trans Activation of the Simian Virus 40 Enhancer

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We describe experiments which demonstrated that the simian virus 40 (SV40) enhancer affects certain transcriptional units differently. We also found that a specific enhancer-transcriptional unit interaction can be regulated by *trans*-acting factors. Using transient assays, we examined the effects of the SV40 enhancer on herpesvirus thymidine kinase (tk) RNA levels when transcription was initiated either by the herpesvirus tk promoter or by an SV40 early promoter-tk fusion. We were unable to detect any effect of the enhancer on transcription from the tk promoter in CV-1 or HeLa cells. However, we found that the addition of T-antigen in *trans* allowed the enhancer to stimulate expression from the tk promoter. This induction by T-antigen did not require T-antigen-binding sites in *cis* and appeared to be an indirect effect. In contrast, tk expression from the SV40 early promoter fusion was greatly stimulated by the enhancer in CV-1 cells. Furthermore, in 293 cells the SV40 enhancer had only a marginal effect on the SV40 promoter-tk fusion, whereas it strongly stimulated tk expression from the tk promoter. Our results raise the possibility that the enhancer function may not show cell specificity per se; rather, the interaction between the enhancer and a specific gene may be responsible for cell specificity. We discuss these observations in terms of the SV40 early gene-to-late gene switch that occurs during SV40 lytic growth.

In general, the *cis*-acting DNA sequences identified for the efficient expression of genes transcribed by eucaryotic RNA polymerase II require a fixed position within a transcriptional unit. In particular, promoters have been classically defined as DNA sites at the 5' ends of genes involved in the initiation of transcription. Although certain distal promoter sequences can function independently of orientation with respect to other elements within the promoter (3, 27, 46), the promoter as a whole can function only in a fixed position and orientation. In this respect enhancer elements (1) are unique in that they can function in an orientation- and position-independent manner; they have been found 5' (15, 16, 18, 26, 47, 65) and 3' (41) to the coding sequences which they affect, and in some of the immunoglobulin genes an enhancer has been found within an intron (2, 20, 49). How enhancers augment the steady-state level of RNA from a gene to which they are linked is still a matter of conjecture, although it has been shown recently that enhancers stimulate the formation of an active transcription complex at the promoter (60, 63).

In general, enhancers display cell type specificity; that is, they appear to function more efficiently in some cell types than in others (9, 13, 14, 33, 36, 37). This probably reflects a requirement for cellular factors for activity; different enhancers may, at least in part, interact with different cellular factors. Thus, enhancer function may be regulated by the availability of these specific factors. Indeed, regulation of enhancer function has been implicated in several viral and cellular systems. The polyomavirus enhancer is non-functional in undifferentiated cells and becomes active only after differentiation is induced (19, 33). Similarly, the immunoglobulin heavy chain enhancer has been shown to be nonfunctional until the final stages of hematopoietic cell differentiation (2, 20, 49). Finally, the enhancerlike element contained within the long terminal repeats of mouse mammary tumor virus has been shown to be under positive

regulation (11, 39). Therefore, the regulation of the level or activity of factors which interact with enhancer sequences may play a major role in the regulation of expression of both cellular and viral genes.

Curiously, the simian virus 40 (SV40) enhancer has little or no effect on the expression of certain genes, yet it has marked effects on others, even when assays are performed in the same cell type. For example, the SV40 enhancer can stimulate in *cis* the expression of genes such as the β -globin (1) and conalbumin (47) genes 20- to 100-fold, whereas in side-by-side assays α -globin expression is stimulated only slightly by the SV40 enhancer (59). Moreover, whereas in CV-1 cells the SV40 enhancer augments gene expression from the SV40 promoter, our data indicate that the SV40 enhancer has very little effect on the herpes simplex virus type 1 thymidine kinase (tk) gene in transient expression assays. This apparent gene specificity for SV40 enhancer function may be the result of one or both of the following processes: (i) it may appear that an enhancer does not affect a gene because the step in transcription which the enhancer stimulates is not the rate-limiting step for expression of that gene; and (ii) the ability of an enhancer to stimulate expression of a gene may depend on precise interactions of gene-specific transcription factors and the enhancer. To investigate the specificity of the SV40 enhancer further, we studied the effects of the SV40 enhancer on tk gene expression when RNA production was mediated by two different promoters, the SV40 early promoter and the herpes simplex virus tk promoter. We examined the enhancer effects on these two promoters in several cell backgrounds which expressed different viral regulatory proteins (e.g., SV40 T-antigen and adenovirus E1A). Our findings indicate that enhancer-dependent RNA expression from the tk promoter can be stimulated by SV40-T antigen and possibly by adenovirus E1A. Our results clearly imply that different genes can be independently regulated by their interactions with the same enhancer. In addition, the apparent cell specificity of

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an enhancer may actually reflect the cell specificity of an enhancer-promoter pair.

MATERIALS AND METHODS

Plasmid construction. pMLTK was constructed by inserting the 3.4-kilobase *Bam*HI fragment containing the tk gene (44, 62) into the *Bam*HI site of pML2 (42). The direction of transcription of the tk gene is the same as that of the *tk* gene in pBR322. SV40 *Hind*III fragment C from pMK16-SV40 6-1, which contained a 6-base pair deletion at the *Bgl*II site (23), was inserted into the *Hind*III site of pMLTK in both orientations so that either the early promoter, pTKSVE⁺O⁻, or the late promoter, pTKSVL⁺O⁻, was oriented in the same direction as tk transcription. pTKSVL⁺ΔSK20⁻ was constructed by partially digesting pTKSVL⁺O⁻ with *Sph*I and then digesting the preparation with *Kpn*I. The ends were treated with T4 DNA polymerase and then ligated. pTKSVL⁺ was identical to pTKSVL⁺O⁻ except that wild-type SV40 *Hind*III fragment C was inserted into the *Hind*III site of pMLTK. pTKSVE2 was constructed by inserting the *Bam*HI tk fragment into the *Bam*HI site of pMLRIIGΔ*Hind*III (42) so that the tk gene was in the same orientation as the SV40 early promoter. pTKACT was constructed by inserting the *Hind*III tk fragment (44) into the *Hind*III site of pACT. pACT was constructed by S. Saragosti and was described by Weiher and Botchan (64). The starting plasmid for this construction was an SV40 early promoter deletion (4) containing a *Bam*HI linker at position 113. The *Bam*HI (position 113)-to-*Pvu*II (position 270) fragment was cloned into the *Bam*HI site of pBR322 by using *Bam*HI linkers. pSVTKEO⁻ was constructed by inserting *Hind*III fragment C from pMK16-SV40 6-1 into the *Hind*III site of pMLTKΔpro. pMLTKΔpro was constructed by partially digesting pMLTK with *Bam*HI and then digesting the preparation with *Bgl*II to remove the promoter region of the tk gene. pTKSVER was constructed by inserting the *Bgl*II-to-*Bam*HI structural tk gene into the *Bam*HI site of pMLRIIGΔ*Hind*III so that SV40 early transcription was in the same direction as tk transcription.

Cell culture and transfection. CV-1, COS-7, HeLa, and 293 cells were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum, whereas tsCOS cells were maintained in Dulbecco modified Eagle medium containing 5% fetal calf serum. For transfection, the HeLa and 293 cells were split 1:8 24 h prior to transfection, whereas the CV-1, tsCOS, and COS-7 cells were split 1:6. For the introduction of DNA into CV-1, COS-7, and tsCOS cells a modified version of the DEAE-dextran procedure of Sompayrac and Danna (55) was used. A 2-μg portion of each plasmid in 0.5 ml of a solution containing 0.5 mg of DEAE-dextran per ml in Tris-buffered saline was added to each 100-mm dish. The mixture was layered onto cells that had been washed previously with phosphate-buffered saline and Tris-buffered saline. The plates were incubated at 33°C for 5 h, during which time the plates were tilted every 20 min. The plates were then washed with Tris-buffered saline and phosphate-buffered saline, and Dulbecco modified Eagle medium containing 5% fetal calf serum and 100 μM chloroquine was added. After incubation at 37°C for 5 h the cells were washed, Dulbecco modified Eagle medium containing 10% fetal calf serum was added, and the plates were left at 37°C until the RNA was isolated. The tsCOS cells were placed at either 33 or 40°C following transfection. For transfection of DNA into HeLa and 293 cells (and COS-7 cells for the experiment shown in Fig. 9) we used the modified calcium

phosphate precipitation method (66); 10 μg of each plasmid was used per 60-mm dish.

RNA isolation and analysis. Total cellular RNA was isolated by using the sodium dodecyl sulfate-proteinase K procedure described by Favalaro et al. (17). In experiments in which the bacterial chloramphenicol acetyltransferase (CAT) gene from the metallothionein promoter (MT-CAT) was used as an internal control, the cells were trypsinized prior to sodium dodecyl sulfate-proteinase K treatment; one-half of the cells were used to assay for CAT activity, and RNA was extracted from the other half. A 40-μg portion of RNA was used for hybridization to the appropriate single-stranded probe. S1 hybridizations were done by using the method of McKnight (44), except that hybridization was performed at 55°C for 5 h in aqueous buffer containing 0.75 M NaCl, 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.0), and 1 mM EDTA and 2,000 U of S1 (P-L Biochemicals, Inc.) per ml were used during S1 treatment at 32°C for 2 h. For analysis of tk RNA a 131-base pair *Eco*RI-to-*Bgl*II single-stranded probe was prepared as described by McKnight (44). For analysis of the SV40 early RNA we prepared a pBR322 probe that contained homology to the pBR322 sequences between the SV40 and tk sequences. Briefly, pBR322 was digested with *Bam*HI, labeled at the 5' end, digested with *Dde*I, and then run on a 5% strand separation gel. The resulting 445-base probe was then used in an S1 analysis; the SV40 tk RNA protected a fragment that was 375 base pairs long from nuclease digestion. The protected fragments from tk 5' S1 mapping were run on a 10% polyacrylamide sequencing gel, whereas a 5% gel was used for SV40 early RNA quantitation. The level of protected probe was quantitated by densitometer scanning of the autoradiogram. In addition, an endpoint titration in which a series of 1:2 dilutions of S1-treated enhancer-plus tk RNA were run adjacent to enhancer-minus S1-treated tk RNA was used to accurately determine the level of enhancement. The CAT assays used as a control (see Fig. 5) were performed as described previously (21), except that 75 μg of protein was used in a 12-h incubation.

RESULTS

Effect of SV40 regulatory sequences on tk expression in CV-1 and COS-7 cells. We initially used SV40 *Hind*III fragment C (Fig. 1a), which contained, in addition to the enhancer, the early and late promoters, T-antigen-binding sites, and a 6-base pair deletion at the origin of replication. The deletion at the origin of replication prevented the replication of SV40, but did not prevent binding of T-antigen in vitro to its normal binding sites (12). *Hind*III fragment C was placed in both orientations relative to the tk gene (Fig. 1b); in our experiments we included both pTKSVE⁺O⁻ (2) and pTKSVL⁺O⁻ (3) to insure that we were studying an orientation-independent enhancer effect. In our experiments we also included pMLTK (1), which contained no SV40 sequences, as an enhancer-minus control.

To determine the cell backgrounds in which we could detect an enhancement of tk-specific RNA in a transient assay, we used several established cell lines. We initially examined the effects on tk expression in T-antigen-producing cell line COS-7; COS-7 cells are CV-1 cells that are stably transformed by SV40 (22). Using a DEAE-dextran procedure (55), we transfected pTKSVE⁺O⁻, pTKSVL⁺O⁻, and pMLTK into both CV-1 and COS-7 cells. The RNA was isolated 48 h after transfection, and the level of tk-specific RNA was measured by 5' S1 analysis. The sizes of the protected DNA fragments (Fig. 2a, arrow) were

FIG. 1. Structure of the SV40 control region and the SV40-tk construction. (a) Region containing the SV40 early promoter and enhancer, as well as the origin of replication and T-antigen-binding sites. (b) SV40 *Hind*III fragment C, which contained a 6-base pair deletion at the *Bgl*II site, was subcloned into the *Hind*III site of pMLTK in both the early and late promoter orientations to give pTKSVE⁺O⁻ and pTKSVL⁺O⁻. The same fragment (*Hind*III fragment C) was inserted into the *Hind*III site of pMLTK Δ pro to give pSVTKEO⁻, in which the SV40 early promoter drove tk transcription. pSVTKER contained SV40 early promoter sequences between the *Eco*RII site at position 160 and the *Hind*III site at position 5171 upstream from the tk structural gene.

determined by electrophoresis relative to known markers; these fragments corresponded to the specific tk RNA starts that have been shown previously to be the authentic tk starts (44). SV40 *Hind*III fragment C had little effect on tk transcription in CV-1 cells. The basal levels of tk RNA for both the enhancer-minus (Fig. 2a, lane 4) and the enhancer-plus (Fig. 2a, lanes 5 and 6) constructions were identical, as revealed by the longer exposure in Fig. 2b. However, as shown in Fig. 2a and quantitatively in Fig. 2c, in COS-7 cells tk expression was stimulated about 15-fold by SV40 *Hind*III fragment C at various DNA input levels. Enhancement was measured by an endpoint dilution analysis relative to the tk control plasmid, as described in Materials and Methods. The effect of the SV40 sequences on tk transcription in COS-7 cells was orientation independent (Fig. 2a, lanes 2 and 3), as

expected for an enhancer effect. Furthermore, it is clear that in the absence of the SV40 regulatory signals (pMLTK) the levels of tk-specific starts were equivalent in CV-1 and COS-7 cells (Fig. 2b, lanes 1 and 4).

The inability of the SV40 enhancer to stimulate tk transcription in CV-1 cells contrasts with previous observations which showed that the enhancer is absolutely required for SV40 early promoter activity (9, 26). Therefore, it is possible that the SV40 enhancer was unable to function in our CV-1 cell line to enhance expression or, alternatively, that the SV40 enhancer functioned with differing efficiencies on different promoters (i.e., the SV40 enhancer exhibited promoter specificity). To differentiate between these two possibilities, we fused the SV40 enhancer-promoter to the tk structural gene so that tk expression was driven from the

FIG. 2. Analysis of tk RNAs from SV40-tk constructions transfected into CV-1 and COS-7 cells. (a) pMLTK, pTKSVL⁺O⁻, and pTKSVE⁺O⁻ transfected into CV-1 and COS-7 cells. The RNA was isolated 48 h posttransfection, and the level of tk RNA was determined by 5' S1 nuclease analysis as described in Materials and Methods. A 40- μ g portion of total RNA was used in each hybridization experiment. The position of the two specific tk RNA 5' starts

SV40 early promoter (Fig. 1b, pSVTKEO⁻). As an early promoter enhancer-minus control we used the SV40 *Eco*RII (position 160)-to-SV40 *Hind*III (position 5171) fragment which contained the SV40 early promoter and a deleted 72-base pair repeat (pSVTKER) (Fig. 1b). The use of the tk gene as a marker for SV40 early promoter expression allowed comparison between specific enhancer-promoter interactions; differences in the potential internal or 3' properties of the transcription units in question were alleviated. Although the RNA expressed from the SV40-pBR322-tk chimera may have had a different stability than intact tk RNA, the use of a pBR322 probe to quantitate SV40 early promoter activity allowed accurate measurement of the steady-state levels of transient SV40 early promoter-dependent RNA and certainly allowed us to compare the differences induced by the presence of the enhancer on the chimeric tk gene transcript. We transfected pSVTKEO⁻ and pSVTKER into CV-1 cells at two different DNA concentrations (2 and 5 μ g) and quantitated expression by performing an S1 analysis at 48 h posttransfection. We assayed the total level of SV40 transcription by using a probe which was homologous to the pBR322 sequences lying between the SV40 and the tk sequences (see Fig. 10).

The effect of the enhancer on SV40 early promoter activity is shown in Fig. 3. The enhancer-plus construction, pSVTKEO⁻ (Fig. 3, lanes 2 and 3), showed a strong expression signal (Fig. 3, arrow). In contrast, the enhancer-minus construction (Fig. 3, lanes 4 and 5) showed no transcription, except for trace levels at the high input DNA concentration (lane 5). Therefore, the SV40 enhancer functioned efficiently to stimulate SV40 early promoter activity in the same cells in which the same enhancer appeared to stimulate tk promoter expression inefficiently.

Stimulation of tk transcription is due to T-antigen. The enhancement of tk expression in COS-7 cells suggests that there are specific factors present in COS-7 cells which are not present in CV-1 cells. One of the apparent differences between CV-1 and COS-7 cells is the effect induced by SV40 proteins. However, other unknown, non-T-antigen-related cellular differences between CV-1 and COS-7 cells could be responsible for the observed enhancement of expression. In order to show that viral proteins are involved in the stimulation of tk expression, we cotransfected SV40-containing plasmid pMK16-SV40 6-1 (which is origin minus, but expresses wild-type T-antigen [23]) into CV-1 cells along with the SV40 tk constructions. The results of an RNA analysis are shown in Fig. 4. The results obtained with pTKSVL⁺O⁻ (Fig. 4, lane 1) confirmed our previous finding that the enhancer was unable to function effectively in CV-1 cells. However, cotransfection of pTKSVL⁺O⁻ with pMK16-SV40 6-1 resulted in a sevenfold enhancement of tk expression (Fig. 4, lane 2). Cotransfection of the T-antigen plasmid

is indicated by an arrow. Lanes 1 and 4, S1-treated RNA from cells transfected with pMLTK; lanes 2 and 5, RNA from cells transfected with pTKSVL⁺O⁻; lanes 3 and 6, RNA from cells transfected with pTKSVE⁺O⁻. (b) Longer exposure of the tk-specific RNA starts shown in panel a. (c) Dose-response curve for enhancer-plus (●) and enhancer-minus (○) plasmids in COS-7 cells. Increasing concentrations of each plasmid were transfected into COS-7 cells, and the levels of specific tk RNA were determined by a 5' S1 analysis at 48 h posttransfection. The levels of RNA were determined following gel analysis by densitometer scanning of the autoradiogram. The relative levels of tk RNA were plotted versus input levels; the level of enhancer-minus tk RNA at an input level of 10 μ g of DNA was arbitrarily given the value of 1.

with constructions lacking SV40 regulatory sequences had no effect on tk expression (Fig. 4, lane 3). The enhancement of tk expression by cotransfection with pMK16-SV40 6-1 was due not merely to dilution of an enhancer-specific repressive component in CV-1 cells by the T-antigen-bearing plasmid (6, 51). This was shown by cotransfecting the enhancer-plus tk plasmid (Fig. 4, lane 1) with a plasmid containing only SV40 regulatory sequences; no stimulatory effect was detected.

In order to demonstrate that the observed effect of SV40 on enhancer-dependent tk expression was due to large T-antigen, we used a construction containing a *tsA* allele (*tsA1609*) expressed from a Rous sarcoma virus (RSV) long terminal repeat (50). The thermolabile T-antigen expressed by RSV-*tsA1609* was defective in binding to the origin and in initiating replication at the nonpermissive temperature (40°C). The enhancer-plus (pTKSVL⁺O⁻) and enhancer-minus (pTKSVL⁺ΔSK20⁻ [see Fig. 7]) plasmids were cotransfected along with the RSV-*tsA1609*-containing plasmid into CV-1 cells. Following transfection, the cells were placed at either 33°C (permissive temperature) or 40°C (nonpermissive temperature) until the RNA was isolated 48 h posttransfection. A construction expressing MT-CAT was also included as a control against which to normalize transfection efficiencies. The activity of the metallothionein promoter does not appear to be affected by T-antigen in transient assays (C. Gorman, personal communication). The results of the tk-specific S1 analysis and the control CAT assays are shown in Fig. 5. Enhancer-dependent tk transcription was stimulated by T-antigen at 33°C (Fig. 5, lane 1) but not at 40°C (lane 2), whereas enhancer-independent tk expression was not dramatically affected by T-antigen at

FIG. 3. Effect of the SV40 enhancer on SV40 early promoter expression in CV-1 cells. Enhancer-plus (pSVTKER⁺) and enhancer-minus (pSVTKER⁻) SV40 promoter-tk fusion plasmids (see Fig. 1) were transfected into CV-1 cells at two different concentrations (2 and 5 μg). The RNA was isolated 48 h posttransfection and quantitated by S1 analysis. A 445-base *Bam*HI-to-*Dde*I pBR322 probe was used, which contained homology to a 375-base region between the SV40 and tk sequences in the two constructions (see Fig. 10). The position of the specific SV40-tk RNA is indicated by an arrow. Lane 1, Untreated probe; lane 2, pSVTKER⁺ (2 μg); lane 3, pSVTKER⁺ (5 μg); lane 4, pSVTKER⁻ (2 μg); lane 5, pSVTKER⁻ (5 μg).

FIG. 4. Effect of T-antigen on the SV40 enhancer in CV-1 cells. An SV40 plasmid, pMK16-SV40 6-1, was cotransfected with either pTKSVL⁺O⁻ (lane 2) or pMLTK (lane 3). A plasmid containing the SV40 enhancer was cotransfected with pTKSVL⁺O⁻ (lane 1). The RNA was isolated 48 h posttransfection and analyzed by 5' S1 nuclease mapping. The positions of the two tk RNA starts are indicated by arrowheads.

either 33°C (lane 3) or 40°C (lane 4). tk starts could be seen in longer exposures. Therefore, we concluded that the observed stimulation of tk expression in CV-1 cells (and presumably COS-7 cells) is due to the presence of T-antigen.

The effect of T-antigen on SV40 *Hind*III fragment C-dependent tk transcription could be a direct effect mediated by T-antigen binding to SV40 sequences or could be an indirect effect produced by T-antigen regulation of cellular factors. In an attempt to differentiate between these two possibilities, we assayed enhancer effects in tsCOS cells. These cells contain integrated copies of a mutant T-antigen gene (*tsA1609*) expressed from an RSV long terminal repeat (50). The tsCOS cells were grown at the permissive temperature (33°C) and then shifted to the nonpermissive temperature (40°C) immediately following transfection. The results of the RNA analysis are shown in Fig. 6. We detected the same level of enhancement at both the permissive and nonpermissive temperatures with the enhancer-plus plasmid (Fig. 6, lanes 3 and 4). Furthermore, the orientation of the SV40 sequences had no effect on transcription at the permissive (Fig. 6, lanes 5 and 7) or nonpermissive (lanes 6 and 8) temperature.

In general, functions of T-antigen are not separated by temperature-sensitive mutations. This is because the known temperature-sensitive mutants are degraded at the nonpermissive temperature and consequently all biochemical activities of T-antigen decrease concomitantly. This is equally true for the *tsA* allele, *tsA1609*, used in this study (50). Therefore, the results described above suggest that the

FIG. 5. Effect of a *tsA* allele on enhancer-dependent tk expression in CV-1 cells. pTKSVL⁺O⁻ and pTKSVL⁺ΔSK20⁻ were cotransfected into CV-1 cells along with an RSV *tsA*1609 construction, and the cells were incubated at either 33°C (permissive temperature) or 40°C (nonpermissive temperature). The RNA was isolated at 48 h posttransfection, and tk RNA was quantitated by a 5' S1 analysis. The positions of the two tk-specific RNAs are indicated by arrowheads. As an internal control for transfection efficiencies, MT-CAT was also included in the transfection experiment. The results of the CAT assays are also shown for each transfection, although the percentage of conversion was determined prior to S1 analysis and the level of RNA in each hybridization was adjusted accordingly. Lane 1, pTKSVL⁺O⁻, 33°C; lane 2, pTKSVL⁺O⁻, 40°C; lane 3, pTKSVL⁺ΔSK20⁻, 33°C; lane 4, pTKSVL⁺ΔSK20⁻, 40°C. CM, Chloramphenicol; A and B, 1-acetate and 3-acetate forms of chloramphenicol, respectively.

function of T-antigen in the stimulation of tk expression augmented by SV40 *Hind*III fragment C is an indirect effect of viral proteins. Thus, it appears that functional T-antigen is not required at the time of tk expression, but presumably is

FIG. 6. Effect of the SV40 enhancer on tk transcription in tsCOS cells. Two plates of tsCOS cells grown at the permissive temperature (33°C) were transfected with the appropriate plasmid. One plate was then immediately switched to the nonpermissive temperature (40°C), and the other plate was left at the permissive temperature. RNA was isolated at 48 h posttransfection, and the level of tk RNA was determined by a 5' S1 nuclease analysis. Lanes 1 and 2, RNA from cells transfected with pMLTK; lanes 3 through 6, RNA from cells transfected with pTKSVE⁺O⁻; lanes 7 and 8, RNA from cells transfected with pTKSVL⁺O⁻. Lanes 1, 3, 5, and 7 contained RNA from cells grown at 33°C, whereas lanes 2, 4, 6, and 8 contained RNA from cells grown at 40°C.

required sometime prior to expression for the effect to be manifested. However, even though the mutation renders the protein inactive for replication and autoregulation and the steady-state levels of T-antigen decrease (50), it is possible that a small population of T-antigen directly participates in the stimulation of expression.

Mapping of the enhancerlike effect. Although we found that SV40 sequences stimulate tk expression in a manner suggestive of their known enhancer function, it is possible that other SV40 sequences are responsible for the stimulation of expression from the tk promoter or work in concert with the enhancer. For example, transcription factors binding to the SV40 early promoter could migrate to the tk promoter in a bidirectional manner. Therefore, using deletion analysis, we mapped the DNA sequences that are responsible for this stimulation of tk transcription. We especially wanted to delete the origin region containing the T-antigen-binding sites to test the possibilities that the stimulatory role of T-antigen was mediated by binding to the SV40 origin and

FIG. 7. Mapping of sequences necessary for T-antigen-dependent stimulation of tk transcription. A condensed diagram of SV40 *HindIII* fragment C is shown. The regions of SV40 maintained in the plasmid constructions are indicated by solid lines. (a) pTKSVL⁺O⁻ contained *HindIII* fragment C with the mutant origin. pTKSVL⁺ΔSK20⁻ contained a deletion between the *Kpn*I site at position 294 and the *Sph*I site at position 121. pTKSVL⁺ contained replication-competent *HindIII* fragment C. pTKSVER contained SV40 sequences from the *Eco*RII restriction site (position 160) to the *HindIII* restriction site (position 5171). pTKACT contained sequences between position 113 and the *Pvu*II site at position 270. (b) The replication-incompetent plasmids were transfected into COS-7 cells, and the replication-competent plasmids were transfected into tsCOS cells grown at the permissive temperature and then shifted to the nonpermissive temperature following transfection. Total RNA was isolated at 48 h posttransfection, and the level of tk-specific RNA was determined by 5' S1 nuclease mapping. Lane 1, S1-treated RNA from cells transfected with pTKSVL⁺O⁻; lane 2, RNA from cells transfected with pTKSVL⁺ΔSK20⁻; lane 3, RNA from cells transfected with pTKSVL⁺; lane 4, RNA from cells transfected with pTKSVER; lane 5, RNA from cells transfected with pTKACT; lane 6, RNA from cells transfected with pTKSVL⁺ΔSK20⁻.

that aberrant replication increased transcription. Furthermore, we wanted to determine whether the presence of a functional origin of replication would have a major effect on tk expression in the absence of replication, as reported previously for the SV40 late promoter (7, 8). As we found that SV40 *cis*-acting sequences can augment tk expression in tsCOS cells at the nonpermissive temperature, this last question could be addressed by using tsCOS cells. The SV40 control region deletions used to map the stimulatory function are shown in Fig. 7a.

The ability of the SV40 sequences to stimulate tk transcription was determined either by transfection of the plasmid constructions into COS-7 cells for the replication-defective plasmids or by transfection into tsCOS cells at nonpermissive temperatures for the replication-competent plasmid constructions. Therefore, replication, with its complicating effects upon expression, was not a variable in these experiments. The results of the S1 analysis are shown in Fig. 7b. Deletion of the sequences essential for SV40 enhancer activity (64) resulted in the loss of stimulation of tk transcription (Fig. 7b, lane 2) compared with enhancer-containing *HindIII* fragment C (lane 1). Sequences containing the early promoter, the T-antigen binding sites, and a functional origin of replication (pTKSVE2) were unable to stimulate tk transcription in tsCOS cells at the nonpermissive temperature (Fig. 7b, lane 3). However, the enhancer-plus and replication-competent plasmid (Fig. 7b, lane 4) showed a level of enhancement similar to that of an enhancer-plus, origin-minus construction in tsCOS cells (Fig. 6). Finally, the SV40 enhancer fragment alone stimulated tk transcription (Fig. 7b, lane 5) compared with the enhancer-minus plasmid pTKSVL⁺ΔSK20⁻ (lane 6). Therefore, mapping the *cis*-acting sequences required for the observed stimulation of tk transcription showed (i) that the enhancer appeared to be the only SV40 element within SV40 *HindIII* fragment C that was capable of stimulating tk transcription in COS-7 cells; (ii)

that the apparent stimulation of enhancer activity did not require known T-antigen-binding sites; and (iii) that a functional origin did not significantly affect expression in the absence of replication.

The experiments described above indicated that the inductive effect of T-antigen on tk expression required an active SV40 enhancer, but they did not prove that the enhancer was the sole element involved. For example, enhancers are known to interact most effectively, although not absolutely, with the most proximal promoter (32). Therefore, as *HindIII* fragment C contains the SV40 early promoter which is highly active in CV-1 cells, it is possible to imagine a situation in which enhancer-promoter communication could be inhibited by the SV40 enhancer-promoter interaction. According to this scenario, T-antigen simply shuts off the early promoter, allowing new enhancer-promoter interactions. We did not believe that this was likely in our experiments since other experiments have shown (50; Rio and Robbins, unpublished data) that autoregulation and DNA replication mediated by T-antigen do not occur at the nonpermissive temperature, whereas enhancer stimulation of tk transcription does occur. Furthermore, enhancer-promoter blocking experiments (15, 32) indicated that the enhancer must be surrounded by two active promoters to block expression of a distal promoter. Since we examined the effect of the enhancer on the tk promoter in different orientations, we do not believe that this was the case in our experiments. Nevertheless, to test this hypothesis definitely, we examined the effect of the 72-base pair repeats alone on tk expression in CV-1 cells in the presence and absence of a functional T-antigen. We cotransfected both pTKACT and pMLTK along with RSV *tsA1609* into CV-1 cells. Following transfection the plates were placed at either 33 or 40°C until RNA isolation 48 h later. Again, as in the experiment shown in Fig. 5, MT-CAT was included as an internal control (data not shown). The results of this experiment are shown in Fig. 8. Consistent with the

FIG. 8. Effect of the enhancer on tk expression was not due to promoter interference. pTKACT and pMLTK were both cotransfected along with RSV *tsA1609* into CV-1 cells. Following transfection the plates were incubated at either 33°C (permissive temperature) or 40°C (nonpermissive temperature). At 48 h posttransfection, the tk RNA was isolated and quantitated by 5' S1 analysis. The positions of the two tk-specific starts are indicated. MT-CAT was included in the transfection experiments as an internal control. Lane 1, pMLTK, 33°C; lane 2, pMLTK, 40°C; lane 3, pTKACT, 33°C; lane 4, pTKACT, 40°C.

observations described above, in our cells pTKACT showed enhancement only at the permissive temperature (Fig. 8, lane 3), whereas pMLTK showed no difference in tk expression at the two temperatures tested. Furthermore, the basal level of tk expression for pMLTK was similar to the level of expression for pTKACT at the nonpermissive temperature. Therefore, these results suggest that the inability of the enhancer to function in CV-1 cells on the tk promoter is not due to blocking of activity by the SV40 early promoter. Moreover, as shown above, SV40 enhancer-dependent expression from the tk promoter can be stimulated in *trans* by a functional SV40 T-antigen. In summary, the results of the temperature shift experiments indicate that the role of T-antigen is indirect and that T-antigen is not needed continuously for induction.

Enhancement of tk expression in 293 cells. The activation of enhancer-dependent tk transcription by T-antigen may reflect the specific activity of T-antigen or, alternatively, may reflect a more general effect of viral *trans* activators upon gene expression. Recent experiments with adenovirus E1A have shown that it can activate transcription from certain viral and cellular genes in an apparently enhancer-independent manner. The phenomenon of *trans* activation by E1A (5, 31) has been demonstrated in particular for the β -globin promoter, where no *cis*-acting enhancer or upstream promoter sequences are required for expression (25), for the SV40 early promoter, where 21-base pair repeats are required for activation (25), and for the E2 gene of adenovirus, where an element required for activation has been identified upstream from the TATA box (29, 30, 48). Additionally, recent experiments have shown that enhancers may

interfere with *trans* activation mediated by E1A (6, 58, 61). Therefore, we wanted to determine whether adenoviral proteins could stimulate the enhancer-dependent transcription of the tk gene, like the effect of T-antigen. We used cell line 293 (24), an adenovirus-transformed human embryonic kidney cell line, for our analysis.

We transfected pTKSVL⁺O⁻ and pTKSVL⁺ΔSK20⁻ into 293 cells, as well as into COS-7 and HeLa cells. The results of the RNA analysis performed with the three cell lines are shown in Fig. 9. We detected a 25- to 30-fold enhancement in 293 cells, whereas in HeLa cells we detected no enhancement; the COS-7 positive control gave a 15-fold enhancement, similar to what we detected previously. These results suggest that in 293 cells there is a stimulation of enhancer-dependent tk transcription analogous to the effect seen in COS-7 cells. The increased level of enhancer-minus tk transcription observed in 293 cells compared with HeLa cells (Fig. 9, lanes 3 and 5) was in part due to the increased uptake of DNA, as determined by Hirt extraction and blot analysis. We are now attempting to determine whether it is E1A alone that stimulates the enhancer activity or whether the stimulatory effect is an unrelated characteristic of 293 cells. However, the enhancement of tk transcription in 293 cells suggests that the *trans* activation of SV40 enhancer-dependent tk transcription is not specific to SV40 T-antigen.

Analysis of the SV40 early promoter in 293 and HeLa cells.

FIG. 9. SV40 enhancer-dependent tk transcription in 293 cells. pTKSVL⁺ΔSK20⁻ and pTKSVL⁺O⁻ were transfected into COS-7, HeLa, and 293 cells. The RNA was isolated at 48 h posttransfection, and tk-specific RNA was quantitated by a 5' S1 nuclease analysis. The positions of the tk-specific bands are indicated by arrowheads. Lanes 1, 3, and 5 contained S1-treated RNA from cells transfected with pTKSVL⁺ΔSK20⁻; lanes 2, 4, and 6 contained S1-treated RNA from cells transfected with pTKSVL⁺O⁻. Lanes 1 and 2, COS-7 cells; lanes 3 and 4, HeLa cells; lanes 5 and 6, 293 cells.

FIG. 10. Effect of the enhancer on SV40 early transcription in HeLa and 293 cells. pSVTKEO⁺ (enhancer plus) and pSVTKER (enhancer minus) were transfected into HeLa and 293 cells, and RNA was isolated and quantitated by S1 analysis diagrammatically (see Fig. 7b and Materials and Methods). When the SV40-tk fusion RNA was hybridized to the 445-base pair *Bam*HI-*Dde*I single-stranded probe, it protected a 375-base pair fragment from nuclease digestion. Lane 1, pSVTKE, HeLa cells; lane 2, pSVTKER, HeLa cells; lane 3, pSVTKE, 293 cells; lane 4, pSVTKER, 293 cells. bp, Base pair.

We also wanted to compare the effects of the SV40 enhancer on the SV40 early promoter (Fig. 3) when it was driving the expression of the tk structural gene with the effects of the SV40 enhancer on the tk promoter in 293 cells.

We transfected the SV40 early promoter-tk fusions pSVTKEO⁺ (enhancer plus) and pSVTKER (enhancer minus) into HeLa and 293 cells and isolated the RNA 48 h posttransfection. We assayed the level of RNA as described above for the experiment shown in Fig. 3 by using a pBR322 probe. The results of the RNA analysis are shown in Fig. 10. In contrast to the effect of the enhancer on the tk promoter, the SV40 early promoter in HeLa cells was dependent on the presence of the enhancer for expression (Fig. 10a, lane 1); no transcription was detected from the enhancer-minus construction (lane 2). Moreover, unlike the tk promoter, enhancer-independent transcription from the SV40 early promoter (Fig. 10a, lane 4) was stimulated in 293 cells, approaching the level of expression from the early promoter in the presence of the enhancer (lane 3). The contrasting effects of the SV40 enhancer on the SV40 promoter-tk fusions and on the intact tk gene in 293 and HeLa cells support the concept that different balances or sets of transcription fac-

tors are required for the two promoter-enhancer interactions in the two different combinations.

DISCUSSION

The results presented above demonstrate that the interaction between the SV40 enhancer and a gene to which it is linked can be induced by the activity of the SV40 T-antigen *in trans*. We found that the SV40 enhancer does not stimulate expression from the tk promoter in transient assays in either CV-1 or HeLa cells. However, in COS-7 and 293 cells we observed stimulation of SV40 enhancer-dependent tk transcription. The stimulation of enhancer activity observed in COS-7 cells is in part due to the presence of T-antigen since cotransfection with a T-antigen-producing plasmid into CV-1 cells also resulted in an increase in enhancer-dependent tk expression. Furthermore, cotransfection with a plasmid expressing the *tsA1609* allele at the nonpermissive temperature did not enhance tk gene expression, whereas at the permissive temperature enhancer-dependent tk expression was stimulated.

We have also found by using deletion analysis that the effect of T-antigen is independent of T-antigen binding to

sites at the SV40 origin. Moreover, we attempted to determine whether the effects of T-antigen are direct or indirect by using cell line tsCOS, which was recently described by Rio et al. (50). Our results suggest that a functional T-antigen is not required at the time of transcription, but is required at some time prior to transcription for the effect to be manifested. Thus, when tsCOS cells are shifted to temperatures at which they do not support SV40 replication, they still show enhancer-dependent tk transcription. These results clearly suggest a model in which T-antigen regulates the level or activity of cellular factors which are able to stimulate enhancer-dependent transcription of the tk gene. Since we were unable to maintain the tsCOS cells at the nonpermissive temperature for extended periods, we were not able to determine whether the observed effect is a permanent or transient one. The indirect nature of this induction of enhancer-dependent tk transcription by T-antigen implies that CV-1 cells may be induced by other means and may explain why other workers have observed some effect of the SV40 enhancer on the herpes simplex virus tk gene (57, 63) in these cells.

We tested the effects of the SV40 enhancer on tk expression by using transient expression assays in CV-1 cells and in HeLa cells in separate experiments over a 2-year period. We detected no significant enhancement of tk transcription by the SV40 enhancer compared with the tk promoter in the absence of SV40 regulatory sequences. Therefore, we do not understand why we have been unable to repeat the previously published observation that the SV40 enhancer is able to stimulate tk expression in HeLa cells (57, 63). We assume that some other variable, such as cell conditions, transfection protocols, or plasmid construction, underlies this discrepancy. In this regard we have learned that R. Triesman and T. Maniatis have also been unable to detect an effect of the SV40 enhancer on tk expression in CV-1 or HeLa cells. Nevertheless, under our conditions we have consistently found that T-antigen induces an enhancer component to tk transcription.

The surprising feature of this induction is that it is clearly gene specific. It has been widely observed that the SV40 promoter can be stimulated by the *cis*-acting effects of its enhancer even in the absence of T-antigen. Accordingly, we have found that when the SV40 early promoter is fused to the tk gene, thereby replacing the tk promoter, tk gene expression is enhancer dependent in both HeLa cells and CV-1 cells. Furthermore, we have found that for these SV40 promoter constructions the SV40 enhancer augmentation of gene expression is markedly reduced in 293 cells, in agreement with other recent reports (25, 58, 59). In contrast, tk promoter expression mediated by the SV40 enhancer is markedly affected in CV-1 cells only in the presence of T-antigen and is augmented by the SV40 enhancer in 293 cells and not in HeLa cells (Fig. 9 and 10). These findings are difficult to reconcile with a simple model for a repressor of SV40 enhancer function in 293 cells, as has recently been suggested (6, 61), but rather suggest that different balances of transcription factors are required for different pairs of genes and enhancers. In this context it seems possible that the establishment of only a partial preinitiation complex may have an apparent negative effect upon transcription. In these situations, therefore, deletion of certain sequences which serve as binding sites for positive transcription factors may actually stimulate the rate at which alternate transcription complexes can be formed. Furthermore, in assessing the cell specificity of enhancerlike sequences, it is clear that the nature of the promoter in question is a critical factor. Thus,

it is not necessarily true that an enhancer has cell specificity; rather, it is the particular enhancer-gene interaction which shows cell-specific responses. Recent observations on the regulation of expression of an immunoglobulin promoter further support the concept that specific interactions between enhancers and promoters are required for cell type-specific expression (43). Even in the presence of the SV40 enhancer, the immunoglobulin V_H promoter has been shown to be inactive in nondifferentiated hematopoietic cells, suggesting that certain promoter-specific factors are also required for enhancer function. Additionally, the activity of a bovine papillomavirus enhancer sequence has been shown to be induced by a bovine papillomavirus *trans*-acting protein, suggesting that specific enhancer factors can be regulated by viral proteins (56).

We propose two models, one quantitative and the other qualitative, to explain the differences observed between the enhancer effects on the herpes simplex virus tk promoter and the effects on the SV40 early promoter. In the quantitative model, we propose that the requirement for enhancer factors for tk expression is greater than the requirement for SV40 early expression; the SV40 enhancer-early promoter interaction is more efficient. Therefore, the SV40 enhancer functions in the analogous manner for each promoter, by using the same enhancer-specific factors which interact with similar transcription factors. Stimulation of the level or function of these transcription factors allows augmentation of tk expression by the SV40 enhancer. The inability of the SV40 enhancer to stimulate the SV40 early promoter in 293 cells may reflect the bypass of enhancer function by stimulation of new, promoter-specific transcription factors which are not involved in tk expression from the tk promoter. Indeed, as stated above, enhancer function may interfere with the specific factors present in 293 cells which allow the SV40 early promoter to function in these cells (6, 61).

In the qualitative model we propose that the SV40 enhancer functions with a slightly different set of transcription factors for the different promoters. Figure 11 shows two hypothetical ways in which this induction may act. At the top of this figure a protein or complex of proteins is interacting with the SV40 enhancer. This enhancer complex interacts ineffectively with the transcription complex at the tk promoter, which leads to no augmentation of tk transcription. After induction by T-antigen, new specific enhancer factors are present (pathway B), which can interact positively with the transcriptional machinery at the tk promoter. Another possibility is that a new set of factors which interact with the upstream promoter sequences are induced (pathway A), which can interact positively with the enhancer complex. The induced factors which interact with the enhancer (pathway B) or the promoter (pathway A) do not necessarily have to be DNA-binding proteins. Therefore, an equally plausible model is that a type of protein which is not a DNA-binding protein is induced; this protein complexes with either the promoter-proximal protein or the enhancer protein. Such tertiary complexes between enhancers and promoters could clearly provide multiple levels for regulation and could readily explain the range of effects seen in different constructions. The model which we present is in some ways equivalent to the entry site model for enhancers in that the protein interacting with the enhancer participates in the initiation of transcription. However, the protein makes contact with the promoter through the bending of the DNA to interact with the closest promoter rather than by migration along the DNA. This bending model readily explains how an apparently asymmetric DNA sequence (64, 65) can interact in both

FIG. 11. Two hypothetical models to explain the effects of the SV40 enhancer on the tk promoter. See Discussion for details.

orientations with respect to the direction prescribed by the promoter.

Neither the quantitative model nor the qualitative model discussed above is exclusive, and either one can also explain why enhancement of the frequency of tk transformation in stable assays in some cell types is observed (10, 13, 40, 57; Robbins and Botchan, unpublished data), whereas no enhancement in RNA levels is detected in certain transient assays (40). If enhancer-promoter factors are limiting, as has been suggested by Schöler and Gruss (51), then a stable assay allows for selection of cells with only a few copies of the enhancer sequence, whereas in transient assays the enhancer is present at a very high copy number and possibly titrates out the specific enhancer factors. Therefore, whether the same factors or different factors are involved in the enhancement of the tk and SV40 early promoters, the stimulation of their level or activity by T-antigen allows for detectable enhancement in transient assays.

T-antigen has been shown previously to stimulate expression of genes transcribed by all three types of eucaryotic RNA polymerases. In particular, several groups of workers have used differential screening to isolate a number of cDNAs representing presumed RNA polymerase II transcripts in SV40-transformed cells (52, 53). SV40 T-antigen has also been shown to stimulate the expression of RNA polymerase I transcription following SV40 infection (38). Furthermore, T-antigen has been demonstrated to stimulate the expression of RNA polymerase III B2 transcripts in

mouse cells (53, 54). Therefore, the wide variety of transcriptional responses to T-antigen suggests that T-antigen activates gene expression in an indirect manner and that these effects are due to the pleiotropic effects of this protein.

What role, if any, does the activation of function of the SV40 enhancer for a promoter play in the normal viral growth cycle? It has been shown that T-antigen can stimulate SV40 late transcription in the absence of DNA replication (7, 8, 28, 34-36). Whereas the activation of late gene transcription is a complex phenomenon that is likely to involve replication which results in amplification of transcription templates, the mapping of the *cis*-acting sequences necessary for activation in the absence of replication clearly implicates the SV40 72-base pair repeats (8, 28, 35). Our own observations support this concept, as we have examined RNA expression from the late promoter when it is linked to the tk structural gene (Robbins and Botchan, unpublished data). We have found that the activation of late gene expression occurs in the same cell backgrounds (COS-7, tsCOS, and 293 cells) as SV40 enhancer-dependent tk expression does. Furthermore, *trans* activation by T-antigen of late promoter function also occurs in tsCOS cells at the nonpermissive temperature, suggesting that T-antigen plays an indirect role. Because of the apparent similarities between activation of late gene expression and activation of enhancer-dependent tk transcription, it is tempting to speculate that the observed effects of T-antigen may reflect the series of events which occur during the switch from early to

late SV40 gene expression. In this regard it is interesting that the SV40 late promoter-enhancer tk gene fusions produce more RNA than the SV40 early promoter-enhancer tk gene fusions do in 293 cells (50a). Recent reports have shown that 293 cells are permissive for SV40 replication, with very low levels of T-antigen required for this replication (J. Lebowski, T. Clancy, and M. Calos, personal communication; D. Lewis and J. Manley, personal communication). It seems plausible that both SV40 and adenovirus early proteins prepare cells for their late modes of action in overlapping ways.

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