Synergistic regulation of human β-globin gene switching by locus control region elements HS3 and HS4

Jörg Bungert, Utpal Davé, Kim-Chew Lim, Ken H. Lieuw, Jordan A. Shavit, Qinghui Liu, and James Douglas Engel[1]

Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60208-3500 USA

Proper tissue- and developmental stage-specific transcriptional control over the five genes of the human β-globin locus is elicited in part by the locus control region (LCR), but the molecular mechanisms that dictate this determined pattern of gene expression during human development are still controversial. By use of homologous recombination in yeast to generate mutations in the LCR within a yeast artificial chromosome (YAC) bearing the entire human β-globin gene locus, followed by injection of each of the mutated YACs into murine ova, we addressed the function of LCR hypersensitive site (HS) elements 3 and 4 in human β-globin gene switching. The experiments revealed a number of unexpected properties that are directly attributable to LCR function. First, deletion of either HS3 or HS4 core elements from an otherwise intact YAC results in catastrophic disruption of globin gene expression at all erythroid developmental stages, despite the presence of all other HS elements in the YAC transgenes. If HS3 is used to replace HS4, gene expression is normal at all developmental stages. Conversely, insertion of the HS4 element in place of HS3 results in significant expression changes at every developmental stage, indicating that individual LCR HS elements play distinct roles in stage-specific β-type globin gene activation. Although the HS4 duplication leads to alteration in the levels of ϵ- and γ-globin mRNAs during embryonic erythropoiesis, total β-type globin mRNA synthesis is balanced, thereby leading to the conclusion that all of the human β-locus genes are competitively regulated. In summary, the human β-globin HS elements appear to form a single, synergistic functional entity called the LCR, and HS3 and HS4 appear to be individually indispensable to the integrity of this macromolecular complex.

[*Key Words*: LCR; competition; HS3; HS4]

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Each of the genes in the human β-globin locus is sequentially activated during embryonic development: the 5′-most (embryonic) ϵ-globin gene is expressed during the first trimester of gestation, the two γ-globin genes during the second and third trimesters, and, shortly after birth, the 3′ adult δ- (minor) and β-globin proteins replace γ-globin chains in tetrameric hemoglobin (Stamatoyannopoulos and Neinhuis 1994). The β-type globin genes are all regulated by the locus control region (LCR), positioned far 5′ of the structural genes themselves (Forrester et al. 1987; Grosveld et al. 1987). The LCR is composed of one constitutive and four tissue-specific DNaseI hypersensitive sites (designated HS1, closest to ϵ-globin, to the constitutive element HS5, lying furthest away; see Orkin 1990, for nomenclature).

The LCR (or smaller units containing the HSs, termed mini- or micro-LCRs) was originally studied as a single contiguous structure, where its effects on single genes or a subset of the genes within the β-globin locus were assayed (e.g., Talbot et al. 1989). Individual LCR elements HS2, HS3, and HS4 were then shown to be independently capable of conferring high level, tissue-specific transcription to linked human β-locus genes in transgenic mice (Curtin et al. 1989; Ryan et al. 1989; Ney et al. 1990; Talbot et al. 1990; Pruzina et al. 1991; Talbot and Grosveld 1991; Lloyd et al. 1992; Morley et al. 1992). Subsequently, it was shown that the HS elements individually elicited markedly different developmental stage-specific activities in constructs harboring several β-locus genes (Fraser et al. 1993). Thus LCR elements were shown to be more than passive amplifiers of globin gene transcription, and rather, were intrinsically capable of conferring a discriminating developmental response in transcriptional control over individual β-locus genes. While the LCR is now generally acknowledged to be an active participant in this program of temporal gene activation, gene-proximal regulatory sequences have also

[1]Corresponding author.

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been known for some time to confer significant tissue-specific activity (Magram et al. 1985; Kollias et al. 1986; Behringer et al. 1987; Trudel et al. 1987; Antoniou et al. 1988). The underlying mechanism by which the LCR modulates the appropriate transcriptional activity of each β-type globin gene during development nonetheless remains obscure.

The nomenclature used to describe the individual behavior of different globin genes during development (hemoglobin switching) was originally applied to the chicken embryonic ϵ- or adult β-globin genes. Autonomously regulated genes are both activated and suppressed in proper developmental time with only an enhancer in *cis*, whereas competitive regulation requires other *cis* elements to be present in a particular construct for proper temporal gene activation and suppression (Choi and Engel 1988). Enver et al. (1990) first showed that competition was also applicable to describing gene regulation in the human β-globin locus. More recent evidence supporting the notion that β-globin locus genes are regulated by a competitive mechanism showed that LCR function is grossly disrupted if a strong transcription unit is placed between human HS1 and HS2 (Kim et al. 1992); however, deletion of HS2 alone from the endogenous mouse β-globin locus results in only a mild phenotype (Fiering et al. 1995). Transgenic experiments examining fragments of the LCR linked to human β-locus genes led to the conclusions that ϵ-globin was autonomously regulated (Raich et al. 1990; Shih et al. 1990), while adult β-globin transcription was competitive (Enver et al. 1990). Evidence for control of the γ-globin genes was, however, contradictory: Two early reports suggested that γ-globin transcription was regulated competitively, whereas subsequent observations provided compelling evidence for autonomy (Behringer et al. 1990; Enver et al. 1990; Dillon and Grosveld 1991).

The disparity in the results analyzing human γ-globin gene regulation could be attributed to differences in the arrangement and number of *cis*-regulatory elements surrounding the gene. Such contradictions arising from the analysis of slightly different DNA constructions could reflect a requirement for mutant transgenes to be examined within a context where all known (as well as perhaps currently unidentified) regulatory elements are present. Thus transformation of mice with YACs containing large segments of contiguous genomic DNA came as a timely technical advance. In independent reports, YAC transformation of the murine germ line resulted in the recovery of tissue-specific control of the tyrosinase or α1(I) collagen genes, where earlier studies had failed to demonstrate complete complementation with smaller (genomic λ or cosmid DNA) clones (Schedl et al. 1993b; Strauss et al. 1993). In contemporary experiments, it was shown that YACs bearing the entire human β-globin locus also resulted in appropriate developmental regulation of the human genes after introduction into the mouse germ line (Gaensler et al. 1993; Peterson et al. 1993). We therefore devised a strategy to create LCR mutations in a YAC bearing the whole human β-globin locus by use of homologous recombination in yeast. YAC DNAs were then isolated from pulse-field gels and injected into fertilized ova to generate transgenic mice. Pups containing intact, unrearranged, single-copy YACs were monitored for the expression of each human β-locus gene at different stages of erythroid development by use of a reverse-transcription–PCR (RT–PCR) assay.

In this report, we describe several explicit tests of a regulatory sequence competition hypothesis formulated for the human β-globin gene locus (Engel 1993). In essence, the model predicted that the stage-specific activation of the genes within the human locus would be achieved by preferential binary association of individual HS sites with (known or theoretical) gene-proximal regulatory elements at various times during erythroid development. DNA looping and direct juxtaposition of distal and proximal elements were proposed to mediate these interactions (Choi and Engel 1988; Gallarda et al. 1989). Each of the mutations revealed new insight into the complex roles that the HS3 and HS4 elements play in the generation of LCR function and in developmental stage-specific competition between human β-globin locus genes and LCR hypersensitive sites.

The present experiments show that deletion of either HS3 or HS4 fundamentally impairs expression of all the β-type globin genes at all erythroid developmental stages, despite the fact that other powerful LCR elements (for example in the case of the HS4 core element-deletion mutant, HS2 and HS3) remain in the YAC transgene. The replacement of HS4 by HS3 appears to fully complement HS4 function at every developmental stage. The converse, however, is not true; when HS4 is substituted for HS3, the expression of every gene at all stages of erythropoiesis is altered, and thus HS4 cannot fully compensate for HS3 function. Moreover, when the wild-type and the HS4 substitution mutant transgenes are compared, alteration of the transcript level of human ϵ- and γ-globin mRNAs in the embryonic yolk sac is reciprocal. This suggests that HS3 and HS4 competitively and collaboratively control the expression of these two genes at the embryonic stage of erythroid development. Taken together, these data reveal that the LCR behaves as a single cooperative unit and that, not only is this synergistic macromolecular complex comprised of the individual HS elements, but also the elements are not always uniquely specified for discrete functions in the complex.

Results

Generation of human β-globin YAC mutants

A201F4 is a 155-kb YAC that contains the entire human β-globin locus (Gaensler et al. 1991, 1993). The YAC initially segregated unstably in the parent yeast strain, and therefore, a clone which stably mitosed was isolated by mating and sporulation. The YAC DNA was then cloned into a bacteriophage λ vector (Maniatis et al. 1982), and individual recombinant phage containing overlapping segments of the β-globin locus were isolated (Fritsch et al. 1980). Recombinants specifying the

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β-globin LCR HS elements and the individual genes were further subcloned into plasmid vectors to facilitate mutagenesis.

The minimal HS4 core element has been localized to a 280-bp *Sac*I–*Ava*I segment of a 3.2-kb parental *Eco*RI–*Hin*dIII fragment (Pruzina et al. 1991), while the minimal HS3 core element was defined as a 225- bp *Hph*I–*Fnu*4HI fragment within a larger 1.9-kb *Hin*dIII fragment (Philipsen et al. 1990; Fig. 1). For the creation of the HS3 replacement mutant, the HS4 core element was amplified by PCR from the parental HS4 subclone with primers that incorporated unique *Xba*I and *Xho*I sites at the ends. The subcloned products of all PCR reactions were verified by sequencing. This 280-bp HS4 minimal element was then used to replace the corresponding HS3 core sequence within the 1.9-kb HS3 *Hin*dIII subclone, which generated the mutant hypersensitive site designated $HS3^43$ (i.e., a 280-bp HS4 core element embedded in HS3-flanking DNA; Fig. 1). A plasmid incorporating the HS3 core element surrounded by HS4-flanking sequences ($HS4^34$) was generated by use of a conceptually identical strategy. Two other plasmids containing deletions of each of the two HS core elements within the bodies of the otherwise unmodified parental fragments were constructed at the same time (see Materials and methods).

To facilitate the generation of mutant β-locus YACs, two manipulations were performed. First, the *URA3* selectable marker gene in the right arm of A201F4 was retrofitted with the *LYS2* gene, thereby inactivating *URA3* in the YAC by homologous recombination (Srivastava and Schlessinger 1991). At the same time, each of the four mutated HS plasmids (described above) was subcloned into the yeast integrative plasmid vector pRS306, containing the *URA3* gene (Sikorski and Hieter 1989). Each pRS306 subclone was then used to transform the (*LYS2*-modified) wild-type human β-globin YAC. For example, $HS3^43$ (Fig. 1) subcloned in pRS306 was digested with *Sau*I and then used to transform yeast bearing the *LYS2*-retrofitted YAC clone (Fig. 2; see Materials and methods). The majority of the yeast colonies growing on selective (trp^-/lys^-/ura^-) medium contained the targeting plasmid integrated at the homologous human β-globin HS site within the YAC, thereby creating an intermediate with the LCR elements arranged (e.g., in replacing HS3 with HS4) in the order: 5′-HS4–[$HS3^43$]–*URA3*–HS3–HS2-3′ (Fig. 2). Selective excision of the targeting plasmid was mediated by growth on medium containing uracil, followed by counterselection on 5-fluoroorotic acid (FOA)-containing plates (lethal to cells retaining the *URA3* marker; see Materials and methods). Excision of pRS306 resulted in reversion to either the parental YAC structure or replacement of the parental HS site by the desired mutant element (Fig. 2; Winston et al. 1983). Thus the number of YACs to be tested for transgene expression characteristics in mice were five (Fig. 3): wild-type (the *LYS2*-retrofitted YAC, designated HS4321), an HS3 duplication (HS3321; incorporating $HS4^34$), an HS3 core element deletion (HS4021; incorporating $HS3^03$), an HS4 duplication (HS4421; incorporating $HS3^43$), and the HS4 core element deletion (HS0321; incorporating $HS4^04$). The structures of the four mutants and the parental wild-type YAC were verified by Southern blotting on both conventional and pulse-field gels (Fig. 4).

Transgenic mice

Two methods have been used to introduce YACs into the mouse germ line. One involved microinjection into

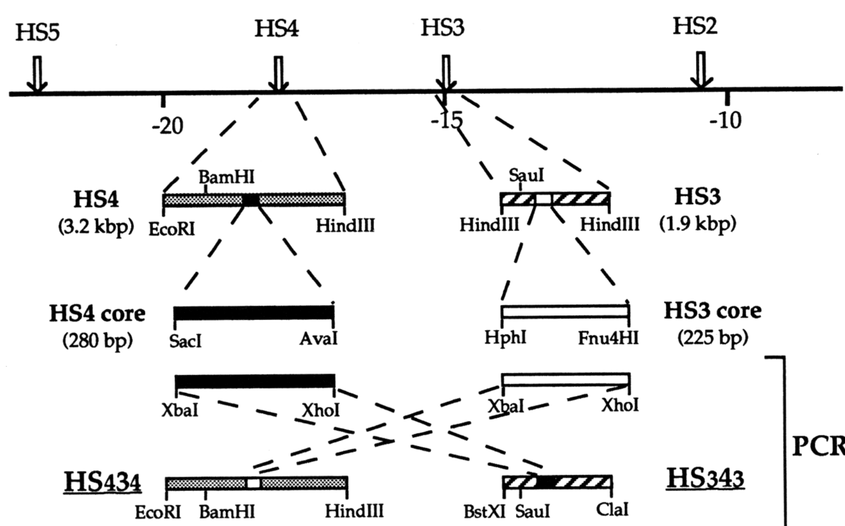

Figure 1. Diagram of the human β-globin LCR and generation of the HS3 and HS4 replacement mutants. The *top* line indicates the positions of the hypersensitive sites in the human β-globin LCR (Talbot et al. 1989). The second line depicts the two subclones (HS3 and HS4) that were used to initiate the yeast targeting mutagenesis strategy (see Materials and methods). These two HS element subclones were used as substrates for PCR amplification of the individual core elements and flanking sequences. The PCR amplification was carried out with primers that incorporated new, unique restriction enzyme sites at a number of positions to aid in subsequent cloning steps (Table 1). Both the HS3 and HS4 core elements (line 3) were amplified from the respective parental plasmids using these primers to incorporate new sites into the cores (line 4), and finally the two core elements were exchanged between the two (appropriately PCR-adapted) parent clones to yield the desired mutant elements $HS3^43$ and $HS4^34$ (line 5).

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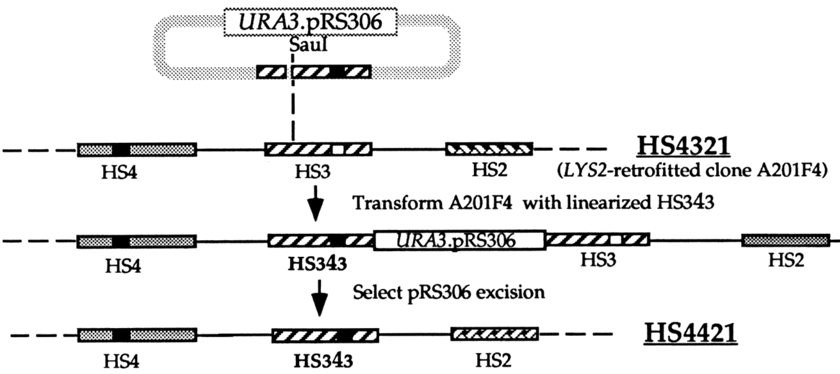

Figure 2. Generation of LCR HS replacement and deletion mutants by use of homologous recombination. In this example, the HS3^{4}3 fragment (generated as outlined in Fig. 1) was subcloned into yeast shuttle vector pRS306 (Sikorski and Hieter 1989). After digestion with *Sau*I (*top* line), it was then used to transform (*LYS2*-modified; see Materials and methods) yeast clone A201F4, harboring the 155-kb human β-globin YAC (line 2; Gaensler et al. 1991). Colonies containing the targeting vector (i.e., growth on Lys$^-$, Trp$^-$, Ura$^-$ medium) were tested for homologous integration by Southern blot analysis (line 3). Individual clones that had undergone homologous recombination were replated on Ura$^+$ medium and then replica plated onto FOA plates (see Materials and methods). Clones growing on FOA were tested individually for the presence of the original YAC (HS4321) vs. the replacement mutant structure (HS4421; line 4) by Southern blots (see Fig. 4).

fertilized murine eggs (Schedl et al. 1993a, b), whereas another involved lipofection-mediated transfer of purified YAC DNA into embryonic stem (ES) cells, followed by blastocyst injection to generate chimeric mice (Strauss et al. 1993). We chose to use the former method because microinjection is reported to result in more frequent integration of intact YAC transgene DNA (Gnirke et al. 1993), and because we planned to use these same mutant YACs for transgenic studies after further mutagenesis.

Wild-type and mutated YACs were isolated after pulse-field gel electrophoresis (PFGE), purified, and microinjected into fertilized murine ova (see Materials and methods; Schedl et al. 1993a,b; Peterson et al. 1993).

After initial PCR identification and then Southern blotting of founder tail DNA to confirm the presence of the transgene, all positive animals were bred to ensure that the transgenes could be stably transmitted to progeny. At least two transgenic lines were analyzed for each of the YACs, arbitrarily referred to below as lines a and b (an additional line, c, was analyzed, which contained the HS4421 YAC mutant transgene). Southern blotting of each of the F_1 or F_2 lines, with probes both flanking and internal to the locus (RI 1.8, RI 3.3, RK29, HS4, HS3, HS2, ε-, γ-, and β-globin; Gaensler et al. 1991; Fig. 3) showed that each contained restriction fragments of the expected size. Fragmented YACs discovered through this analysis were excluded from further consideration. The a

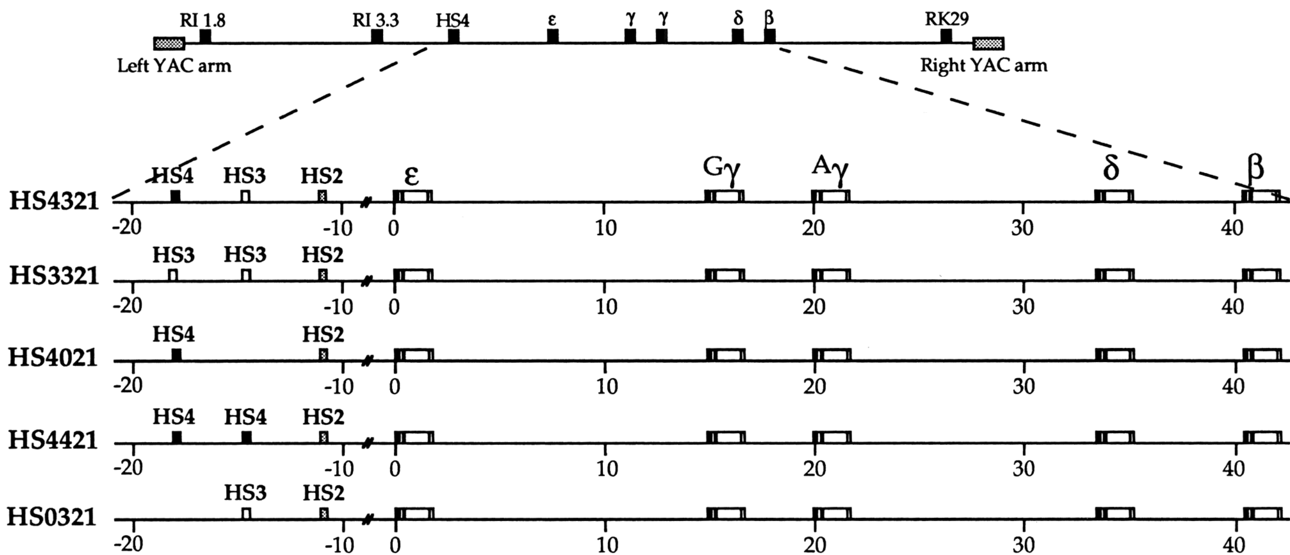

Figure 3. Diagrammatic representation of the LCR mutant YACs. Diagrammatic representations of the five individual human β-globin YACs generated for this analysis are shown. The *bottom* five lines depict the structures of the parental and four mutant YACs studied here, whereas the *top* line depicts the approximate position of all these elements within the YAC borne by A201F4. In addition, other sequences flanking the gene and LCR elements were used as probes in Southern blot analysis to characterize the physical integrity of the locus before and after transgene integration into the germ line of mice (see Results; Fig. 4; data not shown; Gaensler et al. 1991).

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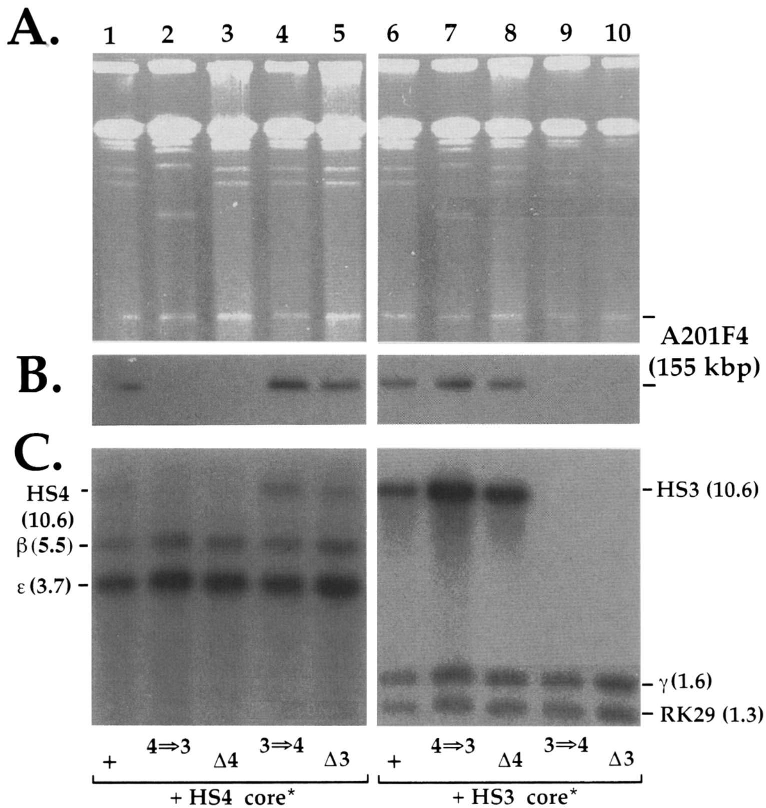

Figure 4. Characterization of the A201F4 YAC HS mutations. All four YAC mutants (HS3321, lanes *2,7*; HS0321, lanes *3,8*; HS4421, lanes *4,9*; and HS4021, lanes *5,10*) generated by use of the strategy outlined in Fig. 2 and depicted diagrammatically in Fig. 3, were analyzed by Southern blotting. (*A*) Confirmation of targeted mutagenesis in each of the YACs was first investigated by PFGE. Examination of ethidium bromide-stained pulse-field gels of the parental and mutated human β-globin YACs showed that the mutant YAC chromosomes are unaltered in size. (*B*) The pulse-field gel shown in *A* was transferred to a nylon membrane and then hybridized with radiolabeled HS4 (lanes *1–5*) or HS3 (lanes *6–10*) core element probes. (*C*) DNA prepared from the wild-type and each of the mutant YACs was digested with *Eco*RI, transferred to nylon filters, and then hybridized to radiolabeled HS4, adult β-globin and embryonic ϵ-globin probes (lanes *1–5*) or HS3, fetal γ-globin and 3′ flanking marker RK29 probes (lanes *6–10*; Fig. 3, Gaensler et al. 1991). Abbreviations representing each of the YACs are (+) HS4321 (lanes *1,6*; the wild-type YAC; Fig. 3); (4 → 3) HS3321; (Δ4) HS0321; (3 → 4) HS4421; and (Δ3) HS4021.

line animals used for breeding and subsequent mRNA analysis were found to contain a single copy of the YAC left and right vector arms, except a single transgenic line in which HS3 had been deleted (HS4021a, Figs. 3 and 7, below), which contained neither (not shown). The b transgenic lines also contained all markers within the locus on contiguous restriction enzyme fragments of the expected size, and virtually identical band intensities. In summary, these data indicated that all of the animals subjected to detailed analysis here contained intact, single-copy YAC transgenes that were transmissible through the germ line.

Human β-globin multiplex PCR assay

To determine the pattern, timing, and abundance of expression of each of the human β-globin locus genes in transgenic mice, RNA isolated from the yolk sacs, fetal livers, or adult spleens of transgenic embryos or animals (see Materials and methods) was analyzed by semiquantitative RT–PCR (Foley and Engel 1992; Foley et al. 1993; Leonard et al. 1993). The level of ϵ-, γ-, and β-globin mRNAs were compared to an internal control, mouse α-globin mRNA, which remains relatively constant during murine gestation (Whitelaw et al. 1990). This RNA analysis method relied on the fact that unique primers for each of the human β-type globin genes could be defined (Fig. 5A; Table 1) that they would give rise to specific amplicons that differed from one another in size, and that these primers would not cross-react, either with one another or with cDNAs produced after reverse transcription of mouse β-type globin mRNAs. We demonstrated that these expectations could be fulfilled in control experiments (Fig. 5B), thus simplifying the analysis by allowing simultaneous (multiplex) assay for accumulation of all three human β-globin transcripts (huϵ, huγ, and huβ) and the endogenous α-globin control (muα) in each RNA sample at every stage of murine erythroid development. The assay is only semi-quantitative, however, because primer sets for each gene may differ subtly in annealing efficiency and could therefore differ in the amount of isotope-labeled deoxynucleotide incorporated (the products are of different length and G+C content); thus the absolute abundance of one transcript compared to another at a different stage of development is not quantitative, but the relative abundance of any individual PCR product at specific developmental stages can be directly compared after normalization to the intensity of the muα globin internal control, the length and G+C content of the PCR products, and the transgene copy number in comparison to the endogenous mouse α-globin genes (see Mateials and methods; Table 2).

RNAs were prepared by use of standard procedures (Chomczynski and Sacchi 1987), from transgenic F_2 or F_3 embryos at 9.5 days post-coitus (dpc) (yolk sac) and 14.5 dpc (liver) or from the spleens of 2- to 6-month-old anemic animals, representing embryonic, fetal or adult de-

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