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Nonspecific DNA Binding Activity of Simian Virus 40 Large T Antigen: Evidence for the Cooperation of Two Regions for Full Activity

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We generated a series of COOH-terminal truncated simian virus 40 large tumor (T) antigens by using oligonucleotide-directed site-specific mutagenesis. The mutant proteins [T(1-650) to T(1-516)] were expressed in insect cells infected with recombinant baculoviruses. T(1-623) and shorter proteins [T(1-621) to T(1-516)] appeared to be structurally changed in a region between residues 269 and 522, as determined by increased sensitivities to trypsin digestion and by altered reactivities to several monoclonal antibodies. These same mutant proteins bound significantly less nonorigin plasmid DNA (15%) and calf thymus DNA (25%) than longer proteins [T(1-625) to T(1-708)]. However, all mutant T antigens exhibited a nearly wild-type level of viral origin-specific DNA binding and binding to a helicase substrate DNA. This indicated that binding to origin and helicase substrate DNAs is separable from about 85% of nonspecific binding to double-stranded DNA. As an independent confirmation that a region distinct from the origin-binding domain (amino acids 147 to 247) is involved in nonspecific DNA binding, we found that up to 96% of this latter activity was specifically inhibited in wild-type T antigen by several monoclonal antibodies which collectively bind to the region between residues 269 and 522. In order to investigate the relationship between the origin-binding domain and the second region, we performed origin-specific DNA binding assays with increasing amounts of calf thymus DNA as competitor. The results suggest that this second region is not an independent nonspecific DNA binding domain. Rather, it most likely cooperates with the origin-binding domain to give rise to wild-type levels of nonspecific DNA binding. Our results further suggest that most of the nonspecific binding to double-stranded DNA is involved in a function other than direct recognition and binding to the pentanucleotides at the replication origin on simian virus 40 DNA.

The simian virus 40 (SV40) large tumor (T) antigen is a multifunctional phosphoprotein of 708 amino acids. It is the only viral protein required for the initiation and elongation phases of SV40 DNA replication in permissive monkey cells (27, 59, 66). In this multistep reaction, T antigen first binds to the GAGGC pentanucleotides in sites I and II of the virus replication origin (48, 69). Subsequently, it catalyzes a duplex opening reaction (melting) at the inverted palindrome in the proximity of site II (3, 39) followed by bidirectional unwinding of the DNA strands (10, 73) with its DNA helicase (58, 72) activity. Initiation and elongation events are dependent on the host cell DNA synthesis machinery, which includes polymerase α-primase complex (36, 53), polymerase δ (43), topoisomerases (10, 76), single-stranded DNA binding protein (14, 74), replication factor C (70), and the proliferating-cell nuclear antigen PCNA (42).

In addition to its function in virus DNA replication during infection, T antigen coordinates the transcription of viral genes by repression of its own synthesis (1, 67) and by transactivation of the late promoter (6, 22). In nonpermissive cells, T antigen induces and maintains the transformed state by a mechanism that most likely involves its interactions with the cellular antioncoproteins p53 (28, 68, 78) and Rb, the product of the retinoblastoma gene (8, 12). T antigen also stimulates cellular DNA synthesis in quiescent host cells (9, 20, 21) and reactivates silent rRNA genes (54, 55).

Many of T antigen's functional domains have been mapped on the linear polypeptide chain. Among these, the origin-binding domain (residues 147 to about 247) (2, 49–52, 61) has been the best characterized. The maximal p53-binding and helicase substrate-binding regions map to residues 272 to 517 (46) and 131 to 517 (75), respectively, and the ATP-binding region maps to amino acids (aa) 418 to 528 (4, 5). The first N-terminal 121 amino acids have been implicated in transactivation (57, 79), and a small region (residues 105 to 114) is responsible for binding to the Rb protein (13).

One activity of T antigen that is less well characterized is its ability to bind nonspecifically to double-stranded and single-stranded DNAs (7, 38, 56). The exact function of this activity is not yet clear, but it has been implicated in origin-specific DNA binding (51). It may also be involved in subsequent steps during viral DNA replication, in the induction of cellular DNA synthesis, or in the activation of rRNA genes. We have mapped nonspecific DNA binding to a region between aa residues 131 and 517 (75). Recently, we showed that at least 5 residues (mapping between positions 149 and 203) within the DNA binding domain were important in binding nonspecifically to double- and single-stranded DNAs (51, 52). All mutations in the domain which significantly reduced nonspecific binding also prevented origin-specific binding (although the converse was not true). This suggested that T antigen first binds to virus DNA nonspecifically and then locates the origin sequences.

Several years ago, Prives et al. (44) reported that a 56-kDa adeno-SV40 hybrid protein (containing T-antigen sequences from about residues 252 to 708) bound calf thymus DNA but could not bind specifically to the SV40 origin. A smaller, 45-kDa protein, with T-antigen sequences from about residues 336 to 708 was unable to bind to any DNA. These

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results defined a region capable of independent binding to calf thymus DNA whose NH_2-terminal end maps between residues 252 and 336. In this present work, we generated a series of COOH-terminal deletions of T antigen in order to better characterize this activity and to study its relationship with origin DNA binding. We report that a region mapping somewhere between residues 269 and 522 appears to interact with the origin-binding domain to give rise to optimal nonspecific binding to double-stranded DNA.

MATERIALS AND METHODS

Cells and viruses and antibodies. *Spodoptera frugiperda* (Sf9) cells were cultured in TNM-FH medium containing 10% heat-inactivated fetal bovine serum (GIBCO) at 27°C as described previously (62). *Autographa californica* nuclear polyhedrosis virus (AcNPV) baculovirus and wild-type T antigen-expressing recombinant baculovirus were generously provided by Robert Lanford (25). All baculoviruses were propagated in Sf9 cells as previously described (62). Anti-T antigen or anti-p53 monoclonal antibodies were obtained from Gooding et al. (16), Gurney et al. (17, 18), Harlow et al. (19), or Lane et al. (24, 35, 77). Anti-T antigen polyclonal serum was from hamsters with SV40-induced tumors.

Plasmids. We constructed pSK(−)SVTC by ligating the cDNA of T antigen with *Bam*HI-linearized SK(−) (Stratagene). The baculovirus-transferring vector, pVL941T, was obtained from Robert Lanford (25). $pSVO^+$ contains sites I and II of the SV40 replication origin, pOS1 contains site I only, and pSVOdl3 contains site II only (59, 60).

Mutagenesis protocol. Mutations were generated in pSK(−)SVTC by annealing oligonucleotides with a mismatch to a uridine-containing single-stranded DNA template as described previously (29). The oligonucleotide was extended with T4 DNA polymerase (New England BioLabs, Inc.), and the resulting double-stranded DNA was sealed with T4 DNA ligase. The DNA was used to transform *Escherichia coli* BMH71-18 strain (International Biotechnologies, Inc.). Single-stranded DNA was prepared from individual colonies and sequenced by using the standard dideoxy procedure with appropriate primers.

Construction of mutant T antigen-expressing recombinant baculoviruses. pSK(−)SVTC harboring a mutation in the cDNA of T antigen was cleaved with *Bam*HI to release the cDNA. It was gel purified and ligated to *Bam*HI-linearized pVL941, a baculovirus-transferring vector (25), by using standard recombinant DNA procedures to generate mutant T antigen-bearing pVL941T. The cDNA was inserted adjacent to the baculovirus polyhedrin promoter and upstream from a defective (minus initiation codon) polyhedrin coding sequence. This allows the production of T antigen but not that of the polyhedrin protein (a component of occlusion bodies). In order to construct recombinant baculoviruses, pVL941T DNA and wild-type baculovirus AcNPV genomic DNA were cotransfected into Sf9 cells by using a calcium phosphate procedure as described previously (62). T antigen-expressing recombinant viruses were detected by using immunofluorescence as a screen and were further purified from wild-type baculovirus by three to four rounds of plaque assays (62) until no occlusion bodies were visualized in infected Sf9 cells.

Immunoprecipitation of T antigens. Sf9 cells were infected with recombinant baculoviruses at a multiplicity of about 10 PFU per cell. At 48 h, unlabeled or L-[^{35}S]methionine-labeled cells were lysed with Nonidet P-40 buffer (26). T antigen was purified from lysates by using immunoprecipitation with various antibodies as indicated in the figure legends and either protein A-agarose or formalin-fixed *Staphylococcus aureus*. For antibody reactivity tests, we performed the immunoprecipitation reactions with lysates that contained about equal amounts of T antigen and an excess of various anti-T antigen monoclonal antibodies. In all cases, a sample of the immunoprecipitated T antigen was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Quantitation was achieved by using standard silver staining followed by scanning with an LKB densitometer.

Preparation of helicase substrate DNA. A 15-mer universal sequencing primer (Bethesda Research Laboratories, Inc.) was annealed to single-stranded M13mp19 DNA template and elongated with [α-^{32}P]dATP and unlabeled dCTP by using the *E. coli Pol*1 fragment (Klenow) (58). The reaction product was purified on a Bio-Gel P-6 spun column (Bio-Rad), and the purified ^{32}P-labeled substrate was used for helicase substrate-binding reactions.

DNA binding assay. DNA binding assays were performed by incubating equal amounts (50 ng) of immunoprecipitated wild-type or mutant T antigen bound to either protein A-agarose beads or *S. aureus* with ^{32}P-labeled DNA. Reactions were carried out in McKay buffer (32) in the presence or absence of competitor calf thymus DNA (as indicated in the figure legends). After a 1-h incubation at room temperature, the bacteria were washed with DNA wash buffer (75), and the radioactivity in the pellet was quantitated by Cerenkov counting.

Tryptic fragment analysis. Sf9 cells were infected with T-antigen gene-bearing recombinant baculoviruses and were labeled with L-[^{35}S]methionine for 18 h before lysis (at 48 h postinfection). T antigen was purified from the lysates by immunoprecipitation with PAb416 (19) and protein A-agarose as described above. The amount of T antigen in the immunoprecipitates was quantitated by silver staining and was found to be proportional to the amount of radioactivity in each preparation. Equal amounts of T antigen were treated with various concentrations of *N*-tosyl-L-phenylalanine chloromethyl ketone-trypsin (Worthington Diagnostic) at 0°C for 45 min, and the reactions were terminated by the addition of aprotinin (100 μg/ml) as previously described (49). The T-antigen fragments, which were either released from the immunoprecipitates or remained attached to the beads, were analyzed by using SDS-PAGE followed by autoradiography.

RESULTS

Generation of C-terminal truncated T antigens. Our initial aim in this work was to define the COOH-terminal end of a putative nonspecific DNA binding domain. However, this proved difficult because there was evidence that the removal of a hydrophobic region of the protein (residues 571 to 588) resulted in the loss of some activities, including complexing with the cellular protein p53 (41, 68) and the transformation of rat REF52 cells (41). Furthermore, a single-amino-acid substitution at residue 584 (Pro to Leu) resulted in a similar loss (41, 63). Consequently, we made several COOH-terminal deletions that did not affect this region as well as some that did. We generated C-terminal truncated SV40 large T antigens (Table 1) by mutagenizing sense codons to one of the stop codons. Mutations were introduced into a plasmid containing the M13 Bluescript (Stratagene) vector and a cDNA copy of T antigen [pSK(−)SVTC]. The mutagenesis

TABLE 1. DNA binding properties of T antigens

T antigen	% of wild-type activity							
	Nonspecific DNA[a]				Viral origin DNA[a,d]			Helicase substrate[e]
	Plasmid[b]		Calf thymus[c]					
	Expt 1	Expt 2	Expt 1	Expt 2	I + II	I	II	
T(1-650)	93	141	97	102	129	118	118	95
T(1-627)	86	143	84	96	95	90	90	100
T(1-625)	126	98	125	100	121	113	97	106
T(1-623)	14	12	21	26	106	60	65	120
T(1-621)	16	10	22	23	103	66	79	123
T(1-599)	16	16	20	22	118	66	78	128
T(1-578)	15	16	28	30	92	63	68	135
T(1-542)	13	10	21	26	85	61	53	147
T(1-516)	12	12	19	26	94	51	89	147
Pro-584–Leu	13	12	ND[f]	ND	92	ND	ND	ND

[a] T antigen was purified by immunoprecipitation with PAb416 and fixed *S. aureus*. The reactions were performed by incubating 50 ng of unlabeled T antigen with 2 ng (expt 1) or 6 ng (expt 2) of ^{32}P-labeled DNA in McKay buffer. After 1 h at room temperature, the bacteria were washed and the radioactivity in the pellet was quantitated by Cerenkov counting.

[b] The DNA was *Taq*I fragment D of pSVO$^+$. It contains vector sequences only. The reactions were performed in the absence of competitor DNA.

[c] The DNA was sheared, and the reactions were performed in the absence of competitor DNA.

[d] The DNA was *Taq*I fragment E (2 ng), which contains sites I and II of the SV40 replication origin (I + II) or separated site I or II. The reactions were performed with a 1,000-fold mass excess of sheared calf thymus DNA as competitor.

[e] T antigen was purified by immunoprecipitation with PAb416 and protein A-agarose beads. The reactions were performed by incubating 100 ng of unlabeled T antigen with 40 ng of ^{32}P-labeled helicase substrate DNA in McKay buffer.

[f] ND, not done.

protocol was similar to the one devised by Kunkel (23) and has been previously described (29).

Expression of mutant T antigens in insect cells infected with recombinant baculoviruses. In order to investigate the effects of these deletions, we expressed the truncated proteins in insect cells by infecting them with recombinant baculoviruses as previously described (62). This system has been successfully used by a number of investigators (25, 37) in order to generate fully functional T antigen. Recombinant baculoviruses containing mutant T-antigen genes were constructed by using homologous recombination between wild-type baculovirus AcNPV genomic DNA and pVL941T, a baculovirus-transferring vector. Mutant T antigens were isolated from insect cell lysates by using immunoprecipitation. The size of each deletion was confirmed by comparing the migration of truncated proteins with known molecular weight standards by using SDS-PAGE (data not shown).

Nonspecific DNA binding activity. We first tested the mutant proteins for nonspecific DNA binding activities by using two DNA substrates: a plasmid-derived fragment of pSVO$^+$(*Taq*I-D) and sheared calf thymus DNA. As shown in Table 1 and Fig. 1, the deletion of all sequences up to and including residue 626 [T(1-625)] had no effect on nonspecific DNA binding. However, in T(1-623) and shorter proteins [T(1-621) to T(1-516)], this activity was significantly reduced to about 15% (plasmid DNA as a substrate) or 25% (calf thymus DNA as a substrate) of the wild-type activity. A similar difference was observed when larger amounts of substrate DNA were used (Table 1, experiment 2). These binding assays were all performed with equal amounts (50 ng) of T antigen. We checked the stabilities of the wild-type and mutant proteins during binding by recovering T antigen from the immunoprecipitates after the reactions. Protein levels were quantitated by using SDS-PAGE followed by silver staining. Negligible differences were found in the amounts of each T antigen before and after the reaction (data not shown), indicating that the reduced levels of nonspecific DNA binding were not due to greater instability of the mutant proteins. Rather, it appears that deletions into a region bounded by residue 624 or 625 depress nonspecific binding activity to double-stranded DNA.

Viral origin-specific DNA binding activity. The viral origin DNA binding domain has been very well characterized by several groups (2, 49–52, 61). It maps from residue 147 to about residue 247 (51). Certain amino acid residues and phosphorylations (34, 47) outside this region have an influence on DNA binding to sites I and II at the origin, but the region by itself is sufficient for sequence-specific binding (2). Since there is strong evidence that nonspecific DNA binding

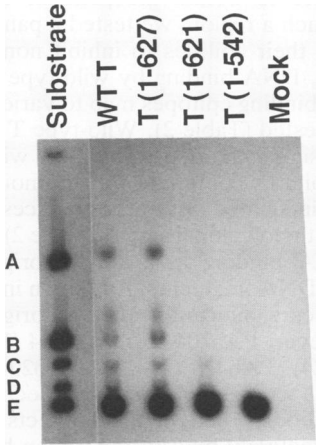

FIG. 1. DNA binding activity of wild-type (WT) and mutant T antigens. Unlabeled T antigen was immunoprecipitated with PAb416. Equal amounts of T antigen (50 ng) were incubated with ^{32}P-labeled *Taq*I fragments (A, B, C, D, and E) of pSVO$^+$ in the absence of competitor DNA. The bound DNA fragments were eluted with 1% SDS and subjected to electrophoresis on a 1.5% agarose gel. Mock, assay of a control precipitate from wild-type baculovirus (AcNPV)-infected insect cells.

activity is important for origin DNA binding (51, 52), we determined the influence of these various deletions on origin-specific DNA binding activity. All truncated proteins still retained wild-type levels of activity to the whole origin (Table 1, I + II; Fig. 1), indicating that the deletions did not significantly disrupt this functional domain. No differences were found between the origin-binding activities of wild-type and mutant proteins when the binding was performed with increasingly smaller amounts (from 50 to 0.08 ng) of T antigen (data not shown). We also tested the binding to separated sites I and II of the replication origin. Although the binding to the separated sites might have been slightly depressed in truncated proteins, the binding to site II was not affected more than the binding to site I (Table 1). Our results suggest, therefore, that the 15% nonspecific DNA binding activity retained by the truncated proteins [T(1-623) to T(1-516)] is sufficient for specific binding to the origin and that nonspecific and origin-specific DNA binding are mostly separable activities.

Helicase substrate-binding activity. Various mutations in the DNA binding domain of T antigen influence both nonspecific double-stranded DNA binding and binding to a helicase substrate (52). This substrate consists of a single-stranded circular DNA to which a short oligonucleotide has been annealed (58), and T antigen apparently prefers to bind to its single-strand–double-strand junction (72). The maximal helicase substrate-binding region has been mapped to residues 131 to 517 (75), the same region that was implicated in nonspecific binding to double-stranded DNA (75). These observations suggest that nonspecific binding to these two substrates is due to the activity of the same region of the protein. However, as shown in Table 1, deletions up to residue 517 had no effect on helicase substrate binding, indicating that this activity, like origin-specific binding, is separable from about 85% of the nonspecific binding activity to double-stranded DNA.

Blocking of nonspecific binding to double-stranded DNA by monoclonal antibodies. The results presented so far and those reported by Prives et al. (44) together suggest that a region distinct from the origin-binding domain is responsible for most of the nonspecific binding activity to double-stranded DNA. In order to obtain independent evidence for the existence of such a region, we tested a panel of monoclonal antibodies for their abilities to inhibit nonspecific, but not origin-specific, DNA binding by wild-type T antigen. Antibodies whose binding epitopes map to various regions of the protein were tested (Table 2). Wild-type T antigen was first purified by using immunoprecipitation with PAb416 (19). Immunoprecipitates containing equal amounts (50 ng) of T antigen were incubated with a large excess of monoclonal antibody or with polyclonal serum (Table 2). They were then tested in DNA binding assays with origin-containing or origin-lacking DNA fragments. As shown in Table 2, none of these antibodies significantly affected origin-specific DNA binding. However, PAb104 (18), PAb114 (18), PAb252 (24), PAb402 (19, 24), PAb420 (19, 24), PAb204 (35), and polyclonal serum all strongly inhibited nonspecific DNA binding. All other antibodies had minimal effects. The inhibitory antibodies collectively bind to the region between residues 269 and 522. Blocking of nonspecific binding, but not of origin binding, was also observed when wild-type T antigen was directly immunoprecipitated with an inhibitory antibody (experiment 2 in Table 2). As observed above, noninhibitory antibodies did not block the activity. We conclude from these data that residues 269 to 522 define a region important for nonspecific, but not origin-specific, DNA binding.

TABLE 2. Effects of antibodies on the DNA binding activity of wild-type T antigen

First and second antibodies	Binding epitope[a]	% of PAb416[b]		D/E ratio
		E (origin specific)	D (non-specific)	
Expt 1[c]				
None		116	106	0.91
PAb419	1–82	57	68	1.19
PAb430	1–82	51	70	1.37
PAb416	83–115	100	100	1.00
PAb100	269–522	113	28	0.25
PAb104	269–522	71	11	0.15
PAb114	269–522	98	8	0.08
PAb252	272–368	80	13	0.16
PAb605	277–698	77	48	0.62
PAb402	333–451	165	5	0.03
PAb413	333–451	81	18	0.22
PAb414	333–451	115	46	0.40
PAb420	333–451	148	4	0.02
PAb205	333–451	84	63	0.75
PAb204	448–509	47	6	0.12
PAb101	523–698	100	54	0.54
PAb405	699–708	71	74	1.04
Polyclonal serum		43	8	0.20
Anti-p53				
PAb122		62	49	0.79
PAb246		76	42	0.55
Expt 2[d]				
PAb416		100	100	1.00
PAb419		99	71	0.72
PAb205		135	81	0.60
PAb104		218	22	0.10
PAb114		199	17	0.08
Polyclonal serum		125	11	0.08

[a] Binding epitopes for the second antibody on T antigen.

[b] In experiment 1, DNA binding was normalized to the reaction containing PAb416 as the second antibody. In experiment 2, DNA binding was normalized to the reaction containing PAb416-immunoprecipitated T antigen.

[c] Wild-type T antigen was purified by immunoprecipitation with PAb416. Where indicated, immunoprecipitates containing 50 ng of T antigen were further incubated with a large excess of a second antibody. The resulting immunocomplexes were washed and subjected to DNA binding reactions with 2 ng of labeled *Taq*I fragment D (nonspecific) or E (origin specific) of pSVO$^+$. Origin-specific reactions were performed with a 1,000-fold mass excess of sheared calf thymus DNA as competitor.

[d] Wild-type T antigen was immunoprecipitated with the indicated antibody. DNA binding reactions were performed as described in footnote *c*.

Evidence for two interacting regions. In order to study further the relationship between this region and the origin-binding domain (residues 147 to 247) for full nonspecific DNA binding activity, we performed viral origin-specific DNA binding assays with increasing amounts of sheared calf thymus DNA as competitor. If this region can bind independently to double-stranded DNA, calf thymus DNA should compete equally (although poorly) for the binding of origin DNA to wild-type and mutant proteins (Fig. 2A) (some competition is expected, since the origin-binding domain can bind calf thymus DNA on its own). However, if this region (region B) cooperates with the origin DNA binding domain (region A) directly or indirectly for full nonspecific DNA binding activity, then calf thymus DNA should compete better for the binding of origin DNA to wild-type than to mutant T antigen (Fig. 2B). As shown in Fig. 2C, at a 100-fold mass excess of competitor DNA, wild type and T(1-627) bound about 30 to 50% less origin DNA than they did without a competitor. Under the same assay conditions, mutants T(1-621) and T(1-542) still retained as much origin-

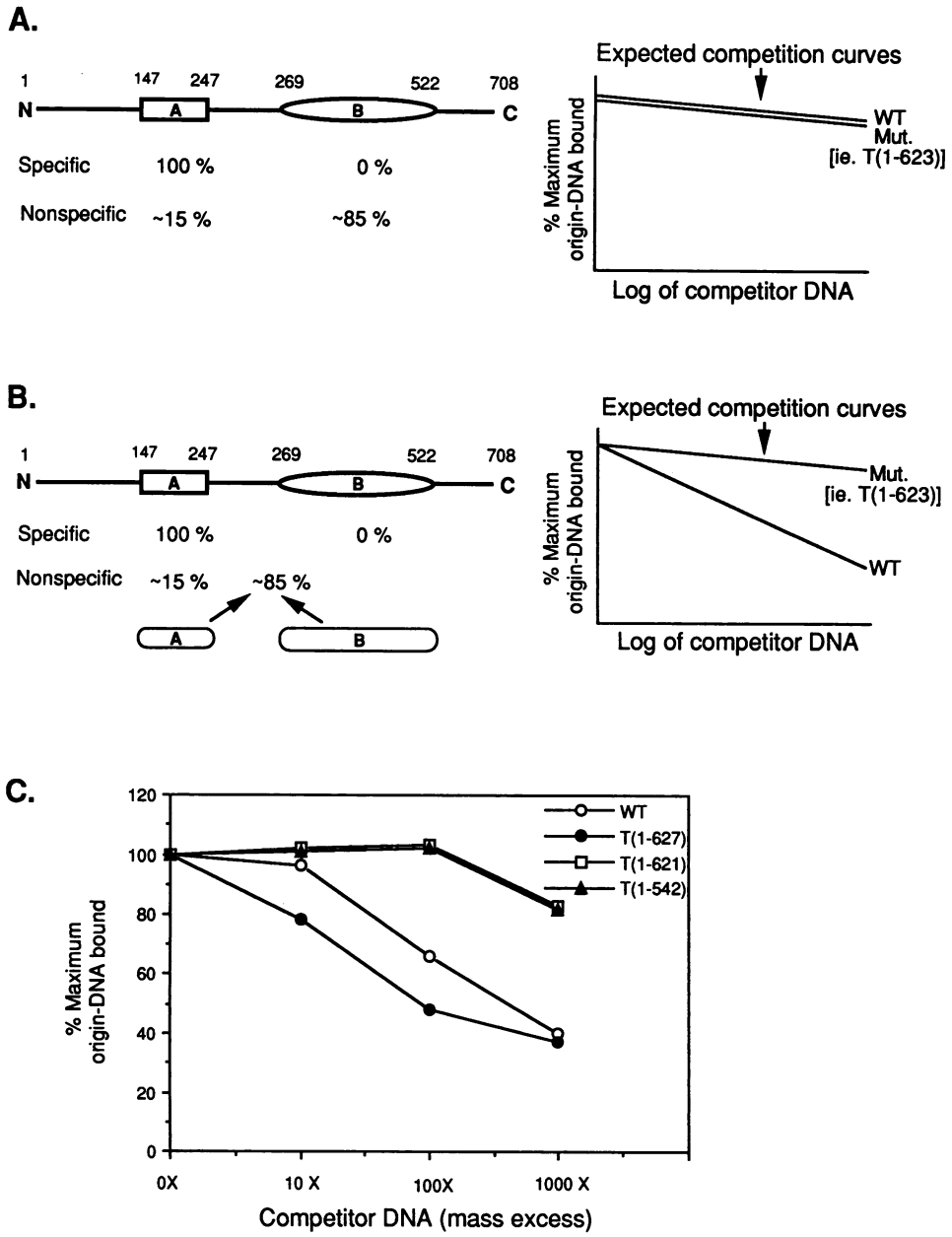

FIG. 2. Models and expected competition curves for two independent regions (A) or interacting regions (B). (C) Competition of viral origin DNA binding by calf thymus DNA. Fifty nanograms of PAb416-immunoprecipitated T antigen was incubated with 2 ng of ^{32}P-labeled origin DNA (*Taq*I-E of pSVO$^+$) in the absence (0X) or presence of various amounts of unlabeled sheared calf thymus DNA. The reaction and quantitation of bound origin DNA were as described in Table 1, footnote *a*.

binding activity as they did in the absence of a competitor. In the presence of a 1,000-fold excess competitor, wild type and T(1-627) bound about 60% less, whereas T(1-621) and T(1-542) bound only about 15% less. Our data therefore suggest that this region most likely cooperates with or influences the origin DNA binding domain in some way to establish wild-type levels of nonspecific DNA binding (model shown in Fig. 2B). It is still possible, however, that this region has a low intrinsic DNA binding activity in order to explain the results of Prives et al. (44).

Probing the structure of T antigen by using proteolysis. Our previous work (75) and the antibody-blocking experiments described above suggest that the C terminus of the nonspecific DNA binding region is at residue 517 or 522. However, we also showed that deletions C terminal of this site depress nonspecific DNA binding. One possible explanation for this discrepancy is that COOH-terminal deletions alter the structure of an upstream region of the molecule. In order to probe for changes in the structure of the mutant T antigens, we examined their sensitivity to trypsin digestion. As shown in Fig. 3, wild-type T antigen was cleaved at low concentrations of trypsin (10 μg/ml) after residue 130 to generate two fragments, a small N-terminal fragment (aa 1 to 130) and a large C-terminal fragment (aa 131 to COOH) (49). Some of the larger fragment was cleaved further after residue 517 to generate a 46K fragment (aa 131 to 517) (49). Higher con-

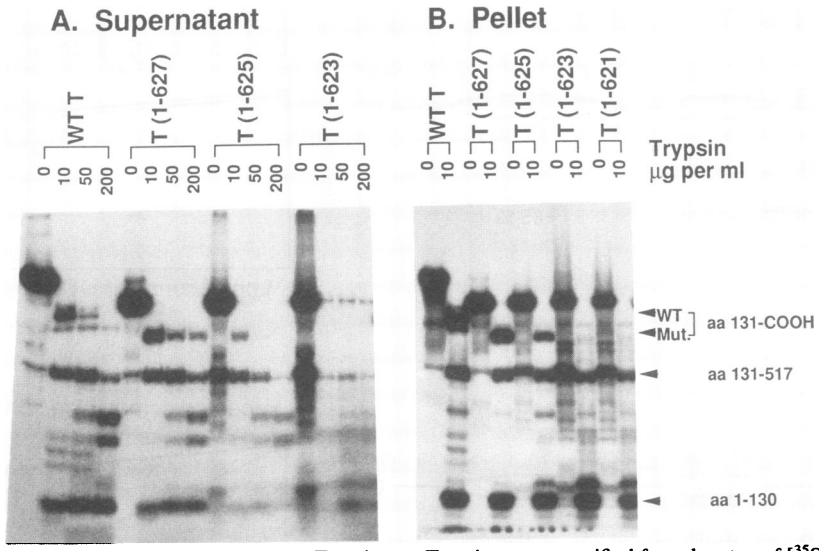

FIG. 3. Tryptic fragments of wild-type (WT) and mutant T antigens. T antigen was purified from lysates of [^{35}S]methionine-labeled insect cells infected with recombinant baculovirus by immunoprecipitation with PAb416 and protein A-agarose beads. The trypsinization reactions were performed by incubating T antigen-containing beads with various concentrations of *N*-tosyl-L-phenylalanine chloromethyl ketone-trypsin. After 45 min on ice, the fragments which were released into the supernatant (A) or which remained attached to the pellets (B) were analyzed on a 13% SDS-polyacrylamide gel and then autoradiographed. Undigested T antigen (lanes with 0 μg of trypsin per ml) was obtained, in each case, by eluting the protein with SDS buffer directly from immunoprecipitated beads.

centrations of trypsin (50 or 200 μg/ml) are necessary in order to completely cleave the larger fragment (aa 131 to COOH) (Fig. 3A). The cleavage pattern of the mutant T(1-627) was very similar to that of wild-type T antigen (Fig. 3). The digestion profile of T(1-625) was, however, slightly different. After being digested with 10 μg of trypsin per ml, less of the 131-to-COOH fragment accumulated, and at 50 μg/ml, none remained (Fig. 3A), indicating that this fragment is more sensitive to trypsin than the corresponding wild-type fragment. This effect was even more pronounced in smaller proteins [T(1-623) and T(1-621)]. Although nearly wild-type levels of the 1-to-130 fragment were associated with the immunoprecipitates after trypsinization (Fig. 3B, pellets), none of the 131-to-COOH fragment remained in the supernatant or in the pellets (Fig. 3). It had probably been digested to a number of smaller fragments (visible in the last lane of Fig. 3B). The results suggest that these deletions increase the susceptibility of the 131-to-COOH fragment to trypsin digestion, perhaps by altering the structure of at least part of that region.

Antibody reactivities of T antigens. As a second way of probing for structural changes in mutant proteins, we determined their reactivities to a number of monoclonal antibodies. Four monoclonal antibodies, PAb204, PAb205, PAb104, and PAb114, whose binding epitopes map to residues 269 to 522 (18, 24, 35), were used. All of these antibodies inhibit T antigen's ATPase activity (64, 71) as well as its in vitro DNA replication function (71). By comparing the reactivities of these four antibodies with that of PAb416, we obtained values for relative antibody reactivity to wild-type and mutant T antigens. PAb416 recognizes a region between residues 82 and 115 and reacts with nearly all T antigens recognized by anti-T serum. As shown in Table 3, T(1-627) still retained wild-type-like reactivity. T(1-625) had an altered pattern of reactivity, with slightly more binding to all four monoclonal antibodies. However, it was not too dissimilar from that of wild-type T antigen. The smaller mutant proteins [T(1-623) and T(1-621)] reacted very differently with these antibodies. Both bound extremely well to PAb204 and very poorly, if at all, to PAb205, PAb104, and PAb114 (Table 3). These data indicate that T(1-623) and T(1-621) were significantly altered in structure in the region between residues 269 and 522.

Biochemical properties of mutant Pro-584–Leu. A single point substitution mutant of T antigen (Pro-584 to Leu) has been previously described (41, 63). This mutation results in the loss of a number of biochemical activities (41, 65), presumably because of a structural alteration. We tested this mutant for nonspecific DNA binding, origin-specific DNA binding, and antibody reactivity (Tables 1 and 3). The results indicate that this mutant is probably very similar biochemically and structurally to T(1-623) and shorter proteins.

DISCUSSION

The results presented in this paper show that the binding of T antigen to the SV40 origin and to a helicase substrate is

TABLE 3. Antibody reactivities of T antigens[a]

T antigen	% of PAb416 reactivity			
	PAb204	PAb205	PAb104	PAb114
WT T	3.4	46.6	3.2	14.7
T(1-627)	1.4	62.7	4.0	19.3
T(1-625)	26.3	70.2	13.4	34.7
T(1-623)	105.0	1.8	0	2.6
T(1-621)	84.6	0	0	3.0
Pro-584–Leu	67.0	4	0	0

[a] Approximately equal amounts of T antigen in lysates from infected cells were incubated with an excess of various anti-T antigen monoclonal antibodies and fixed *S. aureus*. A sample of the immunoprecipitates was analyzed by SDS-PAGE followed by silver staining, and the amount of precipitated T antigen was quantitated by scanning with an LKB densitometer. Percents were obtained by comparing the amounts of T antigen recognized by each antibody with that recognized by PAb416.

separable from most of the nonspecific binding to double-stranded DNA. Two independent experiments support this conclusion. First, COOH-terminal deletion mutants bound much less origin-lacking DNA than wild-type T antigen (Table 1 and Fig. 1). Second, wild-type T antigen's nonspecific DNA binding, but not its origin binding, was significantly inhibited with a number of monoclonal antibodies (PAb104, PAb114, PAb252, PAb402, PAb420, and PAb204) which collectively bind to a region mapping to residues 269 to 522 (Table 2). These binding results were reproduced under various conditions, including those used for in vitro DNA replication (11). We were careful to perform the binding assays with equal amounts of protein, and we showed that mutant proteins are just as stable as wild-type proteins during the binding reactions.

Together, these data implicate a region distinct from the origin-binding domain for most (up to ~96%) of nonspecific DNA binding to double-stranded DNA. Origin-binding experiments performed with competitor DNA (Fig. 2) suggest that this second region (region B) does not bind DNA independently. Rather, it cooperates with or influences the origin-binding domain to give rise to wild-type levels of nonspecific double-stranded DNA binding. Our recent work (51, 52) dealing with the mutagenesis of the origin-binding domain strongly supports the conclusion that region B does not act independently. In that study, five different single point substitution mutations in the domain severely reduced (to 1 to 10%) the binding to all DNAs tested, including nonorigin double-stranded DNA.

It was somewhat surprising that binding to a helicase substrate was separable from nonspecific binding to double-stranded DNA. Up until now, these two activities were thought to represent the operation of the same region of the molecule, since both mapped to a similar tryptic fragment (75) and since mutations in the origin-binding domain which affected one always affected the other (52). Mohr et al. (33) showed that origin-specific DNA binding can be separated from binding to a helicase substrate, since a mutation at proline 522 affected only the latter. This suggests that additional sequences outside of the origin-binding domain are needed for helicase substrate binding. Taken together, these data indicate that the three binding activities are not identical; they require slightly different regions of the molecule. However, our data (Table 1) suggest that the sequences responsible for helicase substrate DNA binding are more similar to those involved in origin DNA binding than to those needed for nonspecific binding to double-stranded DNA.

One possible interpretation of all of these data is that the origin-binding domain (residues 147 to 247) forms the core of all interactions with DNA. It is sufficient for origin DNA binding (2) and has nonspecific DNA binding activity (2, 40), but this second activity is only a small percentage (4 to 20%) of that of full-length T antigen. This small amount of nonspecific binding is apparently all that is required for T antigen to bind to origin sequences normally. Additional sequences are, however, necessary for full nonspecific binding to double-stranded DNA and for helicase substrate binding. For nonspecific double-stranded DNA binding, these ancillary sequences map somewhere between residues 269 and 522.

This model is compatible with all known data, with the possible exception of those of Prives et al. (44), who showed that a 56-kDa, but not a 45-kDa, adeno-SV40 hybrid protein has nonspecific binding activity. Their results would suggest that this second region (Fig. 2, region B) can function as an independent nonspecific DNA binding domain, while ours would argue for dependence on the origin-binding domain for activity. Perhaps this region has a small amount of independent binding activity, and our results cannot eliminate that possibility.

It is noteworthy that the changes in structure imparted by some COOH-terminal deletions can be detected by the sensitivity of the molecule to trypsin digestion (Fig. 3) and by significant changes in reactivity to several monoclonal antibodies (Table 3). A fragment of T antigen (residue 131 to the end) is more sensitive to trypsin digestion in mutant than in wild-type T antigen. This suggests that at least one portion of this region is more exposed and therefore more accessible to digestion. The antibody reactivity data support this possibility. They indicate that at least one monoclonal antibody (PAb204) reacts significantly better with mutant than with wild-type T antigen.

The observation that PAb204 reacts well with mutant T antigen (>80%; Table 3) and very poorly with wild-type T antigen (3%; Table 3) is interesting because the same antibody inhibits wild-type T antigen's ATPase and helicase activities (71). There are at least two possible explanations for this. First, it is possible that PAb204 recognizes a minor, ATPase-active form of the protein and inhibits its activity by interfering with the active site. Tack et al. (65) have reported that the ATPase-active form of T antigen is in a new population of molecules and that this new population reacts with PAb204 better than an older population of molecules. In this possibility, COOH-terminal truncations promote the ATPase-active form to the exclusion of others. However, this explanation is unlikely, because our mutants have less, not more, ATPase activity (data not shown). The phenotypically similar mutant (Pro-584–Leu) is deficient in ATPase and helicase activities as well (63). Furthermore, PAb104 and PAb114, which also prefer to bind to newly synthesized T antigen (65), recognize COOH-truncated mutants [T(1-623) and smaller proteins] very poorly, if at all (Table 3).

A second possible explanation for this observation is that the PAb204 epitope (residues 448 to 509) is exposed only transiently in T antigen's ATPase reaction. This could be a reason why the antibody binds poorly to wild-type T antigen yet inhibits the ATPase. In this scheme, the mutant forms of the protein are constitutively in the PAb204-positive conformation and cannot cycle back and forth between alternate conformations. This explanation is compatible with the mutants' reduced ATPase activity and efficient binding with PAb204. It is possible that the alternate conformation is largely recognized by PAb205, since it binds well with wild-type T antigen and poorly with mutant T antigen (Table 3). It is interesting that the behavior of PAb204 and PAb205 toward mutant and wild-type forms of T antigen is reminiscent of the activities of two different antibodies toward wild-type and mutant forms of p53 (15, 77).

We have also examined our mutants for their abilities to oligomerize by using a nondenaturing gradient gel assay (30). In the presence of ATP, wild-type T antigen appears to form large oligomers consisting of hexamers and double hexamers (31). T(1-627), which behaves normally in all activities tested, had a wild-type pattern of oligomerization (data not shown). However, T(1-625) was aberrant in that hexamers did not form in the presence of ATP. The same mutant, however, retained wild-type levels of DNA binding activity (Table 1), probably indicating that the DNA binding assay measures the binding of low-molecular-weight forms (monomers and dimers, etc.). T(1-623) and T(1-621) and Pro-584–Leu were even more defective in the oligomerization assay. These proteins formed large aggregates that barely pene-

trated nondenaturing gradient gels, and it was not possible to determine whether there was a response to ATP (data not shown). This behavior was similar to that observed for certain mutants in the Zn^{2+} finger region (30) and probably suggests that the conformation of these proteins is significantly different from that of the wild type. This agrees well with the results of trypsin digestion and reactivity to monoclonal antibodies.

Our results suggest that most of the nonspecific double-stranded DNA binding activity has a role in something other than direct binding to the pentanucleotides (GAGGC) at the SV40 replication origin. Possibly, this activity is needed for a different step in DNA replication, such as melting of the inverted palindrome. Parsons et al. (39) reported that the melting of double-stranded DNA in this region is independent of the binding to the pentanucleotides in site II. Furthermore, Scheffner et al. (45) have shown that T antigen catalyzes melting and unwinding reactions in an origin-independent manner. Taken together, these results suggest that these reactions could be mediated in part by T antigen's "nonspecific" DNA binding activity. Another possibility is that nonspecific binding is involved in a separate function, such as the induction of cell DNA synthesis or the activation of rRNA genes.

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