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Activation of the human ε- and β-globin promoters by SV40 T antigen

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We have studied the effect of the SV40 T antigen on expression from human globin promoters fused to the bacterial chloramphenicol acetyltransferase (CAT) gene and compared its effect with the SV40 enhancer and the adenovirus E1A protein. We have observed that expression of pεGLCAT and pβGLCAT (the ε-globin or β-globin promoter linked to the CAT gene) was significantly stimulated when cotransfected with a cloned T antigen plasmid into CV-1 cells, indicating that *trans*-activation of the globin promoters was mediated by SV40 T antigen. Transfection of the pβGLCAT-SV (pβGLCAT containing the SV40 enhancer element) into CV-1 cells resulted in a 50–60-fold increase in CAT activity as compared to pβGLCAT (no enhancer). However, cotransfection of the pβGLCAT-SV with the cloned T antigen resulted in an additional increase of CAT expression, which suggests that T antigen and the SV40 enhancer activate globin gene expression independently. We found that T antigen but not E1A could further stimulate the expression of an enhancer-containing plasmid in CV-1 cells; whereas E1A but not T antigen could further stimulate pεGLCAT expression in COS-1 cells which constitutively express the SV40 T antigen. These results suggest that T antigen and E1A also act independently. Deletion analysis showed that the minimum sequence required for a detectable level of stimulation of the ε-globin promoter by T antigen is 177 bp 5′ to the cap site, suggesting that the target sequences for response to T antigen do not reside in the canonical 100 bp promoter region, but rather reside in sequences further upstream, and therefore the cellular factors interacting with T antigen are not the TATA or CAT box binding proteins, but the proteins interacting with upstream regulatory sequences.

INTRODUCTION

The globin genes provide an important system with which to study gene regulation. By sequence comparison and mutation analysis, several promoter elements in the immediate 5′ flanking region have been identified [1,2]. While some of the promoter elements may interact with common transcription factors, it has also been shown that erythroid cells contain globin-specific *trans*-acting factors that are capable of activating the expression of globin genes [3]. To further our understanding of the regulation of globin gene expression, we have studied the sequences upstream of the canonical promoter region (upstream of −100 bp) of ε- and β-globin genes, and have demonstrated that some upstream sequences have positive and negative effects on the ε- and β-globin promoter expression [4,5]. As one of our approaches to studying the regulation of globin gene expression by *cis*-regulatory sequences and *trans*-acting factors, we have chosen to study the ability of the known viral regulatory protein, the SV40 T antigen, to activate the human ε- and β-globin promoters.

The T antigen of SV40 is a multifunctional protein. It is known to autoregulate its own expression [6–11], to activate transcription from the SV40 late promoter [12–15], and to initiate viral DNA replication [16,17]. It has also been reported that T antigen can stimulate other viral genes [18–20]. However, the effect of T antigen on higher eukaryotic cellular promoters has not been studied in detail. The effect of T antigen on the globin promoters and its comparison with other viral regulatory proteins, such as E1A, as well as with the *cis*-acting enhancer, may provide insights into the regulation of globin genes by interactions between regulatory sequences and globin *trans*-acting factors.

In the present study, we find that the T antigen of SV40 can *trans*-activate the human globin promoters in a transient assay system. By comparing the activation effects of T antigen and E1A as well as the *cis*-acting SV40 enhancer, we show that T antigen, E1A and the SV40 enhancer activate the globin promoter independently, suggesting that their actions are mediated by different cellular factors. By using deletion mutants in the ε-globin 5′ flanking region, we found evidence that sequences required for response to T antigen do not reside in the 100 bp promoter region, but in a region further upstream.

MATERIALS AND METHODS

Plasmid constructs

pεGLCAT and pβGLCAT are as described in Fordis *et al.* [21]. These plasmids carry the bacterial chloramphenicol acetyltransferase (CAT) gene under the

Abbreviations used: CAT, chloramphenicol acetyltransferase; pεGLCAT and pβGLCAT, the ε-globin or β-globin promoter linked to the CAT gene; DMEM, Dulbecco's modified Eagle medium.

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control of the human ε- and β-globin gene promoter. Since pεGLCAT and pβGLCAT do not contain the SV40 origin of replication, they do not replicate in the presence of T antigen. A 200 bp SV40 enhancer element, which contains the two 72 bp repeats and two 21 bp repeats, was inserted in the 3′ BamHI site of the CAT gene of pεGLCAT and pβGLCAT to generate pεGLCAT-SV and pβGLCAT-SV respectively. The ε-deletion mutants were constructed by deleting part of the ε-globin 5′ flanking sequences from pεGLCAT using appropriate restriction sites. The restriction enzymes used were MstII, HinfI, BamHI and EcoRV for pεΔ104, pεΔ115, pεΔ177 and pεΔ274 respectively. pRSV-T, in which the SV40 T antigen gene is under the control of the Rous sarcoma virus long terminal repeat, was provided by Dr. Bruce Howard (National Cancer Institute). This plasmid has been used to supply T antigen in transfection studies [20,22]. pE1A was from Dr. Pozzatti *et al.* (National Cancer Institute) [23] and expresses the adenovirus E1A protein.

Tissue culture, transfection and CAT assay

CV-1 cells (ATCC CCL70) and COS-1 cells (ATCC CRL1650) were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal-calf serum. For transfection, cells were plated 24 h prior to transfection at a density of $1.2 \times 10^4/\text{cm}^2$ in a 100 mm-diam. dish. The method described by Wigler *et al.* [24] was used for transfection except that the DNA precipitate was kept on the cells for 7–8 h and the cells were harvested 48 h after transfection. Under our standard conditions, the transfection efficiencies within one experiment were comparable, as determined by Southern blot and slot blot hybridizations following Hirt extraction [25]. CAT assays were performed by the method of Gorman *et al.* [26], except that protein extracts were preheated at 65 °C for 10 min to destroy interfering activity. Each CAT assay contained 500 μg of protein and was incubated for 20 min to 4 h under linear assay conditions. Because of the inherent variability of the transient CAT expression assay, each experiment was repeated several times. The standard deviation of the data among the experiments ranged from 10–40%. However, if several outliers are taken out, the standard deviation can be reduced to 10–25%. Despite this variation, we find that the effects are clear and reproducible.

RNA isolation and analysis

Total RNA was isolated by using a modified procedure of Auffray & Rougeon [27]. Cell monolayers were washed once with cold phosphate-buffered saline. Cells were dissociated from the dish by trypsin-EDTA and washed twice with cold phosphate-buffered saline; and then lysed by adding 3 M-LiCl/6 M-urea and stirred vigorously in a vortex mixer. The cell lysate was stored at −20 °C overnight. The RNA pellet was recovered by centrifugation and dissolved in urea/Tris solution (7 M-urea/10 mM-Tris/HCl, pH 8.0/200 mM-NaCl/2% SDS), extracted twice with phenol/chloroform and precipitated by ethanol. The RNA was then treated with DNase I (Promega Biotec) at a concentration of 30 units/200 μg of RNA at 37 °C for 20 min, followed by phenol/chloroform extraction and ethanol precipitation. RNA analysis was performed according to the manufacturer's recommended procedure (Promega Biotec). The RNA probe was synthesized from pGEMHε plasmid using SP6 RNA polymerase. This plasmid was constructed by inserting the EcoRV-EcoRI fragment spanning the ε-globin gene cap site (from −274 bp to +277 bp with respect to the cap site) of pεGLCAT into the EcoRI-HincII Site of pGEM-4 in the antisense orientation.

RESULTS

SV40 T antigen *trans*-activates the human ε- and β-globin promoters

We used the CAT transient expression assay system to study the effects of the SV40 T antigen on two human globin promoters. Fig. 1 shows a schematic representation of the plasmids used in this study. pεGLCAT-SV and pβGLCAT-SV contain the SV40 enhancer element (see the Materials and methods section) inserted at the

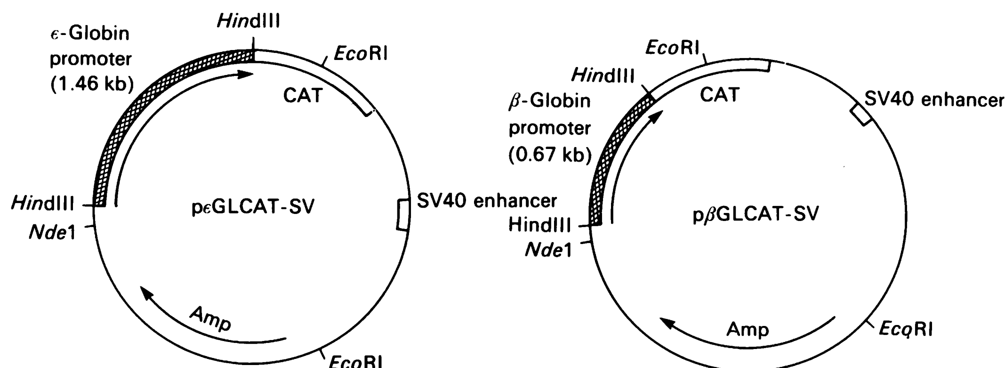

Fig. 1. Structure of the recombinant plasmids pεGLCAT-SV and pβGLCAT-SV

pεGLCAT-SV contains the bacterial CAT gene under the control of a 1.5 kb human ε-globin gene 5′ flanking sequence. The SV40 enhancer element containing the two 72 bp repeats and two 21 bp repeats is located 1 kb downstream of the CAT gene. pβGLCAT-SV is identical to pεGLCAT-SV except it contains a 0.7 kb human β-globin gene 5′ flanking sequence instead of the ε-globin gene sequence. The other two plasmids used in this study, pεGLCAT and pβGLCAT, are described in Fordis *et al.* [21], and are identical to pεGLCAT-SV and pβGLCAT-SV respectively, except each lacks the SV40 enhancer element. None of these plasmids, pεGLCAT-SV, pβGLCAT-SV, pεGLCAT or pβGLCAT, contains the SV40 origin of replication.

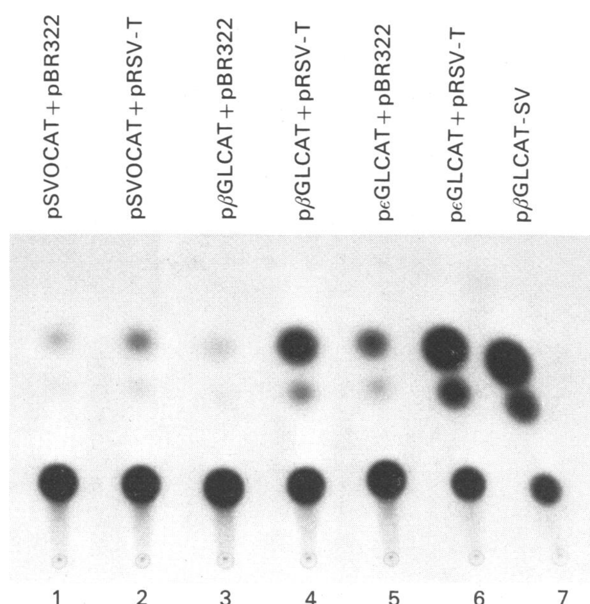

Fig. 2. *Trans*-activation of the human ε- and β-globin promoters by SV40 T antigen in CV-1 cells

CV-1 cells were transfected with 10 μg of the testing plasmids plus 10 μg of either pBR322 or pRSV-T as indicated. At 48 h, protein was extracted and a CAT assay was performed. Each CAT assay contained 500 μg of protein and was incubated for 2 h.

BamHI site located 3′ to the CAT gene. Plasmids pεGLCAT and pβGLCAT, which carry the ε- or β-globin promoter fused to the CAT gene, were described in ref. [21]. We first examined the activity of the human globin promoters in the presence and absence of the SV40 T antigen. Either plasmid pεGLCAT or pβGLCAT was cotransfected with pRSV-T into CV-1 cells. The pRSV-T plasmid contains the SV40 T antigen gene under the control of the Rous sarcoma virus long terminal repeat. In order to maintain the same concentrations of plasmid DNA in each transfection, appropriate amounts of pBR322 were added. After 48 h incubation, protein was extracted from the cells and a CAT assay was performed. Fig. 2 shows that cotransfection with pRSV-T resulted in a great increase in CAT activity for both plasmids compared with that transfected with pεGLCAT or pβGLCAT alone (Fig. 2; compare lanes 3 and 4, 5 and 6). In order to compare the effects of T antigen in *trans* and the enhancer in *cis*, a transfection using pβGLCAT-SV was also included (lane 7). The stimulatory effect of T antigen on pβGLCAT is very similar to that produced by the SV40 enhancer in *cis* (Fig. 2; lanes 4, 7). To determine the transcriptional activity initiated from prokaryotic sequences, pSVOCAT (promoterless) was also cotransfected with pBR322 or pRSV-T (Fig. 2; lanes 1 and 2). The low level of CAT activity from pSVOCAT and the slight increase by T antigen are negligible compared with that of the promoter-containing plasmids (see also Table 1).

To quantify the stimulatory effect of T antigen, acetylated and unacetylated chloramphenicol were counted in a liquid scintillation counter. Table 1 shows that in the presence of T antigen, pεGLCAT and pβGLCAT can be stimulated to increase CAT activity

Table 1. Effects of SV40 T antigen on the human ε- and β-globin promoters in CV-1 cells

The results are presented as means of several separate experiments (numbers in parentheses). In each experiment, the CAT activity derived from transfection with the test plasmid alone was set as 1 unit. The CAT activity derived from transfection with the test plasmid plus pRSV-T was normalized to the test plasmid.

Test plasmid	CAT activity (units)	
	−T antigen	+T antigen
pεGLCAT	1.0 (4)	10.2 (4)
pβGLCAT	1.0 (3)	17.6 (3)
pSV0CAT	1.0 (4)	1.6 (4)

by 10-fold and 17-fold respectively. We conclude that SV40 T antigen can *trans*-activate globin promoters in CV-1 cells.

To examine whether the increased CAT activity was due to an increase in CAT mRNA, we measured the steady-state CAT mRNA level in cells transfected with PεGLCAT by RNase T1 analysis. The probe used in the RNA analysis was synthesized from pGEMHε (see the Materials and methods section). Correctly initiated CAT mRNA would protect a fragment of 277 nucleotides from RNase T1 digestion (Fig. 3*c*). Consistent with the CAT assay results, CAT mRNA was not detected in mock-transfected cells (Fig. 3*a*, lane 1), and was detected at a very low level in cells transfected with pεGLCAT alone (Fig. 3*a*, lane 3). However, a much higher CAT mRNA level was detected in cells cotransfected with pεGLCAT and pRSV-T (Fig. 3*a*, lane 2). In addition to the correctly initiated CAT mRNA band, a number of bands with molecular weight higher than 277 bp were also observed (Fig. 3*a*, lanes 2 and 3). This indicates that there are upstream initiation sites in the ε-globin promoter region. This is consistent with previous reports that multiple upstream RNA initiation sites exist in the ε-globin gene [28].

T antigen and the SV40 enhancer function in an additive manner

We have demonstrated that SV40 T antigen can *trans*-activate the globin promoters, and that the stimulatory effect is comparable to the SV40 enhancer in *cis*. It has been shown that the SV40 enhancer interacts with cellular factors to exert its function [29–31]. Robbins *et al.* [32] found that T antigen or the SV40 enhancer could not stimulate the herpesvirus *tk* promoter separately; the stimulatory effect could only be observed when both T antigen and enhancer were present. We wanted to determine whether the SV40 T antigen and enhancer act independently on the β-globin promoter. Initially, we studied the relationship between plasmid DNA concentration and CAT activity. Increasing amounts of pβGLCAT-SV, containing the SV40 enhancer, were transfected into CV-1 cells, and the level of CAT expression at different concentrations of plasmid was obtained. Fig. 4 shows a typical experiment. The level of CAT activity is plotted against the amounts of plasmid DNA used for transfections. It is clear that the highest

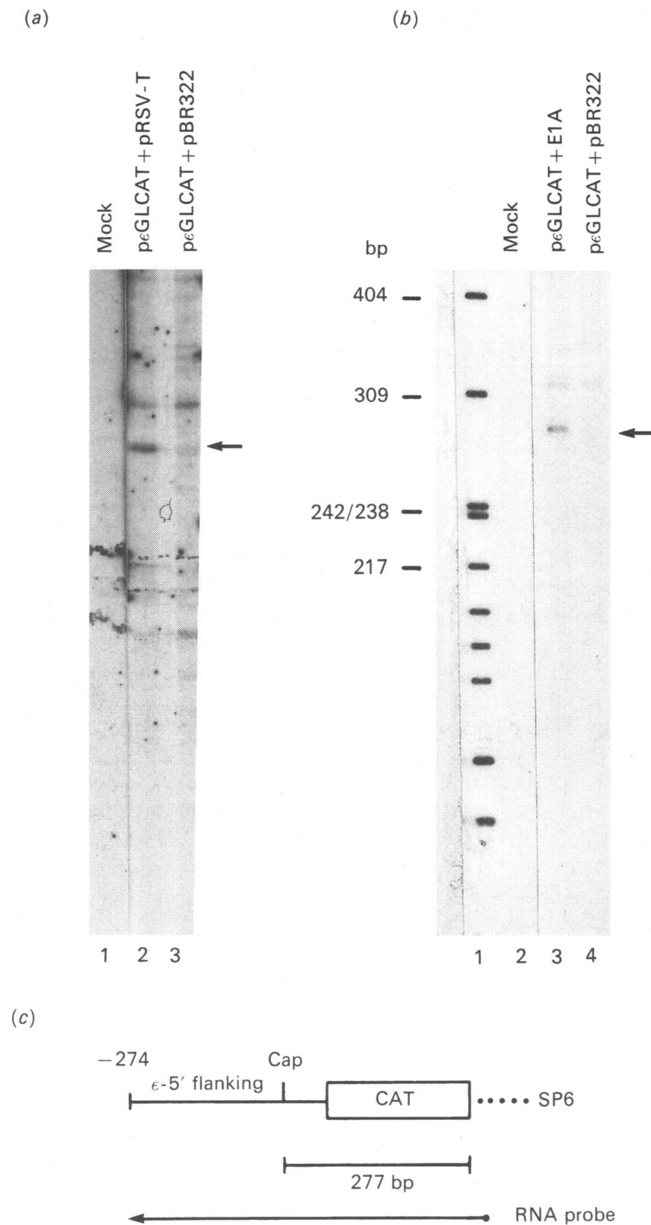

Fig. 3. CAT mRNA levels produced in response to T antigen and E1A in CV-1 cells

CAT mRNA levels were determined by RNA analysis as described in the Materials and methods section. Each sample contained 20 μg of total RNA isolated from transfected cells as indicated. (*a*), Lanes: 1, mock transfection; 2, transfection with 12 μg of pεGLCAT plus 10 μg of pRSV-T; 3, transfection with 12 μg of pεGLCAT plus 10 μg of pBR322. Arrow indicates position of protected probe corresponding to correctly-initiated mRNA. (*b*), Lanes as in (*a*) except lane 3, transfection with 12 μg of pεGLCAT plus 10 μg of pE1A; lane 4, as lane 3 in (*a*). (*c*), Diagram of the RNA probe used for RNA analysis. The 277 bp fragment protected by CAT mRNA from RNase T1 digestion is indicated.

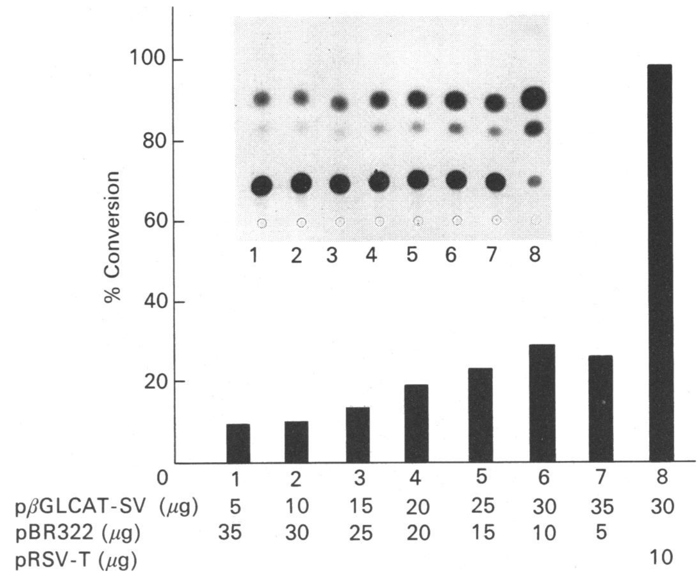

Fig. 4. Additive effects of T antigen and the SV40 enhancer

CV-1 cells were transfected with increasing amounts of pβGLCAT-SV for lanes 1 through 7 as indicated. In each case, the total amount of transfecting plasmid DNA was brought up to 40 μg with pBR322. Lane 8, transfection with 30 μg of pβGLCAT-SV plus 10 μg of pRSV-T. Each CAT assay contained 500 μg of protein and was incubated for 20 min. The inset shows the autoradiogram of the CAT assay and the graph presents the percentage conversion of [^{14}C]chloramphenicol to [^{14}C]acetyl-chloramphenicol.

CAT activity was obtained at 30 μg of plasmid pβGLCAT-SV (Fig. 4, lane 6). Transfection with higher concentrations of pβGLCAT-SV did not result in higher CAT activity, indicating that at 30 μg of plasmid, one or more of the cellular factors necessary for the SV40 enhancer function becomes limiting. Subsequently, we cotransfected 10 μg of pRSV-T plus 30 μg of pβGLCAT-SV into CV-1 cells and observed a more than 4-fold increase in CAT activity (Fig. 4, lane 8) (CAT activity was outside the linear range). This experiment was repeated four times. Even though the absolute CAT activities varied from one experiment to another, stimulation by T antigen occurred in all cases. The above results indicate that T antigen stimulates pβGLCAT-SV expression independently of the enhancer, suggesting interaction with different cellular factors.

T antigen and E1A have different effects on the ε-globin promoter when the SV40 enhancer is present

It has been reported that the early gene product of the adenovirus E1A *trans*-activates various heterologous promoters [20,33–35]. After determining that T antigen can *trans*-activate the human globin promoters, we asked whether the E1A protein can also stimulate the ε-globin promoter. Consistent with the results obtained for other promoters [20,33–35], we found that pE1A could increase the level of steady-state CAT mRNA when cotransfected with pεGLCAT (Fig. 3*b*).

Next we asked whether E1A and T antigen stimulate the ε-globin promoter via the same pathway. We first examined whether E1A, like T antigen, can stimulate the ε-globin promoter to a greater degree when the SV40 enhancer is present. pεGLCAT-SV, containing the SV40 enhancer, was cotransfected with either pRSV-T or pE1A into CV-1 cells. Transfections carried out with either pεGLCAT-SV alone or pεGLCAT-SV plus pE1A

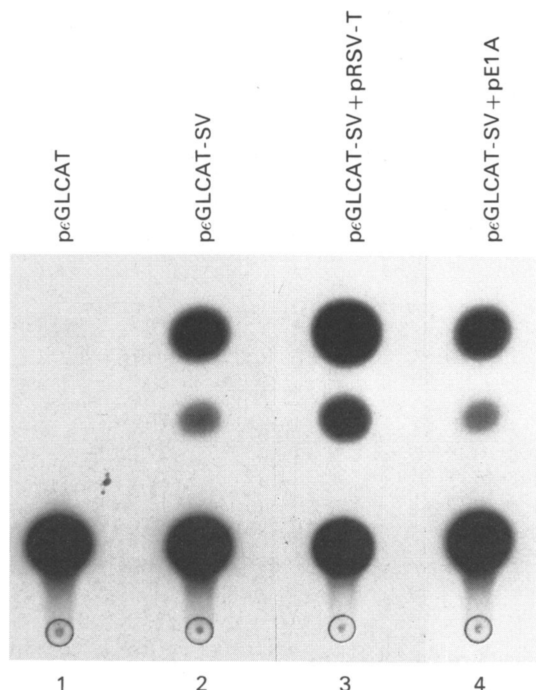

Fig. 5. Differential responses of pεGLCAT-SV to T antigen and E1A

CV-1 cells were transfected with plasmids as indicated. Each transfection experiment contained 12 μg of the testing plasmid, pεGLCAT or pεGLCAT-SV, and 10 μg of pRSV-T or pE1A where indicated. The final amount of plasmid for each transfection was brought to 22 μg with pBR322 when necessary. Each CAT assay contained 500 μg of protein and was incubated for 20 min.

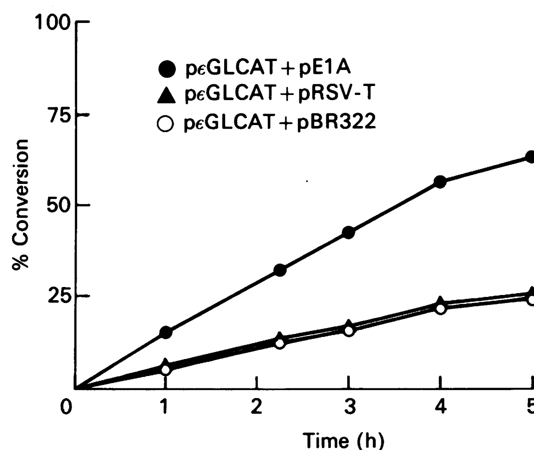

Fig. 6. Different effects of T antigen and E1A on pεGLCAT expression in COS-1 cells

COS-1 cells were transfected with 10 μg of pεGLCAT plus 10 μg of pBR322, pRSV-T or pE1A as indicated. CAT assays were performed after 48 h with 400 μg of protein.

produced the same levels of CAT activity (6 % and 5.5 % conversion respectively; Fig. 5, lanes 2 and 4). In marked contrast, transfection with pεGLCAT-SV plus pRSV-T resulted in a 5-fold increase in CAT activity (Fig. 5, lane 3). This experiment was repeated three times and the same results were observed. Therefore, unlike T antigen, E1A has no effect on pεGLCAT-SV expression. The different response of pεGLCAT-SV to T antigen and E1A indicates that the two viral early proteins *trans*-activate the ε-globin promoter independently.

T antigen and E1A have different effects on the ε-globin promoter in COS-1 cells

Another approach to examining the functional relationship between T antigen and E1A is to test their effects on the human globin promoter in COS-1 cells, which constitutively express the SV40 T antigen. Three parallel experiments were performed. pεGLCAT was cotransfected with pRSV-T, pE1A or pBR322 into COS-1 cells. After 48 h, protein was extracted and CAT activity was measured. This experiment was repeated twice and the same results were obtained. Fig. 6 shows the result of one such experiment. It is clear that cotransfection of pεGLCAT with pRSV-T or pBR322 produced very similar CAT activity. However, cotransfection of pεGLCAT with pE1A resulted in a more than 2-fold increase in CAT level.

To examine whether the increased CAT activity caused by cotransfection with pE1A in COS-1 cells observed above resulted from an increased CAT mRNA level, the steady-state CAT mRNA level was determined by RNAase T1 analysis. In agreement with the results obtained by the enzyme assay, the CAT mRNA level was comparable in cells transfected with pεGLCAT plus pBR322 and pεGLCAT plus pRSV-T (Fig. 7, lanes 3 and 4). In contrast, the CAT mRNA level in cells transfected with pεGLCAT plus pE1A was 2–3-fold higher than that in cells transfected with either pεGLCAT plus pRSV-T or pεGLCAT plus pBR322 (Fig. 7, compare lane 5 with lanes 3 and 4). CAT mRNA was not detected in mock-transfected cells (Fig. 7, lane 2). Therefore, in COS-1 cells, pεGLCAT responds independently to T antigen and E1A, again suggesting that they stimulate pεGLCAT expression by different processes.

Sequences required for T antigen *trans*-activation do not reside in the 100 bp promoter region

In order to determine the sequences responsible for T antigen *trans*-activation, we examined its effect on a number of 5′ deletion mutants of pεGLCAT (Fig. 8). These deletion mutants were cotransfected with pRSV-T or pBR322 into CV-1 cells. Table 2 shows the results. Several observations were made. First, pεΔ274, pεΔ177, pεΔ115 and pεΔ104 had similar basal level CAT activities, but these were much higher (10–14-fold) than that of pεGLCAT (compare the CAT activities in column 2). This suggests that there is a negative regulatory element(s) upstream of 274 bp from the ε-globin gene cap site. Surprisingly, deletion of the CACCC box (pεΔ104) does not significantly decrease the basal level promoter activity. Based on three separate experiments, only a 10–20 % reduction in basal level CAT activity was observed for pεΔ104 compared with that of the other deletion mutants; however, this activity is still 10-fold higher than that derived from pεGLCAT. Secondly, the stimulation by T antigen decreased to 2–3-fold for pεΔ274 and pεΔ177 compared to pεGLCAT, which was stimulated 10-fold. Thirdly, deletions to −115 bp and −104 bp no longer respond to T antigen. These results suggest that the sequences required for T

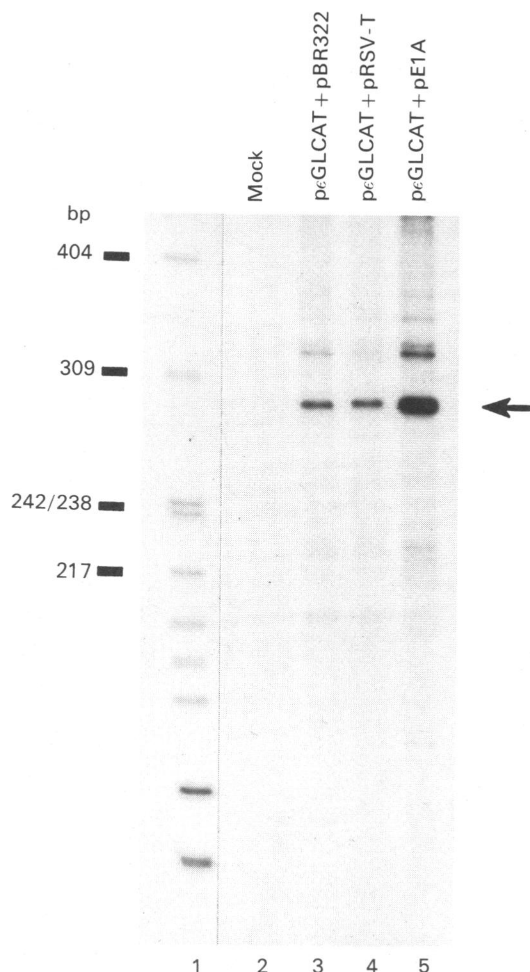

Fig. 7. CAT mRNA levels produced in response to T antigen and E1A in COS-1 cells

The transfection was the same as in Fig. 6. The probe used was the same as in Fig. 3(c). Each lane was loaded with 20 μg of total RNA.

antigen activation are not located in the 100 bp ε-globin promoter region but reside upstream of nucleotide −115.

DISCUSSION

The control of transcription in eukaryotic cells is mediated by interactions of cellular factors and DNA regulatory sequences. The human globin promoters possess the typical higher eukaryotic promoter elements: the TATA box and the CAT box as well as the globin-specific promoter element, the CACCC box [1,2]. In addition, our studies have shown that a number of upstream DNA segments have positive and negative regulatory effects on the ε- and β-globin gene expression [4,5]. In an attempt to understand regulation of these genes, we set out to study the interactions between the regulatory sequences and cellular *trans*-acting factors and their effects on promoter activity. One way to pursue this goal before authentic cellular *trans*-acting factors are available is to look at known *trans*-acting factors. Thus we took advantage of the viral regulatory protein, the SV40 T antigen, to study its *trans*-acting effect on the human ε- and β-globin promoters and to compare its effect with E1A protein and the *cis*-acting enhancer. Our studies demonstrated that in addition to the classical promoter elements (within 100 bp upstream of the cap site), the upstream sequences are also important for globin gene regulation. These upstream sequences are targets of *trans*-acting regulatory factors.

Table 2. Effects of T antigen on pεGLCAT deletion mutants in CV-1 cells

The results are presented as means of several separate experiments (numbers in parentheses). In each experiment, the CAT activity of pεGLCAT plus pBR322 was taken as 1 unit. The CAT activities of deletion mutants plus pBR322 or pRSV-T were normalized to that of pεGLCAT plus pBR322.

Plasmid	CAT activity (units) +pBR322	CAT activity (units) +pRSV-T
pεGLCAT	1.0 (4)	10.0 (4)
pεΔ274	11.8 (3)	22.2 (3)
pεΔ177	12.5 (3)	40.3 (3)
pεΔ115	13.7 (4)	17.0 (4)
pεΔ104	10.3 (3)	9.9 (3)

By using a transient CAT expression system, we have demonstrated that T antigen can *trans*-activate the CAT gene under the control of the human ε- and β-globin promoters in CV-1 cells. RNA analysis showed that the increased CAT enzyme activity resulted from increased CAT mRNA levels. Furthermore, when the SV40 enhancer was present on the globin promoter-CAT plasmid, a higher level of CAT activity was observed when T antigen was present than with the enhancer alone. From several studies with viral systems, it has been suggested that T antigen activates viral promoters by modifying other transcriptional factors rather than by interacting directly with specific DNA sequences [1,36]. By computer sequence analysis, we determined that globin promoters showed little sequence similarity with the T antigen binding sites of SV40 (results not shown). This suggests that T antigen activation of globin promoters is likely to be mediated by cellular transcription factors which bind to the globin regulatory sequences. Enhancers have been known to be targets for interactions with *trans*-acting transcriptional factors [29–31]. Therefore, in the pβGLCAT-SV titration studies where an additive effect was observed for T antigen and SV40 enhancer activation, T antigen could act to increase the level or the activity of the limiting cellular factor(s) required for enhancer function. Alternatively, T antigen could function to increase the level or activity of a different cellular transcriptional factor(s) which is unrelated to the enhancer activity, or to decrease the level or activity of a repressor of the globin promoter. Since we have shown that T antigen alone can *trans*-activate the globin promoters, the latter two possibilities are more likely. If T antigen does increase the level of a transcription factor, it will facilitate the identification and purification of such factor in T antigen producing cells.

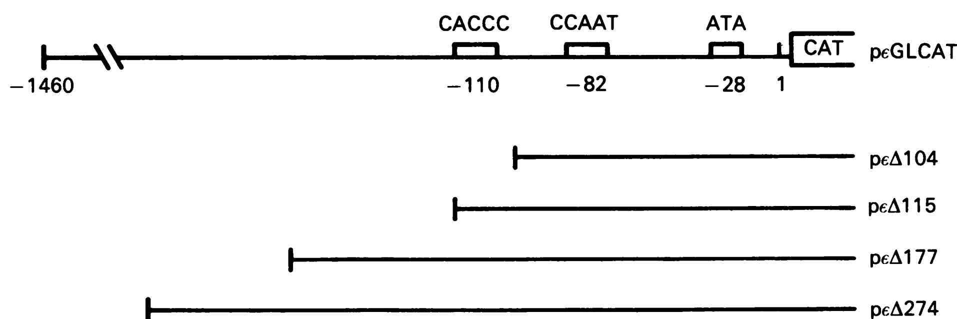

Fig. 8. pεGLCAT and its deletion mutants.

pεGLCAT contains a 1460 bp 5′ flanking sequence of the human ε-globin gene. The relative positions of promoter elements are indicated. pεΔ104, pεΔ115, pεΔ177 and pεΔ274 were created by deleting the upstream region of the ε-globin gene 5′ flanking sequence, leaving 104 bp, 115 bp, 177 bp and 274 bp from the ε-globin gene cap site respectively.

Humphries *et al.* [37] have studied the expression of transfected globin genes in COS-1 cells. While the α-globin gene was shown to express independently of the SV40 enhancer, the β-globin gene required the enhancer. This result is in contrast to our results that the β-globin promoter is activated by T antigen alone in CV-1 cells. The different observations could be due to differences in the plasmid or in the cells. Our plasmid contained a 670 bp 5′ flanking sequence, while a sequence of 2.3 kb was used in their study. To our knowledge, the sequences flanking the β-globin gene have not been studied in detail except the 100 bp promoter region. Thus any hidden sequences having a regulatory function, as in the ε-globin gene, could influence the promoter activity. Alternatively, since COS-1 cells contain most of the SV40 genome, it is not identical to CV-1 cells transfected with pRSV-T.

We have compared the *trans*-activation effects of T antigen with another viral early gene product, the adenovirus E1A protein. E1A resembles the T antigen in that it can *trans*-activate the early transcriptional units of adenovirus [38–40] and stimulate transcription of heterologous promoters [20,33–35]. Recently, E1A was also found to repress the activity of enhancers from SV40, polyoma virus, E1A itself [41,42] and the cellular gene IgH [43]. In the present study, we demonstrated that T antigen and E1A *trans*-activate the ε-globin promoter independently. When pεGLCAT was linked to an SV40 enhancer (pεGLCAT-SV), the addition of T antigen produced an additional stimulatory effect on the ε-globin promoter. In contrast, cotransfection with pE1A had no effect on the expression of pεGLCAT-SV. The different responses of pεGLCAT to T antigen and E1A in COS-1 cells further support the above conclusion. In COS-1 cells, pεGLCAT produced a high basal level of CAT activity. The addition of pRSV-T had no further effect on promoter activity. However, when pE1A was cotransfected with pεGLCAT into COS-1 cells, a 200–300% higher CAT activity was observed compared with pεGLCAT alone (Fig. 6). The fact that the same level of CAT activity was observed upon cotransfection with either pRSV-T or pBR322 suggests that the endogenous T antigen in COS-1 cells is sufficient to activate pεGLCAT expression to a saturation level, and thus additional T antigen would have no effect on CAT expression. E1A, on the other hand, can further stimulate the expression of pεGLCAT in COS-1 cells, suggesting that the *trans*-acting effects of T antigen and E1A are mediated by different cellular factors.

In their study of the E1A *trans*-activation effect, Langner *et al.* (44) have reported that pSV0CAT, which contains no eukaryotic promoter, was able to express the CAT gene in cells (e.g. 293 cells) expressing the E1A protein. This was due to CAT mRNA initiated from prokaryotic sequences. Indeed, we have also observed a certain level of pSV0CAT expression in COS-1 cells. However, if a eukaryotic promoter is inserted 5′ to the CAT gene, the CAT transcripts were correctly initiated from the eukaryotic promoter. This is demonstrated in the RNA analysis of COS-1 cells transfected with pεGLCAT (Fig. 7).

The pεGLCAT deletion analysis reveals the presence of a negative regulatory element upstream from −274 bp of the ε-globin promoter. We have evidence which suggests that this negative regulatory element consists of a 200 bp fragment and acts in a position and orientation independent manner (silencer) (ref. [4], and S. X. Cao & A. N. Schechter, unpublished work). Its significance in the regulation of ε-globin gene expression is currently under investigation. Our deletion analysis showed that while the deletion of the negative regulatory element increased the basal level CAT expression, it reduced the level of activation by T antigen from 10-fold (pεGLCAT) to 2–3-fold (pεΔ274 and pεΔ177). It appears that part of the effect of T antigen is to antagonize the upstream negative regulation. Further deletions toward the cap site (pεΔ115 and pεΔ104) abolished the response to T antigen. Therefore our results suggest that sequences upstream of the 100 bp promoter region are necessary for T antigen activation. We have also examined several deletions upstream of −274 bp (results not shown). While the deletions showed a gradually increased response to T antigen, we were not able to locate a particular sequence responsible for the activation. Our results are distinct from the observation that a 79 bp sequence upstream from the adenovirus E2 cap site is required for full response to T antigen [20]. The different sequence requirements of the ε-globin promoter and E2 promoter for *trans*-activation by T antigen may result from the differences in their promoter structure, and further suggests that the activation is mediated by other cellular factors. Although at the present we do not know the molecular mechanisms involved in the *trans*-activation of

the human globin promoters by T antigen, we have demonstrated that the target sequences of the globin promoter that are responsive to the viral gene product are not the classical promoter elements, but are sequences upstream of the 100 bp promoter region. Therefore the cellular factors interacting with T antigen are not the TATA or CAT box binding proteins, but the proteins interacting with sequences further upstream. We anticipate that this localization should facilitate further characterization of the interaction of these *trans*-acting factors and their *cis* regulatory sequences in the human ϵ-globin 5′ DNA region.

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