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# An N-Terminal Deletion Mutant of Simian Virus 40 (SV40) Large T Antigen Oligomerizes Incorrectly on SV40 DNA but Retains the Ability To Bind to DNA Polymerase α and Replicate SV40 DNA In Vitro

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**A peptide encompassing the N-terminal 82 amino acids of simian virus 40 (SV40) large T antigen was previously shown to bind to the large subunit of DNA polymerase α-primase (I. Dornreiter, A. Höss, A. K. Arthur, and E. Fanning, EMBO J. 9:3329–3336, 1990). We report here that a mutant T antigen, T83-708, lacking residues 2 to 82 retained the ability to bind to DNA polymerase α-primase, implying that it carries a second binding site for DNA polymerase α-primase. The mutant protein also retained ATPase, helicase, and SV40 origin DNA-binding activity. However, its SV40 DNA replication activity in vitro was reduced compared with that of wild-type protein. The reduction in replication activity was accompanied by a lower DNA-binding affinity to SV40 origin sequences and aberrant oligomerization on viral origin DNA. Thus, the first 82 residues of SV40 T antigen are not strictly required for its interaction with DNA polymerase α-primase or for DNA replication function but may play a role in correct hexamer assembly and efficient DNA binding at the origin.**

The large T antigen of simian virus 40 (SV40) is the only viral protein essential for the replication of SV40 DNA in permissive cells (for reviews, see references 7, 10, 11, 55, and 63). T antigen has evolved an impressive array of functions to direct the efficient replication of the viral DNA, including site-specific DNA binding to the SV40 origin region; ATPase, helicase, and DNA-unwinding activities; and direct protein-protein interactions with cellular replication factors, such as DNA polymerase α-primase and the single-stranded DNA-binding protein RP-A (8, 17, 18, 44). In the SV40-infected cell, T antigen also interacts with cellular regulatory proteins and transcription factors such as pRb, p107, p130, p53, p300, TBP, AP-2, and TEF-1 (2a, 28, 34a, 71; reviewed in references 10, 19, 20, and 21). Correlation of deletion and point mutants of T antigen with these specific biochemical activities has led to the identification of a number of functional domains in the polypeptide, such as the minimal SV40 origin DNA-binding domain (1), a helicase domain (69), a zinc finger motif (34), binding sequences for ATP, pRb, and p53 (reviewed in references 10, 20, 21, and 55), and several regions contributing to the stimulation of cellular proliferation by T antigen (12, 14). However, most of the T antigen protein appears to be required for SV40 DNA replication activity in vivo (11).

Interactions of T antigen with the cellular DNA polymerase α-primase, an important event in early stages of viral DNA replication (8, 9, 13, 15, 17, 48, 63), have been mapped to two regions of T antigen. Initial experiments showed competition between polymerase α-primase and p53 for binding to T antigen, suggesting an interaction of polymerase α-primase with the C terminus of T antigen (22). This interpretation was supported by the finding that monoclonal antibodies against T antigen whose epitopes mapped within the p53 binding domain blocked T antigen-polymerase α-primase binding (8, 18, 23, 27, 58, 61). However, it was also shown by modified immunoblotting experiments that T antigen bound specifically to the catalytic p180 subunit of the polymerase α-primase complex and that a binding site for p180 was located within the first 82 residues of T antigen (15, 17, 18).

The notion that the polymerase α-primase interaction with the N-terminal domain of T antigen could be functionally significant is in agreement with the observations that some mutations in the coding sequences of the first exon of T antigen caused defective viral SV40 DNA replication in vivo (26, 43, 52, 54). However, interpretation of these results must include the possibility that mutant T antigen proteins were unstable in vivo or that mutant T antigen may have been defective for oligomerization or other biochemical functions required for replication. D2-T, a hybrid protein composed of residues 115 to 708 of SV40 T antigen and more than 100 N-terminal residues derived from an adenovirus protein and from an unknown source (2), was only 20 to 30% as active as wild-type T antigen in DNA replication in vitro (35). Since several other properties of D2-T were aberrant and it is unknown what effects the "foreign" residues have on the protein, the role of the N-terminal residues in T antigen cannot be deduced from these data. Thus, the role of the N-terminal 82 residues in viral DNA replication remains unclear. It has been reported that under some circumstances the N terminus of the related polyomavirus large T antigen was dispensible for polyomavirus

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origin DNA replication (25). However, since the domain organizations of SV40 and polyomavirus T antigens are somewhat different, direct comparisons between the structure and function of these proteins are not always possible. We have now reexamined the question of whether the N terminus of SV40 T antigen is required for its individual biochemical activities and for SV40 DNA replication in vitro by using a truncated T antigen mutant, T83-708 (previously called Tx) (1, 46).

Recombinant-baculovirus-expressed T antigen (37) has proved highly active in cell-free SV40 DNA replication assays (39, 49), as a result of the replication-active under-phosphorylated form of the protein expressed in this system (33). Therefore, we compared the replication-related biochemical activities of wild-type and T83-708 T antigens purified from insect cells. Both T antigens bound to DNA polymerase α-primase. Peptides comprising the N-terminal T antigen residues alone also bound polymerase α-primase, implying that two independent regions of T antigen must interact with DNA polymerase α-primase. The ATPase, helicase, and SV40 site I DNA-binding activities of T83-708 were nearly equivalent to those of wild-type T antigen. However, the core origin DNA binding and in vitro DNA replication activities of T83-708 were reduced compared with those of wild-type T antigen. Hexamer assembly of T83-708 in the presence of ATP was inefficient, resulting in appreciable amounts of T83-708 monomers and trimers as well as the hexamers detected with the wild-type protein and some higher oligomeric structures. In the presence of ATP and SV40 origin DNA, only aberrant higher-oligomer forms of T83-708 were detected.

In summary, our results suggest that the N-terminal region of T antigen is involved in correct oligomerization of T antigen on SV40 origin DNA and stable site II DNA binding, thus enhancing the assembly of or stabilizing replication-competent T antigen-DNA complexes.

## MATERIALS AND METHODS

**Cells and antibodies.** The human cell line 293 was cultured in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal calf serum (FCS). *Spodoptera frugiperda* cells (Sf9) and *Autographa californica* nuclear polyhedrosis virus (AcNPV) were kindly provided by Max Summers (Texas A&M University) and Walter Doerfler (Institute for Genetics, Cologne, Germany). The Sf9 cells were grown in either spinner or monolayer cultures at 27°C in Excell-401, a serum-free defined medium (JRH Scientific), or TC-100 (GIBCO-BRL) with 10% FCS.

Antibodies were purified by protein A-agarose affinity chromatography as described previously (18) from mouse hybridoma cell lines producing T-antigen-specific monoclonal antibodies PAb419 (31), PAb101 (29, 30), and PAb204 (36). 2CT25, a monoclonal antibody specific for the p180 subunit of DNA polymerase α, was used for detection of p180 in the enzyme-linked immunosorbent assay (ELISA) (16).

**Proteins.** The baculovirus vector vAc373Tx was constructed by ligating an N-terminal oligonucleotide linker encoding a methionine codon and lysine 83 to second-exon DNA sequences of wild-type SV40 T antigen (via the *Pfl*MI site; note that the published linker sequence is incorrect) (1, 46). Recombinant baculoviruses were used to express wild-type and truncated T83-708 in Sf9 insect cells. After insect cells were harvested at 40 h post-infection, the T antigen protein was purified by PAb101 immunoaffinity chromatography (3, 33). The concentrations of T antigens were usually 0.5 to 0.9 mg/ml (wild type) and 0.3 to 0.5 mg/ml (T83-708), as determined spectrophotometrically (24). Preparations were greater than 90% pure, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie brilliant blue staining. Purification of both wild-type T and T83-708 antigens from infected Sf9 cells yielded preparations with some variation in activity. For example, when T83-708 was harvested late after infection, it bound poorly to origin DNA and was unable to replicate SV40 DNA in vitro or to bind DNA polymerase α-primase (18).

T antigen peptides T1-130 and T131-259 were purified from *Escherichia coli* extracts. T1-130 was expressed from a pUC9 vector and purified by immunoaffinity chromatography with PAb419 as described previously (1, 18). T131-259 was expressed as an N-terminal glutathione-*S*-transferase (GST) fusion after cloning of a *Bcl*I-*Eco*RI fragment from the pUC9 vector pKT131-259 (34) into the *Bam*HI and *Eco*RI sites of pGEX-3X (62). After purification on glutathione-agarose beads (Sigma, Deisenhofen, Germany), the fusion protein was eluted from the matrix with 5 mM glutathione and cleaved with factor Xa protease (Boehringer, Mannheim, Mannheim, Germany) for 24 h at 27°C, and the cleavage was monitored by gel electrophoresis.

Calf thymus DNA polymerase α-primase was purified by immunoaffinity chromatography as described previously (17, 50).

**DNA polymerase α-primase binding assay.** ELISAs were carried out as described previously with phosphate-buffered saline (PBS) or replication buffer (30 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-KOH [pH 7.8], 0.5 M dithiothreitol [DTT], 7 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid [EGTA], 4 mM ATP, 10 mM phosphoenolpyruvate, 0.76 μg of pyruvate kinase) (17, 18, 58). Plates were coated with 1 μg of purified protein to produce the solid-phase substrate, and binding of the second protein was detected by incubation with a purified specific monoclonal antibody at 1 μg per well in PBS or replication buffer overnight at 4°C. Detection was completed with rabbit anti-mouse immunoglobulin G (IgG) coupled to horseradish peroxidase (1:100 in 3% bovine serum albumin [BSA], 100 μl per well) and the soluble chromogenic substrate 2,2-azino-bis-(3-ethyl-benzothiazoline-6-sulfonic acid). After 15 to 20 min at room temperature, the resulting green color was evaluated spectrophotometrically at $A_{405}$.

**SV40 DNA replication in vitro.** The plasmids pSVori and pUC-HS, containing the whole SV40 origin region (64, 66), were used as templates for the replication assays, which were performed essentially as described before (45, 58, 66). The reaction mixture contained 50 to 75 ng of pSVori or pUC-HS; 30 mM HEPES-KOH (pH 7.8); 0.5 M DTT; 7 mM $MgCl_2$; 1 mM EGTA (pH 8); 4 mM ATP; 0.8 mM (each) CTP, GTP, and UTP; 0.1 mM (each) dATP and dGTP; 5 μCi each of [$\alpha^{32}$P]dCTP and dTTP (3,000 Ci/mmol); 10 mM phosphoenolpyruvate; 0.76 μg of pyruvate kinase; 50 to 500 μg of S100 cytoplasmic protein extracted from human 293 cells; and T antigen proteins as indicated. The reaction mixture had a final volume of 50 to 60 μl. Aliquots of the mixture were incubated for 90 min at 37°C, and the reaction was stopped by the addition of 0.7% SDS, 20 mM EDTA, and proteinase K (0.2 mg/ml), followed by phenol extraction, gel filtration, and ethanol precipitation. Resuspended DNA was linearized with *Eco*RI, digested with *Dpn*I, and electrophoresed in 0.8% agarose gels and then subjected to autoradiography (not shown). DNA synthesis was quantitated by determining the ethanol-precipitated radioactivity in 4 μl of the SDS-treated sample.

**Enzymatic assays.** ATPase assays (3) were performed in a 20-μl reaction mixture containing 50 mM Tris-HCl (pH 7.8), 100 mM NaCl, 1 mM DTT, 2% glycerol, 66 nM [γ-$^{32}$P]ATP (3,000 Ci/mmol), and 1 to 1,000 μM ATP. Reaction mixtures were incubated at 37°C and analyzed after 15, 30, 45, 60, and 90 min. Test samples (1 μl) were applied to polyethyleneimine-cellulose plates (Brinkmann) for thin-layer chromatography in 0.75 M $NaH_2PO_4$. Phosphate release was measured by analysis of the plates by Betagen counting. Activation of ATPase was assayed with 5 μg of purified T antigen or T83-708 protein adjusted to pH 7 and preincubation with 2 mM β-methylene-5′-adenosine triphosphate (AMP-PCP) for 30 min at 30°C (3). Free AMP-PCP was removed by gel filtration through Biogel P10 (Bio-Rad) equilibrated in ATPase buffer. The ATPase activity of the eluate was tested at 5 and 10 μM ATP.

DNA helicase assays (66) used single-stranded, circular DNA (M13mp19) annealed to a 19-base complementary oligonucleotide as the substrate. The annealed primer was 3′-end labeled with Klenow polymerase and three nucleotides, dGTP, dCTP, and [α-$^{32}$P]dATP, to extend the primer. After purification, the DNA hybrid substrate (8 ng) was combined with 2 mM ATP, reaction buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 0.5 mM DTT, BSA [10 μg/ml]), and purified T antigen proteins in a volume of 20 μl. After incubation at 37°C for 1 h, reactions were stopped by adjusting the mixture to 0.33% SDS and 30 mM EDTA. Percent release of radioactive primer from DNA was analyzed after electrophoresis in 10% nondenaturing polyacrylamide gels followed by autoradiography and densitometric analyses or by excising the bands from gels dried onto DE-81 paper (Whatman) and scintillation counting.

**Oligomerization assay.** Hexamer formation was assayed as described before (51) with slight modifications. Briefly, 2 μg of T antigen was incubated in a 10-μl mix containing 30 mM HEPES-KOH (pH 7.8), 7 mM $MgCl_2$, and 1 mM DTT with or without 4 mM AMP-PNP for 30 min at 37°C. In some experiments, SV40 origin DNA, pUC-HS (45, 64), was added at a concentration of 0.5 mg/ml. Then glutaraldehyde was added to a final concentration of 0.2%, and incubation was continued for a further 5 min. Unreacted glutaraldehyde was quenched by adding 0.1 volume of 10 mM HEPES-KOH (pH 7.9)–100 mM glycine and a further 5-min incubation. When DNA was present, DNase I was added to a final concentration of 0.1 U/μl, and incubation was continued for a further 15 min at 37°C. Complexes were analyzed by native gel electrophoresis in 5 to 15% gradient gels (Bio-Rad, Munich, Germany) with 25 mM Tris–19 mM glycine buffer and Western blotting.

**Mobility shift DNA-binding assays.** ATP-dependent assembly of T antigen on core origin DNA was investigated as described before (65). A DNA fragment (*Eco*RI-*Hin*dIII from pOR) (45) containing only the minimal origin was 3′ labeled with Klenow enzyme to a specific activity of 2,500 cpm/fmol. The labeled DNA (8 fmol) was incubated in a 10-μl reaction mixture in binding buffer (30 mM HEPES-KOH [pH 7.8], 7 mM $MgCl_2$, 1 mM DTT, 40 mM creatine phosphate, 0.2 mg of creatine kinase per ml, 4 mM AMP-PNP, 10 ng of pBluescript KSII per μl, 1 mg of BSA per ml) with the indicated amount of T antigen for 30 min at 37°C. Protein was cross-linked to DNA by the addition of glutaraldehyde to a final concentration of 0.2% and a further 5-min incubation. To the reaction

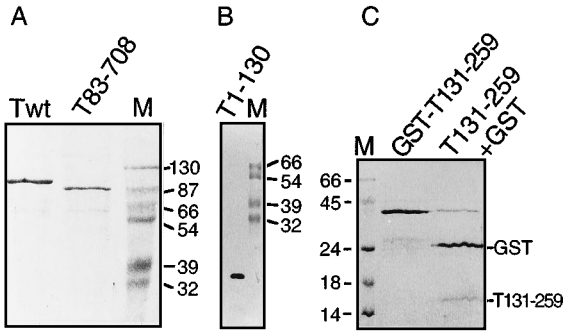


FIG. 1. Purified T antigen proteins from recombinant baculovirus-infected insect cells and from plasmid-transformed *E. coli*. Proteins (1 μg) were electrophoresed in 10% (A) or 15% (B and C) denaturing polyacrylamide gels and stained with Coomassie brilliant blue. The molecular masses of marker proteins are shown in kilodaltons (lanes M). (A) Immunoaffinity-purified wild-type T (Twt) and T83-708 antigens from baculovirus-infected Sf9 cells; (B) immunoaffinity-purified T antigen peptide T1-130 from *E. coli*; (C) fusion protein GST-T131-259 and products after cleavage with protease factor Xa. Equimolar amounts of the cleaved products were present in the peptide mixture loaded into the gel, but T131-259 stained less strongly than GST.

mixture, 0.2 volume of loading buffer (10 mM HEPES-KOH [pH 7.8], 2% Ficoll 400, 0.2% bromophenol blue, 0.2% xylene cyanol) was added, and protein-DNA complexes were resolved by electrophoresis in 3.5% native polyacrylamide gels in TBE (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA) at 200 V. The gel was dried and autoradiographed. Protein-complexed DNA was quantitated by densitometry of the autoradiograms. The relative affinities of wild-type and mutant T antigens for pOR DNA can be estimated by the point of half-maximal saturation by plotting the fraction of shifted DNA versus the protein concentration (see Fig. 8) (6, 32, 57).

**Immunoprecipitation DNA-binding assay.** Plasmids pONwt (site I) and p1097 (site II) (45) were digested with restriction enzymes and end labeled with $^{32}P$ by using T4 polynucleotide kinase. Both sets of DNA fragments (100 ng each) were added together with purified PAb101 antibody (500 ng) and either purified wild-type T or T83-708 antigen (50, 100, and 200 ng) and incubated at 37°C for 30 min. The buffer used was 30 mM HEPES-KOH (pH 7.8)–7 mM magnesium acetate–1 mM DTT–1 mM EGTA–80 mM KCl–20 mM phosphoenolpyruvate–1.6 μg of pyruvate kinase–10 μg of BSA–4 mM ATP. Addition of salt to the replication buffer decreases nonspecific DNA binding. Immunoprecipitated protein-DNA complexes were isolated and analyzed as described previously (45). Substitution of PAb416 for PAb101 yielded similar results.

## RESULTS

**Expression of T83-708 and N-terminal regions of T antigen.** Since preliminary experiments indicated that expression of the second exon of T antigen was relatively inefficient in *E. coli* and barely detectable in monkey cells (1) (data not shown), we chose to express T83-708 in insect cells by using a recombinant baculovirus. Several preparations of wild-type and T83-708 T antigens were purified by immunoaffinity chromatography and characterized by SDS gel electrophoresis followed by Coomassie blue staining. T83-708 migrated faster than wild-type T antigen (about 84 versus 94 kDa) (Fig. 1A), confirming the predicted size of the truncated protein. Both proteins were detected in immunoblots by PAb101, which recognizes a C-terminal epitope, but only wild-type protein was detected by the N-terminus-specific monoclonal antibody PAb419 (data not shown). Two N-terminal peptides derived from wild-type T antigen were expressed in *E. coli* and used to assess the interaction of T antigen with DNA polymerase α-primase as described below. Figure 1B and C shows Coomassie blue staining of the purified peptide T1-130 and GST-fused T131-259 plus its cleavage products.

**Two domains of T antigen bind DNA polymerase α-primase.** Binding of the N-terminal T antigen peptides to native DNA polymerase α-primase was measured in an ELISA. Soluble DNA polymerase α-primase bound to immobilized wild-type T antigen and the T1-130 peptide (Fig. 2A), confirming that a region of T antigen in the N terminus was sufficient for this interaction (18). Neither a peptide encompassing the minimal DNA-binding domain, T131-259 (18, 34), nor BSA, included as a negative control, bound to polymerase α-primase under the same conditions. With T83-708 and the wild-type T antigen in the solid phase, DNA polymerase α-primase bound to both T83-708 and wild-type T antigen regardless of the buffer used in the assay (Fig. 2B and C). To confirm these results, we measured binding of soluble T83-708 and wild-type T antigens to DNA polymerase α-primase immobilized on ELISA plates (Fig. 2D). We found that both T antigens bound DNA polymerase α-primase.

Therefore, we concluded that T antigen has two independent binding sites for DNA polymerase α-primase, one located within the N-terminal region, and a second located between residues 83 and 708. The DNA-binding domain played no direct role in these interactions, suggesting that the C-terminal binding site for polymerase α-primase may be located between residues 260 and 708.

**SV40 DNA replication activity of T antigen deletion mutant T83-708.** These experiments posed the question of whether one or both of the polymerase α-primase binding sites in T antigen were essential for assembly of a replication-proficient complex on SV40 origin DNA. This was tested by using purified T83-708 and wild-type T antigen in cell-free SV40 DNA replication assays. The products of both reactions were resistant to *Dpn*I digestion, but T83-708 was reproducibly less active than wild-type T antigen (data not shown). DNA synthesis was quantitated by using equal amounts of the two T antigen proteins (0.6 μg) in the presence of increasing concentrations of 293 cell extract (Fig. 3A). Although it lagged behind by about 50%, the replication activity of T83-708 increased like that of wild-type T antigen (Fig. 3A). When the 293 extract titration experiment was repeated with 0.6 μg of wild-type T antigen and 3 μg of T83-708, essentially identical amounts of replication products were obtained (Fig. 3B). These results suggest that T83-708 is able to replicate SV40 DNA in vitro with an activity two- to fivefold lower than that of the wild-type protein.

**Comparison of ATPase and helicase activities of T83-708 and wild-type T antigen.** To identify the biochemical defect(s) that could be responsible for the reduction in replication activity of T83-708, we directly compared a number of replication-related activities of purified T83-708 with those of wild-type T antigen. While the ATP binding site of T antigen resides in the C terminus, the N terminus may contain sites of interaction between T antigen subunits that contribute to ATPase and helicase activity (3, 4). We compared the ATPase activity of several preparations of T83-708 with that of the wild-type T antigen and determined the $K_m$ and the $V_{max}$ of both proteins at a range of ATP concentrations from 0.5 μM to 1 mM. The $K_m$ for T83-708 (12 μM) was consistently lower than that for wild-type T antigen (20 μM), indicating that the truncated protein had a slightly higher affinity for the substrate ATP. $V_{max}$ was 0.3 and 0.11 pmol/s for wild-type T and T83-708, respectively. The efficiency of T83-708 ATPase activity was two- to threefold lower than that of the wild-type T antigen preparations ($K_{cat}/K_m$, 370 versus 590 $M^{-1}$ $s^{-1}$). We also tested whether the allosteric effect of ATP on T antigen ATPase activity was affected by the N-terminal truncation. Treatment of wild-type T antigen with AMP-PCP and magnesium stimulated the ATPase activity twofold. T83-708 ATPase activity was stimulated 1.8-fold by treatment with AMP-PCP, which was not significantly different from the wild-type value of 2.0. For comparison, a replication-defective T antigen mutant had a

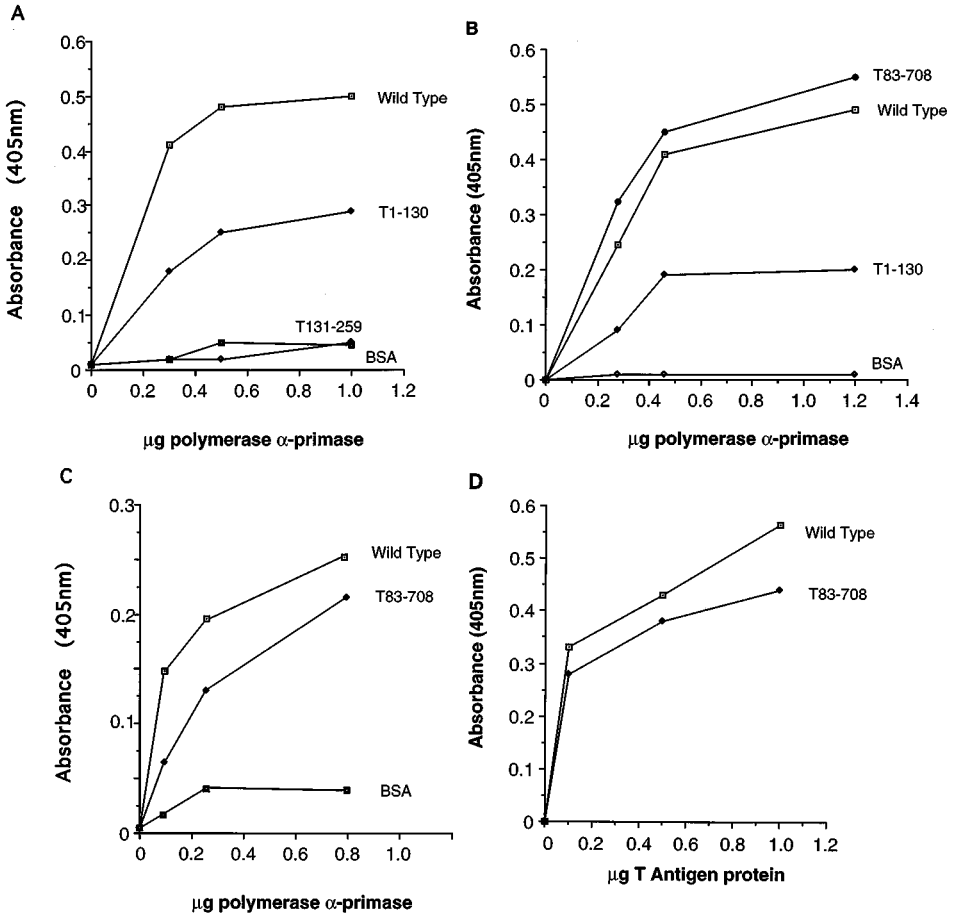


FIG. 2. Mapping the DNA polymerase α-primase binding regions in SV40 T antigen by ELISA. (A) Purified wild-type T antigen from baculovirus-infected Sf9 cells and T antigen peptides from *E. coli*, T1-130 and T131-259, were used in the solid phase. BSA served as a solid-phase control. DNA polymerase α-primase binding to the solid-phase proteins was detected with monoclonal antibody 2CT25, specific for the p180 subunit. (B) The solid-phase substrates were purified wild-type T antigen, T83-708, T1-130, and BSA. DNA polymerase α-primase binding to the solid-phase proteins was detected as for panel A. (C) The experiment for panel B was repeated, with T antigen, T83-708, and BSA in the solid phase, except that PBS was replaced by replication buffer. (D) Purified DNA polymerase α-primase was used in the solid phase, and binding of purified wild-type T and T83-708 antigens was detected with PAb101 antibody.

maximal value of 1.1 (66). Thus, the mutant T antigen resembled the wild-type protein in its interaction with ATP in this assay.

Domain mapping of the DNA helicase activity of T antigen has shown that the region associated with ATP binding and at least part of the DNA-binding region are required for helicase activity (68, 69). We measured the helicase activity of the T83-708 protein by assaying its ability to displace a labelled oligonucleotide from partial duplex DNA. T83-708 activity was about 75% as efficient as wild-type T antigen at concentrations of between 150 and 600 ng and less efficient at lower or higher concentrations (Fig. 4). Therefore, T83-708 was able to undergo the protein-protein and protein-DNA interactions required for the hydrolysis of ATP and melting of double-stranded DNA, but somewhat less efficiently than wild-type T antigen.

**Oligomerization of T83-708.** The next question was whether the deletion of the N terminus in T83-708 affected the allosteric interaction of T antigen with ATP that is associated with the formation of T antigen hexamers and double hexamers. Figure 5 presents evidence that both wild-type T and T83-708 protein samples contained a mixture of monomer, dimer, trimer, and hexamer species (lanes 1 and 3). As expected, after incubation with Mg/AMP-PNP, wild-type T antigen formed predominantly hexamers, and monomers, dimers, and trimers were hardly detectable (lane 2). However, significant amounts of monomers, trimers, and hexamers as well as novel higher oligomeric structures (indicated by the arrow and asterisk in lane 4) were observed with T83-708 protein in the presence of Mg/AMP-PNP.

In these assays, double hexamers of T antigen were not detected, even when ATP was substituted for the nonhydrolyzable AMP-PNP, unless DNA was included. Addition of subsaturating amounts of DNA resulted in small amounts of wild-type T antigen double hexamers and increased amounts of the aberrant higher oligomeric form of T83-708 (data not shown). These results suggested that T83-708 oligomerizes into hexamers less efficiently than wild-type T antigen. T83-708 also formed higher oligomeric structures of unknown subunit composition that are not observed with wild-type T antigen.

**Site-specific DNA binding of T83-708.** Since T antigen protein-protein interactions play an important role in its ability to bind SV40 origin DNA, particularly site II, we tested the site-specific DNA-binding activity of T83-708. For direct comparison of the binding of T83-708 to site I versus site II DNA, we used an immunoprecipitation assay containing both DNA binding sites under replication conditions plus 80 mM KCl (45). The inclusion of 80 mM KCl greatly reduces nonspecific

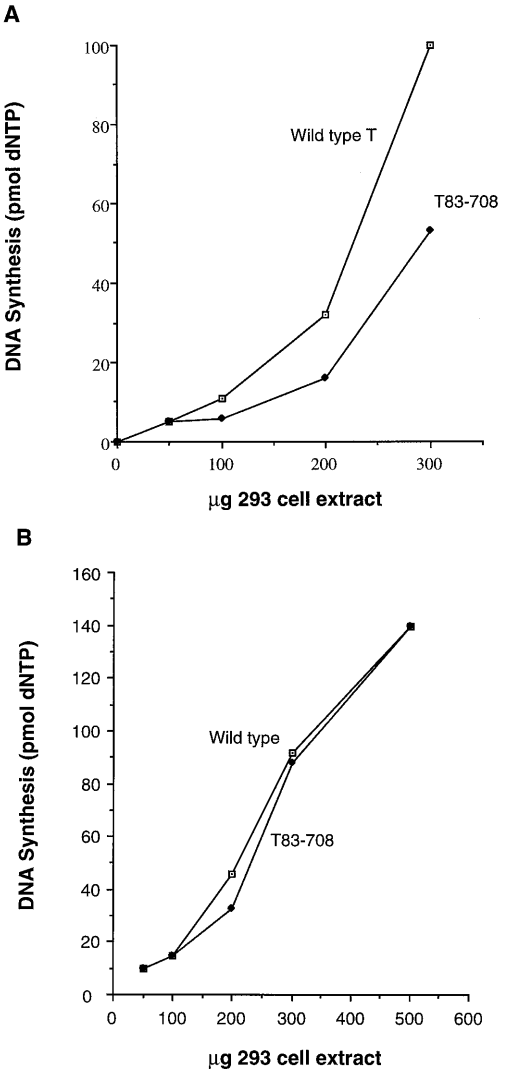


FIG. 3. T antigen and T83-708 SV40 DNA replication activity in vitro. (A) Replication assays were performed with 0.6 μg of wild-type T or T83-708 protein and increasing amounts of S100 extract from human 293 cells, as indicated. The DNA synthesis detected by ethanol precipitation (dNTP incorporation) is shown as a function of T antigen added to the reaction. (B) Replication assays were performed with 0.6 μg of T antigen or 3 μg of T83-708 and increasing amounts of S100 extract from human 293 cells, as indicated.

DNA binding, which interferes with quantification of site II binding. We found that T83-708 bound about 25 to 50% as much site I DNA as wild-type T antigen (Fig. 6, lanes 1 to 3 versus lanes 4 to 6), while T83-708 bound to site II DNA only 5 to 10% as well as wild-type T antigen. This result indicated that T83-708 had a quantitative defect in DNA binding, particularly with site II DNA.

**T83-708 multimers with more than 12 subunits bind origin DNA.** The DNA binding of T83-708 was analyzed directly in replication buffer by band shift experiments, which at the same time assessed whether its origin DNA-binding defect was related to its ability to assemble as a hexamer or double hexamer on site II DNA. DNA complexes containing T83-708 migrated more slowly than those containing full-length T antigen, and indeed some material remained in the pockets of the gel (Fig. 7). We conclude that the shifted bands of T83-708 probably contain more than 12 subunits of the protein.

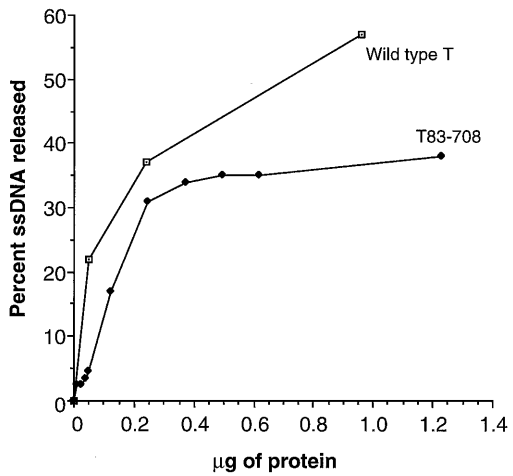


FIG. 4. Helicase activity of wild-type T antigen and T83-708. DNA helicase activity was quantitated by counting the radioactivity in the released oligonucleotide excised from a gel and expressed as a percentage of the total counts in the lane. The 100% value was obtained by boiling the template to release all of the oligonucleotide, and the value for the negative control was the percent oligonucleotide released after incubation with BSA or T antigen without ATP. ssDNA, single-stranded DNA.

Gel band shift assays were also used to determine the concentration of T antigen proteins resulting in half-maximal binding of DNA fragments containing the minimal origin sequences (site II); (Fig. 8) (6, 66). Both wild-type T antigen and T83-708 showed sigmoidal binding curves, suggesting cooperative binding in both cases. Wild-type T antigen reached half-saturation at a concentration of 4 nM, whereas 60 nM T83-708 was required to bind half of the input DNA. Thus, in agreement with the immunoprecipitation assay (Fig. 6), T83-708 exhibits a 15-fold reduction in its ability to assemble as an oligomeric complex on site II DNA compared with the wild-type protein.

## DISCUSSION

T antigen interactions with DNA polymerase α-primase were previously shown to require a C-terminal region of T antigen overlapping the region which binds the cellular protein

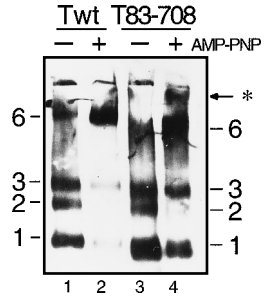


FIG. 5. Oligomeric state of T antigen and T83-708. Wild-type T antigen (Twt, lanes 1 and 2) and T83-708 (lanes 3 and 4) (3 μg) were incubated in the absence (lanes 1 and 3, −) or the presence (lanes 2 and 4, +) of Mg/AMP-PNP. Samples were cross-linked with glutaraldehyde and subjected to native gel electrophoresis in a 4 to 15% gradient polyacrylamide gel. Proteins were transferred to nitrocellulose filters and then incubated with a mixture of antibodies PAb204 and PAb101. Proteins were visualized by electrochemiluminescent detection. The left and right margins show the oligomeric state of wild-type T antigen and T83-708, respectively. The arrow and asterisk indicate higher oligomeric structures that were observed only with T83-708.

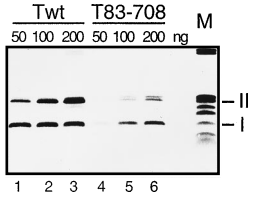


FIG. 6. Specific SV40 DNA-binding activity of T83-708. Immunoprecipitation assays with the indicated amounts wild-type T and T83-708 were performed with nonspecific site I and site II DNA fragments together in replication buffer. Complexes were immunoprecipitated with PAb101 antibody, and the DNA was prepared for electrophoretic analyses in 1.5% agarose gels. The binding of site I and site II DNA was quantitated by densitometric analysis of autoradiograms. Aliquots of labeled DNA fragments were loaded into the side wells as markers (lane M), and the positions of site I- and site II-containing DNA fragments are indicated.

p53 (22, 61). Subsequently it was shown that a binding site for DNA polymerase α-primase was located in the first 82 residues of T antigen (18). These apparently conflicting reports are resolved by the data presented here, which confirm the presence of a second binding site. We show that separate peptides encoded by the N terminus and by the second exon had polymerase α-primase binding activity, while a peptide comprising only the DNA-binding domain had none. While polymerase α-primase bound less efficiently to the N-terminal peptide than to wild-type T antigen, it bound as well to T83-708 as to the wild-type protein. Thus, T antigen appears to have two binding sites for DNA polymerase α-primase: a strong binding site located within residues 260 to 708, and a secondary N-terminal site located within the first 82 residues. Several other examples of proteins with more than one binding site for a specific protein partner have been reported recently, such as the pocket proteins, adenovirus E1A, YY1, TBP, and TEF-I (2a, 34a, 38; reviewed in references 10 and 45).

Both polymerase α-primase binding sites in T antigen may contact the p180 subunit of polymerase α-primase, since in protein blots, specific interaction with the p180 catalytic subunit of the blotted renatured polymerase α-primase was detected with wild-type T antigen, T1-82 (18), and T83-708 (not shown). The existence of two independent polymerase α-primase binding domains in T antigen may also reflect interactions between T antigen and more than one subunit of the polymerase α-primase complex. More recent analyses of these interactions with native proteins by ELISA showed that wild-type T antigen bound to p180, to p68, and to the catalytic primase subunit p48 (5, 9; unpublished data). The interaction of polyomavirus T antigen with primase may play a role in the species specificity of polyomavirus DNA replication (5). No evidence of T antigen binding to the other primase subunit p58 was detected, but it cannot be ruled out. More detailed analyses will be required to map these individual protein-protein interactions and explore their functional relevance.

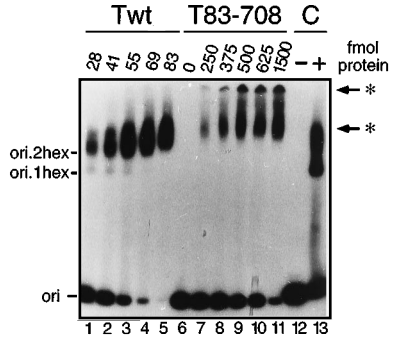


FIG. 7. Titration of DNA binding by T antigen and T83-708 on SV40 origin DNA. The indicated amounts of wild-type T antigen (lanes 1 to 5) and T83-708 (lanes 6 to 11) were incubated with a labeled origin-containing (site II) DNA fragment. Free and bound DNAs were resolved in a 3.5% polyacrylamide gel. The autoradiogram shows the position of double-hexamer-bound DNA (ori.2hex) and single-hexamer-bound DNA (ori.1hex) observed with wild-type T antigen, and free DNA (ori), as indicated on the left. Shifted DNA obtained with T83-708 and material remaining in the well are indicated by an arrow and asterisk on the right. Control reactions (C) were carried out with no protein (lane 12) or with 68.75 fmol of a mutant T antigen (T124A; lane 13), which inefficiently forms double hexamers on origin DNA (45) and thus serves as a marker for single and double hexamers in this reaction.

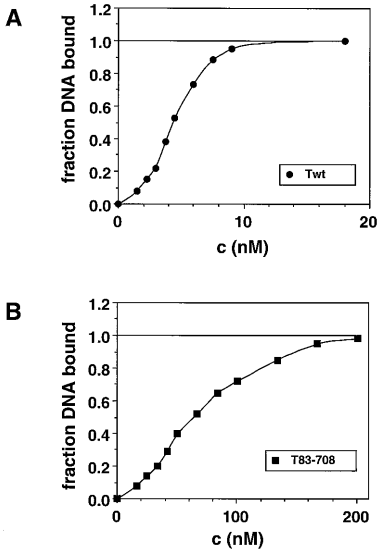


FIG. 8. Saturation isotherms of T antigen and T83-708 binding to DNA. Band shift assays performed as for Fig. 7 were quantitated densitometrically, and the fraction of bound DNA was plotted as a function of protein concentration (*c*) for wild-type T antigen (A) and T83-708 (B). Saturation occurred with 95 to 99% of the DNA shifted in both cases.

The interaction of T antigen with DNA polymerase α-primase is thought to play a key role in the initiation of SV40 DNA replication (13, 15, 17, 48, 63). One way to assess the functional importance of the two independent DNA polymerase α-primase binding sites was to determine whether deletion of the first 82 amino acids of T antigen would result in a protein with replication activity. T83-708 clearly replicated SV40 DNA in vitro (Fig. 3), albeit at a reduced level, suggesting that the DNA polymerase α-primase binding site in the N terminus of T antigen may be functionally redundant. Alternatively it may serve to stabilize or enhance the interaction mediated by the C terminus.

That the C-terminal region of T antigen was sufficient for replication activity at the SV40 origin was consistent with published evidence (1, 3, 4, 34, 68, 69; reviewed in references 10 and 21) and correlated with similar results obtained with polyomavirus T antigen (25). Since T83-708 replication activity was less efficient than wild-type replication activity, it may be that this particular truncation alters the structure of the rest of the polypeptide. Our results suggest that it cannot be severely altered, but we cannot rule out the possibility that a larger or a smaller N-terminal truncation of T antigen might have replication activity equivalent to that of the wild-type protein.

**Deletion of the amino-terminal 82 residues affects the oligomerization and assembly of T antigen on the core origin DNA.** We investigated the role of deleting T antigen residues 2 to 82 in stabilizing interactions among T antigen subunits, particularly hexamer and double hexamer formation. The unidirectional helicase activity of T83-708, which requires single hexamers (59, 67), was not greatly reduced compared with that of wild-type T antigen at lower protein concentrations, suggesting that a significant fraction of the T83-708 hexamers observed in solution (Fig. 5) may be functionally active. The reduced activity observed with higher concentrations of T83-708 protein may reflect aberrant oligomerization at higher protein concentrations.

Both immunoprecipitation and mobility shift DNA-binding experiments showed a reduction in the site II binding activity of T83-708 (Fig. 6 and 8), yet interestingly, binding was still cooperative, as revealed by the titration experiments shown in Fig. 8. However, unlike complexes containing wild-type T antigen, all the detectable origin DNA–T83-708 complexes appeared to contain more than 12 monomers (Fig. 7). Our data suggest that the N terminus plays a role in limiting the complex to 6 or 12 subunits, particularly during origin DNA binding, but this remains to be confirmed by the analyses of more deletion and point mutations in this region. Excessive oligomerization of T83-708 might account for its reduction in the minimal origin (site II) DNA-binding activity compared with wild-type T antigen (Fig. 6 and 7). In contrast, binding to site I, which requires a T antigen dimer, was not so severely compromised.

The strong reduction in the origin DNA-binding activity of T83-708 and the aberrant stoichiometry of the T83-708 complexes on the origin raise the question of why the SV40 DNA replication activity of the mutant is not more severely compromised. Several possible explanations for the replication activity of the aberrant DNA-T antigen complexes can be imagined. Perhaps such complexes retain sufficient structural integrity to be partially active in DNA replication or small amounts of double hexamers are present which are not detectable in the band shift assay. Alternatively, the cellular replication proteins which interact directly with T antigen in the replication assay could guide the assembly of correct, functionally active T83-708 replication complexes on the origin DNA. In this case, it would be informative to determine the ratio of T antigen molecules to one or more cellular replication proteins in the preinitiation complexes. Experiments to measure the stoichiometry of such interactions are under way.

Discrepancies between loss of individual biochemical functions and loss of DNA replication activity have also been reported for other T antigen mutants (40, 41, 43, 60, 70). In contrast with our results, however, the DNA replication activity of the mutant in each of these cases was more severely compromised than the individual functions.

**Other functions of the N-terminal region of T antigen.** The first 82 residues of T antigen encode several other functions that are essential in SV40-infected cells but not apparent in cell-free replication assays. Attempts to determine whether T83-708 has viral DNA replication activity in vivo were unsuccessful because of its poor expression in monkey cells, perhaps due to properties of the T83-708 protein or its intronless mRNA. However, T antigens with point mutations in the N-terminal domain have been reported to be DNA replication defective in vivo (26, 43, 54) and defective in immortalization, transformation, virion production, transactivation (52, 53, 56, 72; reviewed in reference 10), and stimulation of cellular DNA synthesis (12). Deletion of T antigen residues 2 to 82 resulted in a transformation-defective phenotype in rodent cells that could be complemented in *trans* by small t antigen, which has the same first 82 amino acids as large T antigen (46). An adenovirus E1A mutant defective in p300 binding and induction of adenovirus or host cell DNA synthesis is complemented by wild-type T antigen but not a mutated T antigen lacking residues 17 to 27 (10, 47, 54, 71), suggesting that T83-708 may also have this defect. Certainly further work is required, particularly with different N-terminal deletion mutants, to understand the functions associated with the amino terminus of T antigen.

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