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Expression of a β-Globin Gene Is Enhanced by Remote SV40 DNA Sequences

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Summary

We have studied the transient expression of a cloned rabbit hemoglobin β1 gene after its introduction into HeLa cells. Two and one-half days after transfection using the calcium phosphate technique, we extracted RNA from the entire cell population and analyzed it by the S1 nuclease hybridization assay. Transcripts were barely detectable when β-globin gene–plasmid recombinants were used. However, 200 times more β-globin gene transcripts were found when the β-globin gene recombinants also contained SV40 DNA, and 90% of these transcripts (about 1000 per cell) had the same 5′ end as authentic rabbit globin mRNA. In the latter case, abundant production of β-globin protein was readily detected in a fraction of transfected cells by immunofluorescent staining. Enhancement of globin gene expression was dependent on SV40 sequences acting in cis, but independent of the viral origin of DNA replication. The enhancing activity was associated with the 72 bp repeated sequence element located at the beginning of the viral late gene region. Viral DNA fragments containing the transcriptional enhancer element could act in either orientation at many positions, including 1400 bp upstream or 3300 bp downstream from the transcription initiation site of the rabbit β-globin gene. These studies define a class of DNA elements with a mode of action that has not been heretofore described. The activation of genes by specific enhancer elements seems to be a widespread mechanism that may be used for the regulation of gene expression.

Introduction

The DNA sequence requirements for transcription of eucaryotic genes by RNA polymerase II in vivo are being intensively studied (Grosschedl and Birnstiel, 1980a, 1980b; Benoist and Chambon, 1980, 1981; Gluzman et al., 1980; Dierks et al., 1981a, 1981b; Faye et al., 1981; Gruss et al., 1981; Guarente and Ptashne, 1981; Mellon et al., 1981). Most of the DNA important for efficient transcription in vivo seems to be present within some 100 bp upstream from the transcription initiation site. For at least some genes, sequences far more than 100 bp upstream from the initiation site were found to influence transcription in vivo. Grosschedl and Birnstiel (1980b) identified a "modulator" of transcription, a segment of DNA in front of an H2A histone gene that is required for its efficient transcription. Transcription of the early genes of SV40 depends on DNA sequences around 200 bp upstream from the initiation sites, in a region of two directly repeated 72 bp sequence motifs (Benoist and Chambon, 1981; Gruss et al., 1981; M. Fromm and P. Berg, personal communication).

We show a 200-fold increase in the level of correctly initiated transcripts from a rabbit β-globin gene when it is linked to SV40 DNA. The DNA segment that was found to enhance the expression of the β-globin gene, for convenience referred to as the enhancer, was found to be associated with the 72 bp repeated sequence motif of SV40 mentioned above. Most interestingly, the viral "enhancer" can act over very long distances, and independent of its orientation. Thus the 72 bp repeat region does not act solely as an upstream promoter component of the SV40 early genes, as could be inferred from the work of Benoist and Chambon (1981) and Gruss et al. (1981). Apart from its biological significance, the enhancer phenomenon can also be exploited for the construction of high-level expression vectors for mammalian cells.

Results

Transient Expression of the Rabbit β-Globin Gene in HeLa Cells

We tested transcription of a cloned hemoglobin β-chain gene from the rabbit (Maniatis et al., 1978; obtained from T. Maniatis) using a transient expression assay in HeLa cells. Subconfluent cell monolayers were transfected with recombinant plasmids by a modification of the calcium phosphate coprecipitation technique described by Wigler et al. (1978). The recombinants contained a 4.7 kb long segment of rabbit chromosomal DNA encompassing the β1-globin gene (Figure 1A). RNA was extracted from the transfected HeLa cells after 2½ days (60 hr) and analyzed by the S1 nuclease hybridization assay (Berk and Sharp, 1977; Weaver and Weissmann, 1979). Efficient globin gene transcription was observed with the clone pSVK+, a recombinant in which the 4700 bp Kpn I fragment with the rabbit β-globin gene had been inserted into the Kpn I site of SV40 DNA in an SV40–pBR322 recombinant (Figures 1B and 1C). Most of these transcripts had a 5′ end indistinguishable from that of authentic rabbit β-globin mRNA (Figures 2B and 2C lanes 1 and 2). Transfection with the plasmid pβ2×, a recombinant of the same size with two globin genes but no SV40 DNA (Figure 1F), resulted in only 0.5% of the level of β-globin gene transcripts (Figures 2B and 2C lanes 3) as compared with transfection with the pSVK+ clone. By comparing the high levels of transcripts from pSVK+ with the β-globin mRNA standard, we estimated that there were 1000 to 1500 correct globin-gene transcripts per cell. If we consider a transfection efficiency of 10%–15%, this means that

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in every successfully transfected cell there were 5,000 to 10,000 globin-gene transcripts. Equally high levels of correct transcripts (Figure 2D) were obtained with a derivative of the pβ2× plasmid, designated pβSV(−)β, which had a complete copy of SV40 DNA inserted between the two globin genes (see legend to Figure 1F).

There exists the formal possibility that the globin RNA in HeLa cells was generated from longer transcripts by nucleolytic cleavage at the cap site. However, there is good evidence for eucaryotic genes in general, and for mammalian β-globin genes in particular, that the 5′ ends of messenger RNAs are the sites of transcription initiation (Contreras and Fiers, 1981; Grosveld et al., 1981a; Hofer and Darnell, 1981), findings which are supported by in vitro transcription

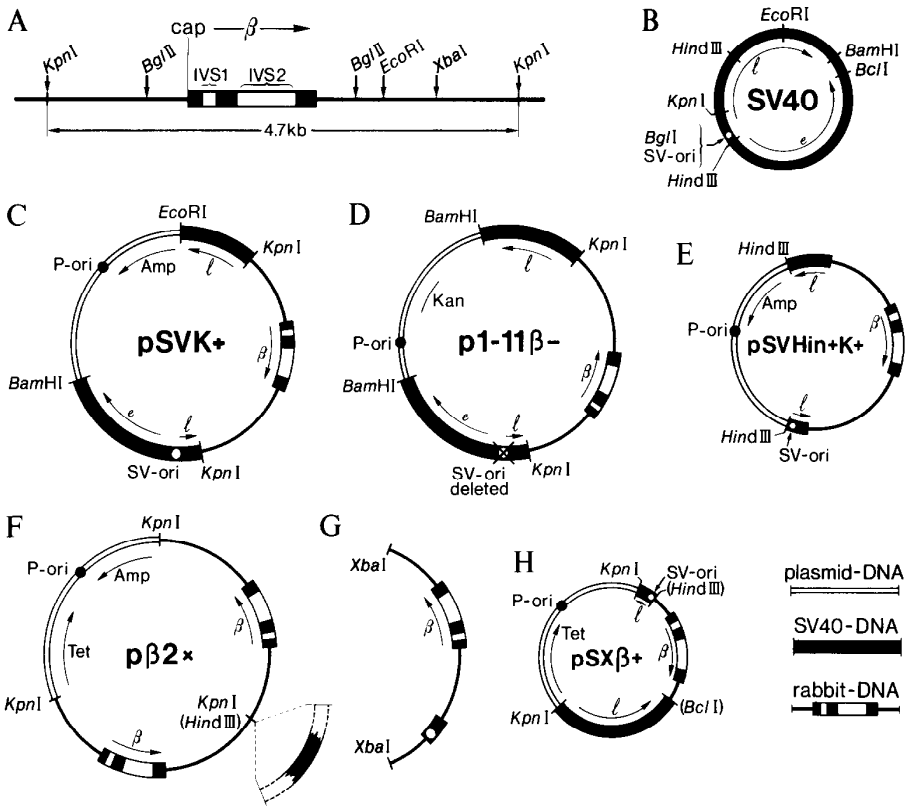

Figure 1. Recombinant DNAs Containing the Rabbit β-Globin Gene

(A) Schematic representation of the genomic rabbit hemoglobin β1 gene (Maniatis et al., 1978). Solid line: noncoding DNA. Solid bars: coding sequences. Open bars: intervening sequences (IVS1 and IVS2). Vertical arrows: restriction sites where SV40 sequences were integrated and were found to enhance globin gene expression (see text).

(B) Map of SV40 DNA with some of the restriction sites used for the construction of our recombinants. The DNA is 5243 bp long; nucleotide positions mentioned in the text are the distances from the replication origin in a clockwise fashion according to Appendix A of Tooze (1980).

(C) Map of pSVK+; the 4.7 kb Kpn I globin gene fragment was cloned into the single Kpn I site found in the SV40 late region of the previously constructed recombinant plasmid pBSV−early (Schaffner, 1980). The globin gene was also cloned in the opposite orientation into pBSV−early, yielding the recombinant pSVK−.

(D) Map of p1-11β−; the 4.7 kb globin gene fragment was cloned into the Kpn I site of a mutant SV40 DNA lacking 58 bp at the origin of replication (Gluzman et al., 1980).

(E) Map of pSVHin+K+; the Hind III C fragment of SV40 containing the integrated β-globin gene was obtained from pSVK+ and was cloned into the plasmid pJC-1 (pJC-1 is a derivative of pBR322; see Experimental Procedures).

(F) Map of pβ2×; the 4.7 kb globin gene fragment was cloned as a dimer insert into the plasmid pJC-1 (see Rusconi and Schaffner, 1981, where this recombinant was also referred to as pJKd−). For further experiments, putative "enhancer" DNAs of various size were cloned into the Kpn I site between the two globin genes; some of these DNAs were inserted by means of Hind III linkers (see text). One of the pβ2× derivatives, the clone pβSV(−)β, was obtained by insertion of a complete Kpn I–cut SV40 genome between the two globin genes such that the SV40 early region and the β-globin genes were transcribed from opposite DNA strands.

(G) Map of pβ366(−)β cleaved with Xba I; example of a linear DNA used for transfection. The 366 bp SV40 Hind III–Kpn I fragment was inserted between the globin genes of pβ2× by means of Hind III linkers. Digestion of the resulting clone, pβ366(−)β, with Xba I liberated one globin gene with the SV40 DNA insert.

(H) Map of pSXβ+; a 2.1 kb Bgl II fragment with the globin gene replacing the SV40 early region was inserted between the Bcl I site and the Hind III site near the origin of replication.

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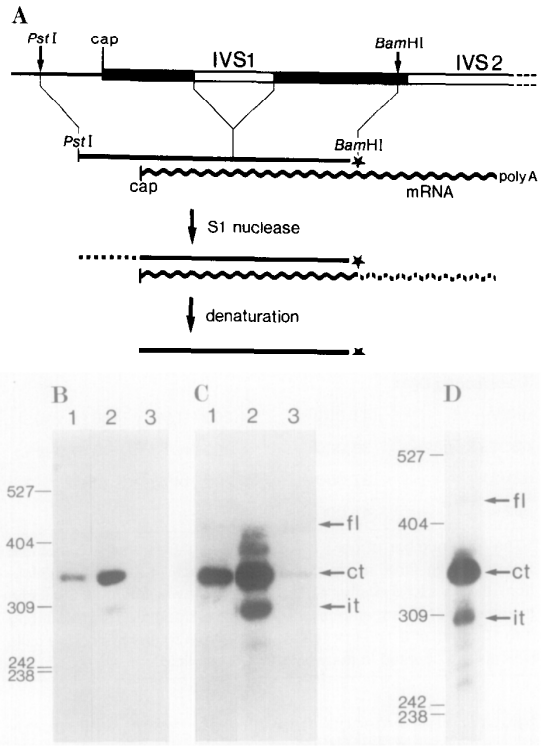

Figure 2. Detection of the Correct 5′ Terminus of Globin RNA

(A) S1 nuclease mapping scheme (Weaver and Weissmann, 1979). For this experiment, a globin gene clone lacking the first intervening sequence (IVS1; see Figure 1A; Weber et al., 1981) was used as a radioactive probe (for further details see Rusconi and Schaffner, 1981). DNA end-labeled at the Bam HI site was hybridized to unlabeled RNA, treated with S1 nuclease, denatured, fractionated by gel electrophoresis and autoradiographed.

(B and C) Autoradiographs of the same gel after 6 hr and 48 hr of exposure, respectively. (Lanes 1) Hybridization to 0.2 ng rabbit β-globin mRNA. (Lanes 2) Hybridization to RNA from 2.5 × 10^6 HeLa cells transfected with the β-globin–SV40–pBR322 clone pSVK+ (Figure 1C). (Lanes 3) Hybridization to RNA from the β-globin-gene recombinant plasmid pβ2×, which does not contain SV40 sequences (Figure 1F). The intensity difference in the major band between lanes 2 and 3 was measured to be 200-fold. This was done by scanning a series of autoradiographs of different exposure times to minimize any nonlinear relation between radioactivity and blackening of the x-ray film.

(D) Hybridization to RNA from 10^6 HeLa cells transfected with the clone pβSV(−)β. Exposure time was 40 hr.

fl: full-length input DNA (453 nucleotides). ct: fragment with correct terminus, mapping 354 nucleotides upstream from the Bam HI site. it: incorrect terminus, about 306 nucleotides upstream from the Bam HI site. Numbers to the left: size and position of marker DNA fragments (pBR322 digested with Hpa II; Sutcliffe, 1978).

studies (Proudfoot et al., 1980; Grosveld et al., 1981b; Hagenbüchle and Schibler, 1981). We therefore conclude that in our HeLa cell assay we are observing correct transcription initiation at the β-globin-gene cap site. In addition to the correct 5′ terminus, we have also detected low levels of transcripts with an "incorrect" 5′ end in our S1 nuclease assay that mapped about 48 nucleotides downstream from the correct terminus. Such transcripts are not a peculiarity of our assay: rabbit β1-globin genes from different sources have been introduced into a variety of vertebrate cells, and the same incorrect terminus described above has also been observed in these other systems (Wold et al., 1979; Dierks et al., 1981a, 1981b; Rusconi and Schaffner, 1981). Most of the globin-gene transcripts in HeLa cells were not only correctly initiated, but also quantitatively processed to mRNA. We monitored splicing of the first intervening sequence by the S1 nuclease assay as outlined in Figure 2A, using a similar end-labeled DNA probe, which, however, contained the first intron (see also Rusconi and Schaffner, 1981). All transcripts were found to be correctly spliced, since they protected the probe only from the intron–exon junction to the labeled end. They were also polyadenylated (most of them were selectively bound to oligo[dT]-cellulose), and were translated within the HeLa cells to an abundant peptide that comigrated with authentic rabbit β globin in gel electrophoresis (data not shown).

An indirect immunofluorescence assay was also used to examine β-globin production. Sixty hours after transfection, cells were fixed and stained with sets of antibodies so that the SV40 large tumor antigen (T antigen) and β globin could be detected. Fluorescence microscopy indicated the presence of T antigen as a green, nuclear fluorescence. A red, cytoplasmic fluorescence indicated β globin (Figure 3). After transfection with pSVK+ (Figure 1C) or other similar recombinants, T antigen could be detected in 10%–35% of the cells, and of those, 2%–10% (0.7%–1.4% of all cells) showed globin gene expression. The higher sensitivity for detection of T antigen over β globin in this assay may be due to a higher stability of T antigen and/or to a higher antibody titer. In this assay, the number of fluorescent cells is correlated with the level of transcripts, but the relation is probably not linear. At high levels of β-globin gene expression, differences in transcription efficiency may be underestimated with the immunofluorescence assay. However, immunofluorescence was a clear-cut indicator of enhanced globin gene expression: transfection of HeLa cells with globin-gene plasmids without SV40 sequences never yielded any cells with specific cytoplasmic fluorescence (although low levels of globin gene transcripts could be detected by the S1 nuclease assay; see Figure 2). The immunofluorescence assay was therefore used for all further experiments.

To determine the best conditions for assaying the expression of the rabbit β-globin gene, we performed time-course experiments with two β-globin–SV40–plasmid recombinant clones. One was the pSVK+ clone used previously; the other clone, p1-11β−, whose construction is outlined below, contained SV40 DNA from a replication-defective mutant. These were transfected into HeLa cells that were then assayed by

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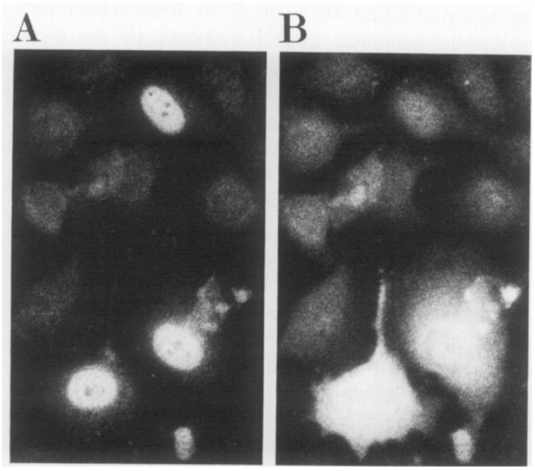

Figure 3. Production of T Antigen and of β Globin Monitored by Immunofluorescence

HeLa cells were fixed with methanol 60 hr after transfection with the β-globin–SV40–pBR322 clone pSVK+ (Figure 1C). The cells were stained by means of indirect immunofluorescence for T antigen (fluorescein; green) and for β globin (rhodamine; red). By switching filters we could screen a given cell for T-antigen production as well as for β-globin production.

(A) Cell sample with three cells having fluorescent nuclei, indicating the presence of SV40 T antigen.

(B) The lower two of the T-antigen-positive cells in (A) are also positive for rabbit β globin, as revealed by cytoplasmic rhodamine fluorescence. Bar = 50 μm.

immunofluorescence for production of both T antigen and β globin. The results with p1-11β− are shown in Table 1. The clone pSVK+ gave essentially the same results (data not shown). It was found that T-antigen and β-globin levels were highest at 2 to 2½ days after transfection and rapidly declined thereafter.

Analysis of the SV40 "Enhancer" Effect

To determine whether the SV40 sequences acted in cis or in trans, we cotransfected HeLa cells with a mixture of the cloned DNAs pBSV3× (Schaffner, 1980) and pβ2×, containing three tandem copies of SV40 DNA and two tandem copies of the β-globin gene, respectively. The cells in an 8 × 8 mm field were analyzed by immunofluorescence. Though the usual number of cells (4800; 13% of all cells) expressed T antigen, only three cells were β-globin-positive. In a parallel transfection the β-globin–SV40 recombinant pβSV(−)β gave rise to 4200 T-antigen-expressing and 466 globin-expressing cells in an area of equal size (Table 1). If the SV40 sequences were acting in trans, one would expect about 450 rather than 3 globin-expressing cells in the mixing experiment, since cells that are "competent" to express transiently one kind of DNA from a mixture of two transfected DNAs are found to express the other DNA also (see footnote to Table 2). The same "competence" phenomenon has already been well documented in cell-transformation experiments (Wigler et al., 1979). The three globin-positive cells in our mixing experiment described above (Table 1) are likely to be the result of intracellular recombination between the transfected plasmids, since unrelated mixed DNAs used for cell transformation can be linked together within the recipient cell eventually to form large DNA entities (Pellicer et al., 1980; Perucho et al., 1980).

Table 1. Production of T Antigen and β Globin in Transfected HeLa Cells

	Total Cells (8 × 8 mm)[a]	Cells Positive for T Antigen	Cells Positive for β Globin
Days after Transfection with Clone p1-11β−			
2	33,000	12,000 (35%)	242 (0.7%)
2½	32,000	11,000 (34%)	265 (0.8%)
3½	29,000	4,900 (17%)	53 (0.2%)
5½	35,000	2,500 (7%)	11 (0.03%)
8	31,000	600 (2%)	0
Transfecting DNA			
pβSV(−)β	41,000	4,200 (10%)	466 (1.1%)
pBSV3×, pβ2×	38,000	4,800 (13%)	3 (0.008%)
pSV3×	37,000	5,200 (14%)	0
pβ2×	41,000	0	0

[a] The total number of cells in an 8 × 8 mm area was extrapolated from counting three areas of 0.145 mm² each (400-fold magnification); that is, a total number of 150 to 300 cells was counted. Similarly, the number of T-antigen-positive cells was extrapolated from counting 7 to 12 areas of 0.145 mm² each. The number of globin-positive cells was always counted in the whole 8 × 8 mm field.

We next wanted to determine if enhanced expression of the globin gene was a copy-number effect resulting from the activity of an SV40 replicon. This possibility did not appear very likely, since the replication of SV40 is severely inhibited by cis-acting plasmid sequences (Lusky and Botchan, 1981). In all our experiments such plasmid sequences were present whenever a complete SV40 replicon was linked to the β-globin gene, and analysis of pSVK+ DNA from transfected HeLa cells by Southern blot hybridization (Southern, 1975) did not indicate any replication of this recombinant (data not shown). In addition, we tested viral DNAs that either lacked a functional origin of replication or did not contain coding sequences for T antigen, both of which are required for SV40 replication (Tegtmeyer and Ozer, 1971; Gluzman et al., 1980). Cloned mutants of SV40 with deletions of 9 bp and of 58 bp at the origin of DNA replication were provided by Y. Gluzman (Gluzman et al., 1980; see Figures 4d and 4e). Both mutants, after transfection into CV1 monkey cells, produce normal amounts of functional T antigen but are unable to replicate. The

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globin-gene Kpn I fragment was cloned into the Kpn I site of the mutant SV40 DNAs 6-17 (9 bp deleted) and 1-11 (58 bp deleted) to yield the clones p6-17β+ and p1-11β− (Figure 1D). HeLa cells were transfected and assayed by immunofluorescence for production of T antigen and rabbit β globin. Both p6-17β+ (data not shown) and p1-11β− (Table 1) efficiently expressed the rabbit β-globin gene.

To determine if T-antigen expression is required for the enhancing effect, we tested the 1118 bp SV40 Hind III C fragment (map positions 5171 to 1046 according to Appendix A of Tooze [1980]; see Figures 1B and 4b), a segment containing the origin of replication and adjacent "late"-region sequences but no sequences coding for T antigen. This fragment, containing a β-globin gene inserted at the Kpn I site, was obtained from a complete Hind III digest of pSVK+ (Figure 1C) and was cloned into the Hind III site of the plasmid pJC-1 in both orientations, resulting in the clones pSVHin+K+ (Figure 1E) and pSVHin−K+. The same SV40 Hind III C fragment containing the β-globin gene in opposite orientation was obtained from the clone pSVK− (see legend to Figure 1C), and was also cloned in both orientations into the plasmid pJC-1 to yield the clones pSVHin+K− and pSVHin−K−. The four different recombinants that resulted were transfected into HeLa cells. These plasmids showed a much lower expression of the β-globin gene than the parental recombinants pSVK+ and pSVK− (data not shown). This suggested either that only part of the SV40 enhancing activity was present within the Hind III C fragment, or that the enhancing activity was disturbed by neighboring plasmid sequences. The latter was found to be the case, since digestion of the recombinants with Hind III prior to transfection led to very efficient globin production (Table 2), thus indicating that T antigen is not required. Linear and circular DNAs work equally well in transfection experiments (our unpublished data), presumably because linear DNA can be circularized within the nucleus of the transfected cell (Subramanian, 1979). We also encountered another example of plasmid DNA interference: the enhancing activity of SV40 was not detected when viral DNA was separated from the globin gene on both sides by one 3.7 kb copy of the plasmid pJC-1 (our unpublished data). This kind of interference was not further investigated. Instead, most of the recombinants were made by inserting putative enhancer sequences between the two globin genes of pβ2×. In these molecules, no negative effect from vector plasmid DNA was observed.

Another DNA fragment lacking coding sequences for T antigen was also tested. The small Hind III–Kpn I fragment of SV40 DNA (366 bp, positions 5171 to 294; see Figures 1B and 4c) was inserted between the two globin genes of pβ2× (Figure 1F), in either orientation, by means of Hind III linkers. These DNAs were transfected into HeLa cells either undigested or digested with Kpn I or Xba I to release β-globin dimers or monomers, respectively, containing the 366 bp of SV40 DNA (Figure 1G). All of these DNAs gave enhanced expression of the β-globin gene (data not shown). No globin-positive cells were found with the parental plasmid pβ2×, whether used undigested (Table 1) or digested with Kpn I or Xba I (data not shown).

Table 2. Production of T Antigen and β Globin in Transfected HeLa Cells

	Total Cells (8 × 8 mm)	Cells Positive for T Antigen	Cells Positive for β Globin
Transfecting DNA			
pSVHin+K+ (Hind III–digested)	32,000	0	286 (0.9%)
pSVHin+K− (Hind III–digested)	36,000	0	415 (1.1%)
pSVHin+K− (digested), pBSV3× (Control)	32,000	4200 (13%)	310 (1%)[a]
pBSV3× (Control)	38,000	6600 (17%)	0
pSVK+ (Control)	42,000	6200 (15%)	438 (1%)
PSXβ+	37,000	0	516 (1.4%)
pSXβ−	30,000	0	333 (1.1%)
72 bp Repeats Present			
2 (Wild-type)	40,000	7900 (20%)	504 (1.3%)
1 (−72 bp)	42,000	6300 (15%)	331 (0.8%)
½ (−105 bp)	36,000	9 (0.03%)	0

[a] Every β-globin-positive cell was also positive for SV40 T antigen.

The phenomenon of enhanced globin gene expression was not restricted to linkage of SV40 and the β-globin gene at their Kpn I sites. A 2.1 kb long Bgl II fragment containing the rabbit β-globin gene was cloned in both orientations into the SV40 early region by means of Xho I linkers (Figure 1H). Both these clones (pSXβ+ and pSXβ−) gave high levels of globin gene expression upon transfection into HeLa cells (Table 2). Viral "enhancer" sequences were also active when inserted at the Xba I or Eco RI sites (Figure 1A) downstream from the globin gene (data not shown).

Localization of the SV40 "Enhancer" Sequences

The 366 bp segment of SV40 DNA that enhances β-globin gene expression contains, apart from the function as origin of replication, which was found to be dispensable, some peculiar structural features: a 17 bp segment containing only the bases adenine and thymine, two repeated-sequence motifs of 21 bp and the 72 bp repeat (Figure 4a). Promoter information for

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