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trans Activation of the Simian Virus 40 Late Promoter by Large T Antigen Requires Binding Sites for the Cellular Transcription Factor TEF-1

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Simian virus 40 (SV40) T antigen stimulates the level of transcription from several RNA polymerase II promoters, including the SV40 late promoter. The mechanism of *trans* activation appears to be indirect since binding of T antigen to specific DNA sequences is not required. However, specific promoter elements that respond to T antigen have not previously been defined. We identified DNA sequences from the SV40 late promoter whose ability to stimulate transcription is induced by the expression of T antigen. In particular, the Sph I + II motifs of the SV40 enhancer can confer T-antigen inducibility to the normally uninducible herpes simplex virus thymidine kinase gene promoter when multiple copies of the sequence are inserted 5′ of the transcription initiation site and TATA sequence. Binding sites for the cellular transcription factor TEF-1 and octamer binding proteins are contained within the Sph I + II motifs, as well as at other positions in the SV40 promoter. To study the role of individual protein-binding sites in *trans* activation by T antigen, mutations were constructed in various TEF-1 and octamer protein-binding sites of the SV40 late promoter. These mutations did not significantly affect basal promoter activity. However, mutation of all three TEF-1 sites prevented detectable activation by T antigen. DNase I footprinting of the mutated promoters with purified proteins demonstrated that inducibility by T antigen correlated with binding affinity of TEF-1 for the DNA and not with binding affinity of an octamer binding protein.

The level of accurate initiation of transcription at RNA polymerase II promoters depends on the activity of sequence-specific DNA-binding transcription factors. The rate of initiation at a particular promoter can be regulated by modulating the activity of the factors that bind to that promoter. The activity of a transcription factor can be altered by changing the amount present in the cell, as in the case of increases in the level of c-*jun* mRNA and AP-1 DNA-binding activity upon treatment of cells with 12-*O*-tetradecanoylphorbol-13-acetate or serum (2, 26). Transcription activity can also be regulated by posttranslational modifications, as in the case of phosphorylation of serine 133 within the cyclic AMP-responsive element binding protein, which affects transcriptional activity without affecting DNA-binding activity of the protein (16).

The simian virus 40 (SV40) early gene products, large tumor (T) and small tumor (t) antigens, are able to activate several viral and cellular RNA polymerase II promoters in transient transfection assays (1, 6, 22, 27, 28, 38, 48). Small t antigen is not a sequence-specific DNA-binding protein, suggesting that its effects are mediated through cellular transcription factors. Although large T antigen is a sequence-specific DNA-binding protein (40), the following observations indicate that specific DNA-binding activity is unnecessary for *trans* activation. First, mutants of T antigen lacking the DNA-binding domain are still capable of activating the adenovirus E2 and SV40 late promoters (47). Second, promoters activated by T antigen, with the exception of the SV40 late promoter and the human heat shock protein 70 promoter (48), lack known T-antigen-binding sites. Finally, a nuclear localization mutant of T antigen which results in T antigen being largely cytoplasmic retained the ability to activate the SV40 early and late promoters (49). These results indicate that *trans* activation by T antigen, like that by t antigen, probably requires a cellular intermediate. Since the promoters activated by large T antigen do not appear to share a common DNA sequence element, it is possible that T antigen can affect the activity of more than one cellular factor. Recent reports describe one cellular protein that is more abundant and differentially modified in SV40-infected CV-1 cells and in T-antigen-expressing COS cells when compared with uninfected CV-1 cells (12, 13).

The goal of the work described here was twofold: to identify a DNA sequence element from the SV40 late promoter that is sufficient for induction by SV40 T/t antigens, and to correlate induction by T/t antigens with the binding of specific cellular transcription factors. We showed that the Sph I + II motifs (51) of the SV40 enhancer can confer responsiveness to T/t antigens upon an otherwise unresponsive promoter. Using site-directed mutagenesis, we found that these motifs, as well as the GTIIC motif (50, 51), each capable of binding the cellular transcription factor TEF-1 (9), are essential for *trans* activation of the SV40 late promoter by T/t antigens. DNase I footprinting analysis of the mutated promoters further supported the hypothesis that TEF-1 is an intermediary required for *trans* activation of the SV40 late promoter.

MATERIALS AND METHODS

Plasmid DNAs. All plasmids were propagated in *Escherichia coli* HB101 and isolated by an alkaline lysis protocol (3). Some plasmids were amplified for 15 h with chloramphenicol before isolation. All plasmids were purified by banding twice in CsCl-ethidium bromide gradients.

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Synthetic oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and purified by electrophoresis through denaturing polyacrylamide gels. Double-stranded oligonucleotides were inserted into the *Bam*HI site at position −54 of the mutant herpes simplex virus thymidine kinase gene (HSV *tk*) LS −59/−49 (31) by standard procedures (3) (see Fig. 1). The sequences of the oligonucleotides are shown below:

AP-1: 5′-gatcGTGACTCAGCGCG (consensus)
CACTGAGTCGCGCctag-5′

AP-2: 5′-gatcCTGGGGAGCCTGGGGACTTTCCACACCCTAAC
GACCCCTCGGACCCCTGAAAGGTGTGGGATTGctag-5′

AP-4: 5′-gatcATTCCACAGCTGGT
TAAGGTGTCGACCActag-5′

LSF-GC: 5′-gatcATGGGCGGAACTGGGCGGAG
TACCCGCCTTGACCCGCCTCctag-5′

LSF-280: 5′-gatcCAGCTGGTTCTTTCCGCCTCA
GTCGACCAAGAAAGGCGGAGTctag-5′

Sph: 5′-gatcCTTGAGATGCATGCTTTGCATACTTC
GAACTCTACGTACGAAACGTATGAAGctag-5′

Recombinant plasmids were isolated that contained from one to six copies of each oligonucleotide. The sequence of the DNA in the promoter region was confirmed for each isolate by the dideoxy nucleotide-sequencing method of Sanger et al. (44), using the enzyme Sequenase (United States Biochemical).

Site-directed mutagenesis of the SV40 late promoter was performed on a single-stranded M13 derivative containing SV40 nucleotides (nt) 4770 to 874, except for a deletion of nt 107 to 179. This region of SV40 includes the origin of DNA replication and both the early and late promoters. Mutagenesis was done with a site-directed mutagenesis kit (Amersham), following the manufacturer's instructions. Subsequently, regions containing the mutations were used to replace the analogous wild-type sequences in pLCATΔ72, which was constructed from pL16nCAT (23) by deleting one of the two 72-bp repeats by using *Sph*I. pLCATΔ72 and the mutant derivatives therefore contain SV40 sequences from nt 5171 to 107 and nt 179 to 333, with the late promoter driving expression of the bacterial chloramphenicol acetyltransferase (CAT) gene. Additionally, the constructs contain a 6-bp deletion at the SV40 origin of replication that prevents DNA replication in mammalian cells (15).

For transfection assays, the Rous sarcoma virus (RSV) long terminal repeat (LTR) directing the expression of the SV40 early region, pRSV-T/t, was used to express high levels of the SV40 tumor antigens (28), and pRSV-CAT (17) was used to control for potential competition for transcription factors between the RSV promoter and the HSV *tk* promoter. Plasmids containing the human growth hormone gene under control of either the HSV *tk* promoter (pTKGH) or the mouse metallothionein I promoter (pXGH5) were used to control for transfection efficiencies (46). Transfections for RNA analysis also included ψwt *tk* DNA as an internal control (31). This DNA contains the HSV *tk* promoter and coding region with a deletion of 20 bp (+16 to +36) in the sequences encoding the 5′ untranslated region of the gene and a 10-bp insertion of a *Bam*HI linker in their place. ptkR1/Rsa was used to prepare RNase protection probes. It consists of a 198-bp *Eco*RI-*Rsa*I fragment from LS −59/−49 that spans the transcription start site, cloned into pBluescript SK+ (Stratagene) cleaved with *Eco*RI and *Eco*RV.

Cell culture and transfection assays. CV-1 cells were maintained in Dulbecco's modified Eagle medium (Hazelton) with 10% (vol/vol) newborn calf serum (GIBCO), 50 U of penicillin G per ml, and 50 μg of streptomycin (GIBCO) per ml. Twenty to 24 h before transfection, approximately 10^6 cells were plated per 10-cm dish. Transfections were performed by the calcium phosphate precipitation method (19). Sixteen to 18 h after the DNA precipitates were added to the plates, the cells were washed twice with Dulbecco's modified Eagle medium and incubated further with Dulbecco's modified Eagle medium containing 10% newborn calf serum. The cells were harvested 44 h after the precipitate was added to the plates. Transfection experiments to be assayed by *tk* RNA analysis included 10 μg of *tk* reporter plasmid DNA, 2 μg of pRSV-T/t or pRSV-CAT, 0.5 μg of pTKGH, 4 μg of ψwt *tk*, and pBluescript SK+ to bring the total amount of DNA to 20 μg for a 10-cm plate. This amount of pRSV-T/t was optimal for *trans* activation of the *tk* reporter plasmids and of the SV40 late promoter (data not shown). Transfection experiments to be assayed by CAT analysis included either 1 or 2 μg of CAT reporter plasmid DNA, 0 or 2 μg of pRSV-T/t, 0.5 μg of pXGH5, and pBluescript SK+ to bring the total amount of DNA to 20 μg per 10-cm plate.

RNA analysis. Total RNA was isolated from the transfected CV-1 cells by the guanidinium isothiocyanate method (8). The RNA was purified by centrifugation at 20°C through a cushion of 5.7 M CsCl in 100 mM EDTA at 35,000 rpm in an SW50.1 rotor. RNA levels were quantitated by the RNase protection assay (32), performed as follows. An antisense *tk* RNA probe was synthesized by transcribing 1 μg of *Eco*RI-digested ptkRsa/R1 with 10 U of T7 RNA polymerase, 3.3 mM each ATP, CTP, and GTP, and 90 μCi of [α-^{32}P]UTP (800 μCi/mmol) at 37°C for 60 min. A total of 2.5×10^5 cpm of this probe were hybridized at 55°C for 16 h with 20 μg of total RNA from transfected cells in a 30-μl reaction mixture containing 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4), 400 mM NaCl, 1 mM EDTA, and 80% deionized formamide. The unhybridized probe was removed by the addition of 28.5 U of RNase T_1 (Calbiochem) per ml in 350 μl of 300 mM NaCl–10 mM Tris (pH 8.0)–1 mM EDTA and incubation for 30 min at 37°C. Resistant reaction products were separated by electrophoresis through a 0.5-mm-thick 6% polyacrylamide gel (20:1, acrylamide-bisacrylamide) containing 7.0 M urea in 89 mM Tris base–89 mM boric acid–2 mM EDTA. The gels were exposed to preflashed Kodak XAR-5 film without drying, and the intensity of the bands was quantitated by using an LKB/Pharmacia Ultroscan II laser densitometer.

CAT analysis. Whole-cell extracts from transfected cells were prepared as described previously (18). Assays for CAT activity were performed by incubating 5 to 15 μl (50 to 150 μg) of extract for 40 min at 37°C in 0.25 M Tris (pH 7.5) containing 0.2 μCi of [^{14}C]chloramphenicol (55 mCi/mmol) and 0.5 mM acetyl coenzyme A. The reactions were stopped by extraction with 1 ml of ethyl acetate. They were spotted on silica gel thin-layer chromatography plates which were then developed in 95% chloroform–5% methanol. The conversion of chloramphenicol to acetyl chloramphenicol was quantitated with a Betascope (Betagen). The results were normalized to the amount of protein in the extracts and the level of human growth hormone secreted into the media of the transfected cells. In some experiments, the normalizations for transfection efficiency and protein concentration were done first, resulting in different volumes of extract being added to each assay. Protein assays were performed by the method of Bradford (5), using the Bio-Rad dye

reagent with gamma globulin as a standard. Growth hormone levels were determined by radioimmunoassays with a kit from Nichols Institute Diagnostics.

DNase I footprinting. Radioactively labeled DNA fragments of identical specific activity from different mutant promoters were prepared by the polymerase chain reaction (43). One primer, extending from SV40 nt 341 to 317, was labeled at nt 341 by using [γ-^{32}P]ATP and T4 polynucleotide kinase. The second primer contained SV40 nt 5172 to 5194. The templates for amplification were either plasmid DNA containing the wild-type or mutant promoters or single-stranded M13 DNA containing these promoters. The 341-bp DNA products of the polymerase chain reaction were purified by electrophoresis through polyacrylamide gels before use. TEF-1 was purified from HeLa cell nuclear extract as previously described (9) except that the single-stranded DNA-cellulose chromatography was omitted. The octamer binding protein was a fusion protein between the Oct-1 POU domain and protein A (PA-Oct1-POU). The protein was purified from an *E. coli* extract by immunoglobulin G column chromatography and was a generous gift from T. Kristie and P. A. Sharp (25).

Binding reactions were performed with 1 ng of labeled DNA fragment and a variable amount of protein and were incubated for 15 min at room temperature in a total volume of 20 μl. The binding reaction mixtures for TEF-1 contained 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 45 mM KCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 1% polyvinyl alcohol, 10% glycerol, and 10 ng of poly(dI-dC) · poly(dI-dC). Binding reaction mixtures for PA-Oct1-POU contained 20 mM HEPES (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 4% Ficoll 400, 0.025% Nonidet P-40, and 100 ng of poly(dI-dC) · poly (dI-dC). After the binding reaction, DNase I (Worthington) was added to a final concentration of 60 ng/ml for a 1-min incubation, and the reactions were stopped by the addition of 20 μl of 400-μg/ml proteinase K–20 mM EDTA–2% sodium dodecyl sulfate. The reaction mixtures were extracted once with phenol-chloroform-isoamyl alcohol (25:24:1), precipitated with ethanol, and electrophoresed through a 0.5-mm-thick 8% polyacrylamide gel (20:1, acrylamide-bisacrylamide) containing 7 M urea, 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA. The gel was exposed to preflashed Kodak XAR-5 film without drying. Levels of protein binding were quantitated from autoradiographs exposed in the linear range of the film with an LKB Ultroscan II laser densitometer.

RESULTS

DNA sequences conferring inducibility by T antigen. To identify T-antigen-responsive promoter elements, we inserted single or multiple copies of defined double-stranded oligonucleotides at position −54 of LS −59/−49, a mutated HSV *tk* promoter directing expression of the HSV *tk* gene (Fig. 1). Previous studies indicated that the HSV *tk* promoter was not inducible by T antigen (41). In this location, the inserted sequences were in the same position as an Sp1 site in the wild-type HSV *tk* promoter (21). The double-stranded oligonucleotides, generally derived from SV40 sequences (see Materials and Methods), included binding sites for proteins that stimulate SV40 late transcription in vitro (AP-1, AP-4, LSF-GC, and LSF-280 [20, 33]) and sequences involved in SV40 late promoter activity in vivo (Sph [29]). In addition, an AP-2-binding site was included in the study

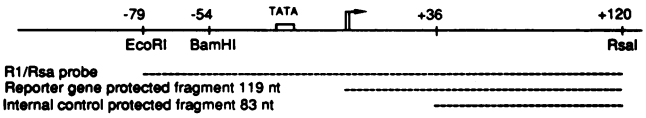


FIG. 1. Reporter plasmids and RNase protection probe. Schematic diagram of the promoter of LS −59/−49 showing the restriction sites used. SV40 oligonucleotides were inserted into the *Bam*HI site at −54. The RNase protection probe is shown below the promoter and is complementary to −79 to +120 (*Eco*RI-*Rsa*I) of LS −59/−49 and to −54 to +120 of reporter plasmids containing an insert at the *Bam*HI site. The probe is complementary to +36 to +120 of the ψwt internal control.

because it was reported that the binding of AP-2 to DNA is inhibited by interaction of T antigen with AP-2 (34).

The resulting HSV *tk* reporter constructs were transfected into CV-1 cells in the presence or absence of a plasmid directing expression of SV40 large T and small t antigens from the RSV LTR (pRSV-T/t). In the absence of pRSV-T/t, a plasmid containing the RSV LTR joined to the bacterial CAT gene (pRSV-CAT) was used to control for potential competition for transcription factors between the RSV LTR and the HSV *tk* promoters. To control for differential transfection efficiencies, a plasmid expressing the human growth hormone gene under the control of the HSV *tk* promoter was also included. Both the levels of *tk* mRNA and the positions of the initiation sites used were determined by an RNase protection assay. A ψwt *tk* plasmid, which contains a small deletion and substitution of sequences in the HSV *tk* coding region, was included as an internal control for the RNase protection assays. Representative data for the AP-1 and Sph constructs are shown in Fig. 2, and quantitation of the data from three separate experiments using these reporter plasmids is given in Table 1.

The products of interest, resulting from HSV *tk* transcripts initiated at the previously mapped positions, are indicated by arrows (Fig. 2), and the product expected for the ψwt *tk* internal control is indicated by the asterisk. The identity of these products was confirmed by analysis of RNA prepared from cells transfected with only a wild-type HSV *tk* gene, HSV 106 (lane 11), or the ψwt *tk* gene (lane 12). Two other major products observed in every lane are artifactual, since they are present in the control lane with only yeast tRNA (lane 13). The slowest-migrating products map to position −79 and result from read-through transcription. Lanes 3 through 10 contain an additional read-through product mapping to position −54 resulting from noncomplementarity between the probe and the reporter plasmids containing an insert at this position. The products that migrate between the read-through products and the artifactual products may be either initiation sites 5′ of the previously described initiation sites or cleavage of hybrids with read-through products at G residues near A+T-rich sequences that are not stably hybridized. In general, these products were induced in parallel with the expected products but were not considered in the quantitation.

Relative to the internal control, the level of *tk* mRNA from LS −59/−49 was unaltered by the expression of T/t antigen (not visible in Fig. 2, but clear in several other experiments; see Table 1). Insertion of a single Sph oligonucleotide did not increase basal promoter activity, and the promoter remained unresponsive to T/t-antigen expression (Fig. 2, lanes 9 and 10, and Table 1). When two or four AP-1-binding sites or five Sph oligonucleotides were inserted, basal promoter activity

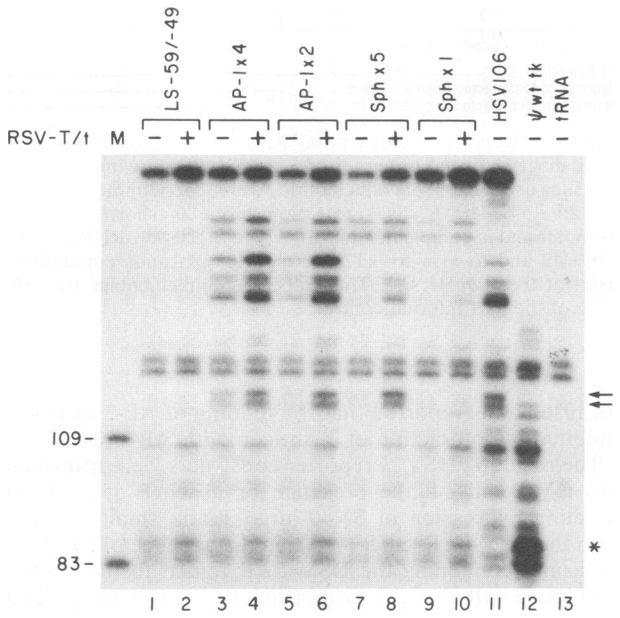


FIG. 2. RNase protection analysis of RNA isolated from cells transfected with reporter plasmids containing AP-1 or Sph sequences. The reporter plasmids indicated above the lanes were transfected into CV-1 cells in the presence (+) or absence (−) of pRSV-T/t. Total RNA from the transfected cells was hybridized to the probe diagrammed in Fig. 1. After digestion to remove unhybridized probe, the protected fragments were separated on a denaturing polyacrylamide gel. The arrows indicate the positions of the authentic HSV *tk* initiation sites, and the asterisk marks the position of the ψwt internal control band. Lanes 1, 3, 5, 7, and 9, RNA from cells cotransfected with 2 μg of pRSV-CAT. Lanes 2, 4, 6, 8, and 10, RNA from cells cotransfected with 2 μg of pRSV-T/t. Lanes 11 and 12, RNA from cells transfected only with HSV 106 (wild-type *tk*) or ψwt *tk*, respectively. Lane 13, as a control, RNase protection was done with 20 μg of yeast tRNA. Lane M, markers (in nucleotides), SV40 DNA digested with *Hin*fI.

increased (Fig. 2, lanes 5, 3, and 7, respectively, and Table 1), and the promoter became T/t antigen inducible (Fig. 2, lanes 6, 4, and 8, and Table 1). Although in Fig. 2 the AP-1 site-containing reporters and the Sph × 5 reporter showed similar responses to T/t antigens, in other experiments the AP-1-containing reporters were not consistently induced by T/t antigens (Table 1). The cause of this variability is not known and was not examined further.

TABLE 1. Quantitation of the results of RNase protection assays[a]

Reporter[b]	−T/t	+T/t	Fold activation[c]
LS −59/−49	1.0	0.9	0.9 (0.5–1.3)
AP-1 × 2 (→→)	2.1	5.7	2.7 (1.1–3.6)
AP-1 × 4 (→→→→)	4.5	6.1	1.4 (1.1–1.8)
Sph × 1 (→)	0.8	1.0	1.2 (0.6–1.3)
Sph × 5 (←←→→→)	3.1	13.0	4.2 (2.5–5.3)

[a] The results were normalized to the level of RNA generated from the ψwt internal control. The level of RNA resulting from LS −59/−49 in the absence of pRSV/T/t was assigned a value of 1.0 in each experiment.

[b] The orientation of the inserts in the reporter plasmids is indicated. Arrows pointing to the right indicate that the insert is in the same orientation as it is in the SV40 late promoter.

[c] The data are averages of three experiments. The ranges of induction observed are indicated in parentheses.

In contrast, when one or two AP-4 sites or one or six AP-2-binding sites were inserted into the HSV *tk* promoter, the basal promoter activity was not detectably changed and the promoter also remained unresponsive to T antigen (data not shown). Additionally, when one or two LSF-GC-binding sites or a single LSF-280-binding site was inserted, the basal promoter activity increased two- to six-fold, but the activity still was not stimulated by expression of T/t antigens (data not shown).

These results indicated that multiple copies of the Sph oligonucleotide, which contains the Sph I + II motifs, were reproducibly able to confer inducibility by T/t antigens to the HSV *tk* promoter.

Mutagenesis of SV40 late promoter. The Sph I + II motifs consist of two adjacent direct repeats of a 9-bp sequence, each capable of binding the cellular protein TEF-1 (9). In addition, an 8-bp sequence at the junction of the two repeats can bind to the octamer binding proteins (4, 42). The role of each of these binding sites in activation by T/t antigens was assessed by making mutations in the Sph I + II motifs that were specific for only a single protein-binding site (9, 42). These mutations were constructed in the context of a derivative of the SV40 late promoter directing the expression of the CAT gene (pLCATΔ72, see Materials and Methods and Fig. 3B). The SV40 late promoter contains a TEF-1-binding site in addition to those within the Sph I + II motifs. This binding site, referred to as GTIIC, maps to the late side of the 72-bp repeats, at nt 263 to 272 (9, 50) (Fig. 3B). To investigate potential redundancy of late promoter elements responsive to T antigen, we mutated each of the three TEF-1-binding sites in pLCATΔ72 individually or in combination with one or both of the other sites to generate double and triple mutants. Similarly, both the octamer-binding site at the junction of the Sph I + II motifs and a second octamer-binding site at nt 190 to 199 (4) were mutated to create a double-octamer mutant (Fig. 3B).

These "wild-type" and mutant reporter plasmids were transfected into CV-1 cells in the presence or absence of pRSV-T/t, and levels of expression from the promoters were measured by quantitation of CAT levels in the resulting cell extracts. A representative CAT assay is shown in Fig. 3A, and averaged data from two to four transfection experiments are represented in Fig. 3B. Mutations in the TEF-1- and octamer-binding sites did not significantly alter the basal activity of the SV40 late promoter. Even promoters containing mutations within all three TEF-1-binding sites retained 60 to 70% of the basal wild-type activity (Fig. 3A, compare lanes 1, 11, and 13). The wild-type promoter in pLCATΔ72 was activated an average of 10.6- to 11.9-fold by T/t antigens. Mutation of one or two TEF-1-binding sites reduced the activation to four- to sixfold (Fig. 3A, lanes 3 to 8). Strikingly, mutation of all three TEF-1-binding sites resulted in a promoter which was not detectably activated by T/t antigens in these assays (Fig. 3A, lanes 11 to 14). The promoters containing mutations intended to interfere specifically with octamer-binding sites reduced *trans* activation to 6.5-fold for OCTCAT2 (Fig. 3A, lanes 17 and 18) and to 3.5-fold for OCTCAT1 (Fig. 3A, lanes 15 and 16). These data demonstrate that sequences within the Sph I + II motifs are not essential for basal activity of the late promoter but are essential for activation by T antigen.

Footprinting of mutant promoters. Interpretation of the results obtained with the mutated promoters is complicated by the fact that the TEF-1- and octamer-binding sites over-

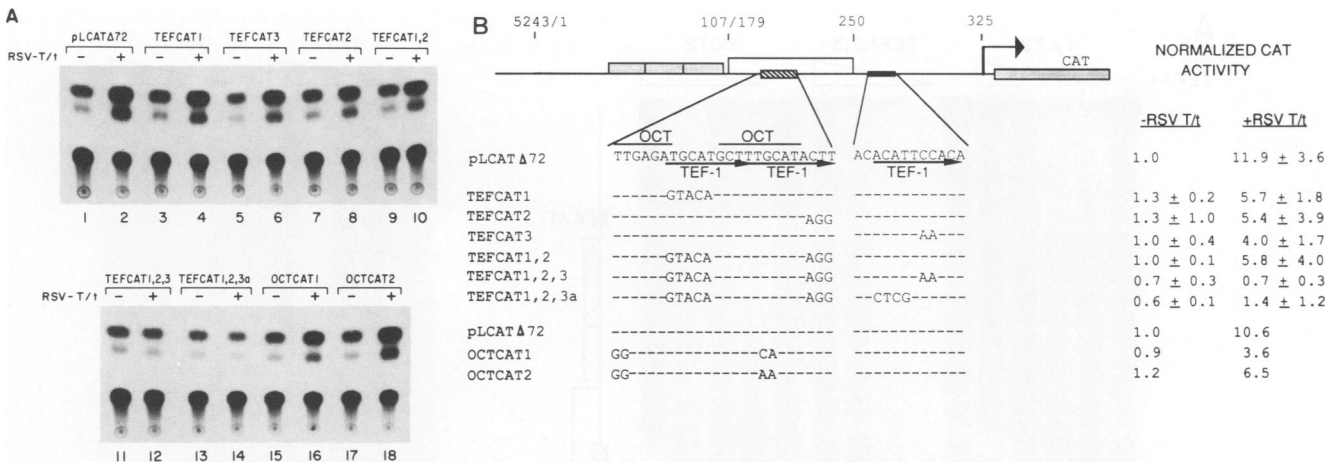


FIG. 3. *trans* activation of the SV40 late promoter mutated in either the TEF-1- or octamer-binding sites. (A) Autoradiograph of a thin-layer chromatography plate from a representative CAT assay of extracts from cells transfected with the mutant late promoters in the presence (even-numbered lanes) or absence (odd-numbered lanes) of T/t antigens. The reporter plasmids are indicated and diagrammed in panel B. (B) Summary of CAT assay results. A schematic diagram of the SV40 late promoter in pLCATΔ72 is shown. The features shown are the single 72-bp repeat (open box), the three 21-bp repeats (shaded boxes), the Sph I + II motifs (hatched box), the GTIIC motif (filled box), and the major late start site (arrow). The nucleotide positions are indicated above the diagram. The sequences encompassing the Sph I + II motifs and the GTIIC motif in the wild-type promoter are shown. Arrows under the sequence represent TEF-1-binding sites, and lines over the sequence represent octamer-binding sites. Below, the mutations in the TEF-1- or octamer-binding sites are shown. The relative level of expression from each of these mutants is shown on the right. The values for CAT activity were normalized for each experiment to the level of CAT activity resulting from pLCATΔ72 in the absence of T/t antigens, so that different experiments could be compared directly. CAT expression from transfection of pLCATΔ72 resulted in 0.3 to 5.0% acetylation of the input chloramphenicol in different experiments. The results for the TEF-1-binding site mutations are the average of four experiments and are presented with standard deviations. The results with the octamer-binding site mutations are the average of two experiments.

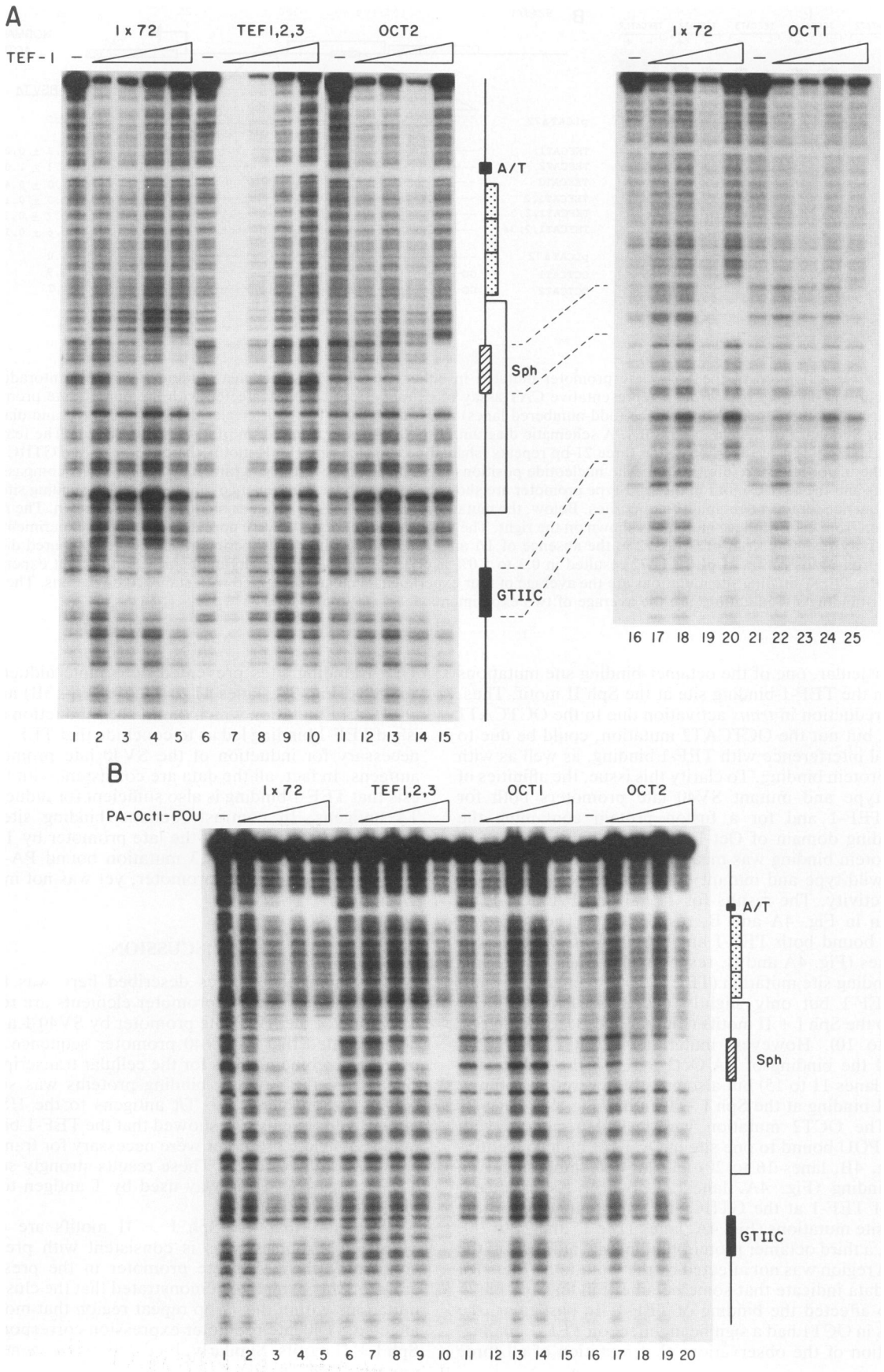
lap. In particular, one of the octamer-binding site mutations lies within the TEF-1-binding site at the Sph II motif. Thus, the large reduction in *trans* activation due to the OCTCAT1 mutation, but not the OCTCAT2 mutation, could be due to differential interference with TEF-1 binding, as well as with octamer protein binding. To clarify this issue, the affinities of the wild-type and mutant SV40 late promoters both for purified TEF-1 and for a fusion protein containing the DNA-binding domain of Oct-1, PA-Oct1-POU, were compared. Protein binding was measured by DNase I footprinting with wild-type and mutant DNA fragments of identical specific activity. The results for TEF-1 and PA-Oct1-POU are shown in Fig. 4A and B, respectively. The wild-type promoter bound both TEF-1 and PA-Oct1-POU at the expected sites (Fig. 4A and B, respectively, lanes 5). A triple TEF-1-binding site mutation (TEF1,2,3) prevented the binding of TEF-1 but only slightly decreased PA-Oct1-POU binding to the Sph I + II motifs (Fig. 4A and B, respectively, lanes 6 to 10). However, mutations in OCT1 not only prevented the binding of PA-Oct1-POU to the Sph region (Fig. 4B, lanes 11 to 15) but also greatly lowered the affinity for TEF-1 binding at the Sph I + II motif (Fig. 4A, lanes 21 to 25). The OCT2 mutation was less detrimental, with PA-Oct1-POU bound to one site in OCT2 at high concentrations (Fig. 4B, lanes 16 to 20) and no detectable effect on TEF-1 binding (Fig. 4A, lanes 11 to 15). As expected, binding of TEF-1 at the GTIIC site was unaffected by the octamer-site mutations (Fig. 4A, lanes 11 to 15 and 21 to 25). Similarly, a third octamer protein-binding site adjacent to the A+T-rich region was not affected by the mutations (Fig. 4B).

These data indicate that some octamer-binding site mutations also affected the binding of TEF-1. In particular, the mutations in OCT1 had a significant effect on TEF-1 binding. Combination of the observations that mutation of all three TEF-1-binding sites prevented detectable induction by T/t antigens (Fig. 3A, lanes 11 to 14, and Fig. 3B) and that the OCTCAT1 mutation which diminished induction also diminished TEF-1 binding led us to conclude that TEF-1 binding is necessary for induction of the SV40 late promoter by T/t antigens. In fact, all the data are consistent with the hypothesis that TEF-1 binding is also sufficient for induction by the T/t antigens. In contrast, octamer-binding sites are not sufficient for activation of the late promoter by T/t antigens because the TEFCAT1,2,3 mutation bound PA-Oct1-POU as well as the wild-type promoter, yet was not inducible by T/t antigen.

DISCUSSION

The aim of the studies described here was to test the hypothesis that specific promoter elements are required for activation of the SV40 late promoter by SV40 T antigen. We demonstrated that an SV40 promoter sequence containing overlapping binding sites for the cellular transcription factor TEF-1 and the octamer binding proteins was sufficient to confer responsiveness to T/t antigens to the HSV *tk* promoter. Additionally, we showed that the TEF-1-binding sites in the SV40 late promoter were necessary for transcriptional activation by T antigen. These results strongly suggest that TEF-1 is part of a pathway used by T antigen to stimulate viral late transcription.

Our result that the Sph I + II motifs are crucial for T-antigen responsiveness is consistent with previous data concerning the SV40 late promoter in the presence of T antigen. May et al. (29) demonstrated that the clustered point mutations within the 72-bp repeat region that most strongly affected SV40 late promoter expression corresponded to the Sph I + II motifs. Similarly, by deletion mutagenesis, Keller


A
1 x 72
TEF1,2,3
OCT2
TEF-1
1 x 72
OCT1
A/T
Sph
GTIIC
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
16 17 18 19 20 21 22 23 24 25
B
1 x 72
TEF1,2,3
OCT1
OCT2
PA-Oct1-POU
A/T
Sph
GTIIC
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

and Alwine (23) identified a 46-bp element within the 72-bp repeats (τ) that is important for late promoter activity. τ includes an Sph motif plus other enhancer DNA sequences. Since both sets of experiments examined late promoter activity in the presence of T antigen rather than induction from a basal level by T antigen, it remained unclear whether these sequences were important for *trans* activation by T antigen. Subsequently, a 20- to 28-kDa protein has been partially purified that binds to τ and is differentially modified in COS cells and infected CV-1 cells when compared with uninfected CV-1 cells (12, 13). The binding site of the protein roughly spans nt 190 to 220, containing two octamer-binding sites and one TEF-1-binding site. It is not known whether modification of this protein affects the level of transcription from SV40 late promoter. We believe that the effect of TEF-1-binding sites on the *trans* activation of the late promoter that we observe is not due to the τ-binding protein. The τ-binding protein protects sequences containing only one of the three TEF-1-binding sites mutated in our experiments, whereas *trans* activation by T antigen was partially dependent on all three sites.

Although Sph and GTIIC motifs are necessary for stimulation of the SV40 late promoter, our experiments do not address whether sequences other than these two motifs might also be necessary. The initial screen for T-antigen-responsive promoter elements with the HSV *tk* promoter may have overlooked some important promoter elements because the assay is biased toward DNA sequences that have inherent promoter or enhancer activity (e.g., Sph motif [10, 37, 45]) rather than sequences that depend on complementing sequences for activity (e.g., GT-1 [10]). Elements such as the GT-1 motif might not be sufficient to confer responsiveness to T antigen even if they were necessary for T-antigen activation in the context of the SV40 late promoter. Other elements may have been overlooked if the spacing between protein-binding sites in the multimerized oligonucleotide constructs was not optimal.

Roles of large T and small t antigens in activation of the SV40 late promoter. In all the experiments described above, both large T and small t antigens were expressed simultaneously. Large T antigen is necessary for activation of the late promoter, but the role of small t antigen is not clear. Small t antigen alone is sufficient to activate the adenovirus E2 promoter, but under identical conditions, no effect is observed on the SV40 late promoter (27). However, a helper role for small t antigen in *trans* activation by large T antigen remains possible.

Comparison of role of TEF-1-binding sites in early promoter and late promoter activity. As part of the SV40 enhancer, both the GTIIC and Sph motifs are important elements of the SV40 early promoter in the absence of T antigen (51), and each element can act as an enhancer when multimers are placed 5′ of the rabbit β-globin transcription initiation site (10, 37, 45). In contrast, none of these elements significantly affected the basal activity of the late promoter in our experiments. However, all three elements are required for maximum activation by T antigen, demonstrating that the basal and T-antigen-inducible elements of the late promoter are separable. These results are surprising because the SV40 enhancer is active in both orientations when joined to the early promoter (51), indicating that the enhancer is capable of being active in the late orientation. This implies that transcriptionally competent factors bound to the enhancer have no detectable effect on the late promoter in the absence of T antigen.

Models of the mechanism of *trans* activation. Some simple models can be proposed that are consistent with all the data. First, TEF-1, or another cellular transcription factor that binds to the same sites, could be induced or modified as a result of T-antigen expression, leading to an increase in late promoter activity. Second, TEF-1 itself may not be affected by T antigen, but another protein that interacts with TEF-1 may be induced or modified. Finally, if late transcription was ordinarily repressed, expression of T antigen could lead to release of the repression. Each of these models is discussed in more detail below.

Induction of the transcription factor AP-1 by 12-*O*-tetradecanoylphorbol-13-acetate or serum, or posttranslational modification of the transcription factor cyclic AMP-responsive element binding protein in response to cyclic AMP, stimulates transcription from promoters containing binding sites for these proteins (2, 16, 26). In the same way, induction of late-gene expression by T antigen may be the result of induction or modification of TEF-1. We performed gel band mobility shift assays to look for differences in TEF-1 binding between cells that express T antigen and those that do not. Binding to both the Sph and GTIIC motifs was examined by using whole-cell extracts from CV-1 cells, SV40-infected CV-1 cells, or CV-1-derived cells that constitutively express T antigen. No obvious differences between the extracts were observed in the amount or mobility of protein-DNA complexes formed with either DNA using the different extracts, including those complexes comigrating with that of purified HeLa TEF-1 (unpublished observations). However, lack of alteration in the mobility of the protein-DNA complexes does not rule out the possibility of posttranslational modification.

The requirement for TEF-1-binding sites in T-antigen *trans* activation does not prove that the target of T antigen is a protein binding to TEF-1-binding sites. Instead, proteins that interact with TEF-1 could be induced or modified by T antigen. The dependence on TEF-1-binding sites could result from TEF-1 and the second protein both being essential for *trans* activation. Although no proteins that interact with TEF-1 have been identified, there is a precedent for this sort of mechanism. Analogous to the results we obtained by mutating the TEF-1-binding sites of the SV40 late promoter, mutations in Oct-1-binding sites in the HSV immediate-early

FIG. 4. DNase I footprinting of TEF-1 and an octamer binding protein on wild-type and mutated SV40 late promoter sequences. DNA fragments derived from the promoters of pLCATΔ72, TEFCAT1,2,3, OCTCAT1, and OCTCAT2 were labeled with ^{32}P at nt 341 and incubated with either purified HeLa TEF-1 (A) or *E. coli*-produced PA-Oct1-POU (B) and then digested with DNase I. Diagrams next to the footprints indicate the positions of the Sph I + II motifs (hatched box), the GTIIC motifs (larger solid box), the 72-bp repeat (open box), and the 21-bp repeats (shaded boxes), based on G sequencing ladders electrophoresed on the gels. The promoter used to prepare each fragment is indicated over each set of lanes (see Fig. 3B for sequences). Left panel of A: no added TEF-1 (lanes 1, 6, and 11) or 0.5 μl (lanes 2, 7, and 12), 1.0 μl (lanes 3, 8, and 13), 2.0 μl (lanes 4, 9, and 14), or 4.0 μl (lanes 5, 10, and 15) of TEF-1. Right panel of A: no TEF-1 (lanes 16 and 21) or 0.15 μl (lanes 17 and 22), 0.44 μl (lanes 18 and 23), 1.3 μl (lanes 19 and 24), or 4.0 μl (lanes 20, 25) of TEF-1. (B) No added PA-Oct1-POU (lanes 1, 6, 11, and 16) or 0.011 μl (lanes 2, 7, 12, and 17), 0.033 μl (lanes 3, 8, 13, and 18), 0.10 μl (lanes 4, 9, 14, and 19), or 0.30 μl (lanes 5, 10, 15, and 20) of PA-Oct1-POU.

genes prevent activation of transcription by the HSV protein VP16 (7, 11, 24). VP16 is a strong activator of transcription, but localization to a promoter requires interaction with Oct-1 (14, 30, 35, 36, 39). T antigen might induce a protein that interacts with TEF-1 in the same manner that VP16 interacts with Oct-1.

The final model invokes release of the late promoter from repression. In this case, active TEF-1 might always be bound to the promoter, but a repressor would prevent it from activating transcription from the SV40 late promoter. Expression of T antigen would displace the repressor and allow TEF-1 to stimulate transcription. If this repressor does exist, it probably does not bind TEF-1-binding sites since mutation of these sites does not cause an increase in the basal level of late transcription.

The mechanism by which T antigen stimulates the SV40 late promoter most likely involves utilization of cellular factors and pathways that are used in normal cellular processes. Understanding of this mechanism should provide significant information about how the activity of RNA polymerase II transcription factors are regulated.

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