JOURNAL OF VIROLOGY, June 1991, p. 2778–2790
0022-538X/91/062778-13$02.00/0

Vol. 65, No. 6

Mapping the Transcriptional Transactivation Function of Simian Virus 40 Large T Antigen

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Received 6 December 1990/Accepted 18 February 1991

T antigen is able to transactivate gene expression from the simian virus 40 (SV40) late promoter and from several other viral and cellular promoters. Neither the mechanisms of transactivation by T antigen nor the regions of T antigen required for this activity have been determined. To address the latter point, we have measured the ability of a set of SV40 large T antigen mutants to stimulate gene expression in CV-1 monkey kidney cells from the SV40 late promoter and Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter. Transactivation, although reduced, was retained by an N-terminal 138-amino-acid fragment of T antigen. Mutants with alterations at various locations within the N-terminal 85 amino acids transactivated the RSV LTR promoter less well than did wild-type T antigen. Most of these were also partially defective in their ability to transactivate the SV40 late promoter. Two mutants with lesions in the DNA-binding domain that were unable to bind to SV40 DNA were completely defective for transactivation of both promoters, while a third mutant with a lesion in the DNA-binding domain which retained origin-binding activity transactivated both promoters as well as did wild-type T antigen. Only a low level of transactivation was seen with mutant T antigens which had lesions in or near the zinc finger region (amino acids 300 to 350). Mutations which caused defects in ATPase activity, host range/helper function, binding to p53, binding to the retinoblastoma susceptibility protein, or nuclear localization had little or no effect on transactivation. These results suggest that the N-terminal portion of T antigen possesses an activation activity. The data are consistent with the idea that the overall conformation of T antigen is important for transactivation and that mutations in other regions that reduce or eliminate transactivation do so by altering the conformation or orientation of the N-terminal region so that its ability to interact with various targets is diminished or abolished.

The large T antigen of simian virus 40 (SV40) is a multifunctional 708-amino-acid phosphoprotein that plays diverse roles during the SV40 infection cycle (for review, see reference 69). Enzymatically, T antigen is able to bind and hydrolyze ATP. Additionally, it binds specifically to pentanucleotide sequences within the SV40 origin of replication and possesses nonspecific DNA-binding activity. Through coordination of these activities, T antigen plays a central role in the initiation of viral DNA replication, by catalyzing the unwinding of viral DNA at the origin, and in elongation, by acting as a helicase at the replication fork (for review, see reference 60). It may also have additional roles in viral DNA replication.

A second major arena of T antigen activity is transcription. T antigen autoregulates the level of SV40 early mRNA through direct binding to sequences on the early side of the origin of replication (2, 30, 40, 62, 65). In addition, T antigen activates transcription from the SV40 late promoter (8, 10, 37) and is also able to transactivate transcription from a variety of other viral and cellular promoters (1, 9, 48, 68, 73). This activity may be responsible for the observed induction of increased levels of enzymes involved in DNA replication (for reviews, see references 78 and 79).

The mechanisms by which some viral transactivators such as adenovirus E1A and herpes simplex virus type 1 VP16 stimulate transcription has been studied extensively. The adenovirus E1A protein acts to stimulate transcription from the E1B, E2, E3, E4, and major late promoters and appears to affect these through multiple mechanisms (43, 63, 85), including modification or induction of cellular transcription factors and direct interaction between cellular factors and the E1A protein (44, 52). The herpes simplex virus type 1 VP16 protein is contained within the herpesvirus virion. Once inside newly infected cells, VP16 acts to stimulate transcription from a set of herpesvirus immediate-early genes. The available data indicate that VP16 stimulates transcription through interactions with protein factors at the promoter (5, 75, 76, 84). VP16 contains an acidic activating domain and has proven to be an exceptionally strong *trans* activator (67).

Less is known about *trans* activation by SV40 large T antigen. Many studies have focused on defining sequence elements within the SV40 late promoter that are involved in mediating transactivation by T antigen (10, 38). Other studies have demonstrated that the presence of T antigen causes alterations in the pattern of cellular transcription factors able to recognize these key sequences, but the mechanism by which these changes are caused is not known (4, 20, 21).

Like the adenovirus E1A protein, SV40 large T antigen has been shown to interact with cellular transcription factors. Mitchell et al. showed that T antigen can form a complex with transcription factor AP-2 and inactivate it (54). The importance of this to transcriptional activation by T antigen is not known. The SV40 regulatory region contains multiple binding sites for transcription factor Sp1. Sp1 mRNA and protein levels are increased 100-fold after infection by SV40 (68), suggesting that some of the genes expressed at higher levels after SV40 infection may be induced indirectly as a consequence of higher levels of SP1 or other transcription factors in SV40-infected cells.

Since only limited work has been done to define the portions of T antigen important for transactivation, we set out to map this activity. T antigen has been subjected to

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extensive genetic analysis, permitting the assignment of many of its activities to distinct regions of the polypeptide. The DNA-binding domain is known to require sequences between amino acids 131 and 259 (3), but DNA-binding activity is affected by the presence of additional sequences, including a zinc finger region near amino acid 300 (34). Much of the C-terminal half of the polypeptide (residues 400 to 600, approximately [11, 12, 14, 55]) constitutes an ATP-binding and ATPase domain that has homology to domains found in many other proteins that also bind and hydrolyze ATP (7). The C terminus of T antigen is not required for the replicative activities of T but provides a host range function; mutants lacking the C terminus produce drastically reduced virus yields in some monkey kidney cell lines (39, 74, 80–82). Regions of T important for nuclear localization (amino acids [aa] 126 to 132 [35, 36, 42]) and for binding to the tumor suppressor protein $p105^{Rb}$ (aa 105 to 115) have also been mapped precisely (15, 18, 49, 56). T antigen binds a second tumor-suppressor protein, p53, but the minimal sequences required for this interaction have not been determined (55, 71). The amino-terminal 130 amino acids of large T are required for transformation of virtually all rodent cell lines examined (73); this portion alone is sufficient to transform C3H 10T1/2 cells, while transformation of most other cell lines requires substantially more T antigen, including most of the first 600 amino acids (59, 77).

In the experiments described below, we measured the ability of a large set of SV40 mutants to stimulate gene expression from the SV40 late and Rous sarcoma virus (RSV) long terminal repeat (LTR) promoters. Mutants with alterations at various locations within the N-terminal 85 amino acids transactivated the RSV LTR promoter less well than did wild-type T antigen. Most of these were also partially defective in their ability to transactivate the SV40 late promoter. Mutants with lesions in the DNA-binding domain that were unable to bind to SV40 DNA were completely defective for transactivation of both promoters. Another mutant with a lesion that mapped within the DNA-binding domain but which retained the ability to bind to the SV40 origin transactivated as well as wild-type T antigen did. Only a low level of transactivation was seen with mutant T antigens with lesions in or near the zinc finger region (aa 300 to 350). Mutations in the C-terminal half of T antigen had little effect on transactivation.

We suggest that the N-terminal portion of T antigen possesses an activation activity and that this activity is sensitive to the physical orientation of this domain with respect to the overall structure of the protein. In addition, we discuss the possible relationships between this function of T antigen and its other activities.

MATERIALS AND METHODS

Plasmids used in this study. Transfection of bacterial cultures (51), analysis of minilysate preparations of DNA (6), and large-scale preparation of purified plasmid DNA (13) were performed by established methods. All plasmids were propagated in *Escherichia coli* HB101.

(i) Reporter plasmids. Both pL16n-CAT (38) and pRSV-CAT are derivatives of pSV0-CAT (27). pL16n-CAT has a 405-bp *Hin*dIII-*Nco*I fragment from the SV40 origin region inserted into the *Hin*dIII site of pSV0-CAT. In this plasmid, the SV40 late promoter region is upstream from chloramphenicol acetyltransferase (CAT) coding sequences. pRSV-CAT (26) contains a 524-bp fragment, including the RSV LTR promoter, inserted into the *Hin*dIII site of pSV0-CAT, bringing *cat* mRNA synthesis under control of the RSV promoter.

In order to facilitate production of large quantities of plasmid DNA, the pBR322 sequences originally in pSV0-CAT and these other derivatives of it were replaced with pUC18 sequences. To accomplish this, pL16n-CAT and pRSVLTR-CAT were digested with *Hin*dIII and *Pst*I or *Pst*I and *Nde*I, respectively. The appropriate fragments, containing the promoter, *cat* gene, and RNA processing signals, were inserted into pUC18. The new plasmids, pUL16n-CAT and pURSVLTR-CAT, respectively, functioned identically to the original plasmids in CAT assays (data not shown).

(ii) Plasmids for producing probes for DNA assays. pGEM-CAT was constructed from pE2-CAT (47) by cutting pE2-CAT with *Sca*I and *Pvu*II. A 516-bp fragment containing the CAT coding region was gel purified by using Geneclean (Bio 101, San Diego, Calif.) and inserted into the *Sma*I site of pGEM1.

(iii) Plasmids containing SV40 mutant genomes. Plasmid pCC2pA contains the entire SV40 genome, opened at its *Eco*RI site and inserted into the *Eco*RI site of pUC18 in an orientation which places the *Bam*HI site of SV40 close to the *Bam*HI site in the pUC18 polylinker. Various SV40 mutants were maintained as recombinant clones inserted into pUC18 in the same orientation as in pCC2pA. p6-1 contains a 6-bp deletion at the *Bgl*I site in the SV40 origin region and therefore is not able to replicate in the presence of wild-type SV40 large T antigen (24; data not shown). To produce replication-defective forms of SV40 variants with mutations in the second exon of the large T antigen coding sequence, each mutation was moved into the p6-1 plasmid by replacing the *Bst*XI-*Apa*I fragment of p6-1 with an equivalent fragment containing the mutation. To produce replication-defective forms of mutants with lesions in the first exon of large T antigen, a small deletion was created at the *Sfi*I site within the origin of replication of each of these mutants by digesting plasmids containing SV40 mutant genomes with *Sfi*I (which cuts only at the site within the SV40 origin), removing the 3′ extensions by using the Klenow fragment of DNA polymerase I and deoxynucleoside triphosphates (dNTPs), and recircularizing with T4 DNA ligase. This procedure deleted 3 bp from the center of the origin of replication. The precision of this mutagenesis was confirmed by determining that the resulting plasmids no longer contained an *Sfi*I site within the origin region and that a *Sac*II site had been created at the site of the deletion. The resulting plasmids were not able to replicate in the presence of wild-type large T antigen (data not shown).

Partial digestion of p6-1 DNA with *Pfl*MI at nucleotide (nt) 4458 of SV40, followed by treatment with the Klenow fragment of DNA polymerase I and four dNTPs, generated blunt-ended linear molecules lacking the 3-nt 3′ extensions produced by *Pfl*MI digestion. The linear DNA was either circularized directly with T4 DNA ligase, generating mutant *dl*2834, or ligated in the presence of an excess amount of 12-bp nonphosphorylated synthetic oligonucleotide linkers, d(TCGCGATCGCGA), as described previously (86), generating *in*2835. *dl*2834 encodes a large T antigen which contains an Asn residue in place of the Thr and Tyr residues at positions 85 and 86 of large T antigen. The T antigen encoded by *in*2835 also lacks the Thr and Tyr residues at positions 85 and 86 and contains, in their place, Ile-Ala-Ile-Ala-Asn.

(iv) Plasmids containing binding sites for SV40 large T antigen. Digestion of pCC2pA (wild-type SV40 genome in pUC18) with *Hin*dIII generated several fragments, including one of 1,118 bp that contains the complete SV40 origin

region, including binding sites I, II, and III (65, 72). p1097 is an SV40 mutant with a 31-bp deletion that removes binding site I (17). Digestion with *Hin*dIII generated an 1,100-bp fragment containing binding sites II and III. pON contains a 19-bp synthetic oligonucleotide version of binding site I (66); a 626-bp *Sal*I-*Hin*dIII fragment of this plasmid was used in DNA-binding studies.

Transactivation assays. CV-1 monkey kidney cells were maintained in Dulbecco's modification of minimal essential medium (GIBCO) supplemented with 10% newborn calf serum (Hyclone, Logan, Utah) and antibiotics. Transactivation assays were performed by cotransfecting CV-1 cells with 4.0 μg of a plasmid containing wild-type SV40 or an SV40 mutant genome, 0.5 μg of a reporter plasmid, and 15.5 μg of salmon sperm DNA. The reporters used contained the bacterial CAT coding region downstream from either the SV40 late promoter of the RSV LTR promoter. We used the calcium phosphate method to introduce the DNAs into cells (28). To monitor transfection efficiency, we included in a parallel plate a control transfection with pSV2APAP DNA (32), encoding placental alkaline phosphatase. After 45 h, cells expressing placental alkaline phosphatase were detected histochemically by using naphthol AS-MX phosphate alkaline solution and fast violet B (both from Sigma Chemical Co., St. Louis, Mo.) as directed by the manufacturer. At 45 h after transfection, we also prepared extracts of transfected cells in the following manner. Cells were washed twice with TS (25 mM Tris Cl [pH 7.5], 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.05 mM $MgCl_2$, 0.7 mM $CaCl_2$), and 1 ml of TS was added to washed plates. Cells were scraped off the plate with a cell scraper, centrifuged briefly in a microfuge, resuspended in RSB (10 mM Tris Cl, 10 mM NaCl, 1.5 mM $MgCl_2$ [pH 7.35]) containing 0.4% Nonidet P-40 (NP-40), and incubated at 0°C for 30 min. The nuclei were pelleted, and the supernatant, containing the CAT activity, was carefully removed.

To assay the amount of CAT enzyme present in each sample, we used a modification of the method of Neumann et al. (58). This assay uses [^{3}H]acetyl coenzyme A (CoA) and measures direct diffusion of the labeled product (acetylated chloramphenicol) into water-immiscible liquid scintillation counting fluid (Econofluor; Du Pont). Only the acetylated derivatives of chloramphenicol are able to enter this organic phase. Portions of cell extracts were added to 100 μl of CAT reaction buffer (125 mM Tris Cl [pH 7.8], 2.5 μCi of [^{3}H]acetyl CoA [200 mCi/mmol; Du Pont, Boston, Mass.] per ml, 1.25 mM chloramphenicol). Since chloramphenicol was present in large excess compared with the concentration of [^{3}H]acetyl CoA, the initial rate of acetylation of chloramphenicol was proportional to the concentration of [^{3}H]acetyl CoA, and the rate constant (K) is a measurement of the amount of CAT enzyme present in each extract. Since all CAT reactions were conducted with the same concentration of [^{3}H]acetyl CoA, the only measurements required were time elapsed since the acetylation reactions began and amount of radioactivity detected in the scintillation fluid. In practice, a set of samples were each counted for 0.1- min several times during a 4-h period, and these data were plotted against elapsed time. Background radioactivity was subtracted, and the rate constants were calculated. This method was standardized and validated by use of a range of concentrations of purified CAT enzyme (Sigma). When using CAT enzyme, the same results were obtained whether or not an aliquot of a mock-transfected extract was added to the reaction mixture.

To determine the amount of plasmid DNA present in transfected cell cultures, the nuclear pellet was solubilized by the addition of Hirt's solution (0.1 M Tris Cl, 0.6% sodium dodecyl sulfate [SDS], 0.1 M EDTA [pH 7.5]) (33), and the plasmid DNA was separated from chromosomal DNA by centrifugation at 4°C in an Eppendorf 5413 centrifuge. The supernatant, containing low-molecular-weight episomal DNA, was phenol extracted, depurinated in 0.25 M HCl for 3 to 5 min, denatured in 0.6 M NaOH–1.5 M NaCl, neutralized with 1 M Tris Cl–1.5 M NaCl, pH 7.0, and applied to nitrocellulose membranes with a slot blot apparatus (Schleicher & Schuell, Keene, N.H.). The membrane was baked in vacuo for 1 h at 80°C. The amount of plasmid DNA in each sample was determined by hybridization of the membrane to a *cat*-specific probe made by oligolabeling (19) a twice-gel-purified 529-bp *Sac*I-*Bam*HI fragment containing part of the CAT protein coding region. The purity of the probe was tested by hybridization to known quantities of either pURSVCAT or p6-1 (origin-defective SV40 cloned into pUC18). The signal generated with p6-1 was less than 0.5% of the signal generated with an identical amount of pURSVCAT DNA.

Autoradiograms of the slot blots were quantitated by using the MasterScan Interpretive Densitometer (Scanalytics, Division of CSP, Inc., Billerica, Mass.).

DNA-binding assays. The DNA-binding activity of mutant SV40 large T antigens were assayed by using extracts from mouse cell lines expressing mutant or wild-type T antigens. Exponentially growing cells were lysed by incubation in EBC (15) (50 mM Tris Cl [pH 8.0], 100 mM NaCl, 0.5% NP-40, 0.1 trypsin inhibitor units of aprotinin per ml [Sigma], added just prior to use) at 4°C for 20 min, and the supernatants were separated from cellular debris by centrifugation in an Eppendorf 5413 centrifuge at 4°C for 15 min. The amount of T antigen present in each lysate was estimated by immunoprecipitation with anti-T antibodies (PAb901 and PAb902; gifts from Satvir Tevethia), followed by Western immunoblot analysis. Western blots were probed with anti-T antibodies PAb901 and PAb902, followed by ^{125}I-labeled goat anti-mouse immunoglobulin antibody (Amersham, Arlington Heights, Ill.). The amount of each cell lysate used was adjusted so that approximately equal amounts of different mutant T antigens were analyzed.

The substrate DNA fragments for binding were produced by digestion of pON with *Sal*I and *Hin*dIII, digestion of p1097 with *Hin*dIII, and digestion of pCC2pA with *Hin*dIII. The DNA fragments were radiolabeled by using the Klenow fragment of DNA polymerase I in the presence of [α^{32}P] dCTP (50 μCi; specific activity, 3,000 Ci/mmol) and unlabeled dATP, dTTP, and dGTP to fill in the 3′ recessed ends. T antigen-containing (or control) cell lysates were mixed with 2 μl of PAb902 ascites fluid and rocked at 4°C for 1 h. Then 20 ml of protein A-Sepharose beads (Pharmacia; a 1:1 suspension in Tris-buffered saline [25 mM Tris Cl [pH 8.0], 120 mM NaCl] containing 10% bovine serum albumin [BSA; Sigma]) was added to the T antigen-antibody mixtures and rocked for 30 min at 4°C. Antibody-T antigen complexes were collected by centrifugation, and the Sepharose beads were washed three times with 1 ml of EBC and once with 1 ml of DNA-binding buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.0], 80 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.2 mg of glycogen per ml, 1 mg of BSA per ml). The immunocomplexes were then incubated on ice for 1.5 h in a total volume of 0.5 ml of DNA-binding buffer containing 10 ng of end-labeled pON fragments and 25 ng of end-labeled pCC2pA or p1097 fragments. The DNA binding reactions

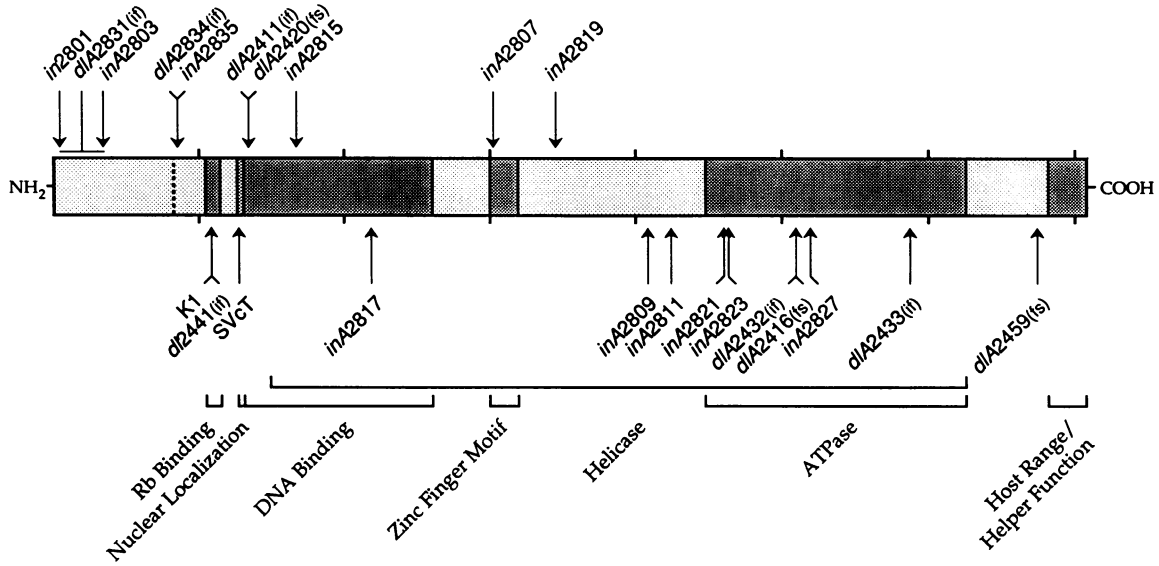

FIG. 1. Map of SV40 large T antigen and mutations used in this study. The protein backbone of T antigen is indicated by the shaded bar. The dark grey shading within the bar indicates domains of known function, which are also indicated by labeled brackets below the map. The exonI-exonII junction at amino acids 82 and 83 is indicated by a vertical dashed line. Vertical spikes on the map indicate each 100 amino acids. In-frame deletion mutations are designated by the suffix -(if), and those which cause frameshift mutations are designated by the suffix -(fs). Mutations which reduce the level of transactivation are indicated above the map, and mutations which do not have a significant effect are indicated below.

were performed in the presence of a 20-fold excess of sonicated salmon sperm DNA and with an excess of labeled fragments, relative to the amount of T antigen. The DNA-protein A-Sepharose beads were then washed three times with phosphate-buffered saline containing 0.4% NP-40 and resuspended in 30 μl of 2× Laemmli's sample buffer (125 mM Tris Cl [pH 6.8], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol [41]). The DNA fragments bound to T antigen were released by heating at 70°C for 15 min, the Sepharose beads were removed by centrifugation for 30 s in a microfuge, and the DNA fragments in the supernatants were analyzed by electrophoresis on 1.5% agarose gels in TBE buffer (89 mM Tris-OH, 89 mM boric acid, 2.5 mM EDTA [pH 8.5]). The gels were dried and exposed to X-ray film (Kodak XAR-5).

RESULTS

Transactivation of the RSV LTR and the SV40 late promoter. We examined the ability of a large set of SV40 large T antigen mutants to activate transcription from the SV40 late and the RSV LTR promoters. The mutants used in these studies are shown in Fig. 1 and contain mutations located throughout the T antigen coding region. The 2400 series mutants were described previously (80, 81) and contain small deletions. Some encode nearly full-length T antigens lacking one to four amino acids at the site of the deletion; in others, the deletion causes a translational reading frame shift, resulting in the production of a T antigen truncated near the site of the deletion. Most of the mutants in the 2800 series contain linkers inserted at various sites within the early region of SV40 (86). A few point mutants were also examined.

Plasmids encoding each of these mutant SV40 T antigens were cotransfected with a *cat* reporter plasmid containing either the SV40 late or RSV LTR promoter sequences into CV-1 monkey kidney cells. Since wild-type T antigen and some of the mutant T antigens used in these studies were competent for viral DNA replication, the SV40 origin of replication was inactivated in all plasmids. Therefore, none of the plasmids could be amplified in the transfected cells.

Both cytoplasmic extracts and nuclear DNA were prepared from each transfected culture. CAT enzyme activity was determined by the method of Neumann et al. (58) and standardized to the amount of reporter plasmid DNA in the transfected cells as determined by slot blot analysis.

We first compared three mutants that produce T antigens truncated after 138 (*dl*A2420), 510 (*dl*A2416), or 676 (*dl*A2459) amino acids. The relative ability of these three mutants (wild-type T antigen = 1.00) to transactivate the RSV LTR promoter and the SV40 late promoter is displayed in Fig. 2. The data indicate that transactivation of the RSV LTR promoter was not affected significantly by truncation at amino acid 510 or 676 within the carboxy-terminal half of T antigen. Transactivation of the SV40 late promoter was reduced slightly, to 60 to 80% of wild-type levels, by truncation at these sites. Less activity on either promoter was seen with the N-terminal 138 amino acids of large T (*dl*A2420), which transactivated only 30 to 50% as well as wild-type SV40 large T.

We extended this study by examining a larger group of mutants that produce T antigens of approximately full length. The results from analyses of mutants with lesions in the C-terminal half of T antigen are shown in Fig. 3. These mutants have deletions or insertions at amino acids 409 (*in*A2809), 424 (*in*A2811), 460 (*in*A2821), 464 (*in*A2823), 509 (*dl*A2432), 520 (*in*A2827), or 587 (*dl*A2433). Each of these transactivated both promoters at 60 to 140% of the level of wild-type T antigen, confirming the conclusion with the truncation mutants (Fig. 2) that sequences within the C-terminal half of the molecule are dispensable for transactivation. This region has been shown to contain the ATP-binding and ATPase domains of T antigen and to play a role in

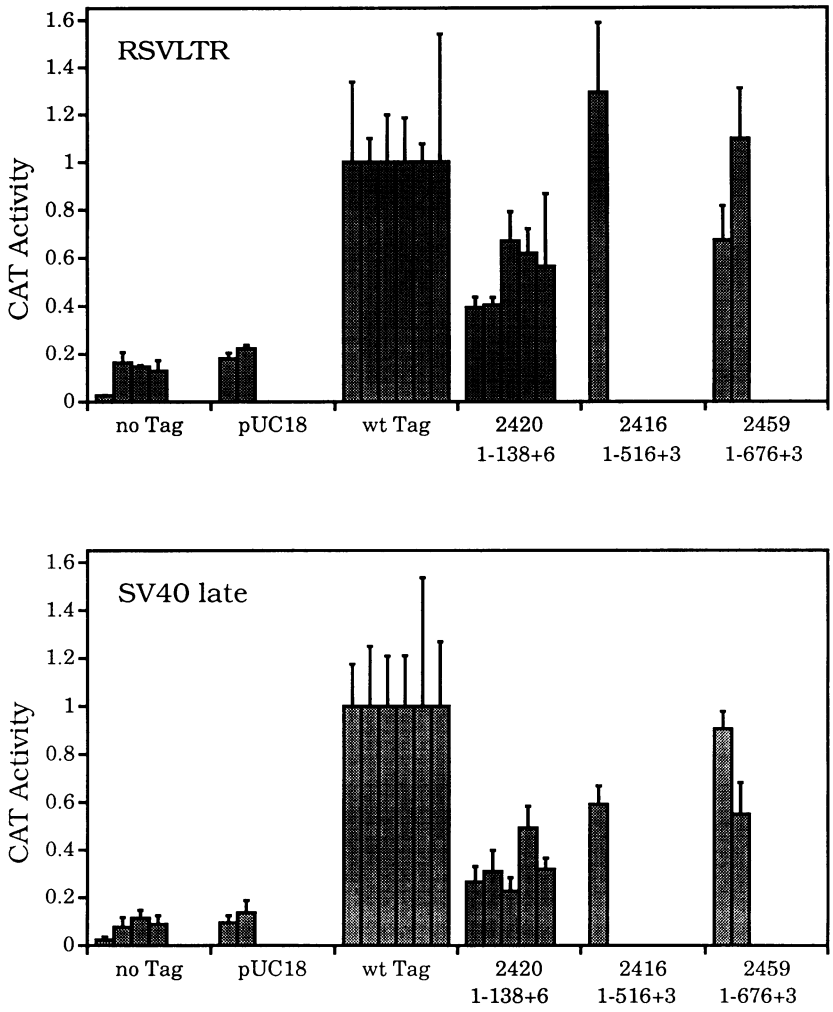

FIG. 2. Transactivation by SV40 large T antigen mutants producing truncated T antigens. In each experiment in Fig. 2 through 6, the transactivation activity of each mutant was measured in triplicate. Each mutant was examined in one to four separate experiments. The data are presented as bar graphs in which each separate experimental analysis of a given mutant is represented as a separate bar and the error bar reflects the standard deviation of the mean of values obtained from the triplicate samples in one single experiment. The identity of each plasmid is indicated under each set of bars. The number of amino acid residues of the T antigen (Tag) produced by each truncation mutant is indicated below the mutant number, as are any additional amino acids encoded in the new reading frame, which are indicated after a + sign. Data are normalized to the level of CAT enzyme produced in the presence of wild-type (wt) T antigen. In the upper graph, the reporter construct was pRSV-CAT (RSV LTR promoter), and in the lower panel it was pUL16nCAT (SV40 late promoter).

transformation of some rodent cell lines and in immortalization of primary mouse embryo fibroblasts.

Data obtained from analyses of mutants with lesions affecting the N-terminal half of T antigen are shown in Fig. 4. Several mutants with lesions within the first 85 amino acids of large T showed significantly reduced transactivation of the RSV LTR (Fig. 4). These included mutants with insertions at amino acids 4 (*in*2801), 34 (*in*A2803), and 85 (*in*2835) as well as one with a deletion from amino acids 4 to 34 (*dl*A2831) or 85 (*dl*2834). Some of these showed similar reduced activity for transactivation of the SV40 late promoter (Fig. 4), while others (*in*2801, *dl*A2831, and *in*A2803) transactivated the SV40 late promoter as well as did wild-type T antigen. This region is absolutely required for transformation of established rodent cell lines (73).

Several mutations in the DNA-binding domain of SV40 T antigen had a more dramatic effect on transactivation. Two mutants, *dl*A2411 (deletion of aa 143 to 146) and *in*A2815 (insertion at aa 168) failed to transactivate either the SV40 late or RSV LTR promoter (Fig. 5). However, a third mutation in the DNA-binding domain (*in*A2817, insertion at aa 219) transactivated as well as wild-type T antigen did.

Mutants with lesions between amino acid 85 and the DNA-binding domain (*dl*2441, K1, and SVcT) also showed wild-type levels of transactivation (Fig. 5). This region is important for nuclear localization of T antigen and for binding to the retinoblastoma protein $p105^{Rb}$; mutants examined included ones defective in each of these two properties. Our results indicate that efficient nuclear localization or binding to $p105^{Rb}$ is not required for transcriptional activation of either the SV40 late or RSV LTR promoter.

SV40 large T antigen contains a zinc finger motif, located between amino acids 300 and 325 (45, 46). This region lies between the minimal DNA-binding domain (aa 131 to 259) and the ATPase/ATP-binding domain (aa 400 to 600). The helicase activity of T antigen requires sequences extending

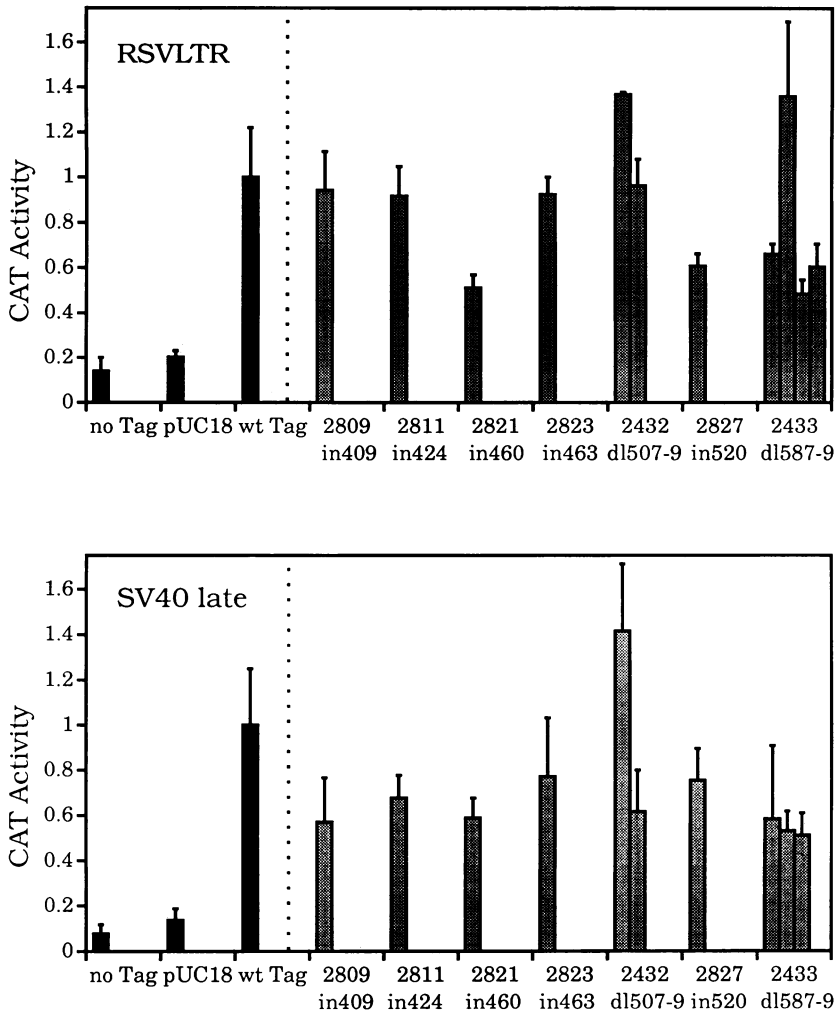

FIG. 3. Transactivation by SV40 large T antigen mutants which produce nearly full-sized T antigens with lesions in the C-terminal half of T antigen. The means of the data from Fig. 2 are indicated to the left of the vertical dotted line in darker shading. For Fig. 3 through 6, the identity of each plasmid is indicated below each set of data, as is the nature of the mutation it carries and the location of the amino acids of T antigen that are affected. The prefix in- represents an insertion of a 12-bp linker; the suffix +in represents a linker inserted at the site of the deletion; the prefix *dl-* represents a deletion of the indicated amino acids; point mutants are indicated by the wild-type single-letter amino acid code, followed by its location and the mutant amino acid which replaces it.

from the DNA-binding domain through the ATPase domain. Coordination between these regions is probably essential for this activity and is a likely function of the zinc finger motif. The transformation function of large T is substantially reduced by mutation of this motif (45, 46). Two mutants containing mutations in this region, one within (*in*A2807, aa 303) and one adjacent to (*in*A2819, aa 346) this motif, transactivated both promoters approximately 30% as well as wild-type T (Fig. 6). These mutants transactivated to a greater extent than negative controls, but their activity was always low.

In summary, sequences within the N-terminal half of T antigen appear to play an important role in transactivation. Transactivation was reduced but not eliminated by mutations within the first exon or within or near the zinc finger region. Transactivation, although reduced, was retained by an N-terminal 138-amino-acid fragment of T antigen, while some mutants with lesions in the DNA-binding domain were completely defective for transactivation of both promoters examined.

Determination of the DNA-binding activity of mutants of SV40 large T antigen. The origin-binding activity of T antigen cannot be absolutely required for transcriptional activation, since a 138-amino-acid N-terminal fragment of T antigen retained substantial activity (Fig. 2). However, since two mutants (*dl*A2411 and *in*A2815) with lesions in the DNA-binding domain were completely defective for transactivation while one (*in*A2817) showed a wild-type level of transactivation (Fig. 5), we wondered whether DNA binding was important for transactivation by mutant T antigens of approximately wild-type size. Therefore, we measured the ability of mutant T antigens to bind to the SV40 origin region.

We performed McKay-type DNA-binding assays (53). To provide adequate quantities of wild-type and mutant T antigens, we prepared extracts of exponentially growing mouse cells expressing various T antigens. The concentration of wild-type and mutant T antigens in these extracts was determined by Western blotting (data not shown) so that approximately equal amounts of each T antigen could be used in DNA-binding experiments.

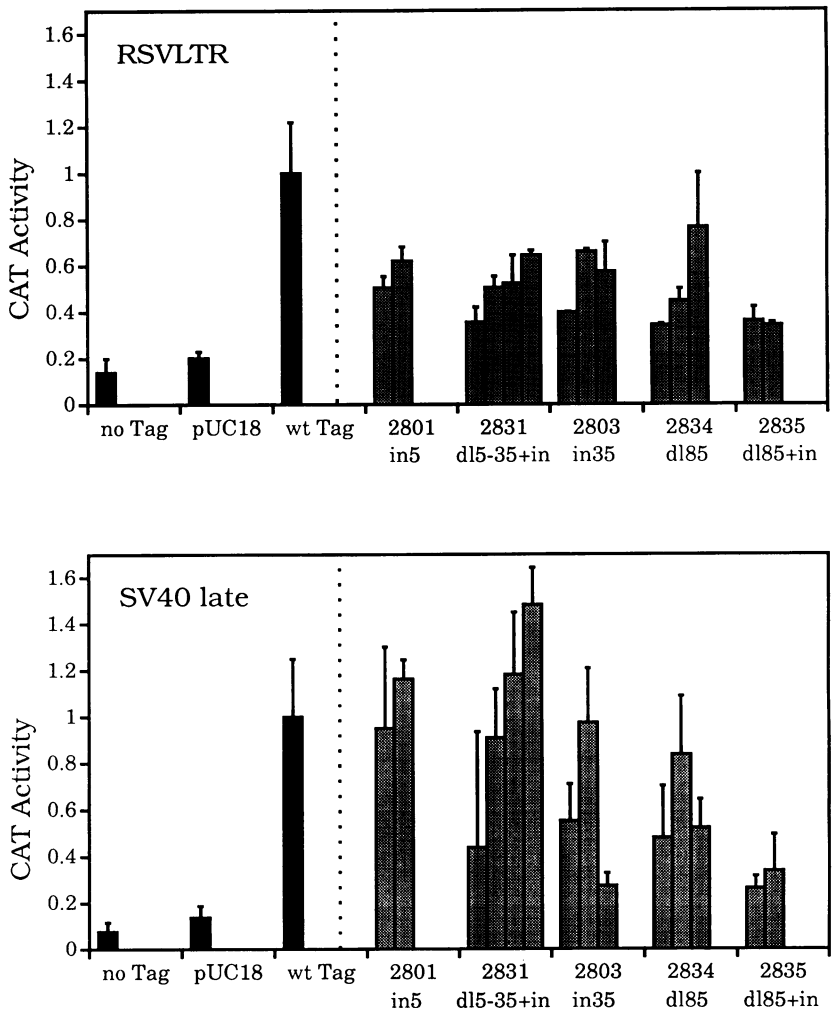

FIG. 4. Transactivation by SV40 large T antigen mutants which produce nearly full-sized T antigens with lesions in the N-terminal half of T antigen. See the legends to Fig. 2 and 3.

Wild-type SV40 DNA contains three large T antigen-binding sites. Site I contains three 5′-GAGGC-3′ pentanucleotides. Site II, which contains four pentanucleotides with two oriented in each direction, is contained within the minimal origin and is essential for SV40 DNA replication. The binding of T antigen to both sites I and II is responsible for repression of the early promoter by T antigen (65). Site III is located on the late promoter side of site II; T antigen binds to site III at a very low affinity, and it was not possible to detect site III binding in this assay. The fragments used for this assay are shown in Fig. 7A. A 1,118-bp *Hin*dIII fragment of wild-type SV40 DNA contains all three T antigen-binding sites. p1097 contains a deletion which removes binding site I; the binding of the 1,100-bp fragment to T antigen represents site II binding, since T binds to site III very weakly. The third substrate for binding was a 626-bp *Sal*I-*Hin*dIII fragment from pON, which contains a synthetic oligonucleotide form of site I inserted into plasmid pAT153. ^{32}P-3′-end-labeled DNA fragments were incubated with immunocomplexes formed between T antigen and monoclonal antibodies PAb901 and PAb902. After incubation, unbound DNA fragments were washed away. The DNA fragments bound to T antigen were then released and analyzed by agarose gel electrophoresis (Fig. 7B).

Wild-type T antigen binds to site II much more weakly than it does to site I or to the complete wild-type origin (reviewed in reference 16). Mutants *in*A2827 and *dl*A2433 have lesions that are outside of the DNA-binding domain of T antigen. They transactivated both the SV40 late and RSV LTR promoters at wild-type efficiency and bound to sites I and II as well as or better than wild-type T antigen. The T antigen encoded by *dl*A2433 is unstable (80). The fact that an equal amount of this T antigen bound more radiolabeled DNA than did wild-type T may reflect the fact that there would likely be more newly synthesized mutant T antigen in the extract. Newly synthesized T antigen is more active for DNA binding (70).

Three mutations which map within the minimum origin-binding domain defined by Arthur et al. (3) were used in this study. Two of these mutants, *dl*A2411 and *in*A2815, did not transactivate either promoter examined, and both failed to bind either site I or II (Fig. 7B). The other mutant, *in*A2817, transactivated the two promoters as efficiently as the wild type and bound both site I and site II at the wild-type level.

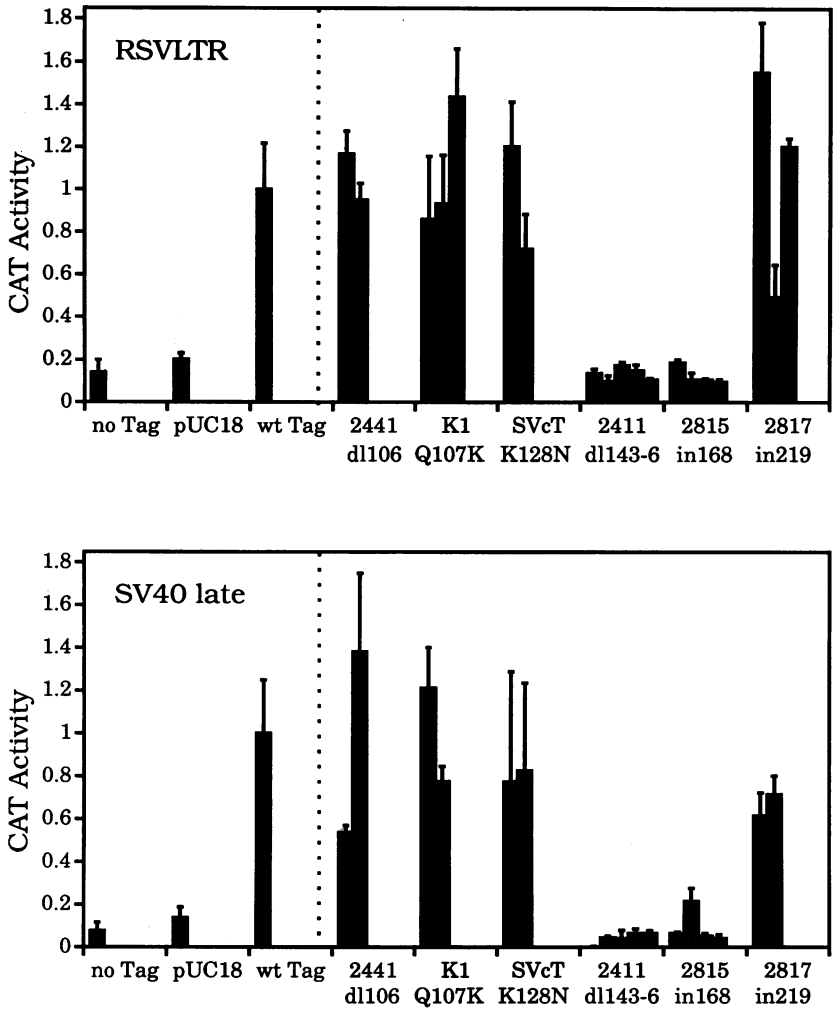

FIG. 5. Transactivation by SV40 large T antigen mutants with lesions in and near the DNA-binding domain. See the legends to Fig. 2 and 3.

Mutant *in*A2807 has a 12-bp insertion within the zinc finger motif (aa 302). This mutant T antigen was unstable (86), failed to bind DNA, and showed a reduced ability to transactivate the promoters studied. Two mutants with lesions in the first exon (*in*2801 and *in*A2803) bound to sites I and II as well as wild-type T did. These results indicated that, for T antigens of nearly full size, the ability to bind to the SV40 origin region or the conformation of the protein required for this binding is important for transactivation.

DISCUSSION

The experiments described in this article were conducted in order to map the transactivation domain of SV40 large T antigen and, by so doing, to provide information essential for determining whether transactivation is related to any of the other activities or properties of large T antigen.

In each experiment, several mutants were compared and a common set of controls were performed, including transactivation by wild-type T antigen, transactivation by a plasmid which encoded no T antigen (e.g., pUC18), and analysis of the level of reporter expression in cells which received only the reporter plasmid and carrier DNA. We noticed that CAT expression could be stimulated two- to fourfold by various plasmids which did not encode any transactivator. These included pUC18, pBluescriptKS+, and pBR322. We do not understand the mechanism of these plasmid effects, but they could reflect the titration of a negative factor by the plasmid, permitting a higher basal level of reporter gene transcription in the absence of transactivator. Since some of the T antigen mutants studied stimulated *cat* expression only slightly (but reproducibly) more than pUC18, caution is required in interpreting these data.

For mutants that transactivated at close to wild-type levels, it is safe to conclude that the mutated region of T antigen was not required for transactivation. When mutants showed levels of transactivation consistently greater than that seen with pUC18 but only 30 to 80% of that of wild-type T, the mutation clearly affected the ability of wild-type T to transactivate. A few mutants examined consistently transactivated to a level only slightly greater than that seen with pUC18. With these (*dl*A2420, *in*A2807, and *in*A2819), we believe that the mutant T antigens retained some transactivation activity but that plasmid-based effects may be responsible for part of the stimulation observed. For the mutants which completely failed to transactivate (*dl*A2411 and *in*A2815), the mutant T antigens likely had no activity at all but may also have been able to prevent the low level of stimulation resulting from plasmid effects.

What portions of SV40 large T antigen are involved in

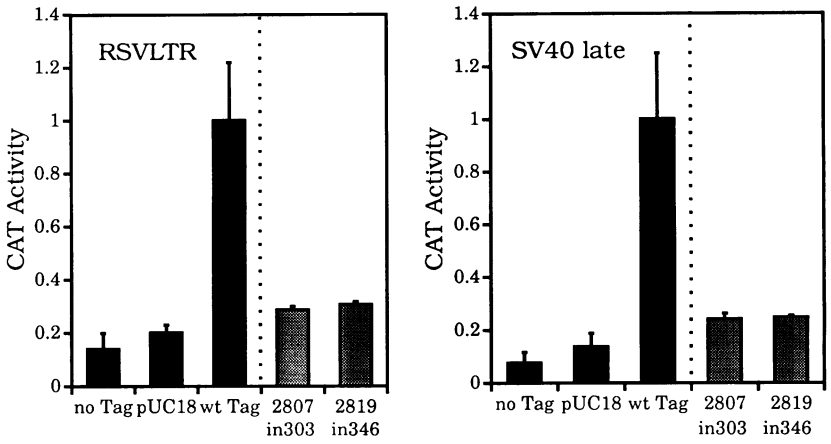

FIG. 6. Transactivation by SV40 large T antigen mutants with lesions in and near the zinc finger region. See the legends to Fig. 2 and 3.

transactivation? The results obtained suggest that the first 85 amino acids are important for transactivation. All mutants examined with lesions in this region showed a reduced ability to transactivate the RSV LTR promoter and some were also reduced in transactivation of the SV40 late promoter. The importance of this region also follows from the finding that a 138-amino-acid N-terminal fragment of T antigen (*dl*A2420) retained the ability to transactivate both promoters, albeit at a low level. Srinivasan et al. reported previously that the N-terminal 121 amino acids of T antigen transactivated the adenovirus E2 promoter (73).

The fact that all of the mutations in this region reduced but did not completely eliminate transactivation of the RSV LTR promoter is reminiscent of observations of mutants within the transactivating regions of Gal4 of *Saccharomyces cerevisiae* (22, 50) and the herpes simplex virus type 1 transactivator, VP16 (67, 83). In both of these, maximal activity was seen when the entire transactivating region was present, but mutants lacking portions of this region retained partial activity. Both Gal4 and VP16 contain acidic transactivating domains, and the overall acidity seems to be more important than the particular sequence of acidic residues.

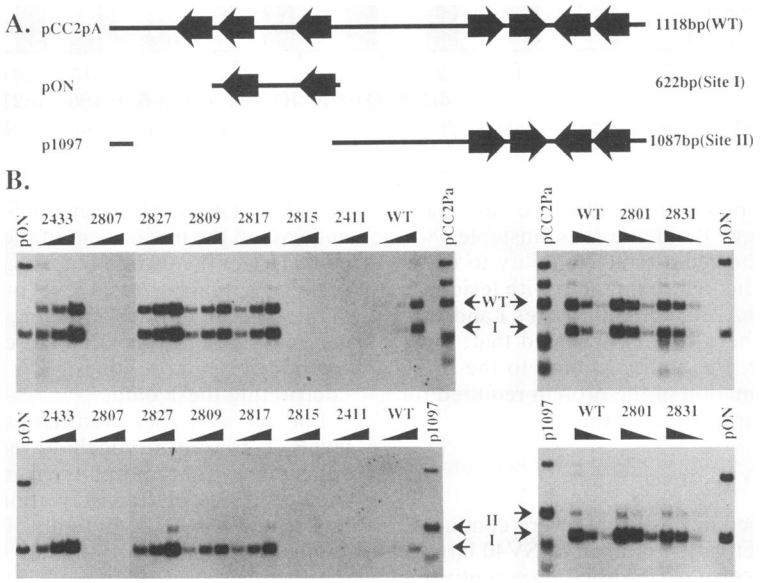

FIG. 7. SV40 origin-specific DNA binding by mutant large T antigens. (A) Plasmids used in the assay: pCC2pA, wild-type (WT) SV40 genome cloned into the *Eco*RI site of pUC18, includes both site I and site II; pON, an artificial site I cloned into pAT153, includes only site I; p1097, an SV40 genome with a 31-bp deletion at the site I cloned into pAT153, contains only site II. Thin lines represent SV40 DNA sequences, and arrows represent the pentanucleotide (5′-GAGGC-3′) consensus sequence. The sizes of the restriction fragments containing the corresponding T antigen-binding site are indicated to the right of each map. (B) Cells expressing mutant T antigens were lysed, and mutant T antigens were immunoprecipitated with PAb902 (two left panels) or PAb901 (two right panels). The immunocomplexes were then incubated with an equal molar amount of ^{32}P-labeled *Sal*I- and *Hin*dIII-digested pON and *Hin*dIII-digested pCC2pA (two upper panels) or *Hin*dIII-digested p1097 and pON (two lower panels). The DNA fragments bound were released in phosphate-buffered saline containing 0.4% NP-40 and resolved on an agarose gel, and the autoradiographs are shown. Four percent of the DNA fragments used in each assay were run in side lanes, and arrows indicate the positions of bound fragments. Three threefold dilutions of T antigens were assayed to ensure that the DNA fragments were in excess. The data were from two different experiments and from multiple gels.

In contrast to the acidic activating domains of VP16 and Gal4, the N-terminal 85-amino-acid portion of large T has a nearly neutral overall charge but is highly hydrophilic (14 acidic and 15 basic residues, two serines and three threonines) and is likely to be located at the surface of T antigen, where it could interact with other macromolecules. Possibly, the inability to find single mutations within this region that eliminate all transactivation activity could indicate that a large surface of T antigen interacts with other protein factors.

The ability of T antigen to transactivate was also affected by mutations in a second region of T antigen, the DNA-binding domain. Of the three mutants examined, the two which failed to bind to the SV40 origin region (*dl*A2411 and *in*A2815) were completely defective for transactivation, while one which retained binding activity (*in*A2817) transactivated as well as the wild type did. Keller and Alwine showed previously (37) that mutant C6 (23), which is defective in DNA binding (61), did not transactivate the SV40 late promoter. These observations raise two important questions. Is the DNA-binding activity of large T important for its role in transactivation? Is there more than one domain of T antigen with transactivation activity, or does a single transactivation domain include sequences that extend into or beyond the DNA-binding domain?

T antigen transactivates a variety of viral and cellular promoters (1). Among them, only the SV40 late promoter constructs contained sites for specific binding of T antigen. Therefore, if binding to DNA were important for transactivation, this would have to be mediated through T antigen's nonspecific DNA-binding activity. Furthermore, two mutant proteins which retained a reduced level of transactivation, the 138-amino acid N-terminal fragment of *dl*A2420 and the full-length protein of *in*A2807, were defective for DNA binding. The truncated protein produced by *dl*A2420 lacks the DNA-binding domain, while the full-length protein produced by *in*A2807 failed to bind to DNA specifically. This suggests that site-specific DNA binding cannot be absolutely required for transactivation by T antigen.

T antigen could contain two distinct transactivation domains—the first with the N-terminal 138 amino acids and the second within the DNA-binding domain. Full transactivation could require the presence of both. Our data indicate that a low level of transactivation occurred when only the first 138 amino acids were present. However, no transactivation was seen with some mutants with lesions in the DNA-binding domain, even though the normal N-terminal 142 (*dl*A2411) or 167 (*in*A2815) amino acids were present. This argues against the existence of two independent transactivation domains. Whether transactivation activity would be displayed by the DNA-binding domain alone or by mutant proteins which were initiated downstream from the normal initiation site will require analysis of additional mutants.

If a transactivation domain were located at the amino terminus, activity of this domain might require a particular conformation or orientation with respect to the remainder of T antigen. Mutants with lesions in the DNA-binding domain which retained DNA-binding activity probably possess an overall conformation similar to that of wild-type T antigen. Mutants such as *in*A2815 which have lost the ability to bind to SV40 DNA and to transactivate the SV40 late and RSV LTR promoters could be defective for both binding and transactivation because of either altered conformation or the specific loss of DNA-binding activity. Distinguishing between these possibilities will also require analysis of additional mutant T antigens.

An additional possibility consistent with the data presented above is that the domain most important for transactivation maps to sequences extending from upstream of amino acid 85 into or through the DNA-binding domain. All mutants with lesions in the first exon retained some ability to transactivate, and these mutations could have altered the overall conformation of the proteins, thereby reducing but not eliminating their ability to transactivate. By this model, amino-terminal fragments of T antigen would be able to transactivate at a reduced level, since they would retain part of a transactivating domain. Since the first 167 residues of *in*A2815 T antigen are wild type and this mutant totally failed to transactivate, this model would also require that this and other mutant T antigens which completely failed to transactivate fold so as to prevent any activating domain from interacting with its target.

Another region where mutations substantially decreased the transactivation activity of T antigen was the zinc finger motif and sequences adjacent to it. Mutants with mutations within (aa 302, *in*A2807) or nearby (aa 346, *in*A2819) showed a reproducible low level of activity. This region is thought to be important for the overall conformation of T antigen and to coordinate the interactions between DNA binding and the ATP-binding/ATPase domains. Mutations in this region could affect the orientation and environment of a specific activation domain and decrease its ability to interact with the targets required for activation of gene expression.

Several mutants which transactivated the RSV LTR less well than did wild-type T transactivated the SV40 late promoter at wild-type levels. This probably reflects the fact that these two transcriptional control regions are quite different (21, 25, 29). Different transcription factors are involved in transcription from these two promoters, and therefore the role played by T antigen in transactivation of each may be different. Consequently, mutant T antigens which show partial transactivation of one promoter may fully transactivate the other if different T antigen-transcription factor interactions are involved.

How does T antigen transactivate? The mechanism by which T antigen transactivates is not understood. We can imagine various possibilities. T antigen could interact directly with transcription factors at the promoter. Currently, there is little evidence for this. Although addition of T antigen to in vitro transcription systems increases the rate of transcription initiation, these increases are smaller than those seen in vivo (31, 57, 64). Alternatively, T could act indirectly. For example, T could act to increase the activity or abundance of one or more transcription factors. Gallo et al. have shown that there are quantitative and qualitative changes in proteins able to bind to critical sequences within the SV40 late promoter in monkey kidney cells expressing T antigen (21). SV40-infected cells contain 100-fold higher levels of Sp1 mRNA and protein than do uninfected cells (68). Genes which respond to increased Sp1 levels would therefore be activated indirectly by T antigen.

What, then, does it mean for a mutant T to transactivate at a reduced level? It could mean that there is less mutant T antigen, but we think this unlikely, since most mutant T antigens have nearly wild-type stability (86) and some T antigens which transactivate well are quite unstable (e.g., *dl*A2433). A second but related possibility concerns post-translational modifications. T antigen is phosphorylated at multiple sites, glycosylated, ADP-ribosylated, and adenylylated. Many of these modifications occur on only a fraction of T antigen molecules, leading to a diverse spectrum of T antigen species. Perhaps only some are active in transacti-

vation, and the relative abundance of different forms could be affected by mutation. Third, if T transactivates through multiple mechanisms, mutants with partial activity could retain wild-type activity for one (or more) but be inactive in another. Finally, mutants with reduced activity could continue to play the normal roles of wild-type T but interact less effectively with the intracellular targets important for this function.

A further question concerns the role of small t antigen in transactivation. Small t has been reported to transactivate the adenovirus E2 promoter but not the SV40 late promoter (47). The fact that some of the mutants were completely defective for transactivation yet produced a wild-type small t suggests that small t was not playing a significant role in transactivation of either of the promoters which we studied. Loeken et al. (47) have suggested that small t can enhance the ability of limiting amounts of large T to transactivate. A related possibility is that small t could enhance the activity of a transactivation-defective T antigen that is produced at normal levels. Additional experiments will be required to examine this possibility. It is worth pointing out that mutants with lesions in the first exon, which showed reduced transactivation activity, encode mutant small t antigens in addition to mutant large Ts.

Are there correlations between transactivation by T antigen and its other activities? Understanding how the transactivation activity of large T is related to its other properties is one of the main purposes of these experiments and others ongoing in our laboratory. The studies presented here suggest that some activities of T antigen are not important to its transactivation function. Mutants defective in binding of retinoblastoma susceptibility gene protein (K-1) (15), in nuclear localization (SVcT [42]), in ATPase activity (*dl*A2432), in p53 binding (*dl*A2433), and in the host range-adenovirus helper function activity (*dl*A2459) transactivated approximately as well as wild-type T antigen.

Previous work has demonstrated that immortalization of primary mouse embryo fibroblasts requires most of large T antigen (77) but that the first 127 amino acids of large T are not needed. Transformation of C3H-10T1/2 mouse cells requires only the N-terminal 120 to 130 amino acids (73), but most studies have shown that transformation of many other mouse and rat cell lines occurs with wild-type efficiency only if most of the first 600 amino acids are present (59, 77). Mutant *dl*A2433 is defective for immortalization of mouse embryo fibroblasts (77, 80) yet transactivates both promoters examined at wild-type levels. Mutant *dl*A2411 was completely defective for transactivation but immortalizes mouse embryo fibroblasts (77) and transforms both mouse 10T1/2 and rat REF-52 cell lines at wild-type efficiency (86a). Together, these data suggest that the ability of mutant T antigens to immortalize and transform is genetically distinct from their transactivation activities. However, we cannot eliminate the possibility that transformation, immortalization, or both require the transactivation of certain cellular promoters; this ability could be lost in mutants which still transactivate the SV40 late and RSV LTR promoters but have lost the ability to immortalize or transform. In addition, mutant T antigens could have a different spectrum of transactivation activities in cell lines of different types or derived from different species.

Caution is clearly required in deciding whether T antigen's ability to transactivate correlates with any of its other properties. Some of these properties are easily measured biochemical activities. Examples include ATP-binding, ATP hydrolysis, and DNA binding. One can determine whether a mutation that affects these properties alters the K_m, the V_{max}, or the binding constant for DNA. Other properties of T, such as immortalization, transformation, and stimulation of quiescent cells to undergo DNA replication, are considerably more complex and represent a phenotypic endpoint which may be several steps downstream from a direct biochemical action of T antigen. For example, if immortalization required the transactivation activity of T antigen, we can imagine that immortalization might not be seen at all if transactivation activity fell below a particular threshold, or, conversely, that nearly wild-type activity for transactivation could be required to obtain immortalization at levels exceeding 10% of wild-type levels.

In summary, our studies show that the sequences in the N-terminal region of large T play a role in transactivation. In the context of a full-length T antigen, mutations in additional regions of the protein also affect transactivation. These other regions may also have transactivating activity. However, we favor the idea that the overall conformation of T is important for transactivation and that this activity will be affected by two sets of mutations. One set includes those that directly modify the portion of the protein which mediates transactivation. The other are mutations which do not affect this region but alter the overall conformation of T antigen and thereby compromise its ability to perform its transactivation function.

ACKNOWLEDGMENTS

The first two authors contributed equally to this work.

We thank the members of our laboratory as well as Peggy Bradley and Ellen Fanning for ongoing discussions and thoughtful criticisms and Nancy Speck for critical reading of the manuscript. We thank Satvir Tevethia for monoclonal antibodies and Jim Alwine, Janet Butel, Ellen Fanning, Bruce Howard, Don Jarvis, David Livingston, and Mary Loeken for plasmids.

This work was supported by a research grant (CA-39253 to C.N.C.), a postdoctoral fellowship grant (CA-08835 to P.W.R.), and a core grant to the Norris Cotton Comprehensive Cancer Center (CA-23018), all from the Public Health Service, National Institutes of Health, National Cancer Institute.

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