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# *cis* and *trans* activation of globin gene transcription in transient assays

(transcription enhancer/simian virus 40/adenovirus/DNA replication/transient expression assay)

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**ABSTRACT** **We examined the effects of the simian virus 40 enhancer sequence on transcription of cloned human α- and β-globin genes shortly after their introduction into cultured mammalian cells. We find that (*i*) detectable transcription of the β-globin gene but not the α-globin gene requires linkage to the enhancer; (*ii*) the enhancer increases the amount of β-globin RNA at least 100-fold but results in only a 5- to 10-fold increase in the amount of α-globin RNA; (*iii*) plasmid replication does not increase the level of β-globin RNA, regardless of linkage to the enhancer, but does result in an ≈50-fold increase in the level of α-globin RNA; (*iv*) the enhancer is not required for and does not increase transcription of either gene in 293 cells, an adenovirus 5-transformed human kidney cell line. We also show that an enhancer sequence is not required for activity of the normally enhancer-dependent simian virus 40 early promoter in 293 cells, indicating that these cells contain a *trans*-acting factor(s) that circumvents the requirement for the enhancer sequence.**

Enhancers are transcriptional regulatory elements that stimulate the expression of genes in *cis* in an orientation- and position-independent manner (for review, see refs. 1 and 2). Although enhancers were first identified in the genomes of animal viruses, sequences with similar properties have been isolated from cellular DNA (3). Recently, enhancer elements have been shown to be involved in the B-cell specific activation of the mouse heavy chain (4, 5) and possibly κ light chain (6) immunoglobulin genes. The activity of enhancers is not restricted to their normal gene partners. For example, the enhancers from both simian virus 40 (SV40) and the mouse immunoglobulin heavy chain gene can stimulate transcription of a rabbit β-globin gene (4, 7).

The mechanism of enhancer action has been studied by using a transient expression assay in which a plasmid carrying the gene of interest is introduced into cultured cells and its transcription is analyzed 30–50 hr later (7, 8). In transient assays transcription of the rabbit (7, 9) and human (10) β-globin gene is dependent on linkage to an enhancer sequence when carried on both nonreplicating and replicating plasmids. In contrast, the human α-globin gene is efficiently transcribed and unaffected by the enhancer sequence when carried on a replicating vector (8, 10). The effect of plasmid replication on α- and β-globin transcription in transient assays has not been investigated systematically.

In this paper we compare the transcription of the α- and β-globin genes in HeLa cells, in which no plasmid replication occurs, and in COS cells (11), which allow efficient replication of plasmids containing an intact SV40 replication origin (8). We also have examined the possibility that globin genes can be activated in *trans* by viral immediate early gene products. This possibility was suggested by the fact that these proteins, which are required for transcriptional activation of other viral genes during lytic infection (12–14), can in some cases act on the genes of other viruses (15) and on certain cellular genes (16). Therefore, we carried out transient expression assays in 293 cells, which constitutively produce adenovirus 5 immediate early gene products (17). Remarkably, in these cells neither gene requires linkage to the SV40 enhancer sequence for transcription. Our results suggest that 293 cells contain a *trans*-acting factor that circumvents the requirement for the function provided by the *cis*-acting SV40 enhancer sequence.

## METHODS AND MATERIALS

**Plasmid Construction.** Vectors πSVHSplac and πSVHPplac (P. Little, personal communication) are derivatives of the miniplasmid πVX (18) carrying the segments of SV40 DNA shown in Fig. 1*A*. Their nonreplicating derivatives πSVHSplacΔ and πSVHPplacΔ were constructed by *Bgl* I digestion, treatment with T4 DNA polymerase, and recircularization, resulting in the deletion of 3 base pairs and concomitant creation of a *Sac* II site. Each vector carries a polylinker sequence to the late side of the SV40 segment. Plasmids of the πSVβΔ128 series (Fig. 1*B*) carry a 2.3-kilobase (kb) *Rsa* I/*Pst* I fragment (20) containing the human β-globin gene together with 128 nucleotides of 5′ flanking sequence, inserted between the polylinker *Bgl* II and *Pst* I sites. Plasmids of the πSVα1 series (Fig. 1*B*) carry a 1.5-kb *Pst* I fragment containing the human $\alpha_1$-globin gene together with 0.5 kb of 5′ flanking sequence inserted into the polylinker *Pst* I site. To construct plasmids containing both the α- and β-globin genes, the 1.5-kb α-globin *Pst* I fragment was inserted into a *Pst* I site located 550 base pairs 3′ to the β-globin gene in plasmids πSVHPβ (21) and πSVHRβ (P. Little, personal communication), which contain and lack the enhancer sequence, respectively. In the resulting plasmids, πSVHPαβ+ and πSVHRαβ+, the genes are in the same transcriptional orientation. To create SV40–α-globin fusion genes (Fig. 1*C*), the SV40 early region *Kpn* I/*Bam*HI, *Sph* I/*Bam*HI and *Nco* I/*Bam*HI fragments (for details, see ref. 22) were separately inserted between the *Sph* I and *Bam*HI sites of pBRd (19); the SV40 *Stu* I/*Bam*HI fragments were then replaced with the α-globin *Nco* I/*Pst* I fragment. Plasmid DNA was prepared as described (23).

**Cell Culture and Transfection.** HeLa, COS7, and 293 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Cells were transfected by coprecipitation with calcium phosphate at 30–50% confluence as described (20, 24); 20 μg of plasmid DNA was used per 9-cm plate. In each case equimolar amounts of the test plasmid and a reference plasmid (carrying either the α- or β-globin genes, as appro-

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Abbreviation: SV40, simian virus 40.

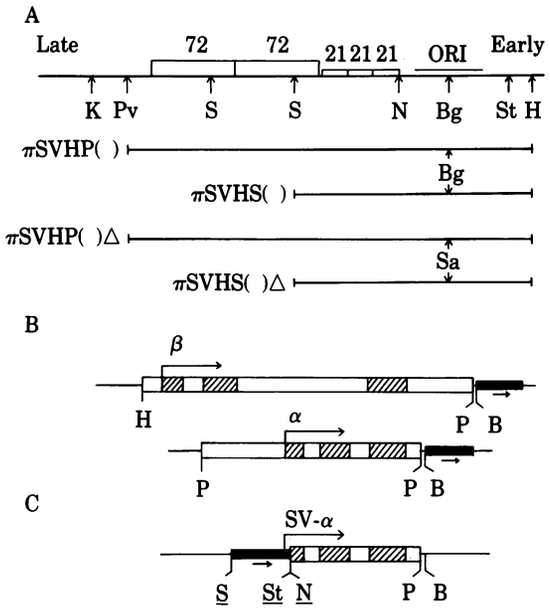


FIG. 1. Plasmid structures. (*A*) SV40 sequences carried on miniplasmid (18) vectors. The SV40 origin of replication region is shown with relevant restriction sites, with the 72- and 21-base-pair (bp) repeated sequences and replication origin indicated. Below are shown the extent of SV40 sequences present in the vectors used in this study; plasmids are named according to the extent of SV40 sequence present and the type of insert. (*B*) Structure of πSVβΔ128 and πSVα1 series of plasmids. Globin DNA sequences are shown as open boxes, with the exons hatched. SV40 sequences are shown as solid blocks with arrows pointing in the direction of early transcription, πVX vector (18) sequences are shown as thin lines. (*C*) Structure of SV40–α-globin fusion genes. Symbols are as in *B*; vector sequences are pBRd (19). K, *Kpn* I; Pv, *Pvu* II; S, *Sph* I; N, *Nco* I; Bg, *Bgl* I; St, *Stu* I; H, *Hin*dIII; P, *Pst* I; B, *Bam*HI; Sa, *Sac* II. Underlined symbols indicate restriction sites destroyed in the plasmid construction.

priate) were transfected to allow for variations in transfection efficiency and RNA recovery. After exposure to the precipitate for 12–16 hr at 37°C, cells were washed twice with warm phosphate-buffered saline and fresh medium was added.

**RNA and DNA Preparation and Analysis.** Total cellular RNA was prepared 40–48 hr after transfection and analyzed by nuclease S1 mapping as described (20, 21). Single-stranded 5′-$^{32}$P-labeled probe DNA fragments spanning the mRNA cap sites of the various genes were used. The following fragments and digestion conditions were used: β globin, **Mst* II–*Hae* III (ref. 20; 500 units/ml); α globin, **Nco* I–*Hae* II (nucleotides −60 to +40; 1,000 units/ml); and SV40–α-globin fusion genes, **Bss*HII–*Sph* I (see Fig. 4; 500 units/ml). DNA replication assays were performed as described (8) with total cellular DNA.

## RESULTS

**Effect of the SV40 Enhancer Sequence on Globin Gene Transcription in HeLa Cells.** We first compared the effect of the SV40 transcription enhancer on the amount of α- and β-globin RNA produced in HeLa cells, where no plasmid replication occurs. The analysis was accomplished by using plasmids containing SV40 sequences on the 3′ side of the globin genes (Fig. 1). To quantitate the amount of correctly initiated globin RNA in transfected HeLa cells, total cellular RNA was analyzed by S1 nuclease mapping. The hybridization probes used in this analysis generated nuclease-resistant DNA fragments 40 and 70 nucleotides in length when hybridized to authentic human α- and β-globin mRNA, respectively (ref. 20; data not shown). Analysis of the transcripts produced by cotransfected β- or α-globin reference plasmids showed that transfection efficiency and RNA recovery were comparable in each case (data not shown).

As shown in Fig. 2*A* α-globin transcripts were detected in HeLa cells in the absence of a linked enhancer sequence; linkage to the enhancer sequence increased 5- to 10-fold the amount of correctly initiated RNA produced (Fig. 2A, lanes 1 and 2). However, as observed in the case of the rabbit β-globin gene (7, 9), transcripts of the human β-globin gene were not detected in HeLa cells unless the plasmid contained the SV40 enhancer (Fig. 2*B*, lanes 1 and 2). Linkage to the enhancer sequence increased the amount of correctly initiated β-globin RNA produced at least 100-fold. On the basis of these and other experiments, we estimate that the level of α-globin RNA produced by plasmids without an enhancer is comparable to the level of β-globin RNA produced by plasmids with an enhancer.

**Effect of DNA Replication on Globin Gene Transcription in COS Cells.** To evaluate the effect of plasmid replication on globin gene transcription, we compared the amount of correctly initiated globin RNA produced in COS cells by plasmids that contain intact or inactivated SV40 replication origins. All plasmids containing intact SV40 replication origins replicated equally efficiently in COS cells; however, replication of those

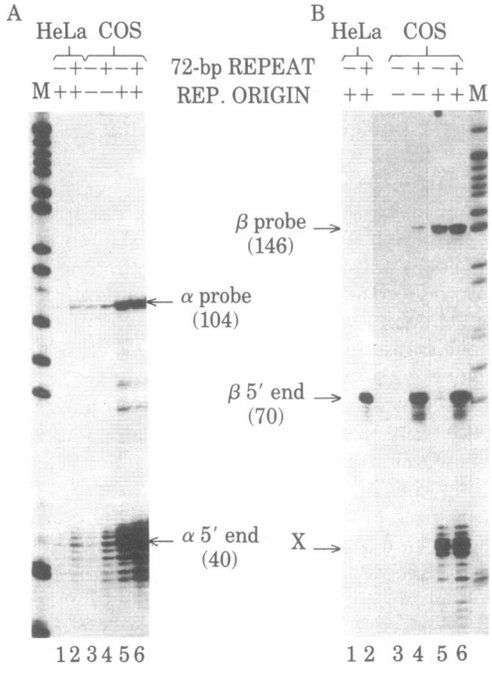


FIG. 2. S1 nuclease analysis of α- and β-globin gene transcription in HeLa and COS cells. The presence of an intact SV40 replication origin and/or 72-base-pair enhancer element is indicated above each lane. (*A*) Analysis of α-globin gene transcription in HeLa (lanes 1 and 2) and COS (lanes 3–6) cells with the *Nco* I/*Hae* II probe. Total cellular RNA (3 or 30 μg) was used for each assay. Lanes: 1, plasmid πSVHSα1 (30 μg); 2, plasmid πSVHPα1 (30 μg); 3, plasmid πSVHSα1Δ (30 μg); 4, plasmid πSVHPα1Δ (30 μg); 5, plasmid πSVHSα1 (3 μg); 6, plasmid πSVHPα1 (3 μg). (*B*) Analysis of β-globin gene transcription in HeLa (lanes 1 and 2) and COS (lanes 3–6) cells with the *Mst* II/*Hae* III probe. Thirty micrograms of total cellular RNA was used in each assay. Lanes: 1, plasmid πSVHSβΔ128; 2, plasmid πSVHPβΔ128; 3, plasmid πSVHSβΔ128Δ; 4, plasmid πSVHPβΔ128Δ; 5, plasmid πSVHSβΔ128Δ; 6, plasmid πSVHPβΔ128Δ. All probe and product lengths are shown on the figure. Primer extension experiments indicate that the additional apparent β globin 5′ ends apparently mapping within the first exon (products X) represent a 3′ splice site (G. C. Grosveld and R. A. Flavell, personal communication). Lanes M show size markers of $^{32}$P-labeled *Msp* I-cut pBR322 DNA.

plasmids containing deleted replication origins was not detected (data not shown).

When the α-globin gene was introduced into COS cells on nonreplicating plasmids, the effect of the enhancer on transcription was similar to that observed in HeLa cells: α-globin RNA was detected in the absence of an enhancer sequence, and linkage to the enhancer resulted in a 5- to 10-fold increase in the amount of RNA produced (Fig. 2*A*, lanes 3 and 4; plasmids). As previously reported (8, 10), a large amount of α-globin RNA was produced when the α-globin gene was introduced into COS cells on replicating plasmids; in some experiments the enhancer also slightly increased the amount of correctly initiated α-globin RNA produced (Fig. 2*A*, compare lanes 5 and 6). Plasmid replication resulted in a 50-fold increase in the amount of α-globin RNA produced (Fig. 2*A*, compare lanes 3 and 4 with lanes 5 and 6).

As observed in HeLa cells, transcription of the β-globin gene on nonreplicating plasmids in COS cells was not detected in the absence of an enhancer (Fig. 2*B*, lanes 3 and 4). When the β-globin gene was introduced into COS cells on replicating plasmids, an enhancer also was required for transcription (Fig. 2*B*, lanes 5 and 6), in agreement with previous observations (10). In addition to correctly initiated β-globin RNA, replicating β-globin plasmids produced large quantities of transcripts initiated at positions 5′ to the mRNA cap site (Fig. 2*B*, lanes 5 and 6; 146 nucleotide products). Comparison of the amount of correctly initiated β-globin RNA produced in COS cells by replicating and nonreplicating β-globin plasmids containing the enhancer shows that plasmid replication leads to essentially no increase in the amount of correctly initiated β-globin RNA produced (Fig. 2*B*, compare lanes 4 and 6).

We conclude that replication of globin plasmids in COS cells substantially increases α-globin transcription irrespective of whether the plasmid contains an enhancer sequence. However, replication does not significantly increase the amount of β-globin RNA produced and does not relieve the requirement of the β-globin gene for a *cis*-linked enhancer sequence.

**Efficient Transcription of Both α- and β-Globin Genes Does Not Require a *cis*-Linked Enhancer Sequence in 293 Cells.** During adenovirus or herpesvirus infection, most viral genes are initially transcriptionally inactive; at later stages of the infection, however, transcription of these genes is activated by *trans*-acting viral immediate–early gene products (12–14). In some cases these proteins can also activate the transcription of heterologous viral (15) and cellular (16) genes. To test whether such gene products might be capable of activating globin gene transcription, we introduced α- and β-globin genes into 293 cells (17), which constitutively express the Ad5 immediate–early region E1A proteins. We first transfected 293 cells with plasmids containing both the α- and the β-globin genes with or without an intact enhancer sequence. No transcripts of the endogenous α- or β-globin genes were detected in mock-transfected 293 cells (Fig. 3, lanes 1 and 4); DNA blotting experiments confirmed that the endogenous β-globin gene in 293 cells was intact (data not shown). In transfected 293 cells, β-globin gene transcription was readily detected in the absence of an enhancer sequence (Fig. 3, lane 2); moreover, transcription was not increased by the enhancer in these cells (Fig. 3; compare lanes 2 and 3). Similarly, the enhancer had no effect on α-globin gene transcription in 293 cells (Fig. 3, lanes 5 and 6). Efficient β-globin transcription in 293 cells occurred when the gene was carried on plasmids lacking SV40 sequences. We also observed enhancer-independent transcription of the β-globin gene in cell lines into which adenovirus 5 early region 1 sequences were introduced by cotransformation with the herpes simplex virus thymidine kinase gene (ref. 25; data not shown).

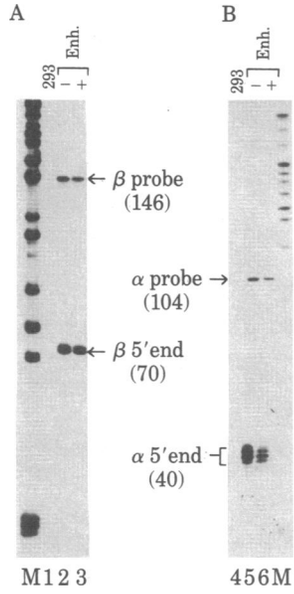


FIG. 3. S1 nuclease analysis of β- and α-globin gene transcription in 293 cells. The presence (lanes 3 and 6) or absence (lanes 2 and 5) of an intact enhancer sequence on the transfected plasmids is indicated. The β-globin *Mst* II/*Hae* III (*A*) or the α-globin *Nco* I/*Hae* II (*B*) probes were used. Lanes: 1 and 4, 40 μg of total cellular RNA from mock-transfected 293 cells; 2 and 5, 40 μg of total cellular RNA from 293 cells transfected with plasmid πSVHRαβ+; 3 and 6, 40 μg of total cellular RNA from 293 cells transfected with plasmid πSVHPαβ+; M, size markers of $^{32}$P-labeled *Msp* I-cut pBR322 DNA. Nuclease-resistant product lengths are indicated.

**The SV40 Enhancer Is Not Required for Activity of the SV40 Early Promoter in 293 Cells.** The results described above suggest that 293 cells might contain factors that are specific for globin gene transcription or that bypass the requirement for an enhancer sequence for all enhancer-dependent genes. To distinguish between these two possibilities we examined the activity of the SV40 early promoter in 293 cells: the SV40 enhancer sequence is normally required for activity of this promoter (26–28). To eliminate the effects of autoregulation of the promoter by large T antigen (reviewed by ref. 21), an SV40 early region gene product, we constructed fusion genes in which the large T antigen protein coding sequences were replaced by those of the α-globin gene. These genes comprise fragments of the SV40 promoter containing various extents of 5′ flanking sequence joined, at a point some 40 nucleotides 3′ to the early mRNA cap sites, to α-globin sequences extending from the translational initiation codon to the 3′ end of the gene (Fig. 1; see *Methods and Materials*).

The transcriptional analysis of the fusion genes in HeLa and 293 cells is shown in Fig. 4. The probe used in this assay is complementary to α-globin mRNA sequences on the 3′ side of the α-globin gene translational initiation codon; therefore, transcripts of the cotransfected α-globin reference plasmid generated nuclease-resistant DNA fragments 60 nucleotides in length, which also appear on the gel ("α reference" products in Fig. 4). In HeLa cells, plasmid pSVKα, which contains an intact enhancer sequence, produced a large amount of RNA that generated S1 nuclease-resistant products of the length expected for transcripts initiated at the normal SV40 early mRNA cap sites (110 nucleotide SV-α products in Fig. 4, lane 1). The plasmid pSVSα, in which the truncated SV40 promoter contains the repeated 21-base-pair element but lacks an intact enhancer sequence, exhibited dramatically reduced promoter activity in HeLa cells (compare lanes 1 and 2 in Fig. 4); a further

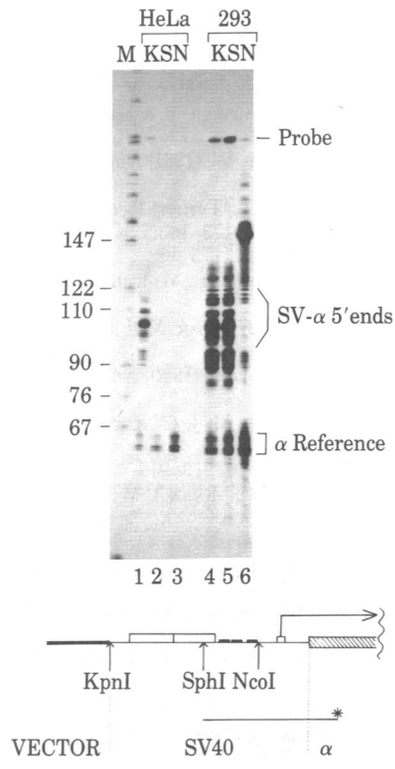


FIG. 4. S1 nuclease analysis of SV40–α-globin fusion gene transcription in HeLa and 293 cells. The *Bss*HII/*Sph* I probe was used. Total cellular RNA (40 μg) was used in each assay. (*Upper*) Lanes: 1–3, HeLa cells; 4–6, 293 cells; 1 and 4, plasmid pSVKα; 2 and 5, plasmid pSVSα; 3 and 6, plasmid pSVNα; M, size markers. Marker lengths are given in nucleotides. K, S, and N indicate that the SV40 sequences extend to the *Kpn* I, *Sph* I, and *Nco* I sites, respectively. (*Lower*) The structure of the fusion gene in pSVKα is shown, with the extent of vector, SV40, and α-globin sequences indicated. The locations of the *Sph* I and *Nco* I sites used to create plasmids pSVSα and pSVNα are shown. The 72-bp repeats are shown as open boxes; the 21-bp repeats, as solid blocks; and transcripts, by an arrow. The extent of the probe is indicated below the gene.

truncation that also removed the 21-base-pair repeats (plasmid pSVNα) completely abolished transcription in HeLa cells (Fig. 4, lane 3). In contrast, in 293 cells, transcription was initiated efficiently at heterogeneous positions centered on the normal SV40 early mRNA cap sites regardless of whether the SV40 promoter contained an intact enhancer sequence (compare plasmids pSVKα and pSVSα; Fig. 4, lanes 4 and 5). However, a truncation that removed both the 72- and 21-base-pair repeated elements (plasmid pSVNα) resulted in total inactivation of the promoter in 293 cells (Fig. 4, lane 6). This truncation also caused an increase in the amount of RNA initiated at positions 5′ to the SV40 early mRNA cap sites, which generated a 150-nucleotide product that mapped the point of divergence between the probe and the template (Fig. 4, lane 6). We conclude that 293 cells contain a *trans*-acting factor(s) capable of relieving the enhancer requirement of both the β globin and SV40 early promoters.

## DISCUSSION

In this study we used transient expression assays to analyze the requirements for transcription of cloned human α- and β-globin genes introduced into nonerythroid mammalian cells. We find that the β-globin gene, but not the α-globin gene, requires linkage to the SV40 enhancer sequence for transcription to be detected in both HeLa and COS cells. In contrast, neither the globin genes nor the SV40 early promoter require a *cis*-linked enhancer for efficient expression in the adenovirus 5-transformed human cell line 293. Moreover, the enhancer sequence does not increase the amount of RNA produced by these genes in 293 cells.

Linkage of the human β-globin gene to the SV40 enhancer sequence results in an increase of at least 100-fold in the amount of correctly initiated β-globin RNA synthesized in HeLa cells, in agreement with previous reports (7, 9). A similar increase in β-globin gene transcription is observed when the gene is introduced into COS cells on a nonreplicating vector. In contrast, linkage of the enhancer to the human α-globin gene results in only a 5- to 10-fold increase in the synthesis of correctly initiated α-globin RNA. One possible explanation for this difference in response is that the α-globin gene itself contains an element analogous to the enhancer sequence. This seems unlikely, however, because the presence of α-globin fragments *cis* to the β-globin gene in a number of different configurations does not activate transcription of the β-globin gene (unpublished data; ref. 10). Alternatively, the α-globin gene may be incapable of responding to the enhancer, or the effect of the enhancer may be attenuated by sequences within or near the gene. A precedent for the latter hypothesis is the observation that certain DNA sequences placed between the SV40 enhancer and a promoter may block transcription enhancement (7, 29, 30). In some cases these effects are due to the presence of promoter sequences within the interposed DNA segments (29, 30). Other genes respond to the enhancer either only weakly (ref. 31; unpublished observations) or in a strongly position-dependent manner (31, 32).

We also examined the effect of plasmid replication on the amount of α- and β-globin RNA produced in transient assays. Each plasmid containing an intact SV40 origin replicates equally efficiently in COS cells regardless of whether an enhancer is present: therefore, enhancer-dependent transcription does not interfere with efficient replication. In the case of the α-globin gene, plasmid replication increases transcription by a factor of about 50. The enhancer has little effect on transcription of the α-globin gene carried on replicating plasmids; similar observations have been made with the α-globin gene carried on a different vector (10) and in the case of the sea urchin histone H2A gene (T. Gerster and W. Schaffner, personal communication). In contrast to the α-globin gene, however, plasmid replication does not affect transcription of the β-globin gene carried on plasmids containing an intact enhancer sequence. Possibly some factor required for enhancer-dependent β-globin gene expression in these cells is limiting; alternatively, replication *per se* may interfere with enhancer-dependent transcription. Replication of β-globin plasmids lacking an intact enhancer appears to increase slightly the amount of correctly initiated β-globin RNA produced, although the sensitivity of our assay was not sufficient to quantitate the increase. At present, therefore, we cannot say whether the increase in transcription observed upon replication of α-globin plasmids is due to a fundamental difference in the properties of the α- and β-globin gene promoters.

To examine the possible effect of adenovirus E1A gene products on globin gene transcription, we performed transient assays in 293 cells, which constitutively produce adenovirus 5 E1A proteins. These proteins are known to act in *trans* to activate transcription from both adenovirus (2, 3) and certain cellular (16) promoters. In contrast to the situation in HeLa and COS cells, neither the α- nor the β-globin gene requires linkage to the SV40 enhancer for transcripts to be detected in 293 cells; moreover, a *cis*-linked SV40 enhancer sequence does not affect transcription of either gene in these cells. Because the SV40

early promoter also does not require an enhancer for activity in 293 cells, we conclude that the factor present in these cells does not specifically activate globin genes but rather circumvents the constraints on promoter activity that render an enhancer sequence necessary for efficient transcription of a transfected gene. The known properties of the E1A gene products strongly suggest that the transcriptional activation that we observe in 293 cells is due to the adenovirus proteins produced by these cells; we show elsewhere that both β-globin and SV40 promoters introduced into HeLa cells are activated by adenovirus and herpesvirus immediate–early gene products (33). Our failure to detect transcription of the endogenous globin genes in 293 cells is of interest because it indicates that these genes are for some reason refractory to the effect of the *trans*-acting factor.

In conclusion, we found that *cis*-acting SV40 sequences have markedly different effects on the transcription of the α- and β-globin genes in transient assays in COS and HeLa cells, but both genes do not require such sequences for efficient and accurate transcription in 293 cells. The relevance, if any, of these observations to the regulated expression of the α- and β-globin genes in erythroid cells remains to be established. However, the involvement of cellular enhancer elements in the regulation of immunoglobulin gene expression (4–6) and the *trans*-activation of cellular heat shock genes by adenovirus infection (16) suggest that the study of the interactions between viral regulatory elements and globin genes may provide useful information regarding possible mechanisms of cellular gene regulation.

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