 rather than RNS1 was the result of an increase in β^A expression per cell or of an increase in the fraction of cells expressing human β-globin. Transduced MEL cells were subcloned by limiting dilution immediately after transduction, avoiding any bias towards favourable chromosomal integration sites as produced by drug selection[5]. The proportion of clones expressing human β-globin varied significantly between the two vectors. One out of ten RNS1 positive clones yielded measurable human β-globin transcripts, in contrast to 12 out of 12 for TNS9 also expressed higher levels of human β-globin than did those bearing RNS1 (P<0.01, Fisher's exact test). Cells bearing TNS9 also expressed higher levels of human β-globin than did those bearing RNS1 (P<0.01, Wilcoxon rank sum test). These findings established that both the level and probability of expression at random integration sites was increased with the TNS9 vector.

EXAMPLE 3

Quantification of Human β-globin mRNA

Total RNA was extracted from MEL, Jurkat and HL-60 cells, or mouse spleen and blood using TRIzol. Quantitative primer extension assays were done using the Primer Extension System-AMV Reverse Transcriptase kit (Promega) with [^{32}P] dATP end-labelled primers specific for retroviral-de-

rived human β-globin (5'-CAGTAACGGCAGACTTCTC-CTC-3', Seq ID No.: 3) and mouse β-globin (5'-TGATGTCT-GTTTCTGGGGTT GTG-3', Seq ID No.: 4), with predicted extension products of 90 bp and 53 bp, respectively. The probes yield products of identical length for β^{maj}, β^{min}, β^{s} and β^{t}. Primers were annealed to 4 μg of RNA and reactions were run according to manufacturer's protocols. Radioactive bands were quantitated by phosphorimager analysis (Bio-Rad). RNA isolated from A85.68 mice[20] was used as positive control. After correction for primer labelling, the human to mouse RNA signal was 29±1% per gene copy in repeated experiments (n>8), in agreement with previous findings based on RT-PCR[20]. Values measured in bone marrow chimaeras that were obtained in separate runs were standardized to the value obtained in the A85.68 RNA sample. In FIGS. 2 and 3*c*, *d*, human β-globin expression is expressed per vector copy and normalized to the endogenous transcripts (accounting for two endogenous alleles). In FIG. 3*b*, human transcripts are reported as the fraction of total β-globin RNA (Huβ/Huβ+Muβ) to reflect absolute contribution of vector-encoded transcripts.

EXAMPLE 4

To investigate the function of the vectors in vivo, we transduced and transplanted murine bone marrow cells without any selection in syngeneic, lethally irradiated recipient mice. Donor bone marrow was flushed from the femurs of 8- to 16-week-old male C57BL/6 or $Hbb^{th3/+mice}$ (Jackson Laboratories) that had been injected intravenously (i.v.) 6 days earlier with 5-flurouracil (5-FU, Pharmacia; 150 mg kg^{-1} body weight). Bone marrow cells were resuspended in serum-free medium, and supplemented with IL-1α (10 ng ml^{-1}), IL-3 (100 U ml^{-1}), IL-6 (150 U ml^{-1}), Kit ligand (10 ng ml^{-1}) (Genzyme), β-mercaptoethanol (0.5 mM; Sigma), *L*-glutamine (200 mM), penicillin (100 IU ml^{-1}) and streptomycin (100 μg m^{-1}), and cultured for 18 h. Recipient mice (11- to 14-week-old C57/BL6 or $Hbb^{th3/+}$ mice) were irradiated with 10.5 Gy (split dose 2×5.25 Gy) on the day of transplantation. Bone marrow cells were pelleted and resuspended in serum-free medium containing concentrated lentiviral supernatant, and supplemented with polybrene (8 μg ml^{-1}), *L*-glutamine (200 mM), penicillin (100 IU ml^{-1}) and streptomycin (100 μg ml^{-1}), and cultured for 6 h. Transduced bone marrow cells (1×10^5 or 5×10^5) were then i.v. injected into each of the irradiated female recipients to establish short-term and long-term bone marrow chimaeras, respectively.

In short-term studies, spleens were removed 12 d after transplantation to extract total RNA and genomic DNA. To monitor long-term chimaeras, two or three capillary tubes of blood were collected every 4-6 weeks, from which genomic DNA, total RNA and haemoglobin were extracted. To examine vector function reliably in long-term animals, erythroid cell populations were purified from spleen. Single-cell suspensions were incubated with rat anti-mouse TER-119 monoclonal antibody (PharMingen). Sheep anti-Rat IgG dynabeads (M-450, Dynal Inc.) Were added to the antibody-coated spleen cells and purified as recommended by the manufacturer. Vector copy number, integration pattern and chimaerism were determined by Southern blot analysis. The fraction of donor DNA relative to recipient was determined by stripping and reprobing the blot with a [^{32}P] dCTP-labelled probe specific for the Y chromosome and normalizing to an endogenous mouse band. Radioactive bands were quantitated by phosphorimager analysis. Sera from five randomly selected long-term bone marrow chimaeras (30 weeks after transplan-

tation) tested negative for HIV-1 gag by RT-PCR using the Amplicor HIV-1 monitor kit (Roche).

Vector copy number and human β-globin RNA transcripts were measured during a 24-week period in mice transplanted with RNS1 (n=8) or TNS9 (n=10) transduced bone marrow. a, Vector copy number was assessed by southern blot analysis of genomic DNA isolated from peripheral blood at weeks 6, 10, 16 and 24. The average vector copy number in peripheral blood cells, measured periodically for 24 weeks (FIG. 4), showed highly efficient gene transfer with both vectors (1.8±0.6 and 0.8±0.6 average vector copies per cell for β-globin transcript levels in the 10-20% range during the same time period. To assess transcriptional activity per vector copy, steady-state RNA transcripts and vector copy number were quantified in pooled CFU-S_{12} and in erythroid TER-119+ spleen cells. Twelve days after transplantation, human β-globin expression per endogenous allele, (FIG. 5*a*). Twenty weeks later these values were 0.5±0.1% (significantly lower than on day 12, P=0.02) and 15.8±0.9% respectively (FIG. 5*b*). These findings established that the larger LCR fragments increased globin expression in vivo and, furthermore, suggested that TNS9 is more resistant to transcriptional silencing than is RNS1.

The levels of TNS9-encoded human β-globin could be produced. Haemoglobin tetramers incorporating vector-encoded human β^A and endogenous murine α-globin (designated Hbb^{hu}) were quantitated in peripheral blood red cell lysates after cellulose acetate gel fractionation. Hbb^{hu} levels accounting for up to 13% of total haemoglobin were found 24 weeks after transplantation (FIG. 3*e*, Table 1 in Supplementary Information). In the same assays, transgenic mice bearing one copy of a 230-kb yeast artificial chromosome (YAC) encompassing the entire human β-globin like gene cluster[20] showed 14% of their total haemoglobin incorporating human β^A. No haemoglobin tetramers containing human β^A were measurable in any of the mice bearing RNS1 (table 1 in Supplementary Information). The proportion of mature peripheral blood red cells expressing human β^A was elevate in most TNS9 bone marrow chimaeras, as shown by dual staining of human β^A and TER-119. In contrast, chimaeras engrafted with RNS1-transduced bone marrow showed highly variable fractions of weakly staining β^A-positive erythrocytes. Normalized to the fraction of circulating β^A-positive mature red cells, the levels of haemoglobin containing lentivirus-encoded β^A were on average 64% of those obtained in the YAC transgenic mice.

EXAMPLE 5

To ascertain that true HSCs were transduced, we carried out secondary transplants using marrow from primary recipients collected 24 weeks after transplantation. The TNS9 and RNS1 vectors were readily detected in all secondary recipients 13 weeks after transplantation. Human β-globin expression was maintained in all recipients of TNS9-transduced marrow. The successful transduction of HSCs was confirmed by integration site analyses. Southern blot analysis was performed on genomic DNA isolated from bone marrow, spleen and thymus of secondary bone marrow transplant recipients collected 13 weeks after transplant (one representative RNS1, and two representative TNS9 secondary transplant recipients are shown). Two endogenous bands are found in the genomic DNA of C57BL/6 (B6) mice.

EXAMPLE 6

In view of the high levels of expression, we tested the extent to which the TNS9 vector could correct the phenotype of thalassaemic cells using β^0-thalassaemic heterozygote mice that lack a copy of their b1 and b2 β-globin genes ($Hbb^{th3/+}$)[21]. These heterozygotes have a clinical phenotype similar to human thalassaemia intermedia and exhibit chronic anaemia (haematocrit 28-30%, haemoglobin 8-9 g dl^{-1}) and anomalies in red cell size (anisocytosis) and shape (poikilocytosis). Fifteen weeks after transplantation with unselected TNS9-transduced $Hbb^{th3/+}$ bone marrow, the haematocrit level, red blood cell count, reticulocyte count and haemoglobin level were markedly improved in five out of five recipient mice (FIG. 6). Anisocytosis and poikilocytosis were markedly reduced in the peripheral blood smears of chimaeras bearing the TNS9 vector. Control mice transplanted with $Hbb^{th3/+}$ bone marrow cells transduced with a vector encoding enhanced green fluorescent protein (eGFP) all remained severely anaemic (n=5, FIG. 6) and maintained their abnormal red cell morphology. These results establish that levels of circulating haemoglobin obtained with TNS9 were indeed therapeutically relevant.

The combined effect of the high efficiency of gene transfer and the absence of vector rearrangements afforded by the recombinant lentivirus carrying the β-globin gene and LCR configuration adopted in TNS9 yielded levels of human β^A expression in the therapeutic range. The higher expression obtained with TNS9 compared with RNS1 was associated with a higher fraction of permissive integration sites in MEL cells and a higher fraction of human β^A-containing red blood cells in bone marrow chimaeras. RNS1, which carries a weaker enhancer, silenced over time whereas TNS9 retained undiminished transcriptional activity over the same time period and in secondary transplant recipients.

Higher levels of murine α_2: human $\beta^A{}_2$ tetramers were obtained in peripheral blood samples from recipients of TNS9-transduced Hbb^{th3+} bone marrow (21±3% of total haemoglobin, n=5, than with $Hbb^{+/+}$ bone marrow (6±4%, n+10). The two groups showed comparable peripheral blood vector copy numbers and levels of human β-globin RNA (0.8±0.2 compared with 0.8±0.6, and 16.8±6% compared with 10.8±7%, respectively). This observation is consistent with a competitive advantage of murine β-globin over human β-globin in associating with murine α-globin[22]. In thalassaemic patients, added human β-chain synthesis would improve the α:β chain imbalance and thus increase red cell survival and ameliorate the ineffective erythropiesis in these patients. In patients with sickle cell disease, transduced β^A chains are expected to have an advantage over the β^S chains produced by both endogenous genes in competing for the available α-chains[23]. Given that patients with S/β-thalassaemia whose HbA represents 10-30% of their total haemoglobin are very mildly affected[1/24], the clinical benefit of such an intervention would be highly significant.

EXAMPLE 7

To investigate long-term expression of the transduced human β-globin genes and its therapeutic efficacy, we generated bone marrow chimeras engrafted with TNS9-transduced $Hbb^{th3/+}$ bone marrow cells (n=5) and studied them over a 40-week period.

Donor bone marrow was flushed from the tumors of 8- to 16-week old male c57/BL6 or $Hbb^{th3/+}$ mice[23] obtain from Jackson Laboratories (Bar Harbor, Me.) that had been injected intravenously (IV) 6 days earlier with 5-flurouracil (5-FU) 150 mg/kg body weight obtained from Pharmacia (Piscataway, N.J.). Bone marrow cells were resuspended in X-VIVO-15 serum-free medium and supplemented with 10 ng/mL interleukin-1 α (IL-1α) 100 U/mL IL-3, 150 U/mL

IL-6, 10 ng/mL Kit ligand obtained from Genzyme (Cambridge, Mass.), 0.5 mM β-mercaptoethanol obtained from Sigma (St. Louis, Mo.), 200-mM $_L$-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Bone marrow cells were then pelleted and resuspended in serum-free medium containing concentrated lentiviral supernatant and supplemented with 8 mg/mL polybrene (Sigma), 200 mM $_L$-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and cytokines as above, and cultured for 8 hours. Transduced bone marrow cells (5×10^5) were then injected IV into each of the irradiated female recipients to establish bone marrow chimeras. Recipients mice (11- to 14-week-old C57/BL6 or $Hbb^{th3/+}$ mice) were irradiated with 10.5 Gy (Split dose 2×5.25 Gy) on the day of transplantation.

Age-matched chimeras engrafted with eGFP-transduced $Hbb^{th3/+}$ (n=5) and $Hbb^{+/+}$ (n=5) bone marrow cells served as controls. Vector copy number was monitored in peripheral blood by quantitive Southern blot analysis, and found to remain stable, between 0.5 and 1.0 copy/cell on average (data not shown). Protein expression was assessed by quantitive hemoglobin analysis, to measure the proportion of hemoglobin tetramers that incorporate human β^A (Hbb^{hu}, mu α_2:hu$\beta^A{}_2$) or murine β-globin (Hbb^{mu}, muα_2:mu$\beta^A{}_2$), and immunofluorescence, to determine the fraction of mature RBCs that contain human β^A protein. Transgenic mice bearing one copy of a 230-kb yeast artificial chromosome encompassing the entire human β-globin-like gene cluster[28] served as reference, showing 14% of their total hemoglobin incorporating human β^A and 100% β^A+RBCs[19,28] Hbb^{hu} accounted for 19% to 22% of the totalhemoglobim in TNS9 chimeras. These levels remained stable up to 40 weeks after transplantation. Over this same time period, the proportion of mature peripheral RBCs expressing human β^A also remained elevated and stable (about 70% to 80%), as shown by dual staining of human β^A and TER-119.

EXAMPLE 8

Long-Term Amelioration of Anemia

The stability of TNS9-encoded β^A expression detected in peripheral blood suggested that long-trem hematologic and systemic therapeutic benefits could be obtained. To investigate whether Hbb^{hu} production would suffice to treat the anemia, we closely monitored hemoglobin parameters over 40 weeks. The marked increase in hemoglobin concentration, RBC counts, and hematocrit was sustained throughout this time period. Control mice that received transplants of eGFP-transduced $Hbb^{th3/+}$ bone marrow cells remained severely anemic, indicating that the transplantation procedure itself did not alter the anemic state. The reticulocyte counts decreased to 5% to 8% in TNS9 treated-chimeras, compared to 19% to 21% in control eGFP-treated $Hbb^{th3/+}$ chimeras and age-matched $Hbb^{th3/+}$ mice, suggesting an increase in RBC life span and a decrease in erythropoietic activity.

EXAMPLE 9

To determine the impact of sustained human β-globin gene expression on hematopoiesis, we studied the degree of splenomegaly (enlargement of the spleen) and EMH in 1-year-old chimeras and age-matched control mice. Spleen weights measured in Tns9-treated $Hbb^{th3/+}$ chimeras were indistinguishable from recipients of eGFP-transduced normal bone marrow, as were the total number of cells per spleen. In contrast, mice engrafted with eGFP-transduced $Hbb^{th3/+}$ bone marrow cells showed spleen weights and total cell numbers that were about 3-fold greater. The correction of spleen

weight in TNS9 bone marrow chimeras corresponded to a concomitant normalization in total hematopoietic progenitor cell content. Spleen CFU-Es, BFUEs, and CFUs-GM were reduced to levels measured in recipients of eGFP-transduced $Hbb^{th+/+}$ bone marrow, whereas they remained elevated in control chimeras engrafted with eGFP-trasduced $Hbb^{th3/+}$ bone marrow cells and in age-matched Hbb^{th3+} mice, as previously observed in another murine model of β-thalassemis.[29]

The regression of EMH was corroborated by morphologic examination of spleen and liver in long-term chimeras and age-match controls. Histopathology of spleens of mice that received transplants of eGFP-tranduced $Hbb^{th3/+}$ marrow was virtually identical to that of spleen from control $Hbb^{th3/+}$ mice. Specifically, the red pulp was significantly expanded, accounting for 80% to 90% of the cross-sectional area, and densely occupied by nucleated erythroid precursors. The white pulp, based on cross-sectional area, was relatively decreased and the marginal zones were obscured by the large number of nucleated RBCs, reflecting major expansion of the red pulp and erythroid precursors. In TNS9-treated chimeras, the amount of red pulp was considerably decreased, accounting for only about 50% to 60% of the cross-sectional area. In addition, the number of nucleated erythroid precursors in the red pulp was decreased. Other immature hematopoietic cells were present in the red pulp, but much less frequently than in the spleens of control $Hbb^{th3/+}$ thalassemic mice. The livers from TNS9-treated chimeras were similar to those of the normal control mice in that no EMH was detected. In contrast, livers from mice engrafted with eGFP-trasduced $Hbb^{th3/+}$ bone marrow cells showed several small foci of intrasinusoidal EMH.

EXAMPLE 10

Toxic iron accumulation in the organs of thalassemic patients is a consequence of RBC destruction and increased gastrointestinal iron uptake. To determine whether sustained expression from the TNS9 vector reduced iron overload, we studied tissue sections of liver and heart, stained using Gomori iron stain. No iron deposition was seen in the livers of normal $Hbb^{+/+}$ control mice, whereas $Hbb^{th3/+}$ mice showed variable amounts of iron, including some large aggregates. TNS9-trasduced treated chimeras demonstrated low to undetectable levels of iron in the livers, whereas iron was readily detected in the livers of all mice that received transplants of eGFP-transduced $Hbb^{th3/+}$ bone marrow cells. No iron accumulation was found in the heart of treated or control mice, as previously observed in another murine model of β-thalassemia,[30] in contrast to what is found in the human disease.[1-3]

EXAMPLE 11

To assess to efficacy of in vivo selection for cells transduced with globin and DHFR-encoding vectors in accordance with the invention, using antifolates the following alternative protocols are used. In protocol 1, the recipient mice are treated daily for 5 days with MTX (25 mg/Kg) and NBMPR-P (20 mg/Kg), starting 6 weeks after administration of transduced bone marrow cells. In protocol 2, the recipient mice are treated daily for 5 days with TMTX (40 mg/Kg) and NBMPR-P (20 mg/Kg), starting 6 weeks after administration of transduced bone marrow cells. In protocol 3, the recipient mice, conditioned with busulphan rather than with gamma-irradiation, are treated daily for 5 days with TMTX (40 mg/Kg) and NBMPR-P (20 mg/Kg), starting 4 weeks after administration of transduced bone marrow cells. (TMTX

(Neutrexin; U.S. Bioscience); >MTX (Methotrexate LPF Sodium; Immunex); NBMPR-P (Nitrobenzylthioinosine 5'-monophpsphate disodium salt; Alberta nucleoside therapeutics). Protocol 3 is in principle the most attractive protocol as the recipients are not irradiated and furthermore not treated with a "myeloablative conditioning regimen". They are treated with a relatively milder conditioning regimen consisting of a "non-myeloablative" dose of busulphan. It is hoped that, in combination with "in vivo selection" mediated by DHFR/TMTX, the recipients could be satisfactorily engrafted without receiving a harsh pre-transplant treatment. This would be the way to go for treating subjects with severe hemoglobinopathies.

3. The vector of claim 2, further comprising a mouse PGK promoter to control the expression of the dihydrofolate reductase.

4. The vector of claim 3, wherein the dihydrofolate reductase is a human dihydrofolate reductase.

5. The vector of claim 4, wherein the human dihydrofolate reductase is a mutant dihydrofolate reductase having increased resistance to antifolates as compared to wild-type human dihydrofolate reductase and differing in amino acid sequence from wild-type human dihydrofolate reductase.

6. The vector of claim 5, wherein the mutant dihydrofolate reductase comprises a mutation at an amino acid corresponding to amino acid 22 of the wild-type sequence and a mutation at an amino acid corresponding to amino acid 31 of the wild type sequence.

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What is claimed is:

1. A recombinant vector comprising a nucleic acid encoding a functional globin operably linked to a 3.2-kb nucleotide fragment which consists essentially of three contiguous nucleotide fragments obtainable from a human β-globin locus control region (LCR), the three fragments being a BstXI and SnaBI HS2-spanning nucleotide fragment of said LCR, a BamHI and HindIII HS3-spanning nucleotide fragment of said LCR and a BamHI and BanII HS4-spanning nucleotide fragment of said LCR, said vector providing expression of the globin in a mammal in vivo.

2. The vector of claim 1, further comprising a nucleic acid encoding a dihydrofolate reductase.

7. The vector of claim 2, wherein the dihydrofolate reductase is a human dihydrofolate reductase.

8. The vector of claim 7, wherein the human dihydrofolate reductase is a mutant dihydrofolate reductase having increased resistance to antifolates as compared to wild-type human dihydrofolate reductase and differing in amino acid sequence from wild-type human dihydrofolate reductase.

9. The vector of claim 8, wherein the mutant dihydrofolate reductase comprises a mutation at an amino acid correspond-

ing to amino acid 22 of the wild-type sequence and a mutation at an amino acid corresponding to amino acid 31 of the wild type sequence.

10. The vector of claim 1, wherein the functional globin is human β-globin.

11. The vector of claim 10, further comprising a nucleic acid encoding a dihydrofolate reductase.

12. The vector of claim 11, further comprising a mouse PGK promoter to control the expression of the dihydrofolate reductase.

13. The vector of claim 12, wherein the dihydrofolate reductase is a human dihydrofolate reductase.

14. The vector of claim 13, wherein the human dihydrofolate reductase is a mutant dihydrofolate reductase having increased resistance to antifolates as compared to wild-type human dihydrofolate reductase and differing in amino acid sequence from wild-type human dihydrofolate reductase.

15. The vector of claim 14, wherein the mutant dihydrofolate reductase comprises a mutation at an amino acid corresponding to amino acid 22 of the wild-type sequence and a mutation at an amino acid corresponding to amino acid 31 of the wild type sequence.

16. The vector of claim 11, wherein the dihydrofolate reductase is a human dihydrofolate reductase.

17. The vector of claim 16, wherein the human dihydrofolate reductase is a mutant dihydrofolate reductase having increased resistance to antifolates as compared to wild-type human dihydrofolate reductase and differing in amino acid sequence from wild-type human dihydrofolate reductase.

18. The vector of claim 17, wherein the mutant dihydrofolate reductase comprises a mutation at an amino acid corresponding to amino acid 22 of the wild-type sequence and a mutation at an amino acid corresponding to amino acid 31 of the wild type sequence.

19. The vector of claim 1, wherein the functional globin is a β-globin.

20. The vector of claim 1, wherein the functional globin is a γ-globin.

21. The vector of claim 1, wherein the functional globin is an α-globin.

22. The vector of claim 1, wherein the vector is a lentiviral vector.

23. A recombinant vector comprising a nucleic acid encoding a functional globin operably linked to a 3.2-kb nucleotide fragment which consists essentially of three nucleotide fragments obtainable from a human β-globin LCR, the three fragments being a BstXI and SnaBI, HS2-spanning nucleotide fragment of said LCR, a BamHI and HindIII, HS3-spanning nucleotide fragment of said LCR, and a BamHI and BanII, HS4-spanning nucleotide fragment of said LCR, wherein the HS3-spanning nucleotide fragment and the HS4-spanning nucleotide fragment are adjacent to each other and the vector further comprises 2 GATA-1 binding sites at the junction between the HS3-spanning and HS4-spanning nucleotide fragments, said vector providing expression of the globin in a mammal in vivo.

24. The vector of claim 23, wherein the vector is pTNS9.

* * * * *

EXHIBIT 2

LEXSEE 580 F. SUPP. 2D 138

WYETH, et al., Plaintiffs, v. JON W. DUDAS, Under Secretary of Commerce for Intellectual Property and Director of U.S. Patent and Trademark Office, Defendant.

Civil Action No. 07-1492 (JR)

UNITED STATES DISTRICT COURT FOR THE DISTRICT OF COLUMBIA

580 F. Supp. 2d 138; 2008 U.S. Dist. LEXIS 76063; 88 U.S.P.Q.2D (BNA) 1538

September 30, 2008, Filed

CASE SUMMARY:

PROCEDURAL POSTURE: In filed an action, claiming that the United States Patent and Trademark Office (PTO) had misconstrued or misapplied *35 U.S.C.S. § 154(b)(2)(A)*, and that the PTO was denying them a portion of the term Congress had provided for the protection of their intellectual property rights.

OVERVIEW: The PTO's view was that any administrative delay under *§ 154(b)(1)(A)* overlapped any three-year maximum pendency delay under *§ 154(b)(1)(B)*: the applicant got credit for "A delay" or for "B delay," whichever was larger, but never A + B. Chevron deference did not apply to the interpretation at issue here. Further, Chevron would not have saved the PTO's interpretation because it could not be reconciled with the plain text of the statute. The operative question under *§ 154(b)(2)(A)* was whether periods of delay attributable to grounds specified in *§ 154(b)(1)* overlapped. The problem with the PTO's construction was that it considered the application delayed under *§ 154(b)(1)(B)* during the period before it had been delayed. That construction could not be squared with the language of *§ 154(b)(1)(B)*, which applied if the issue of an original patent was delayed due to the failure of the PTO to issue a patent within three years. "B delay" began when the PTO had failed to issue a patent within three years, not before.

OUTCOME: The court construed the statute at issue.

LexisNexis(R) Headnotes

Patent Law > Infringement Actions > Exclusive Rights > General Overview
[HN1] *35 U.S.C.S. § 154(a)(2)* establishes a term of 20 years from the day on which a successful patent application is first filed. Because the clock begins to run on this filing date, and not on the day the patent is actually granted, some of the effective term of a patent is consumed by the time it takes to prosecute the application. To mitigate the damage that bureaucracy can do to inventors, the statute grants extensions of patent terms for certain specified kinds of United States Patent and Trademark Office delay, *35 U.S.C.S. § 154(b)(1)(A)*, and, regardless of the reason, whenever the patent prosecution takes more than three years. *35 U.S.C.S. § 154(b)(1)(B)*. Recognizing that the protection provided by these separate guarantees might overlap, Congress has forbidden double-counting.

Patent Law > Infringement Actions > Exclusive Rights > General Overview
[HN2] See *35 U.S.C.S. § 154(b)(2)(A)*.

Patent Law > Infringement Actions > Exclusive Rights

> General Overview

[HN3] *35 U.S.C.S. § 154(b)* provides three guarantees of patent term. The first is found in *§ 154(b)(1)(A)*, the guarantee of prompt United States Patent and Trademark Office (PTO) response. It provides a one-day extension of patent term for every day that issuance of a patent is delayed by a failure of the PTO to comply with various enumerated statutory deadlines: 14 months for a first office action; 4 months to respond to a reply; 4 months to issue a patent after the fee is paid; and the like. *§ 154(b)(1)(A)(i)-(iv)*. Periods of delay that fit under this provision are called "A delays" or "A periods." The second provision is the guarantee of no more than three-year application pendency. Under this provision, a one-day term extension is granted for every day greater than three years after the filing date that it takes for the patent to issue, regardless of whether the delay is the fault of the PTO. *§ 154(b)(1)(B)*. The period that begins after the three-year window has closed is referred to as the "B delay" or the "B period."

Patent Law > Infringement Actions > Exclusive Rights > General Overview

[HN4] See *35 U.S.C.S. § 154(b)(2)(A)*.

Administrative Law > Judicial Review > Standards of Review > Statutory Interpretation

Patent Law > U.S. Patent & Trademark Office Proceedings > General Overview

[HN5] The United States Patent and Trademark Office is not afforded Chevron deference because it does not have the authority to issue substantive rules, only procedural regulations regarding the conduct of proceedings before the agency.

Patent Law > Infringement Actions > Exclusive Rights > General Overview

Patent Law > U.S. Patent & Trademark Office Proceedings > General Overview

[HN6] The authority of the United States Patent and Trademark Office is limited to prescribing regulations establishing procedures for the application for and determination of patent term adjustments under this subsection. *35 U.S.C.S. § 154(b)(3)(A)*

Patent Law > Infringement Actions > Exclusive Rights > General Overview

Patent Law > U.S. Patent & Trademark Office Proceedings > General Overview

[HN7] In *35 U.S.C.S. § 154(b)(2)(C)(iii)* the United States Patent and Trademark Office is given the power to prescribe regulations establishing the circumstances that constitute a failure of an applicant to engage in reasonable efforts to conclude processing or examination of an application--that is, the power to elaborate on the meaning of a particular statutory term. No such power is granted under *§ 154(b)(3)(A)*.

Administrative Law > Judicial Review > Standards of Review > Statutory Interpretation

[HN8] If the statutory text is not ambiguous enough to permit the construction that the agency urges, that construction fails at Chevron's "step one," without regard to whether it is a reasonable attempt to reach a result that Congress might have intended.

Patent Law > Infringement Actions > Exclusive Rights > General Overview

[HN9] In the context of *35 U.S.C.S. § 154(b)(2)(A)*, the only way that periods of time can "overlap" is if they occur on the same day. If an "A delay" occurs on one calendar day and a "B delay" occurs on another, they do not overlap, and *§ 154(b)(2)(A)* does not limit the extension to one day.

COUNSEL: [**1] WYETH, Plaintiff: David O. Bickart, LEAD ATTORNEY, Patricia A. Carson, PRO HAC VICE, KAYE SCHOLER LLP, Washington, DC.

For ELAN PHARMA INTERNATIONAL LIMITED, Plaintiff: David O. Bickart, LEAD ATTORNEY, KAYE SCHOLER LLP, Washington, DC.

For JON W. DUDAS, Honorable, Under Secretary of Commerce, Defendant: Fred Elmore Haynes, LEAD ATTORNEY, U.S. ATTORNEY'S OFFICE, Washington, DC.

JUDGES: JAMES ROBERTSON, United States District Judge.

OPINION BY: JAMES ROBERTSON

OPINION

[*139] *MEMORANDUM OPINION*

Plaintiffs here take issue with the interpretation that the United States Patent and Trademark Office (PTO) has imposed upon *35 U.S.C. § 154*, the statute that prescribes patent terms. [HN1] *Section 154(a)(2)* establishes a term of 20 years from the day on which a successful patent application is first filed. Because the clock begins to run on this filing date, and not on the day the patent is actually granted, some of the effective term of a patent is consumed by the time it takes to prosecute the application. To mitigate the damage that bureaucracy can do to inventors, the statute grants extensions of patent terms for certain specified kinds of PTO delay, *35 U.S.C. § 154(b)(1)(A)*, and, regardless of the reason, whenever the patent prosecution [**2] takes more than three years. *35 U.S.C. § 154(b)(1)(B)*. Recognizing that the protection provided by these separate guarantees might overlap, Congress has forbidden double-counting: [HN2] "To the extent that periods of delay attributable to grounds specified in paragraph (1) overlap, the period of any adjustment granted under this subsection shall not exceed the actual number of days the issuance of the patent was delayed." *35 U.S.C. § 154(b)(2)(A)*. Plaintiffs claim that the PTO has misconstrued or misapplied this provision, and that the PTO is denying them a portion of the term Congress has provided for the protection of their intellectual property rights.

Statutory Scheme

Until 1994, patent terms were 17 years from the date of issuance. *See 35 U.S.C. § 154 (1992)* ("Every patent shall contain . . . a grant . . . for the term of seventeen years . . . of the right to exclude others from making, using, or selling the invention throughout the United States. . . ."). In 1994, in order to comply with treaty obligations under the General Agreement on Tarriffs and Trade (GATT), the statute was amended to provide a 20-year term from the date on which the application is first filed. *See* Pub. L. No. 103-465, § 532, 108 Stat. 4809, 4984 (1994). [**3] In 1999, concerned that extended prosecution delays could deny inventors substantial portions of their effective patent terms under the new regime, Congress enacted the American Inventors Protection Act, a portion of which -- referred to as the Patent Term Guarantee Act of 1999 -- provided for the adjustments that are at issue in this case. Pub. L. No. 106-113, §§ 4401-4402, 113 Stat. 1501, 1501A-557 (1999).

As currently codified, [HN3] *35 U.S.C. § 154(b)* provides three guarantees of patent term, two of which are at issue here. The first is found in *subsection (b)(1)(A)*, the "[g]uarantee of prompt Patent and Trademark Office response." It provides a one-day extension of patent term for every day that issuance of a patent is delayed by a failure of the PTO to comply with various enumerated statutory deadlines: fourteen months for a first office action; four months to respond to a reply; four months to issue a patent after the fee is paid; and the like. *See 35 U.S.C. § 154(b)(1)(A)(i)-(iv)*. Periods of delay that fit under this provision are called "A delays" or "A periods." The second provision is the "[g]uarantee of no more than 3-year application pendency." Under this provision, a one-day [**4] term extension is granted for every day greater than three years after the filing date that it takes for the patent to issue, regardless of whether the delay is the fault of the PTO. [1] *See 35 U.S.C. § 154(b)(1)(B)*. [*140] The period that begins after the three-year window has closed is referred to as the "B delay" or the "B period". ("C delays," delays resulting from interferences, secrecy orders, and appeals, are similarly treated but were not involved in the patent applications underlying this suit.)

> 1 Certain reasons for exceeding the three-year pendency period are excluded, *see 35 U.S.C. § 154(b)(1)(B)(i)-(iii)*, as are periods attributable to the applicant's own delay. *See 35 U.S.C. § 154(b)(2)(C)*.

The extensions granted for A, B, and C delays are subject to the following limitation:

> **(A) In general.**--[HN4] To the extent that periods of delay attributable to grounds specified in paragraph (1) overlap, the period of any adjustment granted under this subsection shall not exceed the actual number of days the issuance of the patent was delayed.

35 U.S.C. § 154(b)(2)(A). This provision is manifestly intended to prevent double-counting of periods of delay, but understanding that intent does not answer [**5] the question of what is double-counting and what is not. Proper interpretation of this proscription against windfall extensions requires an assessment of what it means for "periods of delay" to "overlap."

The PTO, pursuant to its power under *35 U.S.C. § 154(b)(3)(A)* to "prescribe regulations establishing procedures for the application for and determination of patent term adjustments," has issued final rules and an "explanation" of the rules, setting forth its authoritative construction of the double-counting provision. The rules that the PTO has promulgated essentially parrot the statutory text, *see 37 C.F.R. § 1.703(f)*, and so the real interpretive act is found in something the PTO calls its Explanation of *37 CFR 1.703(f)* and of the United States Patent and Trademark Office Interpretation of *35 U.S.C. § 154(b)(2)(A)*, which was published on June 21, 2004, at *69 Fed. Reg. 34238*. Here, the PTO "explained" that:

> the Office has consistently taken the position that if an application is entitled to an adjustment under the three-year pendency provision of *35 U.S.C. § 154(b)(1)(B), the entire period during which the application was pending before the Office* (except for periods excluded under [**6] *35 U.S.C. § 154 (b)(1)(B)(i)-(iii)*), and not just the period beginning three years after the actual filing date of the application, *is the relevant period under 35 U.S.C. § 154 (b)(1)(B) in determining whether periods of delay "overlap" under 35 U.S.C. 154(b)(2)(A).*

69 Fed. Reg. 34238 (2004) (emphasis added). In short, the PTO's view is that any administrative delay under *§ 154(b)(1)(A)* overlaps any 3-year maximum pendency delay under *§ 154(b)(1)(B)*: the applicant gets credit for "A delay" or for "B delay," whichever is larger, but never A + B.

In the plaintiffs' submission, this interpretation does not square with the language of the statute. They argue that the "A period" and "B period" overlap only if they occur on the same calendar day or days. Consider this example, proffered by plaintiff: A patent application is filed on 1/1/02. The patent issues on 1/1/08, six years later. In that six-year period are two "A periods," each one year long: (1) the 14-month deadline for first office action is 3/1/03, but the first office action does not occur until 3/1/04, one year late; (2) the 4-month deadline for patent issuance after payment of the issuance fee is 1/1/07, but the patent does not [**7] issue until 1/1/08, another year of delay attributable to the PTO. According to plaintiff, the "B period" begins running on 1/1/05, three years after the patent application was filed, and ends three years later, with the issuance of the patent on 1/1/08. In this [*141] example, then, the first "A period" does not overlap the "B period," because it occurs in 2003-04, not in 2005-07. The second "A period," which covers 365 of the same days covered by the "B period," does overlap. Thus, in plaintiff's submission, this patent holder is entitled to four years of adjustment (one year of "A period" delay + three years of "B period" delay). But in the PTO's view, since "the entire period during which the application was pending before the office" is considered to be "B period" for purposes of identifying "overlap," the patent holder gets only three years of adjustment.

***Chevron* Deference**

We must first decide whether the PTO's interpretation is entitled to deference under *Chevron v. NRDC, 467 U.S. 837, 104 S. Ct. 2778, 81 L. Ed. 2d 694 (1984)*. No, the plaintiffs argue, because, under the Supreme Court's holdings in *Gonzales v. Oregon, 546 U.S. 243, 126 S. Ct. 904, 163 L. Ed. 2d 748 (2006)*, and *United States v. Mead Corp., 533 U.S. 218, 121 S. Ct. 2164, 150 L. Ed. 2d 292 (2001)*, Congress has not "delegated [**8] authority to the agency generally to make rules carrying the force of law," and in any case the interpretation at issue here was not promulgated pursuant to any such authority. *See Gonzales, 546 U.S. at 255-56, citing Mead, 533 U.S. at 226-27*. Since at least 1996, the Federal Circuit has held that [HN5] the PTO is not afforded *Chevron* deference because it does not have the authority to issue substantive rules, only procedural regulations regarding the conduct of proceedings before the agency. *See Merck & Co. v. Kessler, 80 F.3d 1543, 1549-50 (Fed. Cir. 1996)*.

Here, as in *Merck*, [HN6] the authority of the PTO is limited to prescribing "regulations establishing *procedures* for the application for and determination of patent term adjustments under this subsection." *35 U.S.C. § 154(b)(3)(A)* (emphasis added). Indeed, a comparison of this rulemaking authority with the authority conferred for a different purpose in the immediately preceding section of the statute makes it clear that the PTO's authority to interpret the overlap provision is quite limited. [HN7] In *35 U.S.C. § 154(b)(2)(C)(iii)* the PTO is given the power to "prescribe regulations establishing the *circumstances that constitute* a failure of an applicant [**9] to engage in reasonable efforts to conclude

processing or examination of an application" (emphasis added) -- that is, the power to elaborate on the meaning of a particular statutory term. No such power is granted under *§ 154(b)(3)(A)*. *Chevron* deference does not apply to the interpretation at issue here.

Statutory Construction

Chevron would not save the PTO's interpretation, however, because it cannot be reconciled with the plain text of the statute. [HN8] If the statutory text is not ambiguous enough to permit the construction that the agency urges, that construction fails at *Chevron's* "step one," without regard to whether it is a reasonable attempt to reach a result that Congress might have intended. *See, e.g., MCI v. AT&T, 512 U.S. 218, 229, 114 S. Ct. 2223, 129 L. Ed. 2d 182 (1994)* ("[A]n agency's interpretation of a statute is not entitled to deference when it goes beyond the meaning that the statute can bear.").

The operative question under *35 U.S.C. § 154(b)(2)(A)* is whether "periods of delay attributable to grounds specified in paragraph (1) overlap." [HN9] The only way that periods of time can "overlap" is if they occur on the same day. If an "A delay" occurs on one calendar day and a "B delay" occurs on another, they do not [**10] overlap, and *§ 154(b)(2)(A)* does not limit the extension to one day. Recognizing this, [*142] the PTO defends its interpretation as essentially running the "period of delay" under *subsection (B)* from the filing date of the patent application, such that a period of "B delay" *always overlaps* with any periods of "A delay" for the purposes of applying *§ 154(b)(2)(A)*.

The problem with the PTO's construction is that it considers the application *delayed* under *§ 154(b)(1)(B)* during the period *before it has been delayed*. That construction cannot be squared with the language of *§ 154(b)(1)(B)*, which applies "if the issue of an original patent is *delayed* due to the failure of the United States Patent and Trademark Office to issue a patent within 3 years." (Emphasis added.) "B delay" begins when the PTO has failed to issue a patent within three years, not before.

The PTO's interpretation appears to be driven by Congress's admonition that any term extension "not exceed the actual number of days the issuance of the patent was delayed," and by the PTO's view that "A delays" during the first three years of an applications' pendency inevitably lead to "B delays" in later years. Thus, as the PTO sees it, if [**11] plaintiffs' construction is adopted, one cause of delay will be counted twice: once because the PTO has failed to meet and administrative deadline, and again because that failure has pushed back the entire processing of the application into the "B period." Indeed, in the example set forth above, plaintiffs' calendar-day construction does result in a total effective patent term of 18 years under the (B) guarantee, so that -- again from the PTO's viewpoint -- the applicant is not "compensated" for the PTO's administrative delay, he is benefitted by it.

But if *subsection (B)* had been intended to guarantee a 17-year patent term and *no more*, it could easily have been written that way. It is true that the legislative context -- as distinct from the legislative history -- suggests that Congress may have intended to use *subsection (B)* to guarantee the 17-year term provided before GATT. But it chose to write a "[g]uarantee of no more than 3-year application pendency," *35 U.S.C. § 154(b)(1)(B)*, not merely a guarantee of 17 effective years of patent term, and do so using language separating that guarantee from a different promise of prompt administration in *subsection (A)*. The PTO's efforts to [**12] prevent windfall extensions may be reasonable -- they may even be consistent with Congress's intent -- but its interpretation must square with Congress's words. If the outcome commanded by that text is an unintended result, the problem is for Congress to remedy, not the agency.

JAMES ROBERTSON

United States District Judge

IN THE UNITED STATES DISTRICT COURT FOR THE

DISTRICT OF COLUMBIA

MEMORIAL SLOAN-KETTERING CANCER CENTER, Plaintiff, v. HON. DAVID KAPPOS Under Secretary of Commerce for Intellectual Property and Director of the United States Patent and Trademark Office Office of the General Counsel United States Patent and Trademark Office Defendant.	Civil Action No. 1:09-cv-02282-(JDB)

NOTICE OF VOLUNTARY DISMISSAL

Pursuant to Federal Rule of Civil Procedure 41(a)(1)(A)(i), Plaintiff Memorial Sloan Kettering Center hereby notifies the Court that it voluntarily dismisses, without prejudice, this action against Defendant David Kappos, Under Secretary of Commerce for Intellectual Property and Director of the United States Patent and Trademark Office ("USPTO"), to allow the USPTO to recalculate the patent term adjustment at issue in accordance with the United States Court of Appeals for the Federal Circuit's decision in *Wyeth & Elan Pharma Int'l Ltd. v. Hon. Jon W. Dudas*, Civil Action No. 07-01492 (JR), 591 F.3d 1364 (Fed. Cir. 2010) ("*Wyeth*").

Date: April 7, 2010

Respectfully submitted,

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APR 19 2010

OFFICE OF PETITIONS

In re Patent No. 7,541,179	:
Sadelain et al.	: DECISION UPON REMAND AND
Issue Date: June 2, 2009	: RECONSIDERATION OF
Application No. 10/188,221	: PATENT TERM ADJUSTMENT
Filed: July 1, 2002	: AND NOTICE OF INTENT
Attorney Docket No.64836(51590)	: TO ISSUE CERTIFICATE OF
Title: HumanGlobin Gene and Use	: CORRECTION
Thereof in Treatment of	:
Hemoglobinopathies	

This is a decision following remand from the District Court for the District of Columbia regarding the patent term adjustment indicated on the above-identified patent. The Court remanded this matter to the U.S. Patent and Trademark Office for recalculation of the patent term adjustment in accordance with the decision in Wyeth & Elan Pharma Int'l Ltd. v. Kappos, 591 F.3d 1364 (Fed. Cir. 2010).

The patent term adjustment indicated on the above-identified patent has been recalculated as directed by the Court. The term of the above-identified patent is extended or adjusted by six hundred eighty-two (682) days.

The application is being forwarded to the Certificates Branch for issuance of a certificate of correction indicating that the term of the above-identified patent is extended or adjusted by six hundred and eighty-two (682) days.

Telephone inquiries specific to this matter should be directed to Senior Legal Advisor, Kery A. Fries at (571) 272-7757.

/Kery A. Fries/

Kery A. Fries

Senior Legal Advisor
Office of Patent Legal Administration
Office of Associate Commissioner
For Patent Examination Policy

Enclosure: Copy of DRAFT Certificate of Correction

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT : 7,541,179 B2

DATED : **June 2, 2009** **DRAFT**

INVENTOR(S) : Sadelain et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the cover page,

[*] Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 USC 154(b) by 431 days

Delete the phrase "by 431 days" and insert – by 682 days--

Day : Monday
Date: 4/19/2010
Time: 09:15:06

PALM INTRANET

PTA Calculations for Application: 10/188221			
Application Filing Date:	**07/01/2002**	PTO Delay (PTO):	**803**
Issue Date of Patent:	**06/02/2009**	Three Years:	**0**
Pre-Issue Petitions:	**0**	Applicant Delay (APPL):	**372**
Post-Issue Petitions:	**0**	Total PTA (days):	**682**
PTO Delay Adjustment:	**251**		

File Contents History

Number	Date	Contents Description	PTO	APPL	START
121	04/16/2010	ADJUSTMENT OF PTA CALCULATION BY PTO	251		
115.5	05/13/2009	PTA 36 MONTHS	551		
115	06/02/2009	PATENT ISSUE DATE USED IN PTA CALCULATION			
114	04/28/2009	EXPORT TO FINAL DATA CAPTURE			
113	04/27/2009	DISPATCH TO FDC			
112	04/27/2009	APPLICATION IS CONSIDERED READY FOR ISSUE			
111	04/24/2009	ISSUE FEE PAYMENT VERIFIED			
110	04/24/2009	ISSUE FEE PAYMENT RECEIVED			
109	03/09/2009	FINISHED INITIAL DATA CAPTURE			
108	02/09/2009	SEQUENCE FORWARDED TO PUBS ON TAPE			
107	01/30/2009	EXPORT TO INITIAL DATA CAPTURE			
105	01/26/2009	MAIL NOTICE OF ALLOWANCE			
104	01/16/2009	ISSUE REVISION COMPLETED			
103	01/16/2009	DOCUMENT VERIFICATION			
102	01/16/2009	NOTICE OF ALLOWANCE DATA VERIFICATION COMPLETED			
101	01/16/2009	CASE DOCKETED TO EXAMINER IN GAU			
100	01/15/2009	EXAMINER'S AMENDMENT COMMUNICATION			
99	01/15/2009	NOTICE OF ALLOWABILITY			
98	12/10/2008	DATE FORWARDED TO EXAMINER			
97	12/03/2008	AMENDMENT/ARGUMENT AFTER NOTICE OF APPEAL			
96	12/03/2008	NOTICE OF APPEAL FILED		91	94
95	12/03/2008	REQUEST FOR EXTENSION OF TIME - GRANTED			

94	06/03/2008	MAIL FINAL REJECTION (PTOL - 326)			
93	05/28/2008	FINAL REJECTION			
91	03/18/2008	DATE FORWARDED TO EXAMINER			
90	02/29/2008	RESPONSE AFTER NON-FINAL ACTION		30	86
89	02/29/2008	REQUEST FOR EXTENSION OF TIME - GRANTED			
88	03/17/2008	MAIL EXAMINER INTERVIEW SUMMARY (PTOL - 413)			
87	02/26/2008	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 413)			
86	10/30/2007	MAIL NON-FINAL REJECTION			
85	10/27/2007	NON-FINAL REJECTION			
84	08/02/2007	INFORMATION DISCLOSURE STATEMENT CONSIDERED			
83	09/12/2007	AFFIDAVIT(S) (RULE 131 OR 132) OR EXHIBIT(S) RECEIVED			
82	09/15/2007	DATE FORWARDED TO EXAMINER			
81	09/12/2007	AMENDMENT SUBMITTED/ENTERED WITH FILING OF CPA/RCE			
80	09/15/2007	DATE FORWARDED TO EXAMINER			
79	09/12/2007	REQUEST FOR CONTINUED EXAMINATION (RCE)			
78	09/15/2007	DISPOSAL FOR A RCE / CPA / R129			
77	09/12/2007	NOTICE OF APPEAL FILED		92	66
76	09/12/2007	REQUEST FOR EXTENSION OF TIME - GRANTED			
74	09/12/2007	WORKFLOW - REQUEST FOR RCE - BEGIN			
73	08/10/2007	MAIL EXAMINER INTERVIEW SUMMARY (PTOL - 413)			
72	08/02/2007	MISCELLANEOUS INCOMING LETTER			
71	08/02/2007	INFORMATION DISCLOSURE STATEMENT (IDS) FILED			
70	08/05/2007	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 413)			
69	08/02/2007	INFORMATION DISCLOSURE STATEMENT (IDS) FILED			
68	08/25/2004	INFORMATION DISCLOSURE STATEMENT CONSIDERED			
67	08/25/2004	INFORMATION DISCLOSURE STATEMENT (IDS) FILED			
66	03/12/2007	MAIL FINAL REJECTION (PTOL - 326)			

65	03/05/2007	FINAL REJECTION			
64	12/08/2006	INFORMATION DISCLOSURE STATEMENT CONSIDERED			
63.7	12/08/2006	INFORMATION DISCLOSURE STATEMENT (IDS) FILED		8	61
63	12/08/2006	INFORMATION DISCLOSURE STATEMENT (IDS) FILED			
62	12/18/2006	DATE FORWARDED TO EXAMINER			
61	11/30/2006	RESPONSE AFTER NON-FINAL ACTION		30	56
60	11/30/2006	REQUEST FOR EXTENSION OF TIME - GRANTED			
59	10/23/2006	MAIL EXAMINER INTERVIEW SUMMARY (PTOL - 413)			
58	10/11/2006	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 413)			
57	08/03/2006	CORRESPONDENCE ADDRESS CHANGE			
56	07/31/2006	MAIL NON-FINAL REJECTION			
55	07/24/2006	NON-FINAL REJECTION			
54	10/08/2002	INFORMATION DISCLOSURE STATEMENT CONSIDERED			
53.7	10/08/2002	INFORMATION DISCLOSURE STATEMENT (IDS) FILED			
53	10/08/2002	INFORMATION DISCLOSURE STATEMENT (IDS) FILED			
52	05/15/2006	DATE FORWARDED TO EXAMINER			
51	05/04/2006	RESPONSE AFTER NON-FINAL ACTION		87	45
50	04/04/2006	MAIL NOTICE OF INFORMAL OR NON-RESPONSIVE AMENDMENT			
49	04/04/2006	CORRESPONDENCE ADDRESS CHANGE			
48	04/04/2006	CHANGE IN POWER OF ATTORNEY (MAY INCLUDE ASSOCIATE POA)			
47	03/13/2006	RECEIPT OF ALL ACKNOWLEDGEMENT LETTERS			
46	02/08/2006	DATE FORWARDED TO EXAMINER			
45.1	02/06/2006	INFORMAL OR NON-RESPONSIVE AMENDMENT AFTER EXAMINER ACTION			
45	02/06/2006	RESPONSE AFTER NON-FINAL ACTION		33	42
44	02/06/2006	REQUEST FOR EXTENSION OF TIME - GRANTED			
43	10/12/2005	CORRESPONDENCE ADDRESS CHANGE			
42	10/04/2005	MAIL NON-FINAL REJECTION			

41	10/03/2005	NON-FINAL REJECTION			
40	07/20/2005	REQUEST FOR REFUND			
39	07/25/2005	DATE FORWARDED TO EXAMINER			
38	06/30/2005	SUPPLEMENTAL RESPONSE		0	34
37	07/18/2005	CASE DOCKETED TO EXAMINER IN GAU			
36.7	06/30/2005	ELECTRONIC INFORMATION DISCLOSURE STATEMENT			
36	06/30/2005	INFORMATION DISCLOSURE STATEMENT (IDS) FILED			
35	07/07/2005	DATE FORWARDED TO EXAMINER			
34	06/30/2005	RESPONSE AFTER NON-FINAL ACTION			
33	07/05/2005	CRF IS GOOD TECHNICALLY / ENTERED INTO DATABASE			
32	06/28/2005	CASE DOCKETED TO EXAMINER IN GAU			
31	03/31/2005	MAIL NON-FINAL REJECTION	5		27
30	03/07/2005	NON-FINAL REJECTION			
29	01/11/2005	CASE DOCKETED TO EXAMINER IN GAU			
28	12/29/2004	DATE FORWARDED TO EXAMINER			
27	11/26/2004	RESPONSE AFTER NON-FINAL ACTION		1	22
26	11/26/2004	REFERENCE CAPTURE ON IDS			
25.7	11/26/2004	INFORMATION DISCLOSURE STATEMENT (IDS) FILED			
25	11/26/2004	INFORMATION DISCLOSURE STATEMENT (IDS) FILED			
24	11/26/2004	AFFIDAVIT(S) (RULE 131 OR 132) OR EXHIBIT(S) RECEIVED			
23	11/26/2004	WORKFLOW INCOMING AMENDMENT IFW			
22	08/25/2004	MAIL NON-FINAL REJECTION			
21	08/23/2004	NON-FINAL REJECTION			
20	06/21/2004	DATE FORWARDED TO EXAMINER			
19	06/01/2004	RESPONSE TO ELECTION / RESTRICTION FILED			
18	06/01/2004	WORKFLOW INCOMING AMENDMENT IFW			
17	05/05/2004	MAIL RESTRICTION REQUIREMENT	247		-1
16	05/03/2004	REQUIREMENT FOR RESTRICTION / ELECTION			
15.7	11/17/2003	INFORMATION DISCLOSURE STATEMENT (IDS) FILED			
15	11/17/2003	INFORMATION DISCLOSURE STATEMENT (IDS) FILED			
14	09/08/2003	IFW TSS PROCESSING BY TECH CENTER COMPLETE			

13	07/15/2003	CASE DOCKETED TO EXAMINER IN GAU			
12	10/28/2002	APPLICATION DISPATCHED FROM OIPE			
11	10/24/2002	APPLICATION IS NOW COMPLETE			
10	10/08/2002	ADDITIONAL APPLICATION FILING FEES			
8	10/08/2002	A STATEMENT BY ONE OR MORE INVENTORS SATISFYING THE REQUIREMENT UNDER 35 USC 115, OATH OF THE APPLIC			
7	09/24/2002	LETTER TO APPLICANT - NO GOVERNMENT INTEREST / PATENT TO ISSUE			
6	09/13/2002	NOTICE MAILED--APPLICATION INCOMPLETE--FILING DATE ASSIGNED			
5	09/06/2002	REFERRED BY L&R FOR THIRD-LEVEL SECURITY REVIEW. AGENCY REFERRAL LETTER GENERATED			
4	09/06/2002	CLEARED BY L&R (LARS)			
3	09/05/2002	IFW SCAN & PACR AUTO SECURITY REVIEW			
2	09/04/2002	IFW SCAN & PACR AUTO SECURITY REVIEW			
1	07/01/2002	INITIAL EXAM TEAM NN			

Search Another: Application# Search

EXPLANATION OF PTA CALCULATION

EXPLANATION OF PTE CALCULATION

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UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,541,179 B2
APPLICATION NO. : 10/188221
DATED : June 2, 2009
INVENTOR(S) : Sadelain et al.

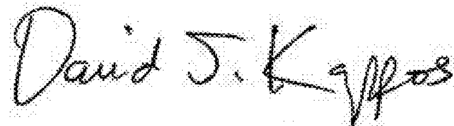
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title Page:

The first or sole Notice should read --

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 682 days.

Signed and Sealed this
Twenty-third Day of August, 2011



David J. Kappos
Director of the United States Patent and Trademark Office

AO 120 (Rev. 08/10)

TO: **Mail Stop 8** **Director of the U.S. Patent and Trademark Office** **P.O. Box 1450** **Alexandria, VA 22313-1450**	**REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK**

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the Southern District of New York on the following

☐ Trademarks or ☒ Patents (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO	DATE FILED	U.S. DISTRICT COURT
1:21-cv-08206-VSB	10/5/2021	for the Southern District of New York

PLAINTIFF	DEFENDANT
Errant Gene Therapeutics, LLC	Memorial Sloan-Kettering Cancer Center and Sloan Kettering Institute of Cancer Research

PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1 7,541,179	06/02/2009	Memorial Sloan-Kettering Cancer Center
2 8,058,061	11/15/2011	Memorial Sloan-Kettering Cancer Center
3		
4		
5		

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

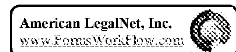
DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE
Ruby J. Krajick	/S/ S. James	10/06/2021

Copy 1—Upon initiation of action, mail this copy to Director Copy 3—Upon termination of action, mail this copy to Director
Copy 2—Upon filing document adding patent(s), mail this copy to Director Copy 4—Case file copy



AO 120 (Rev. 08/10)

TO: **Mail Stop 8** **Director of the U.S. Patent and Trademark Office** **P.O. Box 1450** **Alexandria, VA 22313-1450**	**REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK**

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED 10.21.2021	U.S. DISTRICT COURT for the District of Delaware
PLAINTIFF ERRANT GENE THERAPEUTICS, LLC		DEFENDANT BLUEBIRD BIO, INC.

PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1 7,541,179 B2	6/2/2009	Memorial Sloan-Kettering Cancer Center
2 8,058,061 B2	11/15/2011	Memorial Sloan-Kettering Cancer Center
3		
4		
5		

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	**DATE OF PATENT OR TRADEMARK**	**HOLDER OF PATENT OR TRADEMARK**
1		
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In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director Copy 3—Upon termination of action, mail this copy to Director
Copy 2—Upon filing document adding patent(s), mail this copy to Director Copy 4—Case file copy