MOLECULAR AND CELLULAR BIOLOGY, Feb. 1993, p. 961–969
0270-7306/93/020961-09$02.00/0

Vol. 13, No. 2

Transcriptional Activation by Simian Virus 40 Large T Antigen: Interactions with Multiple Components of the Transcription Complex

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Received 1 September 1992/Returned for modification 27 October 1992/Accepted 12 November 1992

Simian virus 40 (SV40) large T antigen is a potent transcriptional activator of both viral and cellular promoters. Within the SV40 late promoter, a specific upstream element necessary for T-antigen transcriptional activation is the binding site for transcription-enhancing factor 1 (TEF-1). The promoter structure necessary for T-antigen-mediated transcriptional activation appears to be simple. For example, a promoter consisting of upstream TEF-1 binding sites (or other factor-binding sites) and a downstream TATA or initiator element is efficiently activated. It has been demonstrated that transcriptional activation by T antigen does not require direct binding to the DNA; thus, the most direct effect that T antigen could have on these simple promoters would be through protein-protein interactions with either upstream-bound transcription factors, the basal transcription complex, or both. To determine whether such interactions occur, full-length T antigen or segments of it was fused to the glutathione-binding site (GST fusions) or to the Gal4 DNA-binding domain (amino acids 1 to 147) (Gal4 fusions). With the GST fusions, it was found that TEF-1 and the TATA-binding protein (TBP) bound different regions of T antigen. A GST fusion containing amino acids 5 to 172 (region T1) efficiently bound TBP. TEF-1 bound neither region T1 nor a region between amino acids 168 and 373 (region T2); however, it bound efficiently to the combined region (T5) containing amino acids 5 to 383. The Gal4 fusions demonstrated that no region of T antigen could activate a promoter containing Gal4-binding sites, suggesting that T antigen does not contain an activation domain of the type defined by this assay. However, the Gal4 fusion proteins maintained their ability to activate promoters known to be activated by wild-type T antigen. The fusion with region T1, which binds only TBP, modestly activated the SV40 late promoter and the simple TEF-1/TATA promoter. Region T5, which binds both TBP and TEF-1, activated each of these promoters to levels equivalent to that of wild-type T antigen. The correlation between the binding of both TEF-1 and TBP and the ability to mediate wild-type levels of transcriptional activation suggests that T antigen causes activation through direct interactions with multiple factors in the transcription complex.

The simian virus 40 (SV40) early-gene product large T antigen is a potent viral oncoprotein that interacts with a number of cellular proteins known to affect cell growth and gene expression. These interactions may account for the wide variety of functions attributed to T antigen (reviewed in references 14, 28, and 36). One of these functions is the transcriptional activation of both viral and cellular promoters, which was first detected through studies of activation of the SV40 late promoter (Fig. 1A) (7, 24). Subsequently, large T antigen was shown to be a promiscuous transcriptional activator because of its effect on many viral and cellular promoters (1, 17, 31, 42, 52). This activation of cellular promoters may be as significant for the viral life cycle as the activation of the late promoter, since it prepares the cell to complete the viral cycle in permissive infections and may contribute to progression toward transformation in nonpermissive infections.

Within the SV40 late promoter region are binding sites for transcription-enhancing factor 1 (TEF-1 [13]). We and others have shown these sites to be specific upstream elements necessary for T-antigen transcriptional activation of the late promoter (8, 20, 39). We have previously shown that T antigen can activate a relatively simple promoter consisting of repeated TEF-1 binding sites upstream of the β-globin TATA element (20). Such a simple structure may be a common feature of promoters which are transcriptionally activated by T antigen. Promoters consisting of a single upstream transcription factor-binding site, which can be variable in nature, and a downstream TATA or initiator element (TATA-less promoter) can be efficiently activated by T antigen (10a, 17).

Transcriptional activation mediated by T antigen does not require direct binding of the protein to DNA (3, 15, 16, 25, 52). Hence, the most direct effect that T antigen could have would involve protein-protein interactions with transcription factors, the basal transcription complex, or both. In this communication, we present evidence that T antigen can interact both with the TATA-binding factor (TBP) and with TEF-1 but does not bind OCT 1, a factor which T antigen does not utilize for transcriptional activation (20). We find that different regions of T antigen are required for binding TBP and TEF-1. When these two regions are contiguous, their activity is equivalent to that of wild-type (WT) T

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qantigen in transcriptional activation of the SV40 late promoter and a simple promoter containing TEF-1 elements and a TATA box. These data, together with the overall simplicity of promoter structure required for activation by T antigen, suggest that T antigen mediates transcriptional activation by interacting with both upstream-binding factors and components of the basal transcription apparatus. These multiple interactions may stabilize the transcription complex or function in a manner similar to an adaptor or coactivator (4, 5, 37).

MATERIALS AND METHODS

Plasmids. The construction and isolation of all plasmids were done by standard procedures (2). pβGT-TATA (GT-TATA promoter) was constructed by ligation of double-stranded oligonucleotides corresponding to a dimer of SV40 nucleotides 261 to 270 (containing the GTIIc site shown in Fig. 1) with *Bam*HI and *Bgl*II complementary ends and cloned into the *Bam*HI site of pGEM7zf+. A plasmid containing six copies of SV40 nucleotide, 261 to 270, p7zGT17, was digested with *Sac*I and *Xba*I, and the resulting GTIIc site oligomer was ligated into *Sac*I- and *Xba*I-digested p^{β}6xB20 (46), creating pβGT-TATA. pLS102n-CAT, containing the chloramphenicol acetyltransferase (CAT) gene flanked by the SV40 late promoter and polyadenylation site, has been described previously (10). pRSV-Tex contains the large T antigen cDNA under the transcriptional control of the Rous sarcoma virus long terminal repeat (30). pRSV3-BglII, the control plasmid for pRSV-Tex, was generated by removing the T antigen cDNA sequences from pRSV-Tex by cleavage with *Bgl*II and religation of the vector fragment. pCMV contains the cytomegalovirus immediate-early promoter and a *Bam*HI site for cloning of cDNAs for expression; pCMV-T was generated by ligating the T antigen cDNA from *Bam*HI-digested pAC373T2 (35) into *Bam*HI-digested pCMV. pGal4$_5$TK-CAT and pGal4-E1a have both been described previously (32).

Cell culture and transfections. The African green monkey kidney cell line CV-1 was propagated and maintained in Dulbecco's minimal essential medium supplemented with 5% fetal bovine serum at 37°C in 5% CO_2. Cells, less than 9 passages from the time that they were thawed, were plated at 3×10^5 to 5×10^5 cells per 100-mm plate and grown overnight. Monolayers at approximately 80% confluence were transfected with appropriate plasmids by the calcium phosphate precipitation procedure with 12 μg of DNA, 125 mM $CaCl_2$, 25 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 140 mM NaCl, and 0.75 mM Na_2HPO_4 (pH 6.95) (9). At 40 h after transfection, the cells were harvested for RNA or CAT analysis as described below.

RNA preparation and analysis. Total cellular RNA was prepared by the guanidinium thiocyanate method (51). Briefly, cell monolayers were washed three times with phosphate-buffered saline. The cells were lysed by being scraped into 700 μl of guanidine thiocyanate solution (2 M guanidinium thiocyanate, 12 mM sodium citrate, 50% [vol/vol] phenol, 0.2 M sodium acetate, 0.72% β-mercaptoethanol) and then extracted with 500 μl of chloroform. The nucleic acids were precipitated with an equal volume of isopropanol and treated with RNase-free DNase (Boehringer Mannheim). RNase protection assays were performed as described previously (2). β-Globin antisense RNA was prepared by transcription from linearized pSP6β350 (46) with Sp6 RNA polymerase. Properly initiated β-globin RNAs protected a probe fragment of 350 nucleotides (45). Results were quantitated with a Molecular Dynamics Phosphorimager (see also Transfection Standardization, below).

CAT analysis. Cells were harvested as described previously (24), and the cells were lysed by three freeze-thaw cycles. Cell debris was removed by centrifugation, and the CAT enzyme activity was determined on equivalent amounts of extract protein as described previously (19). Results were quantitated with a Molecular Dynamics Phosphorimager.

Transfection standardization. Many transfection studies are internally standardized for transfection variations through the use of a second reporter gene on an independent promoter. For transcriptional activation analyses with T antigen and other viral activators, this is a tenuous protocol because T antigen exerts some level of activation on virtually all promoters (1). We have found that T antigen activated all of the normal promoters used for standardization purposes. Over the years, we have attempted to find a promoter which is not activated by T antigen for use as an internal control. However, promoters which show no activation by T antigen also have such low basal activities that measurements are unreliable. Hence, these promoters are useless as internal controls. Therefore, we have used the approach of multiple repeats of experiments with different cells and different DNA preparations in order to lessen standard transfection variations. Side by side comparisons of data derived with internal standardization and data derived from multiple experiments give results with comparable standard deviations (unpublished observations). In addition, many of the causes of transfection variations can be eliminated by careful preparation of cells and samples, as described above.

The transfection data presented herein represent at least three separate experiments. All transfection experiments included a positive-control sample in which the activating effect of T antigen is determined by using the SV40 late promoter which also contains a functional origin of replication, as in the reporter plasmid pL16-CAT (25). In CV-1 cells, T antigen both activates the pL16-CAT promoter and amplifies the plasmid through replication. This produces a predictably high amount of CAT, with chloramphenicol conversion rates of 1.5%/min/10 μg of extract protein. The results of any transfection experiment in which this control produces levels of chloramphenicol conversion that are significantly different from the norm are eliminated from consideration, since this indicates general transfection anomalies. Such experiments are infrequent, accounting for no more than 1 in 20 experiments.

In the transfection experiments presented, basal CAT activity for the SV40 late promoter, in a 45-min reaction, produced 1.5% ± 0.3% conversion and gave up to 11% ± 0.5% conversion under conditions of greatest activation. The standard deviation of between 0.3 and 0.5% was maintained throughout. For the Gal4-TK promoter, the basal CAT activity in a 60-min reaction produced 1.0% ± 0.3% conversion. This general level of conversion was seen for all of the T-antigen-containing Gal4 fusions, maintaining a relatively constant 0.3% standard deviation; in the case of activation by the Gal4-E1a protein, 40% ± 0.6% conversion was noted. The use of the GT-TATA promoter, requiring RNase sensitivity analysis of product RNA, has been described above. Counts in protected bands from basal and activated samples were quantitated with a Molecular Dynamics Phosphorimager, and fold activations were determined from these data.

In vitro transcription and translation. Capped RNA encoding TEF-1 and TBP was prepared by in vitro transcription (2)

from 1 μg of linearized DNA template, *Bgl*II-digested pXJ40Tef-1A (50) and *Eco*RV-digested pETHIID (23), respectively, with T7 polymerase for 1 h at 37°C. After phenol extraction and ethanol precipitation, RNAs were resuspended in 10 μl of 10 mM Tris–1 mM EDTA (pH 8). From 3 to 5 μl was then translated in vitro with nuclease-treated rabbit reticulocyte lysate (Promega) containing 60 μCi of [^{35}S]methionine (Amersham; >1,000 Ci/mmol) for 1 h at 25°C. Samples (1 μl) were then taken, and [^{35}S]methionine incorporation was determined by trichloroacetic acid precipitation and scintillation counting.

Generation of GST and Gal4 fusion recombinants. pGEX-Tef-1 WT was generated by digesting pXJ40Tef-1 WT (50) with *Pvu*II and *Sma*I and ligating the TEF-1 cDNA fragment to *Sma*I-digested pGEX2T (Pharmacia). pGEXTef5-167 was generated by digesting pXJ40Tef-1 WT with *Pvu*II and *Bam*HI, filling in the 3′ overhang with Klenow polymerase, and ligating the TEF-1 fragment to *Sma*I-digested pGEX2T. pGEXTag WT was generated by ligating the T antigen cDNA from *Bam*HI-digested pAC373T2 (35) and ligation to *Bam*HI-digested pGEX2T. Glutathione-*S*-transferase (GST) fusions of regions T1 to T5 were generated by polymerase chain reaction of the appropriate regions of T antigen cDNA (within pRSV-Tex) with complementary primers containing *Bam*HI (5′) and *Eco*RI (3′) recognition sites which allowed in-frame insertion. After digestion with the appropriate restriction enzymes, the fragments were ligated into *Bam*HI- and *Eco*RI-digested pGEX3X (Pharmacia). Recombinants were assayed for expression of appropriately sized fusion protein. Fusions of Gal4 amino acids 1 to 147 to regions T1 to T7 (see Fig. 5) were generated similarly with complementary primers containing *Bam*HI and *Xba*I restriction sites and ligated to *Bam*HI- and *Xba*I-digested pSG424 (38). Recombinants were sequenced completely and assayed for expression of appropriately sized fusion protein.

Expression and purification of GST fusion proteins. *Escherichia coli* Y1090 (cured of pMC9) transformed with pGEX fusion expression plasmids were grown for 2 h at 37°C with shaking. Protein expression was then induced with 0.1 mM IPTG (isopropylthiogalactopyranoside) for 2 to 3 h at 37°C. Bacteria were lysed by sonication in NETN$^+$ (20 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM tosyl-lysine chloromethyl ketone), and 1-ml aliquots of bacterial supernatant were incubated for 30 min with 25 μl of preswollen glutathione-agarose (Pharmacia). In some experiments, more bacterial supernatant was used for binding to compensate for fusion proteins which were produced in lower amounts in bacteria. The beads were then washed three times with NETN$^+$. For analysis of bound proteins, beads were boiled in sodium dodecyl sulfate (SDS) sample buffer and loaded onto SDS-polyacrylamide gels. Proteins were visualized by silver staining.

Fusion protein binding assays. Glutathione-agarose beads were bound with GST fusion proteins as described above and incubated with 500 μl of NETN$^+$ containing 3% bovine serum albumin for 30 min at 4°C with rocking in order to block nonspecific protein binding to the beads. Then 2.5 × 10^5 trichloroacetic acid-precipitable counts of in vitro-translated TEF-1 or TBP was added to the beads and incubated for 1 h at 4°C with rocking. The beads were then washed five times with NETN$^+$, boiled in SDS sample buffer, and loaded onto 9% polyacrylamide–SDS gels. Proteins were visualized by fluorography (Amplify; Amersham).

Western immunoblot analysis of Gal4 fusion proteins. CV-1 cells were transfected as described above with the various Gal4-T antigen fusion expression plasmids or pCMV-T, expressing WT T antigen. Cells were harvested after 36 h and lysed by boiling in SDS sample buffer. Proteins were separated by electrophoresis on a 12% polyacrylamide–SDS gel and transferred to nitrocellulose as described previously (2). Proteins were detected by incubation with antiserum to a peptide carrying Gal4 amino acids 1 to 147 (anti-Gal4 1–147) or a mixture of monoclonal antibodies recognizing the amino-terminal end of T antigen (PAB 416 and PAB 419) and then visualized with the ECL luminescence system (Amersham). Procedures for transfer, pretreatment, visualization, and stripping of the Western blot for consecutive analyses were as described by the manufacturer.

RESULTS

SV40 T antigen activates simple promoters containing TEF-1 binding sites. In previous studies of the SV40 late promoter (Fig. 1A), we located a specific promoter region through which T antigen mediated transcriptional activation (20). In this article, that region is called the Oct/TEF element (OTE; Fig. 1A and B); it has previously been called the TABS element (16, 20). T antigen efficiently activates a promoter consisting of six repeats of the OTE placed upstream of the β-globin TATA element and the β-globin gene (OTE-TATA promoter, Fig. 1C) (20). As shown in Fig. 1B, the OTE contains overlapping binding sites for octamer factor (Oct) and for TEF-1. Through the use of specific mutations, we previously determined that it was the TEF sites which were necessary for T-antigen-mediated activation (20).

The SV40 late promoter contains a second TEF-1 binding site called the GTIIc site (13), which has a sequence distinct from that of the TEF-1 sites in the OTE (Fig. 1A and B). In studies similar to those described above, we inserted six copies of a GTIIc dimer upstream of the β-globin TATA and gene (GT-TATA promoter; Fig. 1C) and determined whether T antigen could activate through this alternative TEF-1 site, which is not complicated by an overlapping binding site for another transcription factor.

The GT-TATA promoter plasmid was transfected into CV-1 cells alone or cotransfected with pREV-Tex, a plasmid which produces large T antigen (30). Figure 2 shows the RNase protection analysis of the β-globin RNA produced. The expected protected fragment (350 nucleotides) is occasionally detected as a doublet, caused by variations in nuclease T_1 activity. The data show that T antigen caused a five- to sevenfold increase in promoter activity, demonstrating that it can activate the simple promoter containing the alternate TEF-1 site.

T antigen binds to TEF-1 and TBP. The simple promoter structure necessary for T-antigen-mediated transcriptional activation suggested that T antigen may function through direct interaction with the transcription complex. Previous studies had also suggested that T antigen may affect the binding of factors to the OTE (15, 16). To determine whether T antigen interacts directly with TEF-1 and TBP, fusion constructions were prepared (Fig. 3) that contained the glutathione binding site of GST and either (i) full-length WT large T antigen, (ii) full-length WT TEF-1, or (iii) the amino-terminal 167 amino acids of TEF-1, containing the DNA-binding domain (TEFb [50]).

Each GST fusion protein was bound to glutathione-Sepharose and then mixed with in vitro-transcribed/translated [^{35}S]methionine-labeled TEF-1 or TBP. TBP was chosen because it is a basal transcription factor which is known

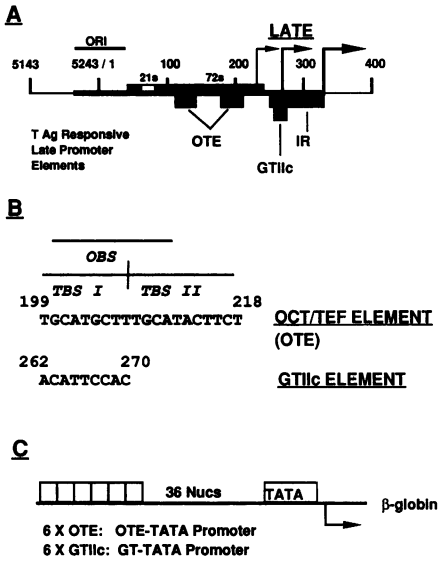

FIG. 1. (A) SV40 promoter and origin regions. SV40 late transcription initiates at numerous heterogeneous start sites, as indicated by the arrows. Much of the initiation arises from an initiator region (IR), which contains repeated elements homologous to the initiator elements defined in the promoters for the terminal deoxynucleotidyl transferase and dihydrofolate reductase genes (33, 41). Upstream elements which have been shown to be necessary for activation of the late promoter by T antigen (T Ag) include the OTE (sequence shown in panel B). The location of this element in relationship to the characterized elements of the SV40 early promoter, the 21- and 72-bp repeats (21s and 72s), is shown. An alternate TEF-1 binding site is contained in the GTIIc element (sequence shown in panel B). In the context of simple promoters considered in this article, the late promoter can be considered a simple promoter containing upstream binding sites for TEF-1 (OTE) and downstream sites for TBP and basal transcription complex interaction at the IR. A simple promoter consisting of only the OTE and the IR is activated by T antigen (17). (B) Sequences of the OTE and GTIIc elements. The OTE is composed of two adjacent TEF-1 binding sites (TBS I and II) overlapped by an octamer-binding site (OBS). In previous studies, we and others have shown that it is the TEF-1 sites which are necessary for transcriptional activation by T antigen (8, 20, 39). The GTIIc element has been shown to be an alternative TEF-1 binding site which has a sequence quite different from the sites in the OTE (13). (C) Simple promoter structure used to examine T-antigen transcriptional activation. Six repeats of the OTE or six repeats of a GTIIc dimer were placed upstream of the β-globin TATA element and the β-globin gene (OTE-TATA and GT-TATA promoters, respectively). The β-globin RNA produced after transfection was analyzed by RNase protection (Fig. 2). Nucs, nucleotides.

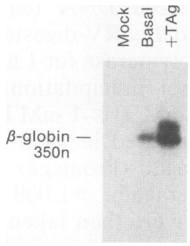

FIG. 2. RNase protection analysis of β-globin RNA produced by the GT-TATA promoter. The simple promoter GT-TATA (Fig. 1C) was transfected into monkey CV-1 cells in the presence of a control plasmid (Basal; pRSV3BglII) or pRSV-TEX, which expresses WT large T antigen (+TAg). RNA from transfected cells was harvested and assayed as described in the text. Size is shown in nucleotides.

to be bound by other viral *trans*-acting proteins, such as adenovirus E1a and herpes simplex virus VP16 (22, 27, 44). After extensive washing, the bound proteins were eluted and analyzed on a 9% polyacrylamide–SDS gel. Full-length bound TEF-1 and TBP are indicated in Fig. 4A; the smaller bands are specific to the RNA used to program the reticulocyte lysate and are most likely due to premature termination of translation in vitro. Figure 4A shows that TEF-1 binds to GST T antigen (T AG WT). In addition, TEF-1 can bind to GST fusions with both TEF-1 (TEF WT) and the amino terminus of TEF-1 (TEF 5-167). This latter observation, suggesting TEF-1 oligomerization, has been implied by cooperative binding to tandem TEF-1 motifs (13, 50).

Analysis of binding to in vitro-transcribed/translated TBP also shows that T antigen, TEF, and the amino-terminal portion of TEF bind TBP better than the glutathione-binding site alone, which had very little affinity for either TEF-1 or TBP.

As a control, we have also determined that GST-T antigen does not bind to in vitro-transcribed/translated Oct 1 (Fig. 4B). This factor, whose binding site overlaps the TEF-1 sites in the OTE (Fig. 1), is not utilized by T antigen for transcriptional activation (20). Oct 1 and TEF do interact, a finding which may relate to the positive and negative control functions attributed to the OTE in the SV40 late promoter (20). With other controls, we have also determined that the binding events noted in Fig. 4A and B are stable in medium containing at least 200 μg of ethidium bromide per ml (not shown), indicating that they are true protein-protein interactions and are not due to spurious interactions with contaminating DNA (26).

GST and Gal4 fusions with distinct regions of T antigen. The data in Fig. 4 suggest that both TBP and TEF-1 bind less efficiently to GST-T antigen than to the GST-TEF proteins. However, synthesis of the GST-T antigen in *E. coli* is low because of instability and low solubility in the bacteria (see discussion of Fig. 6, below). Hence, the apparent lower affinity of TEF-1 and TBP may reflect the low production of GST-T antigen. In order to produce more GST fusion protein and to better define the regions of T antigen to which TEF-1 and TBP bind, we constructed GST fusions with regions of T antigen (Fig. 5). The coding regions were prepared by polymerase chain reaction from a T antigen cDNA with primers with in-frame restriction sites for insertion into the GST vector pGEX3 (Pharmacia) or the Gal4 (amino acids 1 to 147) fusion vector pSG424 (38). Regions T1 through T5

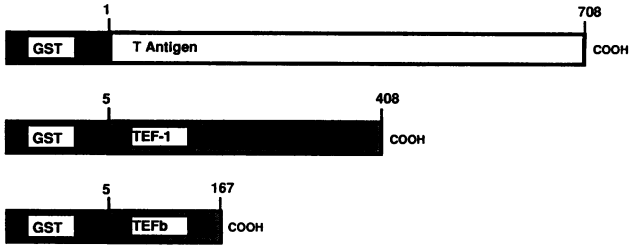

FIG. 3. Structure of fusion proteins made between the glutathione-binding domain of GST and T antigen, TEF-1, and amino acids 5 to 167 of TEF-1, which contain the DNA-binding domain (TEFb [50]).

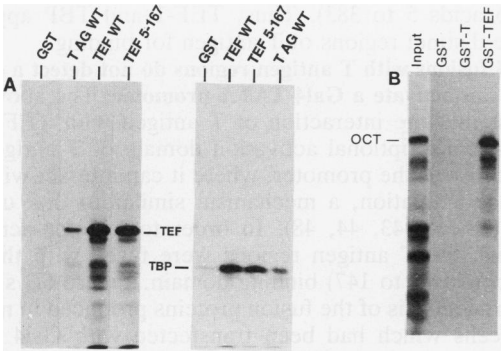

FIG. 4. Binding of in vitro-transcribed/translated TEF-1, TBP, and Oct 1 to GST fusion proteins. (A) In vitro-transcribed/translated [^{35}S]methionine-labeled TEF-1 (left) and TBP (right) were incubated with glutathione-Sepharose beads loaded with the GST binding domain (GST), GST-T antigen (T AG WT), GST-TEF-1 (TEF WT) or GST-TEFb (TEF 5-167). (B) In vitro-transcribed/translated [^{35}S]methionine-labeled Oct 1 was incubated with glutathione-Sepharose beads loaded with the GST binding domain (GST), GST-T antigen (GST-T), or GST-TEF-1 (GST-TEF). The input lane was loaded with one-fifth the amount of ^{35}S-lal led protein used in the binding reactions. Bound protein was eluted and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described in the text.

were fused to the GST binding site; regions T1 through T7 were fused to the Gal4 binding site for use in the transcriptional activation studies discussed below.

Figure 6A shows a silver-stained gel of the various fusion proteins eluted from glutathione-Sepharose by boiling in SDS. In each lane, the position of the full-length fusion protein is indicated by an arrowhead. All of the T antigen fusion proteins suffered some breakdown in *E. coli* despite the addition of several protease inhibitors during isolation (see Materials and Methods). As mentioned above, fusion with WT T antigen produces the least full-length protein; however, the data in Fig. 4 suggest that this is enough to detect binding under our conditions. Full-length forms of fusion proteins with regions T1, T3, T4, and T5 are produced in significantly greater quantities than the fusion with WT T antigen. The T2 fusion protein gives consistently lower production of the full-length form, although its production is often greater than that of the sample shown in Fig. 6A. However, as indicated in Fig. 6A, the amount of full-length

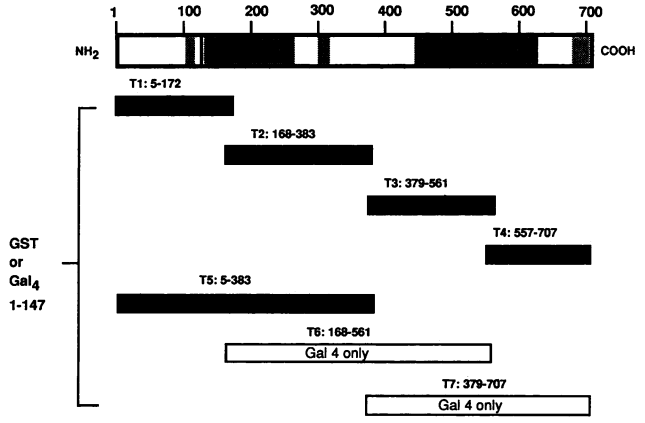

FIG. 5. Regions of T antigen fused to the glutathione-binding domain of GST or to the DNA-binding domain and nuclear localization signal contained in amino acids 1 to 147 of Gal4 (Gal4). The shaded regions T1 to T5 were fused to GST. All of the regions T1 through T7 were fused to Gal4 amino acids 1 to 147. The T antigen regions were produced by polymerase chain reaction with primers with in-frame restriction sites for ligation into the fusion protein expression vectors (see text). The numbers next to the name of each region are the included amino acids. The shaded bands on the full-length T antigen represent, from left to right, the retinoblastoma protein binding domain (amino acids 102 to 115), the DNA-binding domain (amino acids 131 to 259), the putative zinc finger (amino acids 302 to 320), the ATPase domain (amino acids 418 to 627), and the host range region (amino acids 682 to 708); the black bar indicates the nuclear localization signal (14, 28, 36).

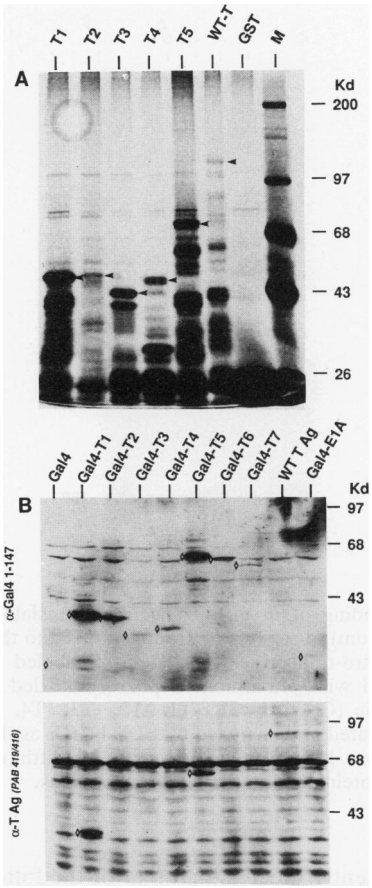

FIG. 6. (A) Silver-stained SDS-PAGE gel of GST fusion proteins eluted from glutathione-Sepharose. GST-T1, -T2, -T3, -T4, and -T5 (Fig. 5), GST-WT T (Fig. 3), and the GST-binding domain alone (GST) were induced in *E. coli*, harvested, and analyzed as described in the text. Arrowheads indicate the positions of each protein. Lane M, size markers. (B) Western analysis of Gal4 fusion proteins. Plasmids encoding the Gal4 DNA-binding domain alone (Gal4), WT T antigen (WT T Ag; not fused to anything), and Gal4 fusion proteins Gal4-T1, -T2, -T3, -T4, -T5, -T6, -T7, and -E1A were transfected into monkey CV-1 cells and harvested after 36 h. Lysates were fractionated by SDS-PAGE and analyzed by Western analysis. In the top panel, the probe was anti-Gal4 1–147 (gift of I. Sadowski), which will detect proteins from all of the expressing plasmids except WT T antigen. In the bottom panel, the same Western blot was stripped and reprobed with a mixture of anti-T-antigen (α-T Ag) monoclonal antibodies PAB 416 and PAB 419, which detect epitopes in the amino-terminal region of T antigen. These epitopes are contained in Gal4-T1, Gal4-T5, and WT T antigen. Open diamonds indicate the positions of each protein.

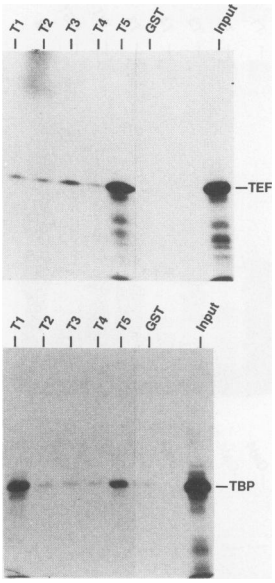

FIG. 7. Binding of in vitro-transcribed/translated TEF-1 (top) and TBP (bottom) to regions of T antigen fused to the GST binding domain. In vitro-transcribed/translated ^{35}S-labeled TEF-1 or TBP was incubated with glutathione-Sepharose loaded with the GST binding domain (GST) or GST-T1, -T2, -T3, -T4, or -T5. Bound protein was eluted and analyzed by SDS-PAGE as described in the text. The input lane was loaded with one-fifth the amount of ^{35}S-labeled protein used in the binding reactions.

T2 is consistently greater than that of the full-length WT T antigen fusion protein and should be sufficient for detection of binding. As a further safeguard, the region of T antigen represented by T2 is also included in T5, which is well expressed as a fusion protein (Fig. 5 and 6A).

TBP and TEF bind to distinct regions of T antigen. The GST fusions with various T antigen regions were bound to glutathione-Sepharose, incubated with in vitro-transcribed/translated [^{35}S]methionine-labeled TEF-1 and TBP, and then analyzed as described above. Figure 7 (top) shows that TEF-1 binds very well to region T5 (which includes regions T1 and T2). In a few experiments, we also detected a small amount of TEF-1 binding to T1, indicating a weak affinity (not shown). However, the larger region, T5, consistently provides stable binding. Hence, the binding domain may be close to or crossing the junction between T1 and T2. In contrast, TBP binds very well to T1 and also to T5, which contains the T1 region.

To eliminate the possibility that the binding we saw with T1 and T5 is nonspecific, caused by overloading the beads with GST fusion protein, we repeated the experiments with up to five times less input protein and found that relative binding levels were maintained (data not shown). In addition, with T2, for which less fusion protein is produced in bacteria (see Fig. 6A), the addition of increased amounts of GST-T2-containing bacterial supernatant to glutathione-Sepharose did not result in detectable binding between T2 and either TEF or TBP (data not shown).

Overall, the above data indicate that TBP and TEF do not occupy identical sites on T antigen. The TBP binding domain is completely within the T1 region, consisting of amino acids 5 to 172, and in fact, the first 82 amino acids appear to be sufficient (see Discussion). However, TEF-1 binding requires additional C-terminal amino acids, as in region T5 (amino acids 5 to 383). Thus, TEF-1 and TBP appear to require distinct regions of T antigen for binding.

Gal4 fusions with T antigen regions do not detect a domain which can activate a Gal4-TATA promoter. The above data suggest that the interaction of T antigen with TEF-1 may bring a transcriptional activation domain of T antigen into the vicinity of the promoter, where it can interact with TBP to cause activation, a mechanism similar to that used by VP16 (18, 38, 43, 44, 48). In order to test for activation domains, the T antigen regions were fused with the Gal4 (amino acids 1 to 147) binding domain. Figure 6B shows a Western analysis of the fusion proteins produced in monkey CV-1 cells which had been transfected with Gal4 fusion-expressing plasmids. The probe was rabbit anti-Gal4 1–147 (top panel) or a mixture of anti-T-antigen monoclonal antibodies (PAB 419 and PAB 416; bottom panel), which recognize epitopes on the amino terminus of T antigen.

The Western blot with anti-Gal4 1–147 shows that the fusion proteins are made in various amounts in CV-1 cells. Gal4-T3 and Gal4-T7 are produced in the lowest amounts; however, each is produced in quantities greater than that of Gal4-E1A, the positive control, which has been shown to activate a promoter containing a TATA element and upstream Gal4 binding sites (32). As shown in Fig. 8, the amount of Gal4-E1A produced significantly activated transcription from such a promoter; hence, we believe that the amounts of Gal4-T3 and Gal4-T7 produced are sufficient to cause activation if they contain an activation domain. Furthermore, in the lower part of Fig. 6B, the same blot was stripped and probed with anti-T-antigen monoclonal antibodies. Only T1, T5, and WT T antigen contain the epitopes recognized by these monoclonal antibodies. The amount of WT T antigen detected is lower than that of Gal4-T1 and Gal4-T5; however, it is comparable to the amounts of Gal4-T3 and Gal4-T7 if a relative comparison of band intensities between the two blots is made. Since this amount of WT T antigen is sufficient to activate the promoters tested below, we believe that the Gal4-T3 and Gal4-T7 proteins produced provide sufficient amounts to detect transcriptional activation domains if such domains are present.

Plasmids expressing the fusion proteins were transfected into CV-1 cells along with pGal4$_5$TK-CAT (40), a reporter plasmid containing five copies of the Gal4 binding site upstream of the TATA element from the herpes simplex virus thymidine kinase (TK) gene and the CAT reporter gene. As shown in Fig. 8 (Gal4-TK promoter), WT T antigen not fused to Gal4 amino acids 1 to 147 did not activate CAT expression from this promoter, while the positive control, Gal4-E1a, caused substantial activation (40-fold), in agreement with previous reports (32). In contrast, none of the Gal4 fusions with T antigen regions T1 to T7 activated CAT expression from pGal4$_5$TK-CAT. Identical results have been noted with a similar promoter containing five Gal4 binding sites upstream of the TATA element from the adenovirus E1b gene (not shown).

Because of the lower production of Gal4-T3, Gal4-T6, and Gal4-T7, additional activation experiments were done in which increasing amounts of the expressing plasmids were transfected in order to produce greater amounts of the fusion proteins; no activation was detected in these experiments either (not shown). These data indicate that T antigen does not contain an activation domain of the type identified by this assay.

T antigen regions which bind TEF-1 and TBP mediate transcriptional activation of the late promoter and the simple GT-TATA promoter. We next asked whether any of the

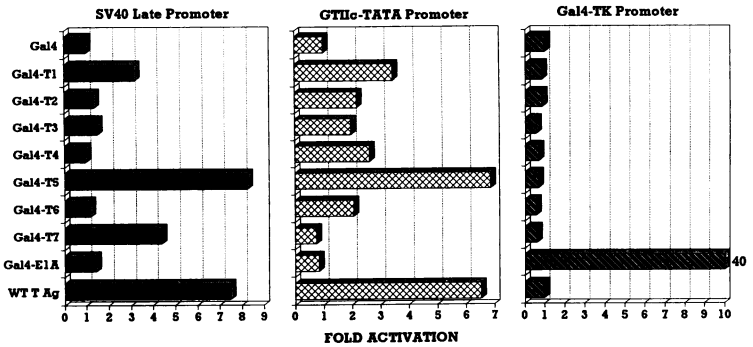

FIG. 8. Promoter activation by Gal4-T antigen fusions. Plasmids expressing either the Gal4 DNA-binding domain alone (Gal4), the Gal4-T antigen fusions (Gal4-T1 to Gal4-T7), Gal4-E1A, or WT large T antigen (WT T Ag, fused to nothing) were transfected into CV-1 cells along with reporter plasmids containing the listed promoters. The SV40 late promoter is contained in the CAT reporter plasmid pLS102n-CAT (10); quantitation of transcriptional activity was based on CAT enzyme activity. Basal percent chloramphenicol conversion was 1.5% ± 0.3% in a 45-min assay, determined from the activity of the reporter plasmid in the presence of the Gal4 binding domain. The GT-TATA promoter (Fig. 1) is contained in a β-globin RNA expression vector; quantitation of transcriptional activity was based on Phosphorimager (Molecular Dynamics) analysis of protected [^{32}P]RNA bands after nuclease protection analyses similar to those shown in Fig. 2. The Gal4-TK promoter is described in the text and is contained in a CAT reporter plasmid; hence, quantitation of transcriptional activity was based on CAT enzyme activity. Basal percent chloramphenicol conversion was 1.0% ± 0.3% in a 60-min assay, determined from the activity of the reporter plasmid in the presence of the Gal4 binding domain. All activities are shown as fold activation over the basal promoter; see Materials and Methods for specifics about transfection standardization and data analysis. The WT-T-antigen-expressing plasmid used in the GT-TATA promoter studies was pRSV-TEX; for the SV40 late promoter and Gal4-TATA promoter studies, pCMV-T was used. Each T-antigen-expressing plasmid functions similarly in activation studies.

Gal4-T antigen fusion proteins retained transcriptional activation functions on promoters known to be activated by T antigen. Under these conditions, the Gal4 binding domain, which provides the nuclear localization signal, would not be utilized for binding. When the fusion proteins were expressed in CV-1 cells cotransfected with the reporter plasmid containing the GT-TATA promoter (Fig. 1C), it was found that the simple TEF-1/TATA promoter could be activated well by Gal4-T5 and moderately by Gal4-T1. Gal4-T5 activation was equivalent to that of WT T antigen (Fig. 8, GTIIc-TATA promoter).

In analogous transfections with an SV40 late promoter-CAT plasmid, pLS102n-CAT (origin defective), Gal4-T5 again activated the promoter as well as WT T antigen did, while Gal4-T1 showed modest activation (Fig. 8). In addition, Gal4-T7 caused some activation of the late promoter; this has not been studied further here but suggests that T antigen may have another transcriptional activation domain, possibly utilized by some of the many other elements of the SV40 promoter region.

For both promoters tested, T1, which binds TBP well, had only a modest activating effect, whereas T5, containing the additional ability to bind TEF-1, activated the promoters to a level equivalent to that of WT T antigen. This suggests that interaction with TBP alone may stimulate transcription modestly but it is the interaction with each protein that allows full activation.

DISCUSSION

We have presented evidence that SV40 large T antigen can interact with a major component of basal transcription complexes, TBP, as well as with an upstream-binding factor, TEF-1, which has previously been suggested to be a significant target for T-antigen-mediated transcriptional activation of the SV40 late promoter (8, 20, 39). That T antigen activates through such protein-protein interactions has been suspected because (i) it is known that direct binding of T antigen to the DNA is not necessary for transcriptional activation (3, 15, 16, 25, 52); (ii) specific factor binding appears to be altered in the presence of T antigen (15, 16); (iii) T antigen requires only a simple promoter structure for activation (17, 20, this study); and (iv) T antigen has been reported to modestly activate transcription in vitro (12, 47).

We find that the amino terminus of T antigen is sufficient to bind TBP. It has been suggested previously that this region contains a minimal transcription activation domain, within the first 85 to 120 amino acids, based on in vivo analysis of point and deletion mutants of T antigen (42, 52). Evidence (35a) suggests that TBP may have two binding regions within region T1, the first between amino acids 5 and 82, which is the region common to both small t and large T antigen, and the second between amino acids 82 and 172. A binding site within the first 82 amino acids may explain the ability of small t antigen to activate a subset of the promoters activated by large T (6, 29, 30). In addition, an interaction with TBP may explain, in part, the promiscuous activation of many promoters by T antigen. It is now established that TBP not only is a required factor for transcription of TATA-containing and TATA-less (41) class II promoters but also affects the activity of class I and III promoters (11, 49). Hence, the interaction between T antigen and TBP has the potential to affect transcription in general in an infected or transformed cell.

The interaction between T antigen and TEF-1 appears to require a different T antigen domain than is needed to bind TBP. This is indicated by the finding that TEF-1 binds very poorly or not at all to regions T1 and T2 (Fig. 5 and 7); however, when T1 and T2 are contiguous, as in region T5, they constitute an effective binding domain for TEF-1. Hence, the TEF-1 binding domain may be close to, or crossing, the junction between T1 and T2 (amino acid 172). Since the regions required for binding TBP and TEF-1 are not identical, it appears possible that T antigen can bind each protein simultaneously. The suggestion that binding to each protein is significant for activation is supported by our findings that T5 activates the GT-TATA and SV40 late promoters to a greater extent than T1 and the recent report

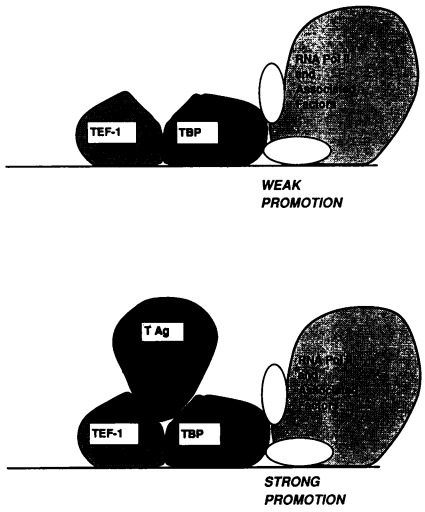

FIG. 9. Models for the interaction of T antigen (T Ag) with transcriptional complexes based on the results presented in this article. See text for discussion. Pol, polymerase.

of in vitro transcriptional activation by T antigen (12). The latter study suggested that the first 272 amino acids of T antigen activated in vitro transcription of the late promoter to the same level as the full-length protein. From our findings, we would predict that this fragment would interact with both TEF-1 and TBP.

Figure 9 suggests a model consistent with our data for the activation of a simple promoter by large T antigen. In the top diagram, the promoter containing TEF-1 and TBP binding sites allows the formation of a TEF-1/TBP transcription complex, which weakly promotes transcription. The interaction between TEF-1 and TBP, shown in the model, is suggested by the binding data in Fig. 4. Additional factor interactions with a cellular adaptor or coactivator may occur. For example, it has been suggested that TEF-1 utilizes a coactivator, the TEF-interacting factor, TIF (50). As shown in the bottom diagram, our data suggest that T antigen interacts with both components of the transcription complex. The simplest working model is that these multiple interactions stabilize the complex, allowing stronger promotion. Alternatively, these multiple interactions may allow T antigen to function in a manner similar to a coactivator or adaptor (4, 5, 37) or to prevent a repressor from interacting with the transcription complex (40).

That T antigen may function through stabilization is also suggested by its lack of an activation domain, as found by using Gal4 (amino acids 1 to 147) fusions with the Gal4-TATA promoter. Hence, T antigen's activation mechanism appears to differ from that of herpes simplex virus VP16, which has a strong acidic activation domain that becomes functional when it is brought to the DNA by the Gal4 binding domain (38) or by interaction with DNA-bound factors (18, 43, 48).

Although the Gal4 fusion proteins fail to activate Gal4-TATA promoters, they do maintain their ability to activate the late promoter and the GT-TATA promoter (Fig. 1). These data confirm that the regions of T antigen needed to bind TEF-1 and TBP correlate with the regions of T antigen needed for transcriptional activation. Specifically, the data in Fig. 8 show that the TBP binding region T1 only modestly activates the GT-TATA promoter and the SV40 late promoter, whereas the region binding both TBP and TEF-1, T5, is significantly better at activation. These findings agree with the model in which multiple contacts between T antigen and the transcription complex can increase promotion, presumably through greater stabilization. Hence, T antigen may be able to affect some promoters through an interaction with TBP; however, additional interactions with upstream-bound factors may significantly increase its ability to affect the transcription complex. This model suggests that T antigen may be able to interact with upstream-binding factors other than TEF-1 and possibly also with coactivators. Although such interactions with additional factors have not yet been well studied, precedence for their existence is suggested by the observation that T antigen can interact with AP-2 in vitro (34).

The binding of TBP by viral transcriptional activation proteins appears to be a recurring feature. It is known that E1a and VP16 interact with this protein (22, 27, 44), and others are suspected (21). Interactions with multiple components of the transcription complex, as shown here with T antigen, may also occur with other viral transactivators. Like T antigen, E1a and VP16 interact with other transcription factors (32, 43, 48). Hence, it appears that a gene expression strategy of some DNA viruses is to affect host and viral transcription directly at the transcription complex. However, the lack of an E1a- or VP16-like activation region in T antigen suggests that the viral proteins accomplish transcriptional modification by different mechanisms once bound to the transcription complex.

ACKNOWLEDGMENTS

We thank the members of the Alwine laboratory for encouragement and helpful discussions throughout the course of this work; Sherri Adams for helpful discussions and critical evaluation of the manuscript; Ivan Sadowski for anti-Gal4-1-147; David Livingston for pRSV-TEX; and Tom Kadesch for *Gal4* fusion vectors and promoter plasmids.

This work was supported by Public Health Service grant CA28379 awarded to J.C.A. by the National Institutes of Health.

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