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

Julian Banerji, Sandro Rusconi and Walter Schaffner
Institut für Molekularbiologie II
Universität Zürich
Hönggerberg, CH-8093 Zürich, Switzerland

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

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

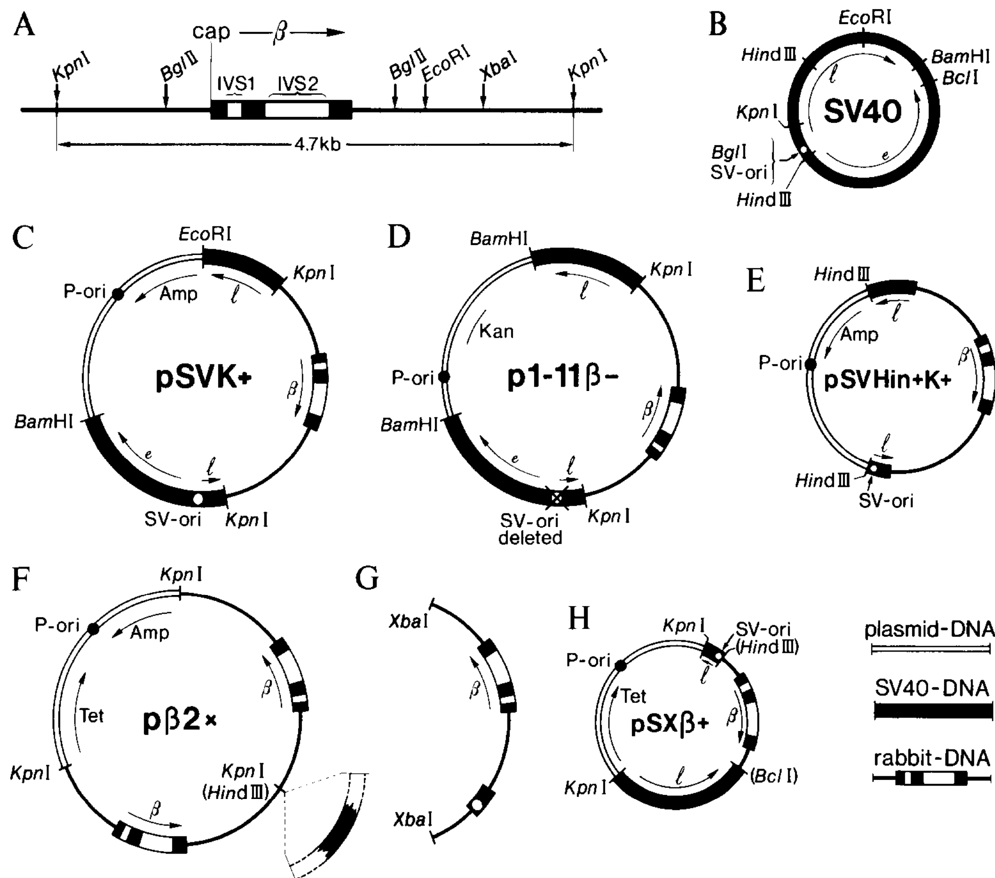

Figure 1. Recombinant DNAs Containing the Rabbit β-Globin Gene

(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.

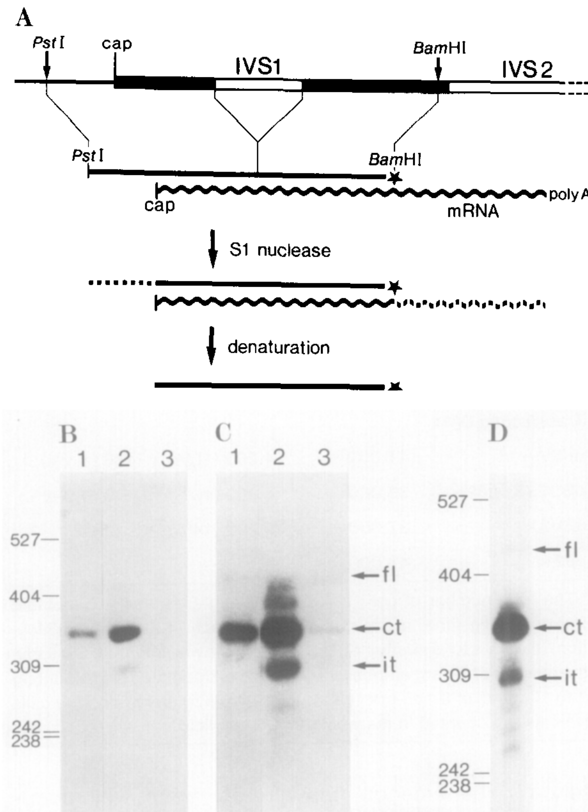

Figure 2. Detection of the Correct 5′ Terminus of Globin RNA

(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

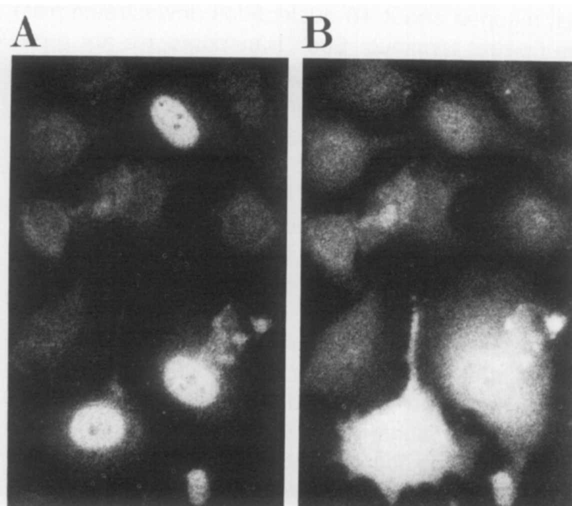

Figure 3. Production of T Antigen and of β Globin Monitored by Immunofluorescence

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.

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 mm^2 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 mm^2 each. The number of globin-positive cells was always counted in the whole 8 × 8 mm field.

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).

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

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

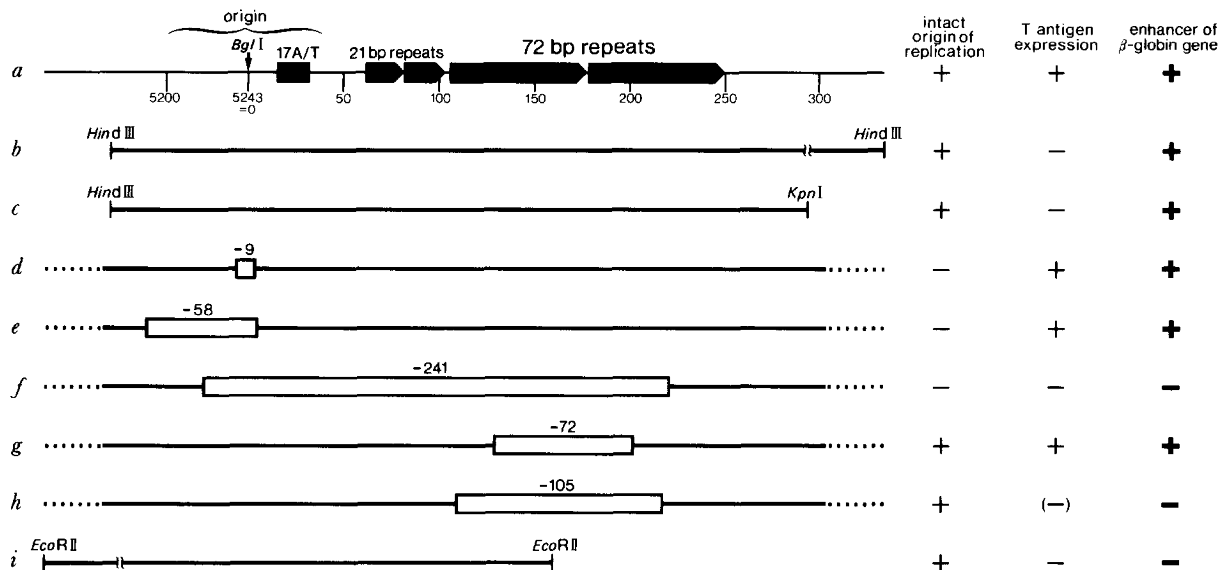

Figure 4. Mapping of the Transcriptional "Enhancer" Region near the SV40 Origin of Replication

(a) Wild-type DNA with the characteristic features of its sequence indicated (nucleotide numbering according to Appendix A of Tooze [1980]).
(b) The 1118 bp Hind III C fragment (map positions 5171 to 1046).
(c) The 366 bp Hind III-Kpn I fragment (5171 to 294).
(d-h) Various deletion mutants constructed by Y. Gluzman (Gluzman et al., 1980; see text for further details).
(i) The 311 bp Eco RII G fragment (5092 to 160; Rio et al., 1980).

the transcription of the viral early and probably also the late region is present within the 366 bp segment. We were able to localize the enhancer activity further by linking the β-globin gene to the cloned Eco RII G fragment of SV40 (Rio et al., 1980; a gift from R. Tjian), and to two SV40 mutants that had deletions located in the 72 bp repeat region (a gift from Y. Gluzman). The deletion mutants had been cloned via the SV40 Eco RI site into the vector plasmid. These recombinants were opened at the Kpn I site within the SV40 DNA, and the β-globin-gene Kpn I fragment was inserted there. The ability of these SV40 deletion mutants to act as long-distance "enhancers" of the globin gene was directly related to the presence of at least one intact 72 bp element. The mutant in which one of the 72 bp repeated motifs had been precisely eliminated by deletion of the small Sph I-Sph I restriction fragment (positions 131 to 202; Y. Gluzman, personal communication) did not show a significant decrease in enhancing activity (Figure 4g; Table 2). However, the mutant with an additional deletion of about 33 bp, removing sequences around the remaining Sph I site as shown in Figure 4h, was unable to enhance β-globin gene expression (Table 2). In addition, the cloned Eco RII G fragment of SV40 (positions 5092 to 160), containing the origin of replication and most of one 72 bp element (Figure 4i), was inserted in both orientations into the Eco RI site 650 bp downstream from the globin gene polyadenylation site. These clones did not show enhanced globin gene activity. Thus we assume that at least one copy of the 72 bp repeated-sequence element is essential for transcriptional enhancement of globin gene expression.

Discussion

We have studied the transient expression of a rabbit β-globin gene by transfecting it into HeLa cells. We found that a small segment of SV40 DNA, including the 72 bp repeated-sequence motif near the origin of replication, enhanced the transcription of the linked β-globin gene by two orders of magnitude, and that the transcripts were translated into hemoglobin β-chain protein. Almost all of the transcripts had the same 5′ end as rabbit β-globin mRNA. Neither SV40 T antigen nor a functional origin of DNA replication was required for the enhancing effect. Therefore both readthrough transcripts from SV40 promoters and a copy-number effect due to replication of a linked SV40 replicon are unlikely to be responsible for the enhanced globin gene expression. It is also unlikely that SV40 sequences act solely by increasing uptake and/or by stabilization of linked DNA, because similar amounts of recombinant DNA were found within the HeLa cell nuclei 60 hr after transfection whether or not SV40 sequences were linked to the β-globin gene (data not shown). Nor is it likely that there is a general stimulation of macromolecular synthesis or a stabilization of mRNA by some diffusible SV40 factor, since SV40 DNA acts only in cis.

The SV40 "enhancer" sequences could activate the promoter of a remote gene by several possible mechanisms, including reorganizing the chromatin

structure; changing the superhelical density of the DNA (reviewed by Smith, 1981); binding to the nuclear matrix (Cook and Brazell, 1975), where viral DNA (Buckler-White et al., 1980) and transcriptional complexes (Jackson et al., 1981) seem to be located; and providing an entry site for RNA polymerase II (discussed by Grosschedl and Birnstiel, 1981).

Another possible mechanism of SV40 DNA action has been suggested by Capecchi (1980), who reported an enhancing effect of SV40 DNA on cell transformation mediated by the herpesvirus thymidine kinase gene. In a transient expression assay, however, the thymidine kinase gene without SV40 DNA appeared highly active. He concluded that the SV40 DNA increased the number of stable transformants by facilitating integration of the thymidine kinase gene into recipient cell DNA. In our transient expression assay the β-globin gene is quite inactive unless linked to the SV40 "enhancer." This could be taken to mean that globin genes have to be integrated into HeLa cell DNA in order to be efficiently expressed. If so, then the cells expressing the β-globin gene should do this in a more stable fashion than the cells expressing T antigen, since the SV40 early region can be well expressed from extrachromosomal DNA (reviewed by Acheson, 1980). However, in our time-course experiment (Table 1) the expression of both β globin and T antigen was transient, and the proportion of globin-positive cells declined even more rapidly than the proportion of T-antigen-positive cells. It should be pointed out that our findings do not argue against a "chromosomal integrator" activity of SV40 DNA, but against requisite integration for efficient globin gene expression.

Whatever the mechanism of globin gene activation observed in our assay, it is a peculiarity neither of the rabbit β-globin gene, nor of SV40 DNA. A histone gene cluster of the sea urchin Psammechinus miliaris (Schaffner et al., 1978) was linked to a pBR322-SV40 vector and transfected into HeLa cells. It was found that the SV40 sequences enhanced the number of transcripts of three histone genes at once (M. Bendig, C. Hentschel and W. Schaffner, unpublished data). Our observations with the SV40 "enhancer" element have been used in other laboratories to obtain enhanced transcription of the human β-globin gene also (Moschonas et al., 1981). The increased transformation frequency of the herpesvirus thymidine kinase gene after linkage to SV40 DNA (see above; Capecchi, 1980; Reddy and Greenspan, 1981) may be due to an enhanced transcription of the thymidine kinase gene.

Polyoma virus contains a functional counterpart to the SV40 72 bp repeat required for viral "early" gene expression (R. I. Kamen, personal communication). We have subcloned a 240 bp segment from this region, and have found that it enhances rabbit β-globin gene expression (J. de Villiers and W. Schaffner, unpublished data). The DNA in the same region is rearranged in polyoma virus mutants that were selected to grow in mouse embryonal carcinoma cells (Katinka et al., 1980, 1981; Fujimura et al., 1981; Sekikawa and Levine, 1981), implicating this region in tissue-specific control of transcription and/or replication.

Even more striking are the functional similarities between the SV40 "enhancer" element and sequences within the long terminal repeat of retroviruses, since retroviruses are organized quite differently from papovaviruses. Avian leukosis virus can activate a cellular oncogene, the so-called *myc* gene, by integrating next to it (Hayward et al., 1981; Neel et al., 1981; Payne et al., 1981). Similar to our findings with the SV40 "enhancer," it appears as if the *myc* gene is activated whether viral DNA is integrated upstream or downstream from it (Payne et al., 1981).

It is tempting to speculate that there are cellular sequences analogous to viral "enhancers." Support for this comes from studies of S. Conrad and M. Botchan (personal communication), who have isolated segments of human DNA that crosshybridize to SV40 DNA. When such a DNA segment was linked to the herpesvirus thymidine kinase gene, in either orientation, the transformation frequency in human thymidine-kinase-negative cells was increased about 20-fold. Enhancer elements of similar function, like the 72 bp repeat of SV40 and the nonrepetitive "enhancer" of polyoma virus, do not show any obvious sequence homology, nor is the 73 bp repeated sequence within the long terminal repeat of Moloney sarcoma provirus (Dhar et al., 1980) similar in primary structure to the SV40 and polyoma virus "enhancers." Crosshybridization to SV40 DNA may therefore yield only a subset of all putative "enhancer" elements of the human genome.

Studies on the organization and expression of the hemoglobin gene family have shown that neighboring genes are activated pairwise during development, thereby defining domains of gene activation of 10–20 kb in length (reviewed by Efstratiadis et al., 1980). The DNA in chromosomes of higher organisms seems to be organized into loop structures of 10–100 kb (Marsden and Laemmli, 1979; Razin et al., 1979), and it was speculated that these chromosome loops constitute the domains of gene activation (Bernards et al., 1979; Bernards and Flavell, 1980). Taken together it appears possible that cellular "enhancers" are activating the genes within each chromosome domain, and that classes of different "enhancers" are involved in the developmental, as well as tissue-specific, expression of genes.

Experimental Procedures

Construction of Recombinant DNA Clones

Cloning in plasmid vectors, including conversion of certain restriction sites with synthetic Xho I, Hind III or Eco RI "linker" DNAs (Collabo-

rative Research) was done as described by Telford et al. (1979). Most of the cloning in bacteria was done with the vector plasmid pJC-1 (a gift from J. Jenkins). pJC-1 is a derivative of pAT153 with its Eco RI site replaced by a Kpn I site. pAT153 (Twigg and Sherratt, 1980) is derived from pBR322 by a deletion of 705 bp (positions 1643 to 2348 of pBR322; Sutcliffe, 1978).

All work involving recombinant plasmids was done under conditions conforming to the standards outlined in the NIH guidelines for recombinant DNA research.

Cell Growth and Transfection

HeLa cells were grown in Dulbecco's modified Eagle's essential medium (GIBCO) containing 2.5% fetal calf serum, 2.5% calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO). Transfections were done according to the method of Wigler et al. (1978) with some modifications; for example, cells were exposed to recombinant DNA for 24 hr instead of 4 hr. Dishes were then washed twice with Tris-buffered saline (Kimura and Dulbecco, 1972) and incubated with fresh medium. Thirty-six hours after addition of the DNA, the cells from a 60 mm dish were trypsinized, and one eighth were used to reseed a 35 mm dish for the immunofluorescence assay, while the rest were reseeded onto a 90 mm dish for RNA extraction. Unless otherwise stated, incubation was stopped 60 hr after addition of the DNA, and the cells were processed for analysis (see below).

Immunofluorescence

Cells in 35 mm plastic petri dishes (FALCON) were rinsed once with Tris-buffered saline (Kimura and Dulbecco, 1972). Two milliliters of cold (−20°C) absolute methanol were added, and the dish was slightly agitated (Mertz and Berg, 1974). The methanol was replaced by another 2 ml cold methanol, and the dish was kept at −20°C for 10 min. The methanol was aspirated, and the dish was allowed to air-dry completely for 15 min in a laminar flow hood. Dried plates were stored at −20°C, or were immediately processed for indirect immunofluorescence. An 8 × 8 mm area in the center of the plate was scratched in with a scalpel blade and was prewetted with PBS-A (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4). A ring of 0.3 ml PBS-A was also placed around the inner edge of the plate to prevent drying of the antibody sample. The PBS-A in the 8 × 8 mm area was replaced by 10 μl of antibody dilution in PBS-A (20% [v/v] goat antibody solution raised against rabbit β-globin, from K. D. Smith; and 5% [v/v] hamster antiserum raised against SV40 T antigen, from F. A. Anderer). The dish was covered with the lid, tilted for a few times and kept at room temperature in a moist atmosphere for 4 to 8 hr with occasional tilting. The dish was washed three times with PBS-A, and kept with PBS-A for 10 min. PBS-A was aspirated, the area around the central 8 × 8 mm square was wiped dry with Kleenex tissue and 60 μl PBS-A were immediately added to the central square (for good results, it was essential not to let the cells dry between the two staining steps). The PBS-A in the central area was replaced by 10 μl of fluorescent antibody solution in PBS-A (10% rhodamine-labeled rabbit antigoat IgG, from Nordic Laboratories; and 10% fluorescein-labeled rabbit antihamster IgG, from GIBCO). The dishes were incubated at room temperature for 3 hr as described above, rinsed three times with PBS-A and incubated with PBS-A for 15 min at room temperature. PBS-A was aspirated, and the 8 × 8 mm area was covered first with a droplet of 90% (v/v) glycerol, 10% Tris-buffered saline, and then with a coverslip before being inspected by fluorescence microscopy. At this stage, dishes could also be stored at −20°C in the dark for several months for repeated inspections. The fluorescence microscope was a Zeiss standard 18 type with epifluorescent condensor and Neofluoar objective. Cells were visible by their slight background staining. We monitored the same cells for rhodamine-stained cytoplasm and for fluorescein-stained nuclei by switching the filters. Cells that had been transfected with DNA lacking coding sequences for SV40 T antigen were also stained with both rhodamine-labeled and fluorescein-labeled antibodies. Since rounded-off mitotic cells and cells that were dead before fixation can bind antibody nonspecifically, double labeling permitted a clear distinction between such artifacts and globin-producing cells: the former were stained with both rhodamine and fluorescein, whereas the latter specifically showed cytoplasmic rhodamine fluorescence.

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References

Acheson, N. H. (1980). Lytic cycle of SV40 and polyoma virus. In DNA Tumor Viruses, J. Tooze, ed. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp. 125–204.

Benoist, C. and Chambon, P. (1980). Deletions covering the putative promoter region of early mRNAs of simian virus 40 do not abolish T-antigen expression. Proc. Nat. Acad. Sci. USA *77*, 3865–3869.

Benoist, C. and Chambon, P. (1981). *In vivo* sequence requirements of the SV40 early promoter region. Nature *290*, 304–310.

Berk, A. J. and Sharp, P. A. (1977). Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell *12*, 721–732.

Bernards, R. and Flavell, R. A. (1980). Physical mapping of the globin gene deletion in hereditary persistence of foetal haemoglobin (HPFH). Nucl. Acids Res. *8*, 1521–1534.

Bernards, R., Kooter, J. M. and Flavell, R. A. (1979). Physical mapping of the globin gene deletion in $(\delta\beta)^0$-thalassaemia. Gene *6*, 265–280.

Buckler-White, A. J., Humphrey, G. W. and Pigiet, V. (1980). Association of polyoma T antigen and DNA with the nuclear matrix from lytically infected 3T6 cells. Cell *22*, 37–46.

Capecchi, M. R. (1980). High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. Cell *22*, 479–488.

Contreras, R. and Fiers, W. (1981). Initiation of transcription by RNA polymerase II in permeable, SV40-infected or noninfected CV1 cells: evidence for multiple promoters of SV40 late transcription. Nucl. Acids Res. *9*, 215–236.

Cook, P. R. and Brazell, I. A. (1975). Supercoils in human DNA. J. Cell Sci. *19*, 261–279.

Dhar, R., McClements, W. L., Enquist, L. W. and Vande Woude, G. F. (1980). Nucleotide sequences of integrated Moloney sarcoma provirus long terminal repeats and their host and viral junctions. Proc. Nat. Acad. Sci. USA *77*, 3937–3941.

Dierks, P., van Ooyen, A., Mantei, N. and Weissmann, C. (1981a). DNA sequences preceding the rabbit β-globin gene are required for formation in mouse L cells of β-globin RNA with the correct 5′ terminus. Proc. Nat. Acad. Sci. USA *78*, 1411–1415.

Dierks, P., Wieringa, B., Marti, D., Reiser, J., van Ooyen, A., Meyer, F., Weber, H. and Weissmann, C. (1981b). Expression of β-globin genes modified by restructuring and site directed mutagenesis. In ICN-UCLA Symposium on Molecular and Cellular Biology, *33*, D. D. Brown and C. F. Fox, eds. (New York: Academic Press), in press.

Efstratiadis, A., Posakony, J. W., Maniatis, T., Lawn, R. M., O'Connell, C., Spritz, R. A., DeRiel, J. K., Forget, B. G., Weissman, S. M., Slightom, J. L., Blechl, A. E., Smithies, O., Baralle, F. E., Shoulders, C. C. and Proudfoot, N. J. (1980). The structure and evolution of the human β-globin gene family. Cell *21*, 653-668.

Faye, G., Leung, D. W., Tatchell, K., Hall, B. D. and Smith, M. (1981). Deletion mapping of sequences essential for *in vivo* transcription of the iso-1-cytochrome *c* gene. Proc. Nat. Acad. Sci. USA *78*, 2258-2262.

Fujimura, F. K., Deininger, P. L., Friedmann, T. and Linney, E. (1981). Mutation near the polyoma DNA replication origin permits productive infection of F9 embryonal carcinoma cells. Cell *23*, 809-814.

Gluzman, Y., Sambrook, J. F. and Frisque, R. J. (1980). Expression of early genes of origin-defective mutants of simian virus 40. Proc. Nat. Acad. Sci. USA *77*, 3898-3902.

Grosschedl, R. and Birnstiel, M. L. (1980a). Identification of regulatory sequences in the prelude sequences of an H2A histone gene by the study of specific deletion mutants *in vivo*. Proc. Nat. Acad. Sci. USA *77*, 1432-1436.

Grosschedl, R. and Birnstiel, M. L. (1980b). Spacer DNA sequences upstream of the T-A-T-A-A-A-T-A sequence are essential for promotion of H2A histone gene transcription *in vivo*. Proc. Nat. Acad. Sci. USA *77*, 7102-7106.

Grosschedl, R. and Birnstiel, M. L. (1981). Delimitation of upstream sequences required for maximal *in vitro* transcription of an H2A histone gene. Proc. Nat. Acad. Sci. USA, in press.

Grosveld, G. C., Koster, A. and Flavell, R. A. (1981a). A transcription map for the rabbit β-globin gene. Cell *23*, 573-584.

Grosveld, G. C., Shewmaker, C. K., Jat, P. and Flavell, R. A. (1981b). Localization of DNA sequences necessary for transcription of the rabbit β-globin gene in vitro. Cell *25*, 215-226.

Gruss, P., Dhar, R. and Khoury, G. (1981). Simian virus 40 tandem repeated sequences as an element of the early promoter. Proc. Nat. Acad. Sci. USA *78*, 943-947.

Guarente, L. and Ptashne, M. (1981). Fusion of an *Escherichia coli* lacZ gene to the cytochrome *c* gene of *Saccharomyces cerevisiae*. Proc. Nat. Acad. Sci. USA *78*, 2199-2203.

Hagenbüchle, O. and Schibler, U. (1981). Mouse β-globin and adenovirus-2 major late transcripts are initiated at the cap site *in vitro*. Proc. Nat. Acad. Sci. USA *78*, 2283-2286.

Hayward, W. S., Neel, B. G. and Astrin, S. M. (1981). Activation of a cellular *onc* gene by promoter insertion in ALV-induced lymphoid leukosis. Nature *290*, 475-480.

Hofer, E. and Darnell, J. E., Jr. (1981). The primary transcription unit of the mouse β-major globin gene. Cell *23*, 585-593.

Jackson, D. A., McCready, S. J. and Cook, P. R. (1981). RNA is synthesized at the nuclear cage. Nature *292*, 552-555.

Katinka, M., Yaniv, M., Vasseur, M. and Blangy, D. (1980). Expression of polyoma early functions in mouse embryonal carcinoma cells depends on sequence rearrangements in the beginning of the late region. Cell *20*, 393-399.

Katinka, M., Vasseur, M., Montreau, N., Yaniv, M. and Blangy, D. (1981). Polyoma DNA sequences involved in control of viral gene expression in murine embryonal carcinoma cells. Nature *290*, 720-722.

Kimura, G. and Dulbecco, R. (1972). Isolation and characterization of temperature sensitive mutants of SV40. Virology *49*, 394-403.

Lusky, M. and Botchan, M. (1981). Inhibitory effect of specific pBR322 DNA sequences upon SV40 replication in simian cells. Nature *293*, 79-81.

Maniatis, T., Hardison, R. C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, G. K. and Efstratiadis, A. (1978). The isolation of structural genes from libraries of eucaryotic DNA. Cell *15*, 687-701.

Marsden, M. P. F. and Laemmli, U. K. (1979). Metaphase chromosome structure: evidence for a radial loop model. Cell *17*, 849-858.

Mellon, P., Parker, V., Gluzman, Y. and Maniatis, T. (1981). Identification of DNA sequences required for transcription of the Human α1-globin gene in a new SV40 host-vector system. Cell *27*, 279-288.

Mertz, J. E. and Berg, P. (1974). Defective simian virus 40 genomes: isolation and growth of individual clones. Virology *62*, 112-124.

Moschonas, N., de Boer, E., Grosveld, F. G., Dahl, H. H. M., Wright, S., Shewmaker, C. K. and Flavell, R. A. (1981). Structure and expression of a cloned β^0 thalassaemic globin gene. Nucl. Acids Res., in press.

Neel, B. G., Hayward, W. S., Robinson, H. L., Fang, J. and Astrin, S. M. (1981). Avian leukosis virus-induced tumors have common proviral integration sites and synthesize discrete new RNAs: oncogenesis by promoter insertion. Cell *23*, 323-334.

Payne, G. S., Courtneidge, S. A., Crittenden, L. B., Fadly, A. M., Bishop, J. M. and Varmus, H. E. (1981). Analysis of avian leukosis virus DNA and RNA in bursal tumors: viral gene expression is not required for maintenance of the tumor state. Cell *23*, 311-322.

Pellicer, A., Robins, D., Wold, B., Sweet, R., Jackson, J., Lowy, I., Roberts, J. M., Sim, G. K., Silverstein, S. and Axel, R. (1980). Altering genotype and phenotype by DNA mediated gene transfer. Science *209*, 1414-1422.

Perucho, M., Hanahan, D. and Wigler, M. (1980). Genetic and physical linkage of exogenous sequences in transformed cells. Cell *22*, 309-317.

Proudfoot, N. J., Shander, M. H. M., Manley, J. L., Gefter, M. L. and Maniatis, T. (1980). Structure and in vitro transcription of human globin genes. Science *209*, 1329-1336.

Razin, S. V., Mantieva, V. L. and Georgiev, G. P. (1979). The similarity of DNA sequences remaining bound to scaffold upon nuclease treatment of interphase nuclei and metaphase chromosomes. Nucl. Acids Res. *7*, 1713-1735.

Reddy, V. B. and Greenspan, D. S. (1981). SV40 DNA sequences enhance efficiency of DNA mediated transformation in the absence of SV40 proteins. Science, in press.

Rio, D., Robbins, A., Myers, R. and Tjian, R. (1980). Regulation of simian virus 40 early transcription in vitro by a purified tumor antigen. Proc. Nat. Acad. Sci. USA *77*, 5706-5710.

Rusconi, S. and Schaffner, W. (1981). Transformation of frog embryos with a rabbit β-globin gene. Proc. Nat. Acad. Sci. USA *78*, in press.

Schaffner, W. (1980). Direct transfer of cloned genes from bacteria to mammalian cells. Proc. Nat. Acad. Sci. USA *77*, 2163-2167.

Schaffner, W., Kunz, G., Daetwyler, H., Telford, J., Smith, H. O. and Birstiel, M. L. (1978). Genes and spacers of cloned sea urchin histone DNA analyzed by sequencing. Cell *14*, 655-671.

Sekikawa, K. and Levine, A. J. (1981). Isolation and characterization of polyoma host range mutants that replicate in nullipotential embryonal carcinoma cells. Proc. Nat. Acad. Sci. USA *78*, 1100-1104.

Smith, G. R. (1981). DNA supercoiling: another level for regulating gene expression. Cell *24*, 599-600.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. *98*, 503-517.

Subramanian, K. N. (1979). Segments of simian virus 40 DNA spanning most of the leader sequence of the major late viral messenger RNA are dispensable. Proc. Nat. Acad. Sci. USA *76*, 2556-2560.

Sutcliffe, J. G. (1978). Complete nucleotide sequence of the E. coli plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. *43*, 77-90.

Tegtmeyer, P. and Ozer, H. L. (1971). Temperature-sensitive mutants of simian virus 40: infection of permissive cells. J. Virol. *8*, 516-524.

Telford, J. L., Kressmann, A., Koski, R. A., Grosschedl, R., Müller, F., Clarkson, S. G. and Birnstiel, M. L. (1979). Delimitation of a

promoter for RNA polymerase III by means of a functional test. Proc. Nat. Acad. Sci USA *76*, 2590–2594.

Tooze, J. (1980). DNA tumor viruses. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Twigg, A. J. and Sherratt, D. (1980). Trans-complementable copy-number mutants of plasmid ColE1. Nature *283*, 216–218.

Weaver, R. F. and Weissmann, C. (1979). Mapping of RNA by a modification of the Berk-Sharp procedure: the 5′ termini of 15S β-globin mRNA precursor and mature 10S β-globin mRNA have identical map coordinates. Nucl. Acids Res. *7*, 1175–1193.

Weber, H., Dierks, P., Meyer, F., van Ooyen, A., Dobkin, C., Abrescia, P., Kappeler, M., Meyhack, B., Zeltner, A., Mullen, E. E. and Weissmann, C. (1981). Modification of the rabbit chromosomal β-globin gene by restructuring and site-directed mutagenesis. In ICN–UCLA Symposium on Molecular and Cellular biology, *33*, D. D. Brown and C. F. Fox, eds. (New York: Academic Press), in press.

Wigler, M., Pellicer, A., Silverstein, S. and Axel, R. (1978). Biochemical transfer of single copy eucaryotic genes using total cellular DNA as donor. Cell *14*, 725–731.

Wigler, M., Sweet, R., Sim, G. K., Wold, B., Pellicer, A., Lacy, L., Maniatis, T., Silverstein, S. and Axel, R. (1979). Transformation of mammalian cells with genes from procaryotes and eucaryotes. Cell *16*, 777–785.

Wold, B., Wigler, M., Lacy, E., Maniatis, T., Silverstein, S. and Axel, R. (1979). Introduction and expression of a rabbit β-globin gene in mouse fibroblasts. Proc. Nat. Acad. Sci. USA *76*, 5684–5688.