Activation of CREB/ATF Sites by Polyomavirus Large T Antigen

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Polyomavirus large T antigen (LT) has a direct role in viral replication and a profound effect on cell phenotype. It promotes cell cycle progression, immortalizes primary cells, blocks differentiation, and causes apoptosis. While much of large T function is related to its effects on tumor suppressors of the retinoblastoma susceptibility (Rb) gene family, we have previously shown that activation of the cyclin A promoter can occur through a non-Rb-dependent mechanism. Here we show that activation occurs via an ATF/CREB site. Investigation of the mechanism indicates that large T can synergize with CREB family members to activate transcription. Experiments with Gal4-CREB constructs show that synergy is independent of CREB phosphorylation by protein kinase A. Examination of synergy with Gal4-CREB deletion constructs indicates that large T acts on the constitutive activation domain of CREB. Large T can bind to CREB in vivo. Genetic analysis shows that the DNA-binding domain (residues 264 to 420) is sufficient to activate transcription when it is localized to the nucleus. Further analysis of the DNA-binding domain shows that while site-specific DNA binding is not required, non-site-specific DNA binding is important for the activation. Thus, CREB binding and DNA binding are both important for large T activation of CREB/ATF sites. In contrast to previous models where large T transactivation occurred indirectly, these results also suggest that large T can act directly at promoters to activate transcription.

Polyomavirus large T antigens have a dual role, acting directly in viral DNA replication and transcription but also functioning to alter host cell signaling. The role of large T antigens in viral DNA replication has been extensively studied. Simian virus 40 (SV40) has provided the major model for establishing the mechanisms of cellular DNA replication, and its replication is still the best understood (8, 70). During a productive infection, murine polyomavirus large T initiates viral DNA replication (23). The interaction of large T with AP-1 enhances origin unwinding (29). In addition, association with histone acetyltransferases is involved in large T-mediated DNA replication (87). By analogy to SV40 (73, 85), large T is also likely to participate in the elongation phase of DNA synthesis. Its role in replication is important in other contexts as well. In transformation it is responsible for integration (16) and excision (4) of the viral genome and can also promote recombination (75, 76).

Large T antigens have broad effects on the host cell. Large T, the major transforming protein of SV40, can participate in the transformation of human cells (30, 31). Polyomavirus large T does not transform by itself, and viruses that make only large T do not cause tumors. Much of the difference in phenotype comes from the obvious interaction of SV40 large T with p53 (41, 44). Although murine polyomavirus large T can interact with p53 phosphorylated on serine 18 (17), polyomavirus tumors show no evidence of a block in p53 function seen in SV40 tumors, and cell lines from polyomavirus tumors retain a normal p53 response to DNA damage (18). Nonetheless, polyomavirus large T can cooperate with other oncogenes such as middle T or ras in the transformation of primary cells (40, 60). Similar complementation can also be seen in tumorigenesis (2). Polyomavirus large T has striking effects on cell phenotype. It induces cellular DNA synthesis (27, 63). In the case of SV40 large T, at least four separate functions contribute to the induction of cellular DNA synthesis (19, 32, 56, 72, 83). Polyomavirus large T immortalizes primary cells (1, 58). It prevents differentiation of either myoblasts (47) or preadipocytes (10). Large T can induce dramatic apoptosis (22, 68). Many of these effects depend on the association of large T with the Rb family of tumor suppressors (24, 34, 42, 47, 68).

Given these phenotypes, it is not surprising that large T affects cellular RNA synthesis. Large T is a transcriptional activator of cellular genes. The first target identified was dihydrofolate reductase (38); since then, many others such as the thymidine kinase (TK), human heat shock protein 70 (hsp70), DNA polymerase alpha (pol α), proliferating-cell nuclear antigen, thymidylate synthase, and cyclin A genes have subsequently been identified as targets (39, 51, 54, 64, 68). Transactivation of the TK, pol α, proliferating-cell nuclear antigen, dihydrofolate reductase, and thymidylate synthase genes requires an intact pRb/p107-binding site on large T and is mediated via the cellular transcription factor, E2F (51). The ability of large T to activate these E2F-responsive genes depends upon the presence of an intact N-terminal DnaJ domain that binds hsp70 (67). As shown most clearly for SV40 large T, the role of this chaperone function is to disrupt E2F-Rb family complexes (77). This kind of activation is therefore indirect and does not imply that large T must be functioning directly at promoters.

Although many genes are activated by large T through Rb-dependent mechanisms, transactivation of cellular and viral
promoters by polyomavirus large T can also occur in the absence of pRb/p107 binding (34, 42, 64). For example, the cyclin A promoter can be activated by large T, even when the E2F site is mutated (68). The basis for these non-Rb-dependent transactivations is not well understood. Studies of SV40 large T have suggested that it is a somewhat promiscuous activator (26).

The nature of both the TATA/Inr element and the upstream sequences can contribute to large T activation of promoters. This has led to a description of SV40 large T as being TBP-associated factor (TAF)-like (14, 15).

The purpose of these studies was to examine Rb-independent mechanisms used by polyomavirus large T to activate promoters. The work focused on the cyclin A promoter, because we had previously shown that large T could activate mutant promoters lacking an E2F site even when large T was unable to associate with Rb family members. This work demonstrates that large T can activate CREB/ATF sites. Further, this activation appears to be mediated by both the ability of large T to interact with CREB family members and the ability of large T to bind DNA in a non-sequence-specific manner.

**Materials and Methods**

Cell lines, NIH 3T3 cells, originally obtained from the American Type Culture Collection (ATCC), were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% calf serum (CS, Invitrogen).

**Plasmids and mutagenesis,** pCMV LT (59), pCMV RB- LT (which contains mutations at the L-X-C-X-E motif of polyomavirus large T) (33), pCMV P34S LT (which contains a mutation in the HLP loop of the J domain) (67), pCMV NT (residues 1 to 259) (33), and pCMV-C (residues 264 to 785) (27) have been previously described. Hemagglutinin (HA)-C-terminal domain (CT) was constructed with an HA epitope N terminal to residues 264 to 785 of large T. HA-LT was constructed by cloning large T cDNA into pOZ (52). A glutathione S-transferase (GST) polyomavirus large T DNA-binding domain (DBD) fusion (GST-DBD) was constructed by inserting a large T fragment that includes amino acids 262 to 421 into the BamHI site of pGEX3X. The large T fragment was generated using primers 5'-GACTAAGGCGGTCTACAGA and 3'-CTTAACTGCTGGAAGCCCGTGTTGATCTC. S306p V538A GST-DBD was identified by screening after forced-misincorporation PCR. The CRE-Luc reporter plasmid (pCRE-Luc) came from Strategene. The CAT reporter constructs UP/NV/40E, ATF/SV/40E, and ATF/Hisp70 were gifts from Robert E. Kingston (81). The Gal4TK-Luc construct is a luciferase reporter plasmid containing five Gal4-binding sites upstream of the thymidine kinase promoter. The E2F-Luc reporter was a gift from Amy Y. Lo (74) from a gift from Antonio Giordano. The ~89/+11 cyclin A luc site was mutated as previously described (68). The other ~89/+11 cyclin A promoter mutants were made with the following primers: for the CHR mutant, 5'-CAATGATCGCCGAATATTTCCAGAAGGACGGCGGCG-3' and 5'-GCGGCCGTTCAGGATGCTAAATTTAAGATTGACCGGGTACCG-3'. The large T fragment was cloned downstream of the SV40 large T nuclear localization sequence directly upstream of the SV40 origin. This mixture was incubated with NIH 3T3 cells (9). Polyclonal anti-T antibody was generated by immunizing rabbits with 3 to 40% confluence in 60-mm-diameter plates and generally transfected with 1.5 μg of reporter together with 333 ng to 1.0 μg of pCMV LT (wild type [WT]) or mutant or Gal4 DNA constructs. pSVA PKA was added in the amount of 0.5 μg. The total amount of DNA on each plate was 5 μg. At 48 h after transfection, cells were harvested by three rounds of freezing-thawing in 250 mM Tris-HCl (pH 7.5)-1 mM EDTA-150 mM NaCl and 5 μl from a 100-μl extract was used for luciferase activity determination (7). Chloramphenicol acetyltransferase assays were performed using standard chromographic techniques (3). Thin-layer chromatography plates (EM Separations) were quantitated using ImageQuant software (Molecular Dynamics) to determine the percentage of acetylated [14C]chloramphenicol versus all forms.

**Immunoprecipitations and immunoblotting,** NIH 3T3 cells were transfected with 3 μg of WT or mutant pCMV LT or 5 μg of WT or mutant pCMV HA-CT in 100-mm-diameter plates. Immunoprecipitations were prepared by washing monolayers in phosphate-buffered saline–1 mM CaCl2–0.5 mM MgCl2, 1 mM MgCl2, 1 mM CaCl2, 10% [vol/vol] glycerol, 1% [vol/vol] Nonidet P-40) for 30 min at 4°C. Cellular extracts were incubated with 5 μl of anti-CREB antibody (catalog no. 06-863; Upstate) and protein A Sepharose beads (Amersham) for 4 h, with rocking, at 4°C. After a wash in T extraction buffer, immunoprecipitates were boiled for 5 min in disassociation buffer (62.5 mM Tris-Cl [pH 6.8], 5% [wt/vol] sodium dodecyl sulfate, 25% [vol/vol] glycerol, 0.0075% [wt/vol] bromophenol blue, 50 μl of [β-mercaptoethanol per ml] and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, samples were blotted onto nitrocellulose and analyzed by immunoblotting (82). Antibodies used in blotting included mouse monoclonal PN116 (33) to detect large T and HA11 (Covance) to detect HA-tagged large T or CT.

**DNA fragment-binding (McKay) assay,** McKay assays (48) were done as previously described (59). Briefly, large T extracts were incubated with 20 ng of 32P end-labeled restriction fragments of an EcoRI-DdeI double digest of pUL11 cyclin A E2F-37/-33 and 5 μl of protein A beads (Amersham) and incubated, with mixing, for 1 h. The beads were washed three times with 1 ml of wash buffer to remove weakly bound DNA from the large T immune complexes. The beads with bound DNA were eluted once with 200 μl of 100 mM ammonium chloride (brought to pH 9.5 with ammonium hydroxide) for 30 min at 37°C. The beads were then rinsed with 200 μl of distilled water, and this was pooled with the ammonium chloride elution. The pooled elutes were then deproteinized by phenol-chloroform and chloroform extractions and were ethanol precipitated. An aliquot of the resuspended material was then run on a 1.8% agarose gel in

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RESULTS

Large T activates the cyclin A promoter through the CREB/ATF site as well as the E2F site. Our work on the induction of apoptosis by large T in myoblasts led us to an examination of the cyclin A promoter (68). Cyclin A activation had long been associated with apoptosis (49, 50), and large T expression in myoblasts had shown to increase the levels of cyclin A (22). The presence of large T was able to activate the cyclin A promoter up to 30-fold in NIH 3T3 cells (66). When cells were serum restricted, as in myoblast differentiation experiments, activation was completely Rb-binding dependent (Fig. 1A). This is not surprising, since there is an E2F site in the cyclin A promoter known to restrict activity during much of the cell cycle (65). This is a case where an E2F site functions as a repressor, as we had observed previously for muscle cells (69). Mutation of the E2F site at −37/−33 (mE2F) rendered the promoter susceptible to large T activation even when large T could not bind Rb (Fig. 1A).

Since large T activated the cyclin A promoter independently of the −37/−33 E2F site, genetic analysis of the cyclin A promoter was carried out to determine the large T target. A series of deletions were made that suggested that activity came from the 5′ end of the promoter (data not shown). To confirm this, point mutations were made in the CHR site, NF1 sites, a putative noncanonical E2F site at −67; and the CREB/ATF site (Fig. 1B). Only the mutation of the ATF site had a significant effect on the ability of large T to transactivate (Fig. 1C). Overall the results suggest that large T deals with the repressing E2F site by binding Rb and activates by using the CREB/ATF site.

In confirmation of the cyclin A result, large T was also able to activate a CRE-luc plasmid that contained four CREB/ATF sites upstream of a basal TK promoter (Fig. 2A). In addition, promoters from a set (80) that consists of different basal and upstream elements were used to test the ability of large T to activate. The results showed that large T could activate a promoter independently of the TATA element when an ATF site is inserted upstream of a heterologous basal promoter (Fig. 2B). For SV40 large T, Damania and colleagues (15) have suggested that large T stabilizes the TATA-binding protein and can transactivate various promoters that have the hsp70 TATA element but not when the SV40 early element is present instead. Figure 2B shows that polyomavirus large T is clearly able to activate either kind of initiation element. Although large T can activate promoters that have ATF sites, such as cyclin A, it does not activate all promoters that have such sites. The cyclin D1 promoter has a functional CREB/ATF site (84) that is not affected by large T (data not shown).

Synergy between large T and the CREB family. ATF sites are the targets of the CREB/ATF family of transcription factors. To get an idea of how large T might be working, we examined whether large T could stimulate transactivation caused by the CREB family. Initially we used CREB fused to the Gal4-DBD. This allowed us to focus on a single CREB/ATF family member and provided an opportunity to use a broad range of CREB mutants. CREB had only a small effect in the absence of PKA to stimulate transcription (Fig. 3A). However, when PKA was coexpressed, CREB became a potent stimulator due to its phosphorylation. As expected, a CREB mutant lacking the critical regulatory phosphorylation site (S119A) was unaffected by coexpression of PKA. Large T showed synergy with CREB in activation even when PKA was not present. Strikingly, large T also showed significant synergy with CREB S119A. This indicates that large T activation is independent of PKA phosphorylation. Exactly the same results

FIG. 1. Cyclin A promoter regulation by large T. (A) Large T activation of the −37/−33 mE2F cyclin A promoter. 3T3 cells were transfected with 1.5 μg of the indicated reporters and 1.0 μg of either WT or Rb-pCMV LT. Cells were placed in 0.2% CS at 24 h after transfection. The luciferase activity was measured 48 h posttransfection. Values represent the activation (n-fold) (Fold Act.) above control means. (B) Schematic of the cyclin A reporter construct. The −89 to +11 portion of the cyclin A promoter was fused to the luciferase gene (65). The various transcription factor-binding sites and their positions are noted. (C) Mapping large T activation to the ATF site. Cells were transfected as described for panel A except with the indicated mutant cyclin A reporters and WT pCMV LT only. Cells were kept in 10% CS throughout the transfection and harvested 48 h later.
FIG. 2. Large T activates CREB/ATF sites. (A) Large T activation of a 4× CRE-containing reporter. Cells maintained under growing conditions (10% CS) were cotransfected with pCMV LT (1.0 μg) and pCRE-Luc (1.5 μg) and assayed for luciferase activity 48 h posttransfection. (B) Large T activation of ATF-containing promoters with different basal and upstream elements. Cells were maintained as described for panel A and transfected with pCMV LT (1.0 μg) and the indicated reporter constructs (3.0 μg). At 48 h posttransfection, a chloramphenicol acetyltransferase assay was performed and the percentage of acetylated [14C]chloramphenicol was measured with thin-layer chromatography and a PhosphorImager. Values represent activation (n-fold) (Fold Act.) above control (CON) levels.

were obtained with Gal4-CREM τ and the S117A CREM τ mutant that cannot be phosphorylated (data not shown). The CREB family member CREM τ appears to be important in the liver for appropriate expression of cyclin A (66). Large T did not stimulate transactivation by Gal4-p53, indicating that this is not some general effect of large T on the Gal4 system (data not shown). A simple interpretation of the ability of large T to affect CREB activation is that it can interact directly with CREB family members. To address this, extracts of NIH 3T3 cells were immunoprecipitated with anti-CREB antibody and blotted for large T. Figure 3B shows that large T was immunoprecipitated by an antibody against CREB, indicating that the two proteins interact. This is not simply due to overexpression of large T after transfection, since the interaction could also be observed in a cell line stably expressing large T (data not shown).

To examine the basis for the large T interaction with CREB, we made use of a series of Gal4-CREB constructs lacking different portions of the CREB protein. As shown in Fig. 3C, CRG, a construct that lacks the DNA-binding and zipper regions of CREB, was activated by large T in the same manner as WT CREB. The CREB molecule contains a kinase-inducible domain (KID), which is PKA dependent, and a constitutive activation domain (CAD), which is PKA independent.

Making use of a series of CRG mutants, we asked whether large T could synergize with CREB through the KID, CAD, or Q domain. Our results show that large T activated the CAD as well as it activates CRG (Fig. 3C), suggesting that the synergy seen between large T and CREB is largely happening via the CAD of CREB. It has been reported that there are three regions within the CAD that are required for constitutive activity of CREB (88). Large T failed to synergize significantly with any of the three regions alone (data not shown), which suggests that multiple CAD determinants may be involved in activation by large T.

**Genetic analysis of large T activation of CREB/ATF sites.** To look more closely at the sequences in large T that are required for CREB/ATF activation, we used constructs that expressed large T fragments in our cyclin A reporter assays. Large T can be readily cleaved with protease between residues 260 and 280, suggestive of a hinge region (31). Fragments consisting of residues 1 to 259 (NT) and 264 to 785 (CT) can be expressed independently and can function autonomously (27) (Fig. 4A). Both NT and CT include multiple domains that have distinct functions. The N-terminal domain contains both the Rb-binding site and the J domain required for E2F activation. It is capable of promoting cellular DNA synthesis in the absence of CT. CT contains a DNA-binding domain (DBD), a Zn-binding element, and an ATP-binding domain. CT is sufficient to drive viral DNA replication in growing cells.

Since NT is well known to affect cell cycle, we first tested its ability to activate the −37/−33 cyclin A promoter. As shown in Fig. 4B, NT lacked activity. When expressed at higher levels, it appeared to be toxic, reducing the values somewhat below control levels (data not shown). Next, NT was extended to include the next structural module in large T, namely, the DBD. Previous studies performed on the basis of deletions showed that residues 282 to 398 were sufficient to allow DNA binding (78). However, the specific activity of large T truncated at residue 398 was substantially lower than that of longer constructs. Also, the SV40 sequence at residue 260, the end of the T-antigen-origin-binding domain (46), corresponds to residue 414 in polyomavirus. From Sunstrom’s data (78), it appeared that constructs ending around residue 420 should be stable. Therefore, the construct 1-420 was made by PCR mutagenesis. This protein is well expressed (Fig. 4B). It also binds polyomavirus origin-containing DNA in a manner similar to that seen with the full-length protein (data not shown). 1-420 was quite active in its ability to activate the −37/−33 cyclin A promoter.

The 1-420 result raised the possibility that CT might also have transcriptional activity toward the −37/−33 cyclin A promoter. When CT was tested at protein levels comparable to large T, it had a modest ability to activate (Fig. 4C). To achieve a level of activation similar to that seen with full-length large T, substantially higher levels of CT were needed. Since it is present both in 1-420 and in CT, it seemed logical to test the function of the DBD directly. This was done by truncating CT to give the construct 264-420. However, the DBD alone (264-420) had little or no activity (Fig. 4C). Immunofluorescence experiments revealed that 264-420, unlike CT, was cytoplasmic (data not shown). This result was quite unexpected, since the two proteins have the same nuclear localization sequence. To try to localize the DBD to the nucleus, the SV40 large T nuclear localization sequence was inserted directly upstream of
the HA tag of the 264-420 construct. The DBD was then able to localize to the nucleus (data not shown). Strikingly, this DBD construct (NLS 264-420) was able to activate the promoter (Fig. 4C), although its activity was lower than that of full-length CT.

Genetic analysis of the CREB synergy yielded similar results (Fig. 4D). NT was without activity. When the DBD was added to NT (1-420), substantial activation was observed. CT could synergize well when expressed at high levels. The nuclear version of the DBD NLS 264-420 was also quite active. Therefore, any large T construct containing the DBD was capable of synergy in the CREB activation assay.

The role(s) of the DBD. The data showed that the DBD had the ability to activate CREB/ATF sites. Genetic analysis was next used to probe the role of the DBD in transactivation. Large T is capable of binding GAGGC sequences through its DBD (12, 13, 55, 62). With the possible exception of the inhibitin promoter (45), it has generally been thought that this activity is unimportant for the ability of large T to transactivate cellular promoters (39). In SV40 large T, the A1 and B2 elements are necessary for sequence-specific recognition of the pentanucleotide within the SV40 origin (71). Serine 306 in polyomavirus large T, judging on the basis of sequence homology to SV40, V358 of polyomavirus large T is in the B2 element. The S306P/V358A double mutant did not bind origin-specific DNA (data not shown). Figure 5B shows that the WT DBD, purified from E. coli, had a large amount of cellular DNA associated with it, as determined by ethidium bromide staining. Pretreatment with DNase I abolished the ethidium staining (data not shown). S306P/V358A, while equally well expressed, had very little DNA associated with it. This suggested that S306P/V358A had relatively little affinity for DNA regardless of sequence.

To examine the connection between DNA binding and activity toward the cyclin A promoter, S306P, the mutant defective in origin binding, was assayed and found to have two-thirds the activity of wild-type large T (Fig. 5C). This suggests that site-specific (GAGGC) binding is not important for large T activation of the cyclin A promoter. This result is not surprising, considering that the cyclin A promoter fragment used in our studies does not contain any GAGGC consensus sequences. The double mutant S306P/V358A retained very little activity (Fig. 5C). This suggests that general affinity for DNA is important for transactivation of the cyclin A promoter by large T.

If nonspecific DNA binding is important with respect to the...
ability of large T to transactivate, then S306P/V358A large T might be able to participate in activation if DNA binding could be provided independently of the polyomavirus DBD. The ability of NT and CT-gal4 fusions to activate the Gal4-TK luciferase promoter was therefore tested. The C-terminal domain of large T was sufficient to cause substantial promoter activation, while NT was without effect (Fig. 6A). The large T mutant defective in non-sequence-specific DNA binding gave a result in this kind of assay that was very different from that seen in experiments measuring CREB/ATF site activation. Gal4-CT S306P/V358A had substantial activity, showing about 50% the activity of WT Gal4-CT. This indicated that it could act as a coactivator if it could get to the promoter. However, the large T S306P/V358A mutant was almost completely defective at activating CREB/ATF sites (Fig. 5C) or at synergizing with CREB or CREM (data not shown). The results again argue that the DNA-binding function of large T is part of its ability to activate these promoters.

Intriguingly, the role of the DBD in large T transactivation of an E2F-containing promoter is different than that seen with the CREB/ATF site of the cyclin A promoter. Figure 6B shows clearly that the addition of the DBD to NT increases its ability to transactivate E2F sites. However, the nuclear 264-420 lacks any activity toward E2F sites (data not shown), presumably...
because it lacks an Rb-binding site. To test the role of non-specific DNA binding in activation of E2F sites, NT was fused at its C terminus to *E. coli* DnaB. The rationale was that DnaB shares two important properties with large T, namely, the ability to bind DNA nonspecifically and the ability to form hexamers. The resulting fusion protein was then tested in both cyclin A and E2F promoter assays (Fig. 6B). While the chimera significantly activated the E2F-containing promoter, it was inactive with respect to supporting cyclin A transactivation. This result is expected from the observation that nuclear 264-420 could provide both the DNA binding and activation activity needed for CREB/ATF sites.

CREB-binding assays suggest that the DBD contributes to the ability to bind CREB. Figure 7A shows that as measured by coimmunoprecipitation, NT associates weakly, if at all, with CREB. The addition of the DBD to 1-420 clearly allowed binding to CREB. Notably, the mutant S306P/V358A large T retained the ability to associate, indicating that nonspecific DNA binding is not required for association with CREB. As shown in Fig. 7B, CT could be coimmunoprecipitated with

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**FIG. 5.** Analysis of large T mutants defective in DNA binding. (A) The LT mutant, S306P, fails to bind to the polyomavirus origin. Cells transfected with 3.0 μg of pCMV LT or pCMV S306P were incubated with 32P end-labeled restriction fragments of an EcoRI-DdeI double digest of pUCori, a plasmid containing the polyomavirus origin. The mixtures were then immunoprecipitated with anti-T serum, and the DNA associated with the large T immune complexes was isolated. Binding to the origin is marked by the presence of the 454-bp EcoRI fragment, denoted by an arrow. Expression of large T and S306P in the cell extracts is shown as a blot probed with PN116 antibody. (B) The S306P/V358A double mutant has a lowered affinity for DNA. Total DNA from *E. coli* cells expressing either WT or S306P/V358A GST fusion proteins was purified and run out on an agarose gel and subjected to ethidium bromide staining. Expression of the GST fusion proteins is shown by Coomassie staining. (C) Non-sequence-specific DNA binding is important for large T transactivation of the cyclin A promoter. Cells were assayed as described for Fig. 4B. Values shown represent activation (n-fold) (Fold Act.) above control (CON) levels. Extracts were blotted with PN116 to detect large T protein.

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**FIG. 6.** The role(s) of the DBD. (A) Large T, when tethered to a promoter, acts as a coactivator. Cells were cotransfected with 1.5 μg of TK-Gal4-Luc reporter and 1.0 μg of Gal4-NT, Gal4-CT, or Gal4-CT S306P/V358A. Luciferase activity was measured 48 h posttransfection, and the values were quantitated as activation (n-fold) (Fold Act.) above control (CON) levels. (B) The role of DBD in E2F site activation is different from CREB/ATF site activation. Cells were cotransfected with either 1.5 μg of −37/−33 mE2F cyclin A reporter or 1.5 μg of E2F reporter and 1.0 μg of pCMV LT, pCMV NT, or pCMV NT/DnaB. Luciferase activity was measured as described for panel A. Values shown represent activation (n-fold) (Fold Act.) above control levels.
CREB. This is consistent with its ability to activate. However, the DBD (264-420), even in its nuclear form, could not be immunoprecipitated with CREB. The overall conclusion from these experiments is that the DBD contributes to a CREB interaction that also involves other determinants N terminal or C terminal to it.

**DISCUSSION**

Most attention on the ability of large T to activate cellular genes has focused on indirect mechanisms (64, 68). The ability of large T to dissociate Rb-E2F complexes (77) generates active E2F that can stimulate a broad spectrum of genes. This work demonstrates large T activation of CREB/ATF sites. More importantly, this indicates another mechanism by which large T can function, namely, by synergizing with transcription factors acting at promoters. In the case of CREB/ATF sites, there are clear precedents for such behavior. ACT (21) can function as a coactivator for CREB in a CBP-independent manner. Recently, Oct1 has been shown to participate in CREB activation of the ATF site in the cyclin D promoter independently of CREB phosphorylation (6).

The DBD of large T is clearly important for the ability of large T to transactivate CREB/ATF sites. Both 1-420 and CT had the ability to activate promoters and to synergize with Gal4-CREB to activate the TK Gal4 promoter. These two constructs share only the large T DBD. When localized to the nucleus, the DBD (NLS 264-420) was active in these assays. It is curious that 264-420 by itself was cytoplasmic and inactive. The sequence from 280-286 contained in this domain is known to be sufficient for conferring nuclear localization on large T (33) and CT (data not shown). Since phosphorylation is known to be able to effect nuclear localization signals (36) and since phosphorylation is known to occur at sites adjacent to the 280-286 NLS (5), it is possible that differences in modification explain the failure of 264-420 to reach the nucleus. In any case, when a SV40 nuclear localization signal was fused to 264-420, it became nuclear and acquired the ability to transactivate.

The ability of large T to interact with CREB/ATF family members is an important element in large T function. Our experiments show directly that large T associates with CREB. Presumably the DBD is involved in the interaction, since 1-420 bound CREB whereas NT did not, CT was shown to bind CREB, while the DBD did not. These results can be interpreted to indicate that more N- or C-terminal sequences act together with determinants in the DBD to bind CREB. When different Gal4-CREB constructs were used to map the effect of large T on CREB, the CAD was found to be responsive to large T. Presumably this contains the primary large T-binding site. Large T likely can associate with other members of the CREB/ATF family. Synergy experiments with Gal4 constructs demonstrated that large T reaches CREM+ and ATF1 but not ATF2 (data not shown). Therefore, large T does not interact with all family members, just as it does not activate all ATF sites. It is entirely possible that the results presented here for CREB/ATF sites may be relevant to other kinds of transcription factor sites as well. Polyomavirus large T has been reported to interact with Jun and Fos (29, 35). It is possible that large T is a kind of scaffold that holds transcription complexes together, interacting with both transcription factors and coactivators.

The ability of large T to associate with DNA is also important. Although large T recognizes GAGGC sequences specifically, an origin-binding-defective mutant like S306P remains active in transcription assays. It is only when the ability to bind DNA nonspecifically is lost (S306P/V358A) that the ability to activate is lost. It is possible that preferences for different flanking DNA sequences might affect whether a particular ATF/CREB site is large T responsive. Three observations support the idea that DNA binding is the function affected by the S306P/V358A mutations. First, modeling based on the structure of the SV40 DBD (46) places these mutations in the A1 and B2 loops (71) that contact the DNA. Second, S306P/V358A retained the ability to associate with CREB in coimmunoprecipitation experiments, which is the other function of the domain. Third, Gal4-S306P/V358A CT retained much of
the activating ability of WT CT. This indicates that the S306P/V358A mutations are only important when no alternative mode of DNA binding is available. We have looked unsuccessfully for changes in CREB gel shifts upon large T expression. Bands corresponding to ATF/CREB/CREM complexes are observed, but there are no differences in results when large T is present. It is likely that this is connected to the fact that it is generally difficult to see large T gel shifts even on polyomavirus DNA and/or to see gel shifts with coactivators such as p300.

The role of the DBD depends on the situation. For E2F activation, the domain contributes to activity, since 1-420 is much more active than NT. However, its role there seems to be nonspecific, since an NT-DnaB chimera could activate E2F sites. In cyclin A activation, the DBD is multifunctional. It has a role in DNA binding, and it allows association with CREB. This is reminiscent of the DBD of SV40 large T, which can interact with TEF1 (28) as well as bind DNA. This point is underscored by the failure of the NT DnaB chimera to activate the cyclin A promoter even though it activates E2F sites. Although the chimera provides the DNA binding, it fails to provide the CREB association. What could large T do if it were present at a promoter because of its association with CREB/ATF and DNA? The results shown in Fig. 3A indicate that large T rendered activation independent of CREB phosphorylation by PKA. Ordinarily CREB activation by PKA involves recruitment of CBP coactivator. Large T, like ACT (21), could associate with PCAF (87), p300 (53), and CBP (11). Since CBP is the coactivator, the simplest interpretation would be that large T recruits CBP in a manner that, unlike that seen with CREB/ATF and DNA, results in large T associating with CAD points toward TAF function. CAD is known to interact with TAF 4 (20). Large T could either enhance or replace the TAF 4 interaction. As mentioned earlier, SV40 large T has been reported to interact with TAFs (14) and TATA-binding protein (27). However, unlike the polyomavirus results, effects were initiator specific. A potentially important mechanism is association with histone acetyltransferases. Large T can associate with PCAF (87), p300 (53), and CBP (11). Since CBP is the coactivator, the simplest interpretation would be that large T recruits CBP in a manner that, unlike that seen with CREB, is independent of CREB phosphorylation. However, there are difficulties with such a view. First, genetics has suggested, as shown by the P671L mutant results, that C-terminal sequences are important for p300/CBP binding (11). This is inconsistent with the activity shown by 1-420 and NLS 264-420. Also, the literature suggests that association of large T with p300 and CBP serves to decrease transcriptional activity (11, 53) whereas large T activates cyclin A through the CREB/ATF (CRE) site. Such sites are known to use CBP/p300 as a coactivator. How could that be, if large T inactivates p300/CBP-mediated transcription? The large T interaction must be functioning in a positive manner.

In summary, the data presented here suggest a new view of large T action with respect to cellular genes in which large T acts directly at promoters. The best analogy would seem to be to results seen with the human T-cell leukemia type 1 Tax protein (25, 89). Tax stimulates human T-cell leukemia type 1 transcription through interaction with viral CREs. These sequences are binding sites for Tax in complex with ATF/CREB family members. Tax binds to the viral CRE sequences through DNA interactions and interactions with the bZip region of CRE-bound CREB (79). The formation of this promoter-bound Tax/CREB complex is critical for the recruitment of the multifunctional cellular coactivators CBP and p300. Future work will determine what cellular genes besides cyclin A might be regulated by polyomavirus in this manner and how they contribute to viral function.

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