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Biochemical Analysis of Mutants with Changes in the Origin-Binding Domain of Simian Virus 40 Tumor Antigen

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The role of the origin-binding domain of simian virus 40 large tumor antigen (T antigen) in the initiation of virus DNA replication was investigated by analyzing the biochemical activities of a series of mutants with single-site substitutions in this region. These activities include origin-specific and nonspecific DNA binding, melting of the imperfect palindromic sequence, untwisting of the AT-rich region, unwinding of origin-containing DNA, helicase activity, and the ability to oligomerize normally in response to ATP. Three classes of T-antigen mutants that are unable to support virus replication in monkey cells are described. Class 1 mutants are unable to bind to the origin of DNA replication but are able to bind to DNA nonspecifically. Class 2 mutants exhibit defective binding to both types of DNA. As expected, mutants in these first two classes are unable to unwind origin DNA. Surprisingly, however, these mutants possess significant levels of melting and untwisting activities, suggesting that these reactions may not be solely dependent on the ability of the protein to recognize origin sequences. Most class 1 mutants oligomerize normally in response to ATP, indicating that their DNA-binding defects are not due to structural alterations but probably to a failure to directly recognize origin sequences. In contrast, class 2 mutants exhibit defective oligomerization. Class 3 mutants bind to origin and nonorigin DNA at near wild-type levels and melt and untwist origin DNA normally but exhibit defective oligomerization and unwinding. These mutants are, however, perfectly able to carry out the helicase reaction, indicating that their unwinding defect is at some step after melting but before a nonspecific helicase is used to separate parental strands during replication. These results therefore suggest that proper oligomerization to correctly position the molecules on the DNA may be more important in initiating unwinding than in bringing about efficient DNA binding, inducing structural changes in the DNA, or carrying out the helicase reaction.

The simian virus 40 (SV40) large T antigen participates in the initiation of SV40 DNA replication by providing several discrete activities. First, it functions as an initiator protein by binding to specific DNA sequences at the viral replication origin (30, 40, 41). These target DNA sequences consist of four GAGGC pentanucleotide repeats located within a 64-bp core origin region (9, 11, 15). Second, T antigen induces structural changes in origin DNA. An imperfect palindromic sequence located on the early side of the recognition pentanucleotides is partially melted by T antigen in the presence of ATP (3, 4, 28). Further, T antigen untwists an AT-rich region located on the late side of the pentanucleotides (3, 8). These structural changes in DNA are thought to require the formation of a bilobed complex consisting of a double hexamer of T antigen in association with the core origin (7, 22). Third, by using its helicase activity, T antigen bidirectionally unwinds the core origin (12, 45, 49) with the participation of the DNA polymerase α-primase complex (5, 43) and replication protein A (6, 42, 48). The interaction of T antigen with these cellular proteins is believed to be essential for the construction of the correct replication complex involved in DNA replication (25). Energy for the helicase reaction is provided by ATP hydrolysis catalyzed by the intrinsic ATPase activity of T antigen (36, 46). Although these steps have been fairly well-defined, there is little understanding of the mechanisms by which they occur.

The origin-binding domain (amino acids 147 to 246) of T antigen has been extensively studied at biochemical and genetic levels (1, 32–35, 38). We have generated a large collection of mutants with conservative single point substitutions throughout this domain (34, 35, and this study). Analysis of these mutants has identified sequence elements that are important for origin binding and nonspecific DNA binding (35). We found three sequence elements (named A, B1, and B2) which appear to contain critical amino acid residues for DNA binding. Element A (residues 147 to 159) contains several sites that are specifically important for origin DNA binding, element B1 (residues 183 to 187) has two residues implicated in nonspecific DNA binding, and element B2 (residues 203 to 207) contains at least one residue important for each activity (35). In our analysis of the origin-binding domain, we noticed that many mutations, although preventing virus replication in monkey cells, had minimal effects on DNA binding. This observation suggested that the mutants were defective at other steps during the initiation of virus DNA replication.

In this study, we obtained information about how T antigen carries out its functions at the replication origin by analyzing a number of replication-negative mutants for their ability to bind DNA, oligomerize, melt the imperfect palindromic sequence, untwist the AT-rich tract, perform the helicase reaction, and unwind origin DNA. The mutants characterized in this study belong to three distinct categories: those with defective origin recognition but normal nonspecific DNA binding, those with impaired binding to both types of DNA, and those with near normal DNA binding but unable to unwind origin-containing DNA like wild-type (WT) T antigen. Analysis of the biochemical activities of these mutants revealed that normal binding to DNA was closely linked with full distortion of the origin (melting and untwisting) and that abnormal oligomerization was associated with defective origin unwinding.

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MATERIALS AND METHODS

Site-directed mutagenesis. Mutations were generated in pBS-SV40 (19) or pSK(−)SVTC (18) by annealing oligonucleotides with a mismatch to an uridine-containing single-stranded DNA template as described previously (19). 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.). Plasmid DNA was prepared from individual colonies and sequenced by standard dideoxy procedures using appropriate primers.

Construction of recombinant baculoviruses. Recombinant baculoviruses were generated as previously described (18) with some modifications. In brief, pVL941T (17) carrying the mutation was made by recloning the mutated cDNA from pSK(−)SVTC. Recombinant viruses were generated by transfection of pVL941T and BaculoGold DNA (Pharmingen) as directed by the manufacturer. T-antigen-expressing recombinant viruses were detected by using immunofluorescence as a screen and were further purified from WT baculovirus by two rounds of plaque assays (39).

Immunoprecipitations. Sf9 cells were infected with recombinant baculoviruses at a multiplicity of infection of about 10 PFU per cell. At 48 h, cells were lysed with buffer B (0.15 M Tris [pH 8.0], 0.15 M NaCl, 0.001 M EDTA, 10% glycerol, 0.5% Nonidet P-40, 0.001 M phenylmethylsulfonyl fluoride) (31). T antigen was purified from lysates by immunoprecipitation with PAb419 (14) and formalin-fixed *Staphylococcus aureus*. A sample of the immunoprecipitated T antigen was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Quantitation was achieved by standard silver staining followed by scanning with an LKB densitometer.

Immunoaffinity purification. For each T-antigen preparation, infected Sf9 cells from two to four T150 flasks were lysed with buffer B at 48 h postinfection. Clarified lysates were incubated with PAb419-coupled protein A-Sepharose beads (Pharmacia) for 2 h at 4°C. For all biochemical assays except for the oligomerization assay, the beads were washed five times with a solution containing 0.05 M Tris (pH 8.0), 0.5 M NaCl, 0.001 M EDTA, 10% glycerol, and 0.5% Nonidet P-40 and then washed five times with the same buffer without Nonidet P-40. For the oligomerization assay, the beads were washed with a solution containing 0.05 M Tris (pH 8.0), 0.5 M LiCl, 0.001 M EDTA, and 10% glycerol. T antigen was eluted with a solution containing 50% ethylene glycol, 0.02 M Tris (pH 8.5), 0.5 M NaCl, 0.001 M EDTA, and 10% glycerol (22) and dialyzed versus a solution containing 0.01 M Tris (pH 8.0), 0.1 M NaCl, 0.001 M EDTA, 0.001 M dithiothreitol, and 50% glycerol. Samples were stored at −20°C. Protein concentrations were estimated by silver staining of acrylamide gels.

Quantitative DNA-binding assays. DNA-binding assays were performed by incubating equal amounts (50 ng) of immunoprecipitated (*S. aureus*-bound) or immunoaffinity-purified WT or mutant T antigen with ^{32}P-labeled DNA. For origin-specific DNA-binding assays, 2 ng of an end-labeled 112-bp *Nco*I-*Hin*dIII fragment of pSVO+ (37) was used. This fragment contains T-antigen-binding sites I and II. For nonspecific DNA-binding assays, 20 ng of the end-labeled 2.7-kb *Nco*I-*Hin*dIII fragment of pSVO+ was used. Both DNA fragments were labeled at their *Hin*dIII sites. Reactions were carried out in 100-μl portions of McKay buffer (0.02 M NaH_2PO_4, 0.15 M NaCl, 0.002 M dithiothreitol, 0.0001 M EDTA, 0.05% Nonidet P-40, 3% dimethyl sulfoxide; pH 6.8) (24) or DNA replication buffer (0.03 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 7.5], 0.007 M $MgCl_2$, 0.04 M creatine phosphate, 0.004 M ATP, 0.001 M dithiothreitol, 20 μg of creatine phosphokinase per ml, 0.1 mg of bovine serum albumin per ml) in the presence (for origin-binding assays) or absence (for nonspecific DNA-binding assays) of 1,000-fold mass excess competitor sonicated calf thymus DNA. Incubations were for 1 h at room temperature (for reactions in McKay buffer) or 37°C (for reactions in replication buffer). For reactions containing immunopurified T antigen, DNA-protein complexes were precipitated with PAb419 and *S. aureus*. The bacteria were washed with DNA wash buffer (0.01 M Tris [pH 8.0], 0.15 M NaCl, 0.5% Nonidet P-40) (50), and the radioactivity in the pellets was quantitated by Cerenkov counting.

DNase footprinting. Immunoaffinity-purified T antigen (0 to 700 ng) was incubated in 100 μl of replication buffer containing 2 ng of the end-labeled 112-bp *Hin*dIII-*Nco*I fragment of pSVO+ described above. The glycerol concentration was 12%. After 1 h at 37°C, the DNA was nicked with DNase (0.25 U/ml) for 5 min at 23°C and purified by phenol-chloroform extractions and ethanol precipitation. The DNA was denatured in formamide sample buffer (21) and applied to a 7% acrylamide sequencing gel. As sequence markers, the same labeled DNA fragment was applied to the gel after treatment with the G-only reaction of Maxam and Gilbert (23).

$KMnO_4$ assays. $KMnO_4$ sensitivity assays were performed essentially as described by Parsons et al. (26). In brief, immunoaffinity-purified T antigen (0 to 1 μg) was incubated with 0.7 μg of pSV01ΔEP DNA (47) in 30 μl of replication buffer. The glycerol concentration was 20%. After 20 min at 37°C, 4 μl of 0.3 M $KMnO_4$ was added and incubation was continued for 4 min at 37°C. Four microliters of β-mercaptoethanol was added, and DNA was extracted once with phenol and purified on a BioGel P6 spin column. About half of the purified DNA was subjected to primer extension exactly as described previously (26). After the samples were loaded on a 7% acrylamide sequencing gel, the gel was dried and exposed to X-ray film overnight. To quantitate the melting and untwisting signals, bands were cut out of the gel and Cerenkov counted. Values were normalized to the amount of radioactivity in background bands (26).

Origin-specific unwinding. The origin-specific unwinding reaction was based on the methods of Goetz et al. (12) and Virshup and Kelly (44) with modifications (45). Immunoaffinity-purified T antigen (0 to 500 ng) was incubated with 1 ng of the same end-labeled 112-bp *Nco*I-*Hin*dIII fragment described above in 20 μl of unwinding buffer (0.001 M Tris [pH 7.5], 0.04 M creatine phosphate, 0.0035 M ATP, 0.002 M $MgCl_2$, 0.001 M dithiothreitol, 0.08 M NaCl, 20 μg of bovine serum albumin per ml, 50 μg of phosphocreatine kinase per ml) containing 500 ng of *Escherichia coli* single-strand binding protein. The final glycerol concentration was 20%. After 1 h at 37°C, the solution was adjusted to 0.5% SDS, 0.02 M EDTA, and 0.2 mg of proteinase K per ml and incubated for 30 min at 37°C. After the samples were heated for 10 min at 65°C, they were applied to a 7% nondenaturing acrylamide gel and subjected to electrophoresis for 7 h at 110 V with cooling at 2°C.

Oligomerization assays. Sf9 cells infected with recombinant baculoviruses were labeled at 32 h postinfection with 20 μCi of L-[^{35}S]methionine per ml. At 44 to 48 h postinfection, T antigen was purified by immunoaffinity as described above and incubated for 30 min at 37°C in replication buffer in the

TABLE 1. DNA-binding properties of T-antigen mutants

Mutant class	Position	Amino acid		% of WT binding to[a]:		Footprint
		WT	Mutant	Nonorigin DNA	Origin DNA	
1	153	Asn	Ser	107	1	Protects at high concn[b]
1	154	Arg	Ser	98	5	Protects at high concn
1	155	Thr	Ser	67	1	Protects at high concn
1	159	Phe	Tyr	62	3	Protects at intermediate concn
1	197	Phe	Leu	79	16	Protects at intermediate concn
1	204	Arg	Lys	109	1	No protection
2	185	Ser	Thr	22	1	Protects at high concn
2	203	His	Asn	0	0	No protection
2	226	Val	Ala	29	3	Some protection at high concn
3	168	Ala	Val	97	66	Close to WT
3	183	Phe	Leu	65	77	Close to WT

[a] These numbers are the averages of two to three determinations of several different T-antigen preparations.
[b] Concentration of T antigen.

presence or absence of 4 mM ATP. The final glycerol concentration was 25%. Proteins were cross-linked with 0.1% glutaraldehyde for 15 min at 37°C and diluted twofold in Laemmli loading buffer (16) without β-mercaptoethanol or SDS. T-antigen oligomers were resolved on a native gradient gel (4 to 25% acrylamide) using a Tris-glycine buffer system (27). Electrophoresis was carried out at 25 mA for 24 h at 4°C with buffer recirculation. Gels were soaked in 1 M sodium salicylate for 45 min, dried, and exposed to X-ray film for 2 to 7 days at −80°C.

Helicase assays. 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 *E. coli PolI* fragment (Klenow) (36). The reaction product was purified on a Bio-Gel P-6 spin column (Bio-Rad). The purified ^{32}P-labeled substrate was incubated with immunoaffinity-purified T antigen (0 to 500 ng) in helicase buffer (36) for 1 h at 37°C. The glycerol concentration was 20%. Reactions were stopped by the addition of SDS to a final concentration of 0.2% and EDTA to a final concentration of 0.05 M. Samples were loaded onto a 12.5% acrylamide gel and subjected to electrophoresis at 100 V for 2.5 h at 3°C.

RESULTS

Quantitative DNA-binding assays. We have generated a large number of virus replication-defective T-antigen mutants carrying mutations in the origin-binding domain (34, 35 and this study) and have cloned about 25 of these genes in recombinant baculoviruses. Some of these mutants were selected for further study because they were representative of changes made in previously identified protein elements (34, 35). Other mutants were chosen because preliminary experiments indicated that they had new and interesting properties. Expression with the baculovirus system was necessary to provide sufficiently large amounts of mutant protein for biochemical analysis. This article describes the detailed characterization of 11 of these 25 mutants, which fall into three distinct classes on the basis of their DNA-binding and unwinding properties. The remaining mutants which fall into three additional classes will be described in a subsequent article (51).

Tables 1 and 2 list the mutants described in this article. In general, mild mutations were introduced at each site. The T-antigen mutants which represent element A (residues 147 to 159) are 153NS, 154RS, 155TS, and 159FY; those for element B1 (residues 183 to 187) are 183FL and 185ST and those for B2 (residues 203 to 207) are 203HN and 204RK (35). The remaining mutations do not fall in easily recognizable protein elements (34, 35).

Immunoaffinity-purified and immunoprecipitated T antigens were assayed for their ability to bind to origin-containing and origin-lacking DNA fragments under replication and nonreplication conditions. At first, a quantitative DNA-binding assay that measures the binding of a labeled DNA fragment to T antigen was used. In this assay, equal amounts (50 ng) of protein were used, and in any one experiment, the binding activity of mutant proteins was always compared with the activity of WT T antigen prepared at the same time. In general, the relative binding activity of a mutant protein under nonreplication conditions closely matched the activity under replication conditions. Similarly, immunoprecipitated and immunoaffinity-purified T antigens had about the same relative activity. Table 1 shows the averages of two to three determinations of several different protein preparations. Class 1 mutants are characterized by the inability to bind specifically to the SV40 origin while maintaining a WT or near WT level of nonspecific DNA-binding activity. Class 2 mutants are identified as having impaired origin-specific and nonspecific DNA binding. Mutant 203HN, in particular, appears to be very defective at interacting with DNA, in agreement with our earlier observations (35). Class 3 mutants, however, appear to be binding DNA at near-normal levels.

DNase footprinting assays. The quantitative DNA-binding data were extended by DNase footprinting assays of immunoaffinity-purified T antigens. Figure 1 shows DNase footprints of selected mutants from each class, and Table 1 summarizes the results for each mutant protein. All class 1 and 2 mutants have aberrant footprints (Fig. 1A and Table 1), in agreement with the results of the quantitative DNA-binding assays. We have already published (35) footprints of the 153NS, 185ST, and 203HN mutant proteins expressed in human 293 cells under the control of the adenovirus major late promoter. These older data are in very good agreement with those obtained with baculovirus-expressed proteins (Fig. 1A and Table 1). Most class 1 and 2 mutants protected the origin from DNase digestion only in the presence of large excesses of mutant protein (Fig. 1A and Table 1). In con-

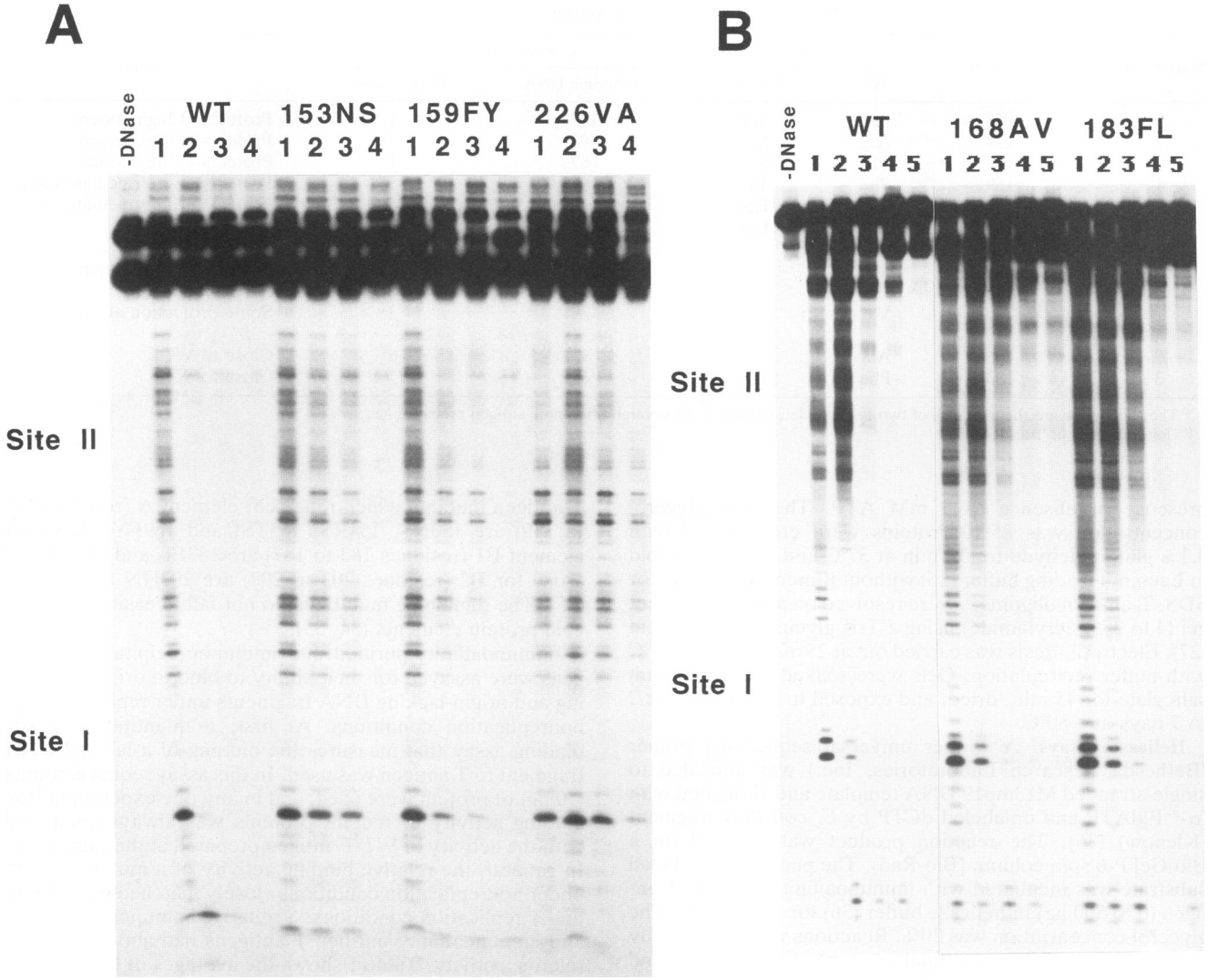

FIG. 1. DNase footprinting of T-antigen mutants. Immunoaffinity-purified T antigen was incubated with end-labeled DNA containing the origin of replication. The samples were treated with DNase, and the DNA was purified by phenol-chloroform extractions and precipitated with ethanol. Samples were applied to a 7% sequencing acrylamide gel. The gel was dried and exposed to X-ray film. (A) Footprints of three class 1 and 2 mutants. For each T-antigen preparation tested, 0, 170, 340, and 680 ng of T antigen were added for the reactions shown in lanes 1 through 4, respectively. (B) Footprints of class 3 mutants. The amounts of T antigen present in the reactions were 0, 63, 125, 250, and 500 ng for lanes 1 through 5, respectively. For panels A and B, one sample received no T antigen or DNase (−DNase). Regions of the gels corresponding to DNA fragments that terminate in T-antigen-binding sites I and II were determined by a G-only sequencing reaction of the same end-labeled DNA (not shown).

trast, both class 3 mutants (168AV and 183FL) have near normal DNA-binding activity as measured by this assay (Fig. 1B). It is estimated that only slightly more mutant T antigen in the reaction would be needed to give the same level of DNase protection as WT T antigen. Results of both DNA-binding assays (Table 1) are consistent with the interpretation that these two mutants have a measurable but very slight defect in DNA binding.

$KMnO_4$ sensitivity assays. When T antigen binds to site II at the replication origin, it induces a structural alteration (untwisting) of the AT-rich tract on the late side of the recognition pentanucleotides and melts the DNA at the imperfect palindrome on the early side (3, 4, 8, 28). These changes can be detected by $KMnO_4$ sensitivity assays (3, 13). In this procedure, $KMnO_4$ oxidizes thymine rings that are improperly base paired. The modification can be detected by primer extension reactions (13).

We investigated whether mutant proteins belonging to classes 1 and 2 are defective in melting and untwisting reactions, as one would predict from their DNA-binding activities, and whether those belonging to class 3 perform these reactions normally. Immunoaffinity-purified T antigen was bound, under replication conditions, to unlabeled plasmid DNA containing the SV40 origin, and the DNA was subjected to $KMnO_4$ oxidation and primer extension. Figure 2A shows results of melting assays of several class 1 and 2 mutants, and Fig. 2B shows results for class 3 mutants. Quantitative results from all mutants are indicated in Table 2. As expected, class 1 and 2 mutants had impaired melting and untwisting activities. Surprisingly, the levels of these

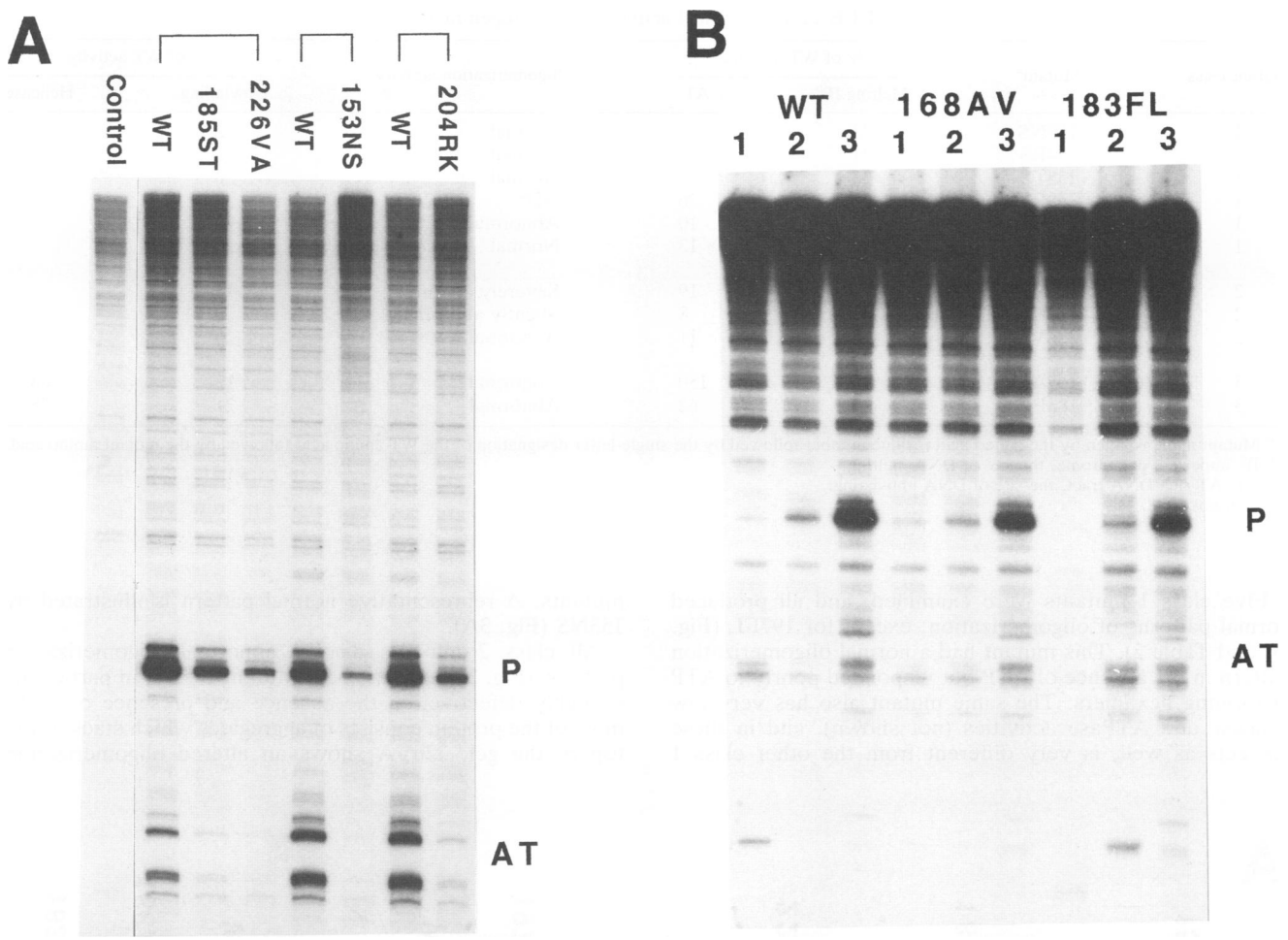

FIG. 2. $KMnO_4$ sensitivity assays of T-antigen mutants. Immunoaffinity-purified T antigen was incubated with pSV01ΔEP DNA, and the samples were treated with $KMnO_4$. DNA was purified by passage through a spin column and then subjected to primer extension analysis. The labeled products were separated on a sequencing gel, and modified residues were determined by dideoxy sequencing using the same primer (not shown). The extent of melting at the imperfect palindrome (P) and untwisting at the AT-rich tract (AT) was quantitated by densitometric tracing and normalized to background bands as described previously (26). (A) $KMnO_4$ assays of some class 1 and 2 mutants. One sample received no T antigen (control). All others contained 1 μg of T antigen. (B) $KMnO_4$ assays of class 3 mutants. Samples contained 125, 250, and 500 ng of T antigen for lanes 1 through 3, respectively.

activities were much higher than one would predict from the mutants' severe defect in origin binding (Table 2). For example, 153NS, 155TS, 185ST, and 204RK displayed about 25% of WT melting and untwisting activities despite having only about 1% of WT's origin-binding activity (Table 1). Mutant 159FY had more than half of the normal melting activity, yet bound only 3% as much origin DNA as WT T antigen did. This observation is consistent, nonetheless, with those of Parsons et al. (26), who determined that the melting and untwisting reactions are not absolutely origin dependent. It is conceivable that T antigen's nonspecific binding to double or single-stranded DNA has a role in this reaction (18), since the mutants were generally less defective in nonspecific than origin-specific binding. Moreover, 203HN, which displayed no detectable nonspecific activity, also had no melting activity (Tables 1 and 2).

In contrast to the results with class 1 and 2 mutants, those in class 3 had normal ability to melt and untwist the origin (Fig. 2B and Table 2). Furthermore, in a subsequent article (51) which will describe three additional classes, all of the mutants that have normal DNA binding also have normal melting and untwisting activities. Hence, we have yet to isolate a mutant which binds DNA normally and fails to fully melt and untwist. These results suggest that the melting and untwisting reactions are a natural consequence of normal binding to DNA.

Oligomerization. The binding of ATP to T antigen induces protein oligomerization. In the absence of ATP, a tetramer is the largest oligomer found, while in the presence of ATP (but in the absence or presence of DNA), T antigen assembles into hexamers and double hexamers (4, 20). We studied the abilities of mutant T antigens to oligomerize normally by using a nondenaturing gradient gel electrophoresis system (20). This assay system is able to resolve a ladder of various oligomers of T antigen, including the presumed hexamers and double hexamers formed in the presence of ATP. Mutant T-antigen proteins were always compared with equal amounts of WT proteins purified at the same time, and at least two separate preparations were made of the mutants with abnormal oligomerization activities.

TABLE 2. Biochemical activities of T-antigen mutants

Mutant class	Mutant[a]	% of WT activity		Oligomerization activity	% of WT activity	
		Melting IP[b]	AT[c]		Unwinding	Helicase
1	153NS	33	5	Normal	2	
1	154RS	31	46	Normal	2	
1	155TS	33	23	Normal	3	
1	159FY	60	26	ND[d]	0	
1	197FL	87	10	Abnormal	3	
1	204RK	25	13	Normal	0	
2	185ST	34	19	Severely abnormal	14	
2	203HN	0	8	Slightly abnormal	0	
2	226VA	8	11	Abnormal	6	
3	168AV	90	150	Abnormal	12	84
3	183FL	85	62	Abnormal	21	75

[a] Mutant name is given by the amino acid residue number followed by the single-letter designation of the WT amino acid followed by the mutant amino acid.
[b] IP, imperfect palindrome, the site of DNA melting.
[c] AT, AT-rich DNA tract, the site of DNA untwisting.
[d] ND, not done.

Five class 1 mutants were examined, and all produced normal patterns of oligomerization, except for 197FL (Fig. 3A and Table 2). This mutant had a normal oligomerization pattern in the absence of ATP but responded poorly to ATP in forming hexamers. The same mutant also has very low helicase and ATPase activities (not shown), and in these respects as well, is very different from the other class 1 mutants. A representative normal pattern is illustrated by 153NS (Fig. 3A).

All class 2 mutants display abnormal oligomerization patterns (Fig. 3A and Table 2). Mutant 185ST, in particular, is highly defective. In the absence and presence of ATP, most of the protein consists of aggregates which stack at the top of the gel. 226VA shows an altered oligomerization

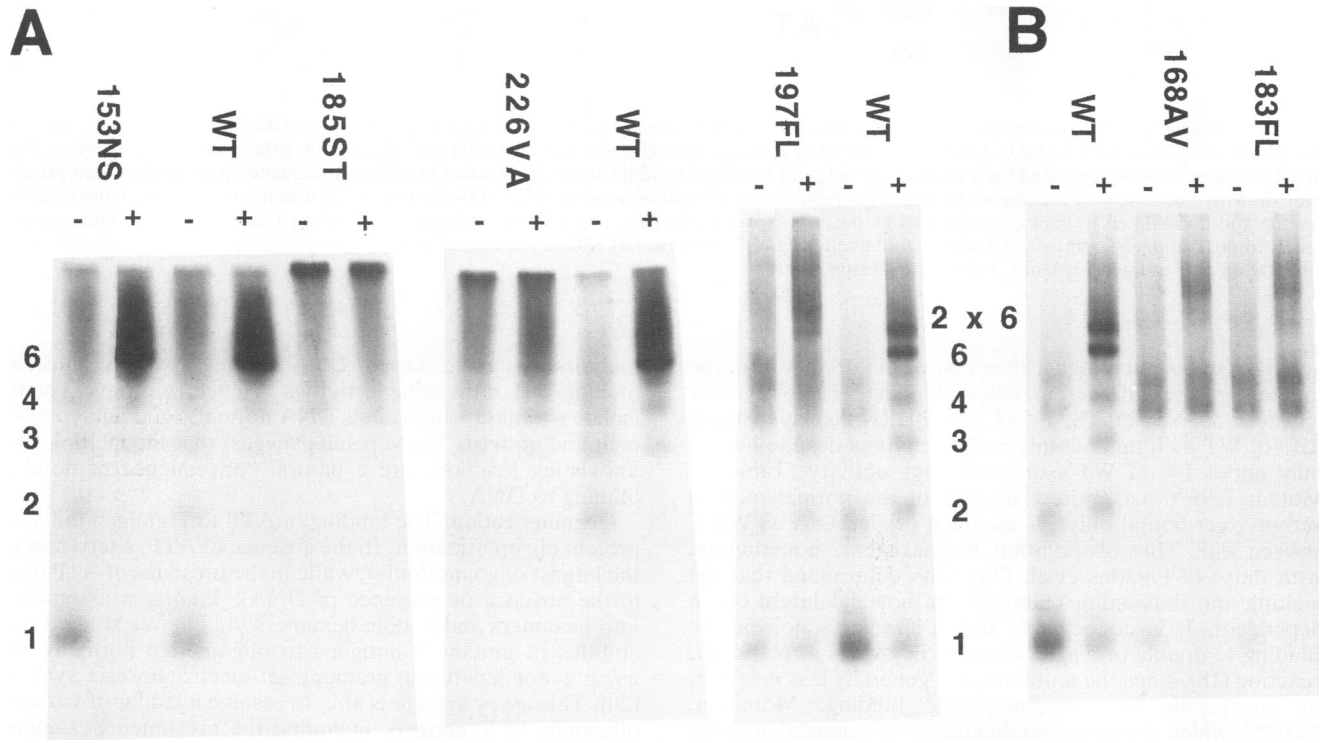

FIG. 3. Oligomerization assays of T-antigen mutants. ^{35}S-labeled immunoaffinity-purified T antigen was incubated in replication buffer in the presence (+) or absence (−) of 4 mM ATP. Samples containing about 15,000 cpm of purified T antigen were applied to a 4 to 25% acrylamide gel gradient and subjected to electrophoresis. (A) Oligomerization of representative class 1 and 2 mutants. (B) Oligomerization of class 3 mutants. A WT control reaction was included for each experiment. The positions in the gel of apparent monomers, dimers, etc. are indicated by the numbers 1, 2, 3, 4, and 6. Possible double hexamers are indicated by 2 × 6.

pattern in the absence of ATP. The monomer and dimer bands are missing, and there are protein aggregates toward the top of the gel. With ATP, a very faint hexamer band as well as additional aggregates can be seen (Fig. 3A).

Both of the class 3 mutants, 168AV and 183FL, exhibit similar defective patterns of oligomerization (Fig. 3B). In the absence of ATP, only very faint monomer and dimer bands are visible. When ATP is present, few if any hexamers and double hexamers are formed, although other bands appear further up in the gel.

Overall, three patterns of abnormal oligomerization were observed. The first pattern, as seen with 197FL, looks fairly normal in the absence of ATP. However, the response to ATP is weak, with much reduced formation of hexamers and double hexamers. In the second pattern, defects in oligomerization are detected in both the absence and presence of ATP. Mutants 168AV, 183FL, and 226VA show only very faint bands of monomers and dimers in the absence of ATP. When ATP is present, very few hexamers and double hexamers are formed, and instead, poorly defined higher aggregates are seen. The third pattern is the most abnormal and is demonstrated by 185ST. In both the presence and absence of ATP, high-molecular-weight aggregates which barely penetrate the gel are detected. This pattern is similar to that reported for mutants in the zinc finger region (20) and for several mutants examined in our laboratory, e.g., 584PL, and mutants truncated at the carboxy-terminal end [e.g., T(1-623)] (18).

Unwinding. When a double hexamer of T antigen is formed at the origin and melts and untwists the correct sequences, it can unwind the origin, probably in association with replication protein A and DNA polymerase α-primase complex (5, 6, 25, 42, 43, 48). This unwinding reaction depends on T antigen's helicase activity (45). An assay that simulates this reaction has been developed (12). This assay measures the unwinding of an origin-containing double-stranded DNA fragment in the presence of *E. coli* single-strand binding protein. This assay is dependent on the replication origin in the sense that WT T antigen fails to unwind a nonorigin DNA sequence under the same conditions (Fig. 4C). Furthermore, helicase-positive (35) mutant T antigens which fail to recognize the origin, but which bind DNA nonspecifically (i.e., most class 1 mutants) do not unwind origin-containing DNA (Fig. 4A and Table 2, 153NS, 154RS, 155TS, 159FY, and 204RK).

Figure 4A and 4B show the unwinding of origin DNA by a number of mutants in classes 1 to 3. Quantitative data are indicated in Table 2. All of the mutants described in this study have defective unwinding origin DNA activities. However, we have isolated a number of other mutant proteins that bind DNA normally and are fully able to unwind this DNA (51). In general, we found that class 1 and 2 mutants are extremely defective at origin unwinding. Class 3 mutants are somewhat less defective.

Helicase activity. It is easy to explain the inability of the DNA-binding defective class 1 and 2 mutants to unwind origin DNA. The unwinding defect of the class 3 mutants which bind, melt, and untwist DNA at near normal levels is, however, harder to explain. Although DNA binding (2, 10) and melting (3, 4) rely on the binding of ATP, ATP hydrolysis is not required, whereas ATP hydrolysis is necessary for unwinding. One possible explanation, therefore, is that the class 3 mutants are defective in ATP hydrolysis and/or helicase activity. We tested this possibility by performing a helicase assay as described previously (36). Figure 5 and Table 2 show that the two mutants in this class have WT

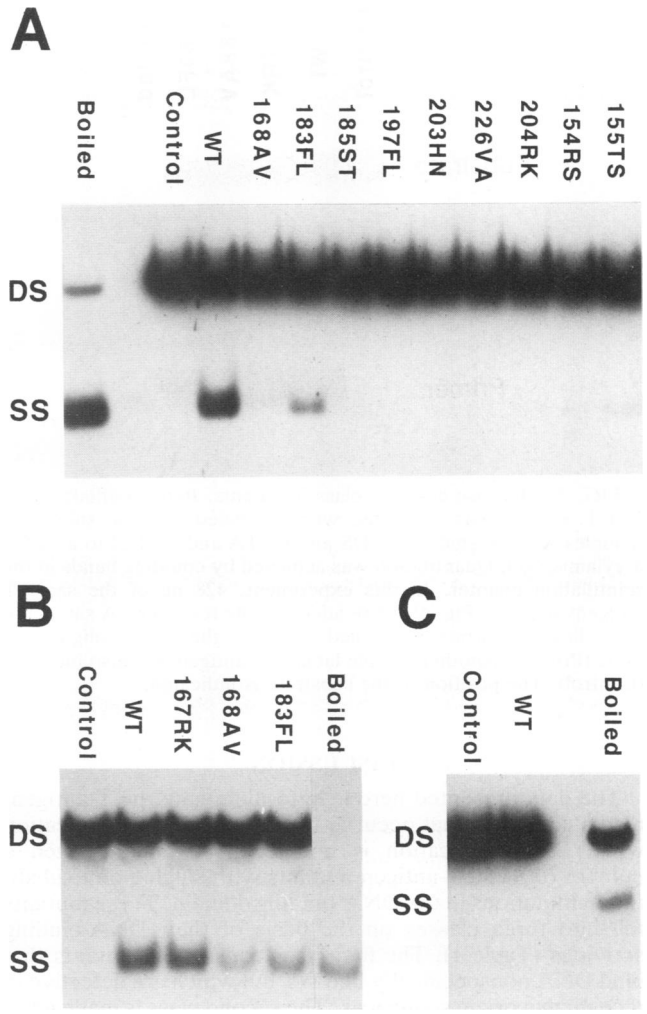

FIG. 4. Origin DNA unwinding assays of T-antigen mutants. Immunoaffinity-purified T antigen was incubated with an end-labeled origin-containing DNA fragment in the presence of *E. coli* single-strand binding protein. Samples were treated with proteinase K, heated, and applied to a nondenaturing 7% acrylamide gel. To quantitate the unwinding signal, bands were cut out and radioactivity was counted by using a scintillation counter. (A) Unwinding assay of representative class 1, 2, and 3 mutants. Each sample received 438 ng of T antigen. (B) Unwinding assay of class 3 mutants. Different preparations of immunoaffinity-purified T antigen were used in panel B than in panel A. In this experiment, an unwinding-positive T-antigen mutant (167RK) was included as a second positive control. Each sample received 428 ng of T antigen in this experiment. (C) Unwinding assay using an end-labeled 183-bp DNA fragment obtained from the promoter region of the rat muscle creatine kinase gene. WT T antigen (428 ng) was used. In each experiment, a sample of the labeled DNA was boiled and applied to the gel (Boiled). Another sample lacking T antigen was also included in each experiment (Control). The positions of the native (double-stranded [DS]) and denatured (single-stranded [SS]) DNAs are indicated.

levels of helicase activity. In a previous publication (35), we demonstrated that the mutants belonging to class 1 (except for 197FL) had normal helicase activity, whereas those of class 2 (which are defective in nonspecific binding) had impaired helicase activity.

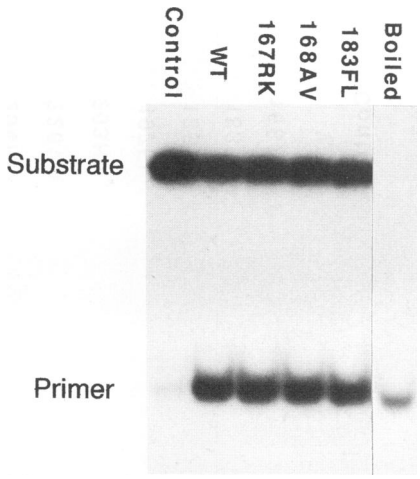

FIG. 5. Helicase assay of class 3 mutants. Immunoaffinity-purified T antigen was incubated with a labeled helicase substrate. Samples were treated with SDS and EDTA and applied to a 12.5% acrylamide gel. Quantitation was achieved by counting bands in the scintillation counter. In this experiment, 428 ng of the same T antigens used for Fig. 4B were added to the reactions. A sample of the helicase substrate was boiled to release the labeled oligonucleotide (Primer). Another sample lacking T antigen was also included (Control). The position of the substrate is indicated.

DISCUSSION

The data presented here help to illuminate the T-antigen-mediated events that occur at the SV40 origin of replication when DNA replication is initiated. We characterized a number of SV40 T-antigen mutants with single point substitution mutations in the DNA-binding domain. These mutants fell into three classes on the basis of their DNA-binding activities (Table 1). The first class consists of mutants that bind DNA nonspecifically like WT but which are defective in recognizing origin sequences. The second class is made up of a number of mutants which fail to bind normally to all DNAs. The third class is composed of two mutants which appear to bind to DNA at close to WT levels but which are defective at unwinding origin-containing DNA (Table 1 and Fig. 1).

By testing the abilities of the mutant proteins to melt the imperfect palindrome and to untwist the AT-rich tract at the origin, we determined that the mutants with defective DNA-binding activity (classes 1 and 2) have somewhat repressed DNA distortion activity. The level of activity was higher than expected, since all of the mutants, except for 203HN, retained a significant ability to melt and untwist despite having extremely defective origin-specific DNA-binding activity. Because the mutants had, for the most part, less impaired nonspecific DNA-binding activity, a nonorigin double-stranded or single-stranded DNA binding activity may be responsible for part or all of the melting reaction. This finding would be consistent with the results of Parsons et al. (26), who determined that T antigen can melt the imperfect palindrome in the absence of other sequences from the origin. Class 3 mutants (and mutants in additional classes with normal DNA binding [51]), however, had close to normal melting and untwisting activities. This result indicates that these reactions are natural consequences of normal binding to DNA.

We evaluated the abilities of these mutants to oligomerize into hexamers and double hexamers like WT T antigen. Class 1 mutants were, except for 197FL, able to form oligomers like WT (Table 2), indicating that these mutants have defective origin binding because they are missing some ability to recognize the origin (e.g., they lack a contact site), and not because they are structurally altered. Class 2 mutants, on the other hand, all had abnormal oligomerization patterns, indicating that they were structurally changed. This structural defect might explain their inability to recognize DNA nonspecifically. One possible exception to this applies to mutant 203HN which had only slightly defective oligomerization activity and yet had very defective DNA-binding activity (Tables 1 and 2).

Interestingly, the class 3 mutants were also unable to oligomerize normally (Fig. 3B and Table 2). Changes in oligomerization patterns were observed in the presence of ATP, but the proper structures were not formed. Although it is possible that these structures are hexameric, their migration in acrylamide gels indicate that they at least have aberrant structures. This observation strongly suggests that the formation of correctly formed hexamers and double hexamers is not essential for near WT levels of DNA-binding, melting, and untwisting activities. The same applies to the helicase activity, since the class 3 mutants have normal helicase activity as well. Wessel et al. (45) and SenGupta and Borowiec (29) identified hexamers and double hexamers as the forms that bind to artificial forks and concluded that they are the structures that normally unwind parental DNA strands. Our results are not inconsistent with this model. They simply imply that although the process normally involves hexameric T antigen, some improperly formed mutant structures can carry out these reactions.

It is perhaps surprising that so many of our mutants oligomerize abnormally. We observed at least three phenotypes of oligomerization defects in mutants with changes in the DNA-binding domain. It is plausible, perhaps even likely, that the DNA-binding domain contains sequences that are directly involved in oligomerization. Alternatively, it is possible that the effects of the mutations on oligomerization are indirect and that the sites of protein-protein interactions reside elsewhere. In either case, the different oligomerization phenotypes probably reflect the mechanism by which T-antigen oligomers form. Mutants like 197FL must predominantly have defective responses to ATP. At least one mutant, 203HN, forms hexamers and double hexamers in the presence of ATP but also aggregates into higher-molecular-weight forms (not shown), suggesting that it has subtle variations in structure. More-defective mutants like 226VA, 168AV, and 183FL have more global structural changes, indicating the involvement of larger regions of the protein in the formation of hexamers. Finally, mutants like 185ST, which are probably partially denatured in the absence or presence of ATP implicate the DNA-binding domain in the overall structure and stability of the protein.

Not surprisingly, class 1 and 2 mutants were unable to unwind origin-containing DNA normally (Table 2 and Fig. 4). However, class 3 mutants, although able to bind and melt at close to WT levels, were unexpectedly defective in origin-unwinding and oligomerization. These proteins must be missing an activity specifically required for opening up origin-containing duplex DNA. This deficiency is not due to a failure to perform the helicase reaction (Fig. 5 and Table 2). These data indicate that the block in these mutants must be at some step after melting but before a nonspecific helicase is needed to separate the two parental strands at the replication forks. It is inferred, therefore, that the unwinding of origin-containing DNA requires the assembly of properly formed

hexamers and/or double hexamers at the origin but that some incorrectly formed structures can substitute for them in DNA binding, melting, untwisting, and helicase activities. This difference reflects, perhaps, the complex task of unwinding DNA in a sequence-specific manner.

The results presented in this article characterize three classes of mutants with changes in the DNA-binding domain. First, we found a close association between the ability of T antigen to bind normally to DNA in the presence of ATP and to structurally distort the DNA. Second, a close relationship was identified between the inability to form hexamers and double hexamers and a deficiency in origin unwinding. A subsequent article (51) will detail the properties of mutants that fall into three additional classes. These other mutants are normal in all of the biochemical activities described in this article but still cannot support virus replication. We find it intriguing that a relatively small region of a protein can be so complex.

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