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The N-Terminal Side of the Origin-Binding Domain of Simian Virus 40 Large T Antigen Is Involved in A/T Untwisting

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We investigated the role of the N-terminal side of simian virus 40 (SV40) large T antigen's origin-binding domain in the initiation of virus DNA replication by analyzing the biochemical activities of mutants containing single point substitutions or deletions in this region. Four mutants with substitutions at residues between 121 and 135 were partially defective in untwisting the A/T-rich track on the late side of the origin but were normal in melting the imperfect palindrome (IP) region on the early side. Deletion of the N-terminal 109 amino acids had no effect on either activity, whereas a longer deletion, up to residue 123, greatly reduced A/T untwisting but not IP melting. These results indicate that the region from residue 121 to 135 is important for A/T untwisting but not for IP melting and demonstrate that these activities are separable. Two point substitution mutants (126PS and 135PL) were characterized further by testing them for origin DNA binding, origin unwinding, oligomerization, and helicase activity. These two mutants were completely defective in origin (form U_R) unwinding but normal in the other activities. Our results demonstrate that a failure to normally untwist the A/T track is correlated with a defect in origin unwinding. Further, they indicate that some mutants with substitutions in the region from residue 121 to 135 interact with origin DNA incorrectly, perhaps by failing to make appropriate contacts with the A/T-rich DNA.

Simian virus 40 (SV40) T antigen is a multifunctional protein and the only viral protein involved in SV40 DNA replication. To initiate DNA replication, T antigen must interact with three distinct DNA elements at the SV40 replication origin (12–14, 43): a central palindrome that has four pentanucleotide repeats, an early-side imperfect palindrome (IP), and a late-side adenine-thymine (A/T)-rich region. T antigen initiates SV40 DNA replication in discrete steps, each of which is a consequence of a defined biochemical activity. First, activated by ATP, T antigen forms a double hexamer which binds to the SV40 origin (34, 65). The protein then melts the DNA in the IP region and untwists the A/T-rich region, leading to a structurally distorted origin (2, 11, 27, 43, 45). After formation of the distorted origin, T antigen functions as a DNA helicase to unwind the origin (22, 64, 68). This reaction requires ATP hydrolysis and a single-strand-specific DNA binding protein (RPA) (30, 59, 66). With closed circular DNA as the substrate, a DNA topoisomerase must be present to relieve the torsional strain generated during unwinding (10, 17). In a subsequent step, the cell DNA polymerase α-primase (8, 18–20, 38, 41) binds to unwound origin complexes through specific protein-protein contacts with T antigen, and perhaps with RPA, and short RNA-DNA primers are synthesized at the origin (6, 16, 42, 62). Then, cellular replication factor C, proliferating cell nuclear antigen, and DNA polymerase δ are recruited to the unwound origin complex, forming the DNA replication forks (21, 59, 60, 62, 63).

As a replication initiator protein, T antigen binds to the SV40 origin and targets two separate regions of the DNA (IP and A/T) for structural distortion. There is little information about how T antigen functions to distort the SV40 origin. Some information about the requirements for origin distortion has come from the study of T antigen's origin-binding domain, which is located between amino acids 147 and 246 (1, 33, 50, 52, 58). In our lab, we have analyzed the biochemical activities of a large series of mutants with single-site substitutions in this region (52–54, 70). Our analysis shows that all mutants that bind normally to DNA also distort the DNA like wild-type T antigen does (53, 70). Furthermore, there are many mutants that bind to origin DNA very poorly and yet distort DNA to significant levels. These observations indicate that structural distortion, although dependent on DNA binding for full activity, is nevertheless distinct from DNA recognition and binding. This suggests that a T-antigen region outside the origin-binding domain is required for structural distortion.

The DNA replication activities of T antigen depend on its state of phosphorylation. Phosphorylation at threonine 124 significantly upregulates its activity (35, 48), whereas phosphorylation at serine 120 and serine 123 function in downregulation (7, 48, 51, 61). Replacement of threonine 124 by alanine led to a complete loss of DNA replication activity in vivo and in vitro (48), and an alanine substitution for serine 120 or 123 dramatically reduced in vivo replication (48). Other substitutions at neighboring sites prevented DNA replication (29), suggesting that the region N terminal to the DNA-binding domain has some crucial function.

We therefore investigated the role of this region in DNA replication by making single point substitution mutations and analyzing their effects on DNA binding, melting of the IP sequence, untwisting of the A/T-rich region, unwinding of origin DNA, oligomerization, and the helicase reaction. The results reveal that the N-terminal side of the DNA-binding domain has a role in A/T untwisting and DNA unwinding but is not required for IP melting activity.

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MATERIALS AND METHODS

Site-directed mutagenesis. Mutagenesis of T antigen was performed as described previously (32) by annealing a synthetic oligonucleotide containing a single mismatch to uridine-containing single-stranded pSK(−)SVTC DNA (31). This oligonucleotide functioned as a primer for complementary strand synthesis by T4 DNA polymerase (New England BioLabs). The newly synthesized second strand was then sealed with T4 ligase. The DNA was used to transform *Escherichia coli* BMH71-18 or JM109. Plasmid DNA was prepared from individual colonies and sequenced by a standard dideoxy procedure with appropriate primers.

Construction of recombinant baculoviruses. Recombinant baculoviruses were generated as previously described (31) with some modifications. For wild-type and substitution mutants of T antigen, a *Bam*HI fragment of pSK(−)SVTC was inserted into the *Bam*HI site of pVL941 (Pharmingen). This generated the baculovirus transfer vector pVL941T. For N-terminal deletion mutants of T antigen, cDNA was generated by PCR amplification of specific regions of the T-antigen gene by using wild-type pSK(−)SVTC as a template and two primers bearing a *Bam*HI or *Eco*RI site at the 5′ end. The PCR DNA was purified from agarose gels, cleaved with *Bam*HI and *Eco*RI, and ligated to *Bam*HI- and *Eco*RI-cleaved pVL1393 DNA to make transfer vector pVL1393T. Recombinant baculoviruses were generated by cotransfection of the baculovirus transfer vector and BaculoGold DNA (Pharmingen) in *Spodoptera frugiperda* (Sf9) cells as described by the manufacturer. T-antigen-expressing recombinant viruses were detected by immunofluorescence and were further purified by two rounds of plaque assays.

Immunoaffinity purification. Immunoaffinity purification of T antigen was performed as previously described (53). Two T150 flasks of Sf9 cells were infected with recombinant virus at 10 PFU per cell. At 44 to 48 h postinfection, the cells were washed with TD buffer (25 mM Tris, 136 mM NaCl, 0.7 mM Na_2HPO_4 [pH 7.4]) and lysed with buffer B (150 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 [NP-40], 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol [DTT] [pH 8.0]). The cellular extract was clarified by centrifugation and incubated with Biogel P6 resin to remove sticky and aggregated proteins. The supernatant was then incubated with protein A-Sepharose beads cross-linked with the monoclonal antibody pAb101 (25, 26). The beads were then washed with wash buffer 1 (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40 [pH 8.0]) and wash buffer 2 (same as wash buffer 1 but without NP-40). T antigen was eluted in 250-μl aliquots with elution buffer (50% ethylene glycol, 20 mM Tris, 500 mM NaCl, 1 mM EDTA, 10% glycerol [pH 8.5]). Eluted T antigen was dialyzed overnight against dialysis-storage buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, 50% glycerol, 1 mM DTT [pH 8.0]) and stored at −20°C. The concentration of the purified protein was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and silver staining of the gel.

$KMnO_4$ assay. Structural distortion (melting and untwisting) of the SV40 origin was detected by $KMnO_4$ oxidation of thymidine residues in distorted DNA regions as described by Borowiec and Hurwitz (2). Four hundred to 1,600 ng of immunoaffinity-purified T antigen was incubated with 0.5 μg of SV40 origin-containing plasmid pSVOΔEP (67) under binding conditions (40 mM creatine phosphate [di-Tris salt, pH 7.8], 7 mM $MgCl_2$, 1 mM DTT, 4 mM ATP) for 60 min. $KMnO_4$ was added to a final concentration of 6 mM, and the mixture was incubated for an additional 4 min at 37°C. The oxidation reaction was stopped by the addition of mercaptoethanol to 1.0 M. The 30-μl reaction mixture was diluted to 55 μl with water, and the DNA from the reaction mixture was purified on a Sephadex G-50 column. The DNA was subjected to primer extension analysis with a ^{32}P-end-labeled *Eco*RI pBR322 sequencing primer (Life Technologies) and Klenow enzyme. The DNA was denatured and subjected to electrophoresis on a DNA sequencing gel. The positions of the modified thymidines on the gel were determined by comparison with dideoxy DNA sequencing products of the same plasmid DNA. The melting and untwisting activities were quantitated by cutting out the appropriate bands, followed by scintillation counting. Values were normalized to the amount of radioactivity in background bands.

Gel shift assays. The binding of T antigen to the SV40 core origin and to the late-half origin was tested by gel shift assays. For core origin DNA binding, a ^{32}P-end-labeled *Nco*I-*Hin*dIII fragment of pOR1 (15) was used. For late-half origin DNA binding, we used an *Eco*RI-*Hin*dIII fragment from pLH (44). Both reactions were carried out in the presence of a 100-fold mass excess of competitor sonicated calf thymus DNA in DNA replication buffer (30 mM HEPES [pH 7.5], 7 mM $MgCl_2$, 40 mM creatine phosphate, 4 mM ATP, 1 mM DTT, 20 μg of creatine phosphokinase per ml, 0.1 mg of bovine serum albumin per ml). The reactions were performed with 200 to 800 ng of immunoaffinity-purified T antigen at 37°C for 30 min. The DNA-protein complexes were then cross-linked with 0.1% glutaraldehyde for an additional 10 min at 37°C and subjected to electrophoresis at 25 mA for 2 to 3 h on a native 4% polyacrylamide gel in TBE (0.089 M Tris, 0.089 M boric acid, 0.02 M Na-EDTA). The gels were dried and exposed to X-ray film. The regions of the gel representing bound and unbound DNA were cut out and quantitated by Cerenkov counting.

Form U_R unwinding. T-antigen-dependent form U_R unwinding reactions were based on the methods of Dean et al. (10) and Bullock et al. (5). Reactions were conducted under replication conditions (67), and reaction mixtures contained 30 μl of crude HeLa cell extracts (12.3 mg of protein/ml), 1.0 μg of immunoaffinity-purified T antigen, and 0.75 μg of pSV01ΔEP (67). After a 15-min incubation, the reactions were terminated by the addition of 6 μl of stop mixture (14 mM EDTA, 0.25 mg of *N*-lauroylsarcosine per ml, 0.45 mg of proteinase K per ml). DNA was purified by phenol-chloroform extraction and subjected to electrophoresis through a 1.8% agarose gel containing chloroquine (1.5 mg/ml) and Tris-acetate-EDTA buffer (46) for 14 h at 2.8 V/cm. The gel was stained for photography as described previously (10).

Oligomerization assay. Four hundred nanograms of immunoaffinity-purified T antigen was incubated for 10 min on ice in 30 μl of replication buffer (30 mM HEPES [pH 7.5], 7 mM $MgCl_2$, 4 mM creatine phosphate, 1 mM DTT, 20 μg of creatine phosphokinase per ml, 20% glycerol) and in the presence or absence of 4 mM ATP. Proteins were cross-linked with 0.1% glutaraldehyde for an additional 15 min at 37°C and diluted twofold in Laemmli loading buffer without β-mercaptoethanol and SDS (44). T-antigen oligomers were resolved on a 4 to 20% native gradient gel (Bio-Rad) for 16 h at 100 V and 4°C with buffer recirculation using a Tris-glycine buffer system without SDS. The proteins were blotted onto a nitrocellulose membrane and detected by reaction with pAb101 and ECL reagent (Amersham).

Helicase activity assay. The helicase activity assay was performed by incubating 200, 400, and 800 ng of purified T antigen with 2 ng of a ^{32}P-labeled *Nco*I-*Eco*RI fragment of pIR/AT (43) in 20 μl of unwinding buffer (10 mM Tris [pH 7.5], 40 mM creatine phosphate, 3.5 mM ATP, 2 mM $MgCl_2$, 1 mM DTT, 80 mM NaCl, 20 μg of bovine serum albumin per ml, 50 μg of phosphocreatine kinase per ml) containing 500 ng of *E. coli* single-stranded binding protein. After 1 h at 37°C, the reaction was terminated by the addition of EDTA to 20 mM, SDS to 0.5%, and proteinase K to 0.2 mg/ml, and incubation was continued for 30 min at 37°C. The unwound DNA was resolved by electrophoresis on a 7% acrylamide gel for 1,000 V · h at 2 to 3°C and visualized by autoradiography of the dried gel. The DNA bands were cut out and counted by scintillation counting.

TABLE 1. Structural-distortion activity of T-antigen mutants

Amino acid residue no. and wild type	Mutation	Mutant name	Distortion activity (% of wild type)		Replication in vivo[a]
			IP melting	A/T untwisting	
120 Ser	Ala	120SA	95	95	Low
121 Gln	Leu	121QL	84	50	Not known
123 Ser	Ala	123SA	78	80	Low
126 Pro	Ser	126PS	86	14	Negative
127 Lys	Thr	127KT	90	40	Negative
135 Pro	Leu	135PL	60	8	Negative

[a] Data from Kalderon and Smith (29) and Schneider and Fanning (48).

RESULTS

We have previously found that all T-antigen mutants carrying mutations in the origin-binding domain which bind to DNA normally also structurally distort origin DNA normally (53). In fact, some mutants with minimal binding activity have measurable distortion activity (53). In addition, wild-type T antigen can weakly melt the IP sequence at the origin even in the absence of a specific binding sequence (44). These observations suggest that the region of T antigen needed for structural distortion maps outside of the origin-binding domain (53). On the amino-terminal side of the origin-binding domain, there is a cluster of phosphorylation sites at residues 120 to 124 that participates in DNA binding and DNA replication (48). To further characterize the role of this region in DNA replication, we made several point substitution mutations between amino acid residues 120 and 135 (Table 1). Some of these mutations have been shown by others to affect virus DNA replication in vivo (Table 1) (29, 48). The mutant genes were cloned into recombinant baculoviruses, and mutant T antigens were isolated from infected insect cells by immunoaffinity chromatography. As a first test, we examined the purified proteins for the ability to bind to origin-containing DNA, and none were defective (data not shown, but see Fig. 3A).

The N-terminal side of the DNA-binding domain of T antigen is involved in A/T untwisting. We then determined if these

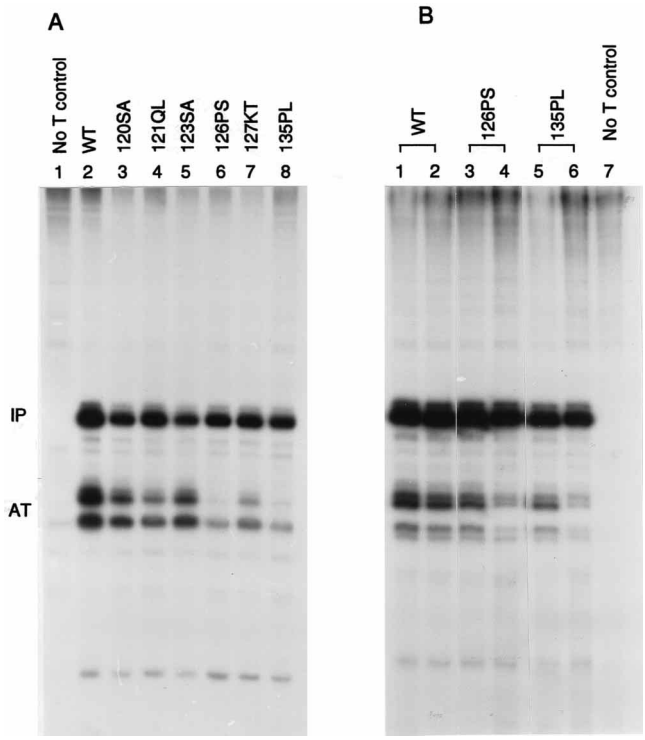

FIG. 1. SV40 origin DNA structural-distortion assays of wild-type and mutant T antigens. Immunoaffinity-purified T antigen was incubated with pSV01ΔEP DNA for 1 h at 37°C in replication buffer and treated with $KMnO_4$. DNA was purified by passage through a Sephadex G-50 spin column and then subjected to primer extension analysis. The ^{32}P-labeled products were resolved on a sequencing gel, and modified residues were determined by dideoxy sequencing with the same primer. (A) $KMnO_4$ assays with 400 ng of wild-type and mutant T antigens 120SA, 121QL, 123SA, 126PS, 127KT, and 135PL. (B) $KMnO_4$ assays with 800 (lanes 1, 3, and 5) and 400 (lanes 2, 4, and 6) ng of wild-type and mutant T antigens 126PS and 135PL. The melting (IP) and untwisting (AT) signals are shown.

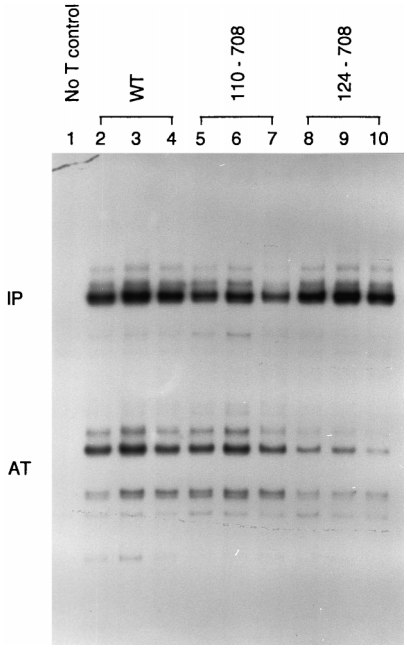

FIG. 2. SV40 origin DNA structural-distortion assays of wild-type and N-terminal deletion mutant T antigens. $KMnO_4$ assays with 1,600 (lanes 2, 5, and 8), 800 (lanes 3, 6, and 9), and 400 (lanes 4, 7, and 10) ng of wild-type or mutant T antigens are represented. The melting (IP) and untwisting (AT) signals are shown.

mutants were able to distort the structure of the replication origin. When T antigen binds to the core origin, it melts the DNA at the IP on the early side of the recognition pentanucleotides and untwists the A/T-rich region on the late side (2, 11, 27, 45). These structural changes are sensitive to $KMnO_4$ modification, which in turn can be detected by primer extension reactions (4, 23). Immunopurified T antigens were incubated under replication conditions with supercoiled plasmid DNA containing the SV40 origin. The DNA was subjected to $KMnO_4$ oxidation and primer extension. Figure 1A shows that mutants 121QL, 126PS, 127KT, and 135PL (lanes 4, 6, 7, and 8, respectively, and Table 1) have reduced A/T untwisting activity but normal (or near normal) IP melting activity. Analysis of additional mutants with single substitutions between amino acids 110 and 119 showed no defect in structural distortion (data not shown).

We selected the two most defective mutants, 126PS and 135PL, and repeated the $KMnO_4$ sensitivity assay with different amounts of T antigen. Results were quantitated by scintillation counting. 126PS has normal melting activity but less than 50% of the wild type's untwisting activity at the higher concentration of T antigen (Fig. 1B, lane 3) and about 20% of the expected untwisting activity at the low concentration of T antigen (Fig. 1B, lane 4). 135PL may have slightly reduced melting activity, but it has less than 40% of the wild type's untwisting activity at the high concentration of T antigen (Fig. 1B, lane 5) and about 10% at the low concentration (Fig. 1B, lane 6). These results suggest that the T-antigen region from amino acid 120 to 135 is involved in A/T untwisting but not in IP melting.

To confirm the importance of this region in A/T untwisting, we generated N-terminal deletion mutants 110–708 and 124–708 and cloned them into recombinant baculoviruses. Purified mutant proteins were tested for structural distortion activity (Fig. 2). Mutant 110–708, which contains a deletion of the first 109 amino acids, carries out both reactions (melting and untwisting) normally, whereas 124–708 melts the IP region normally but is severely depressed in its ability to untwist the A/T region. These results support the conclusion that the region from amino acid residue 121 to 135 is important for A/T untwisting.

Mutant T antigens bind to the replication origin normally. Since the melting and untwisting reactions require binding to the replication origin, we tested the untwisting-defective mutants 126PS and 135PL for the ability to bind the origin. Two DNA fragments were used as binding substrates: a site II-containing *Hin*dIII-*Nco*I fragment obtained from pOR1 (15) and a *Hin*dIII-*Eco*RI fragment obtained from pLH (44) containing the late half of the origin. The first DNA fragment contains all three DNA elements from the virus minimal origin, whereas the second contains only two of the four central pentanucleotide recognition sequences and the A/T-rich track. Different amounts of T antigen were incubated with labeled substrate in replication buffer (57) at 37°C for 30 min and then cross-linked with glutaraldehyde. The T antigen-DNA complexes were separated from free DNA by gel electrophoresis. Figure 3 shows that the mutants bind to the core origin and to the late half in a manner similar to the wild type at all concentrations of T antigen. Based on comparisons with published data (3, 44), the fastest- and slowest-migrating gel-shifted bands in Fig. 3A probably correspond to hexamers and double

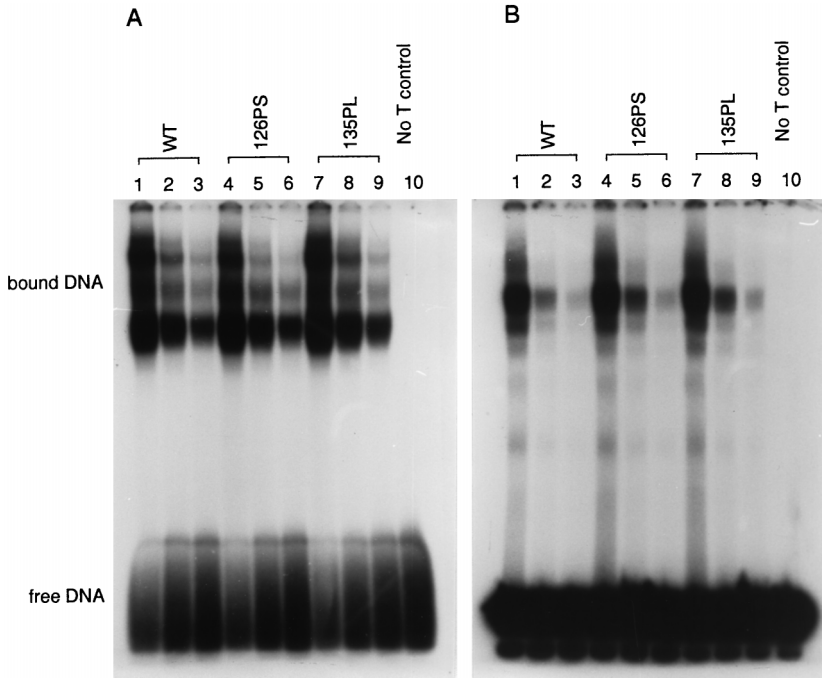

FIG. 3. SV40 origin DNA gel shift assays of wild-type and mutant T antigens. Samples contained 800 (lanes 1, 4, and 7), 400 (lanes 2, 5, and 8), and 200 (lanes 3, 6, and 9) ng of immunoaffinity-purified T antigen. T antigen was incubated with a ^{32}P-labeled *Nco*I-*Hin*dIII fragment of pOR1 (A) or an *Eco*RI-*Hin*dIII fragment of pLH (B) for 30 min at 37°C in replication buffer containing a 100-fold mass excess of competitor sonicated calf thymus DNA. The protein-DNA complexes were then cross-linked with 0.1% glutaraldehyde for 10 min at 37°C. The samples were subjected to electrophoresis at 25 mA for 2 to 3 h on a native 4% polyacrylamide gel in TBE.

hexamers bound to the core origin fragment. The hexamer is most likely bound to the early half of the origin, based on its higher affinity to that side of the origin (44). The band between the hexamers and double hexamers may represent a hexamer bound to the early side and low-molecular-weight oligomers bound to the late side. This intermediate is missing in binding reactions performed in the absence of calf thymus DNA (not shown). Reactions performed with the late half (Fig. 3B) indicate that the major gel-shifted species consists of hexamers bound to DNA, but lower-molecular-weight oligomers are present as well. In all cases, there was very little difference between the DNA-protein complexes formed with wild-type and mutant T antigens. Mutant 126PS may be slightly altered in its ability to form double hexamers on DNA, but 135PL is completely normal in this regard (Fig. 3A).

A/T untwisting-defective mutants are negative in form U_R unwinding. After T antigen structurally distorts the origin, it then functions as a helicase to unwind the DNA (10, 55). Unwinding of DNA can be measured by the amount of partially unwound circular DNA (form U_R) generated in the presence of a HeLa cell extract. We tested whether the untwisting-defective substitution mutants 126PS and 135PL were able to carry out the form U_R unwinding reaction. Both were completely inactive (Fig. 4), probably indicating that their defect in A/T untwisting prevents them from properly unwinding origin-containing circular DNA.

A/T untwisting mutants oligomerize normally. T antigen's oligomerization into hexamers and double hexamers is important for the initiation of unwinding (34, 64). To exclude the possibility that the mutants' defect in unwinding is due to abnormal oligomerization, we examined their ability to form oligomers in the presence or absence of ATP. Oligomers were cross-linked with glutaraldehyde and subjected to electrophoresis through a nondenaturing gradient gel (Fig. 5). The results show that mutant T antigens have the same oligomerization profiles as wild-type T antigen. With all three proteins, monomers were undetectable and more hexamers were formed in the presence of ATP. Higher-molecular-weight species, probably including double hexamers, were also present in all samples in the presence of ATP (Fig. 5). This indicates that the substitution mutations in 126PS and 135PL did not change the ability of the proteins to oligomerize and that the defect in A/T untwisting does not correlate with an inability to oligomerize or form double hexamers.

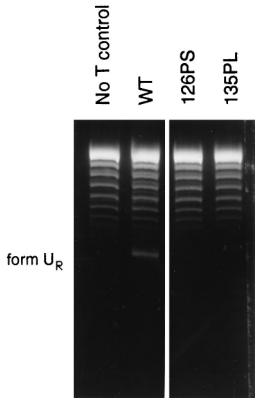

FIG. 4. Form U_R unwinding by wild-type and mutant T antigens. The topological isomers of pSV01ΔEP generated after 15 min of incubation of the plasmid in crude HeLa cell extracts with 1.0 μg of T antigen or no T antigen (control) are shown. Samples were analyzed on a 1.8% agarose gel containing chloroquine (1.5 mg/ml). The position of form U_R is indicated.

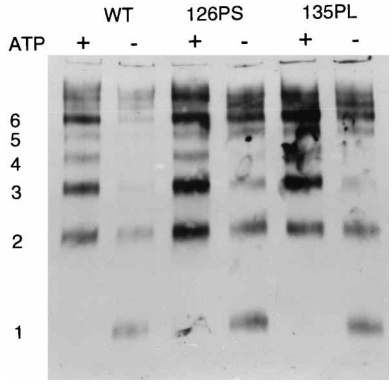

FIG. 5. Oligomerization assays of wild-type and mutant T antigens. Immunoaffinity-purified T antigen was incubated in the presence of replication buffer with (+) or without (−) ATP. Samples were subjected to electrophoresis in a 4 to 20% native gradient polyacrylamide gel. Proteins were visualized by Western blotting. The positions in the gel of apparent monomers to hexamers are indicated by the numbers 1 to 6, respectively.

A/T untwisting-defective mutants are normal in helicase activity. We compared the helicase activities of wild-type and mutant T antigens by testing their ability to unwind a DNA fragment missing the pentanucleotide binding sites (47, 56). Unwinding was carried out under relaxed conditions in which binding to origin sequences was not required. Unwound single-stranded DNA was detected by gel electrophoresis (Fig. 6). The two point mutants and wild-type T antigen have similar helicase activities at all three protein concentrations tested. Therefore, the defect in form U_R unwinding by these mutants is not due to an inability to perform the helicase reaction. The helicase reaction is catalyzed by double hexamers (64), further supporting our data that the mutants are not defective in oligomerization. In addition, since the helicase activity of T antigen requires a large region of the protein which includes the DNA-binding and ATPase domains (66, 69), the mutations at residues 126 and 135 do not affect the activity of a large part of the protein molecule.

DISCUSSION

In this study, we showed that several mutants of T antigen with point substitutions between amino acids 121 and 135 are partially defective in untwisting the A/T track but not in melting the early IP sequence at the SV40 origin of replication. Consistent with this, a deletion mutant missing the first 123 residues (124–708) has a similar defect, whereas a mutant with a deletion of the first 109 amino acids (110–708) is normal in both activities. These results suggest that the N-terminal side of the origin-binding region including residues 121 to 135 is involved in A/T untwisting but not in IP melting. This provides evidence that the structural distortion reactions that take place on the two sides of the GAGGC recognition pentanucleotides are separable. This finding is not unexpected, since the two DNA sequences, as well as the reactions that take place at the two sites, are different. Furthermore, Borowiec et al. (4) found that the melting and A/T untwisting reactions respond differently to changes in temperature and ATP concentrations. Together these data argue that T antigen interacts with these two regions of the origin differently.

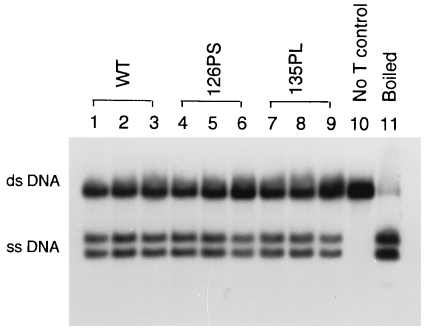

FIG. 6. Helicase activity assays of wild-type and mutant T antigens. Samples contained 800 (lanes 1, 4, and 7), 400 (lanes 2, 5, and 8), and 200 (lanes 3, 6, and 9) ng of immunoaffinity-purified T antigen. T antigen was incubated with a ^{32}P-labeled *Nco*I-*Eco*RI fragment of pIR/AT at 37°C for 60 min in unwinding buffer in the absence of NaCl and in the presence of *E. coli* single-stranded binding protein. Samples were treated with proteinase K and subjected to electrophoresis on a native 7% polyacrylamide gel in TBE. The positions of double-stranded (ds) and single-stranded (ss) DNA are indicated.

Further characterization of two A/T untwisting-defective mutants (126PS and 135PL) showed that these mutants oligomerize normally and bind core origin DNA and late-half DNA at close to normal levels. In addition, since the mutants are normal in helicase activity, they are most likely not severely altered in structure. Our results indicate that the defect in A/T untwisting is not due to an inability to form a hexamer on either side of the origin. Mutant 135PL appeared to be perfectly able to form double hexamers on DNA, while 126PS may have a minor defect in hexamer-hexamer interaction in the presence of DNA (Fig. 3A). However, our results are consistent with the interpretation that both mutants are blocked at a step after the formation of double hexamers at the origin.

Although the mutant T antigens bind to the origin with wild-type efficiency and give rise to the same gel-shifted bands, it is possible that the binding interaction with the late half of the origin is abnormal. Binding to the origin is dominated by the central pentanucleotide element, and a possible defect in contacting the A/T track would not be detected. It is therefore likely that the deficiency in A/T untwisting is due to an inability to make the correct interactions with the DNA at the A/T track. This possible mechanism is consistent with Borowiec's results (3), which showed that nucleotide substitutions at the A/T track inhibit A/T untwisting but not binding to the mutated origin by T antigen. Binding of T antigen with the A/T track alone has not been detected (reference 43 and unpublished observations), so this simple test cannot be used to test our model.

The 126PS and 135PL mutants are completely defective at unwinding a circular plasmid containing the SV40 origin (form U_R unwinding). This form U_R unwinding defect is not due to an absence of helicase activity, since the mutants can unwind a DNA fragment like the wild type can (Fig. 6). Our interpretation is that their form U_R unwinding defect is caused by a failure to untwist the A/T track normally. Consistent with this, we found that deletion mutant 124–708, which is also defective in A/T untwisting, is unable to generate form U_R, whereas 110–708 carries out this reaction normally (data not shown). Our conclusion is in agreement with previous studies showing that mutations at the A/T track that inhibit A/T untwisting (3) also dramatically reduce the ability of wild-type T antigen to generate form U_R (9).

The region of T antigen implicated in A/T untwisting is phosphorylated at several sites. Phosphorylation of threonine 124 is essential for DNA replication (35, 48), whereas phosphorylation of serine 120 and serine 123 reduces DNA replication activity (24, 40, 48, 61). Substitution of alanine for serine at either of the latter two sites has no effect on melting and untwisting activities (Fig. 1 and Table 1), consistent with the

inhibitory effect of phosphorylation. Mutations of threonine 124 have been well characterized (36, 39). Replacement of threonine with alanine has no significant effect on structural distortion but depresses origin binding (48) and inactivates origin unwinding (36, 39). It has been proposed that phosphorylation of that site enhances the binding of four subunits of T antigen to the four pentanucleotide repeats at the origin (37) and enhances hexamer-hexamer interactions (36, 37, 39). The point mutants studied here are defective in structural distortion but bind origin DNA normally and therefore have a different phenotype than the Thr-124 mutants. The function of phosphothreonine 124 must therefore be different from that of residues 121, 126, 127, and 135.

Although a small region N terminal to the DNA-binding domain is implicated here in untwisting the A/T track, other regions of the protein must be involved in the structural distortion reactions. Structural distortion is mediated mostly by contacts with the sugar-phosphate backbone of the IP and A/T elements (49); therefore, nonspecific DNA binding may be important for this activity. We have previously determined that two regions of the protein mediate nonspecific DNA binding (31). One region maps to the DNA-binding domain (residues 147 to 246) and the second maps to residues 269 to 522. We have found that monoclonal antibody pAb413 (28), which binds to an epitope located between residues 333 and 451 and inactivates nonspecific DNA binding (31), strongly inhibits structural distortion (data not shown) but does not interfere with origin DNA binding (31). The binding site for pAb413 appears, therefore, to be required for structural distortion. The results of this study imply that the pAb413 binding region and the region around residues 121 to 135 must cooperate with one another for correct distortion of the viral origin of DNA replication.

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REFERENCES

1. **Arthur, A. K., A. Höss, and E. Fanning.** 1988. Expression of simian virus 40 T antigen in *Escherichia coli*: localization of T-antigen origin DNA-binding domain to within 129 amino acids. J. Virol. **62:**1999–2006.
2. **Borowiec, J., and J. Hurwitz.** 1988. Localized melting and structural changes in the SV40 origin of replication induced by T-antigen. EMBO J. **7:**3149–3158.
3. **Borowiec, J. A.** 1992. Inhibition of structural changes in the simian virus 40 core origin of replication by mutations of essential origin sequences. J. Virol. **66:**5248–5255.
4. **Borowiec, J. A., F. B. Dean, and J. Hurwitz.** 1991. Differential induction of structural changes in the simian virus 40 origin of replication by T antigen. J. Virol. **65:**1228–1235.
5. **Bullock, P. A., W. S. Joo, K. R. Sreekumar, and C. Mello.** 1997. Initiation of SV40 DNA replication in vitro: analysis of the role played by sequences flanking the core origin on initial synthesis events. Virology **227:**460–473.
6. **Bullock, P. A., Y. S. Seo, and J. Hurwitz.** 1991. Initiation of simian virus 40 DNA synthesis in vitro. Mol. Cell. Biol. **11:**2350–2361.
7. **Cegielska, A., I. Moarefi, E. Fanning, and D. M. Virshup.** 1994. T-antigen kinase inhibits simian virus 40 DNA replication by phosphorylation of intact T antigen on serines 120 and 123. J. Virol. **68:**269–275.
8. **Collins, K. L., and T. J. Kelly.** 1991. Effects of T antigen and replication protein A on the initiation of DNA synthesis by DNA polymerase α-primase. Mol. Cell. Biol. **11:**2108–2115.
9. **Dean, F. B., Y. Borowiec, Y. Ishimi, S. Deb, P. Tegtmeyer, and J. Hurwitz.** 1987. Simian virus 40 large tumor antigen requires three core replication origin domains for DNA unwinding and replication in vitro. Proc. Natl. Acad. Sci. USA **84:**8267–8271.
10. **Dean, F. B., P. Bullock, Y. Murakami, C. R. Wobbe, L. Weissbach, and J. Hurwitz.** 1987. Simian virus 40 (SV40) DNA replication: SV40 large T antigen unwinds DNA containing the SV40 origin of replication. Proc. Natl. Acad. Sci. USA **84:**16–20.
11. **Dean, F. B., and J. Hurwitz.** 1991. Simian virus 40 large T antigen untwists DNA at the origin of DNA replication. J. Biol. Chem. **266:**5062–5071.
12. **Deb, S., A. L. DeLucia, C. P. Baur, A. Koff, and P. Tegtmeyer.** 1986. Domain structure of simian virus 40 core origin of replication. Mol. Cell. Biol. **6:**1663–1670.
13. **Deb, S., A. L. DeLucia, A. Koff, S. Tsui, and P. Tegtmeyer.** 1986. The adenine-thymine domain of the simian virus 40 core origin directs DNA bending and coordinately regulates DNA replication. Mol. Cell. Biol. **6:**4578–4584.
14. **Deb, S., S. Tsui, A. Koff, A. L. DeLucia, R. Parsons, and P. Tegtmeyer.** 1987. The T-antigen binding domain of the simian virus 40 core origin of replication. J. Virol. **61:**2143–2149.
15. **DeLucia, A. L., S. Deb, K. Partin, and P. Tegtmeyer.** 1986. Functional interactions of simian virus 40 core origin of replication with flanking regulatory sequences. J. Virol. **57:**138–144.
16. **Denis, D., and P. A. Bullock.** 1993. Primer-DNA formation during simian virus 40 DNA replication in vitro. Mol. Cell. Biol. **13:**2882–2890.
17. **Dodson, M., F. B. Dean, P. Bullock, H. Echols, and J. Hurwitz.** 1987. Unwinding of duplex DNA from the SV40 origin of replication by T antigen. Science **238:**964–967.
18. **Dornreiter, I., W. C. Copeland, and T. S.-F. Wang.** 1993. Initiation of simian virus 40 DNA replication requires the interaction of a specific domain of human DNA polymerase α with large T antigen. Mol. Cell. Biol. **13:**809–820.
19. **Dornreiter, I., L. F. Erdile, I. U. Gilbert, D. von Winkler, T. J. Kelly, and E. Fanning.** 1992. Interaction of DNA polymerase alpha-primase with cellular replication protein A and SV40 T antigen. EMBO J. **11:**769–776.
20. **Dornreiter, I., A. Hoss, A. K. Arthur, and E. Fanning.** 1990. SV40 T antigen binds directly to the catalytic subunit of DNA polymerase α. EMBO J. **9:**3329–3336.
21. **Eki, T., T. Matsumoto, Y. Murakami, and J. Hurwitz.** 1992. The replication of DNA containing the simian virus 40 origin by the monopolymerase and dipolymerase systems. J. Biol. Chem. **267:**7284–7294.
22. **Goetz, G. S., F. B. Dean, J. Hurwitz, and S. W. Matson.** 1988. The unwinding of duplex regions in DNA by the simian virus 40 large tumor antigen-associated DNA helicase activity. J. Biol. Chem. **263:**383–392.
23. **Gralla, J. D.** 1985. Rapid "footprinting" on supercoiled DNA. Proc. Natl. Acad. Sci. USA **82:**3078–3081.
24. **Grasser, F. A., K. Mann, and G. Walter.** 1987. Removal of serine phosphates from simian virus 40 large T antigen increases its ability to stimulate DNA replication in vitro but has no effect on ATPase and DNA binding. J. Virol. **61:**3373–3380.
25. **Gurney, E. G., R. O. Harrison, and J. Fenno.** 1980. Monoclonal antibodies against simian virus 40 T antigens: evidence for distinct subclasses of large T antigen and for similarities among nonviral T antigens. J. Virol. **34:**752–763.
26. **Gurney, E. G., S. Tamowski, and W. Deppert.** 1986. Antigenic binding sites of monoclonal antibodies specific for simian virus 40 large T antigen. J. Virol. **57:**1168–1172.
27. **Han, F. X., and L. H. Hurley.** 1996. A model for the T-antigen-induced structural alteration of the SV40 replication origin based upon experiments with specific probes for bent, straight, and unwound DNA. Biochemistry **35:**7993–8001.
28. **Harlow, E., L. V. Crawford, D. C. Pim, and N. M. Williamson.** 1981. Monoclonal antibodies specific for simian virus 40 tumor antigen. J. Virol. **39:**861–869.
29. **Kalderon, D., and A. E. Smith.** 1984. In vitro mutagenesis of a putative DNA binding domain of SV40 large-T. Virology **139:**109–137.
30. **Kenny, M. K., S. H. Lee, and J. Hurwitz.** 1989. Multiple functions of human single-stranded-DNA binding protein in simian virus 40 DNA replication: single-strand stabilization and stimulation of DNA polymerase α and δ. Proc. Natl. Acad. Sci. USA **86:**9757–9761.
31. **Lin, H.-J. L., R. Upson, and D. T. Simmons.** 1992. Nonspecific DNA binding activity of simian virus 40 large T antigen: evidence for the cooperation of two regions for full activity. J. Virol. **66:**5443–5452.
32. **Loeber, G., R. Parsons, and P. Tegtmeyer.** 1989. The zinc finger region of simian virus 40 large T antigen. J. Virol. **63:**94–100.
33. **Luo, X., D. G. Sanford, P. A. Bullock, and W. W. Bachovchin.** 1996. Solution structure of the origin DNA-binding domain of SV40 T-antigen. Nat. Struct. Biol. **3:**1034–1039.
34. **Mastrangelo, I. A., P. V. C. Hough, J. S. Wall, M. Dodson, F. B. Dean, and J. Hurwitz.** 1989. ATP-dependent assembly of double hexamers of SV40 T antigen at the viral origin of DNA replication. Nature (London) **338:**658–662.
35. **McVey, D., L. Brizuela, I. Mohr, D. R. Marshak, Y. Gluzman, and D. Beach.** 1989. Phosphorylation of large tumour antigen by cdc2 stimulates SV40 DNA replication. Nature (London) **341:**503–507.
36. **McVey, D., S. Ray, Y. Gluzman, L. Berger, A. G. Wildeman, D. R. Marshak, and P. Tegtmeyer.** 1993. cdc2 phosphorylation of threonine 124 activates the origin-unwinding functions of simian virus 40 T antigen. J. Virol. **67:**5206–5215.
37. **McVey, D., B. Woelker, and P. Tegtmeyer.** 1996. Mechanism of simian virus 40 T-antigen activation by phosphorylation of threonine 124. J. Virol. **70:**3887–3893.
38. **Melendy, T., and B. Stillman.** 1993. An interaction between replication

protein A and SV40 T antigen appears essential for primosome assembly during SV40 DNA replication. J. Biol. Chem. **268:**3389–3395.
39. **Moarefi, I. F., D. Small, I. Gilbert, M. Hopfner, S. K. Randall, C. Schneider, A. A. R. Russo, U. Ramsperger, A. Arthur, H. Stahl, T. J. Kelly, and E. Fanning.** 1993. Mutation of the cyclin-dependent kinase phosphorylation site in simian virus 40 (SV40) large T antigen specifically blocks SV40 origin DNA unwinding. J. Virol. **67:**4992–5002.
40. **Mohr, I. J., B. Stillman, and Y. Gluzman.** 1987. Regulation of SV40 DNA replication by phosphorylation of T antigen. EMBO J. **6:**153–160.
41. **Murakami, Y., and J. Hurwitz.** 1993. DNA polymerase alpha stimulates the ATP-dependent binding of simian virus tumor T antigen to the SV40 origin of replication. J. Biol. Chem. **268:**11018–11027.
42. **Nethanel, T., and G. Kaufmann.** 1990. Two DNA polymerases may be required for synthesis of the lagging DNA strand of simian virus 40. J. Virol. **64:**5912–5918.
43. **Parsons, R., M. E. Anderson, and P. Tegtmeyer.** 1990. Three domains in the simian virus 40 core origin orchestrate the binding, melting, and DNA helicase activities of T antigen. J. Virol. **64:**509–518.
44. **Parsons, R. E., J. E. Stenger, S. Ray, R. Welker, M. E. Anderson, and P. Tegtmeyer.** 1991. Cooperative assembly of simian virus 40 T-antigen hexamers on functional halves of the replication origin. J. Virol. **65:**2798–2806.
45. **Roberts, J.** 1989. Simian virus 40 (SV40) large tumor antigen causes stepwise changes in SV40 origin structure during initiation of DNA replication. Proc. Natl. Acad. Sci. USA **86:**3939–3943.
46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd. ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
47. **Scheffner, M., R. Wessel, and H. Stahl.** 1989. Sequence independent duplex DNA opening reaction catalyzed by SV40 large T antigen. Nucleic Acids Res. **17:**93–106.
48. **Schneider, J., and E. Fanning.** 1988. Mutations in the phosphorylation sites of simian virus 40 (SV40) T antigen alter its origin DNA-binding specificity for sites I or II and affect SV40 DNA replication activity. J. Virol. **62:**1598–1605.
49. **SenGupta, D. J., and J. A. Borowiec.** 1994. Strand and face: the topography of interactions between the SV40 origin of replication and T-antigen during the initiation of replication. EMBO J. **13:**982–992.
50. **Simmons, D. T.** 1986. DNA-binding region of the simian virus 40 tumor antigen. J. Virol. **57:**776–785.
51. **Simmons, D. T., W. Chou, and K. Rodgers.** 1986. Phosphorylation down-regulates the DNA-binding activity of simian virus 40 T antigen. J. Virol. **60:**888–894.
52. **Simmons, D. T., G. Loeber, and P. Tegtmeyer.** 1990. Four major sequence elements of simian virus 40 large T antigen coordinate its specific and nonspecific DNA binding. J. Virol. **64:**1973–1983.
53. **Simmons, D. T., R. Upson, K. Wun-Kim, and W. Young.** 1993. Biochemical analysis of mutants with changes in the DNA-binding domain of simian virus 40 tumor antigen. J. Virol. **67:**4227–4236.
54. **Simmons, D. T., K. Wun-Kim, and W. Young.** 1990. Identification of simian virus 40 T antigen residues important for specific and nonspecific binding to DNA and for helicase activity. J. Virol. **64:**4858–4865.
55. **Stahl, H., P. Droge, and R. Knippers.** 1986. DNA helicase activity of SV40 large tumor antigen. EMBO J. **5:**1939–1944.
56. **Stahl, H., M. Scheffner, M. Wiekowski, and R. Knippers.** 1988. DNA unwinding function of the SV40 large tumor antigen. Cancer Cells **6:**105–112.
57. **Stillman, B. W., and Y. Gluzman.** 1985. Replication and supercoiling of simian virus 40 DNA in cell extracts from human cells. Mol. Cell. Biol. **5:**2051–2060.
58. **Strauss, M., P. Argani, I. J. Mohr, and Y. Gluzman.** 1987. Studies on the origin-specific DNA-binding domain of simian virus 40 large T antigen. J. Virol. **61:**3326–3330.
59. **Tsurimoto, T., M. P. Fairman, and B. Stillman.** 1989. Simian virus 40 DNA replication in vitro: identification of multiple stages of initiation. Mol. Cell. Biol. **9:**3839–3849.
60. **Tsurimoto, T., and B. Stillman.** 1991. Replication factors required for SV40 DNA replication *in vitro*. II. Switching of DNA polymerase α and δ during initiation of leading and lagging strand synthesis. J. Biol. Chem. **266:**1961–1968.
61. **Virshup, D. M., A. A. Russo, and T. J. Kelly.** 1992. Mechanism of activation of simian virus 40 DNA replication by protein phosphatase 2A. Mol. Cell. Biol. **12:**4883–4895.
62. **Waga, S., and B. Stillman.** 1994. Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication in vitro. Nature (London) **369:**207–212.
63. **Weinberg, D. H., K. L. Collins, P. Simancek, A. Russo, M. S. Wold, D. M. Virshup, and T. J. Kelly.** 1990. Reconstitution of simian virus 40 DNA replication with purified proteins. Proc. Natl. Acad. Sci. USA **87:**8692–8696.
64. **Wessel, R., J. Schweizer, and H. Stahl.** 1992. Simian virus 40 T-antigen DNA helicase is a hexamer which forms a binary complex during bidirectional unwinding from the viral origin of DNA replication. J. Virol. **66:**804–815.
65. **Wiekowski, M., P. Dröge, and H. Stahl.** 1987. Monoclonal antibodies as probes for function of large T antigen during the elongation process of simian virus 40 DNA replication. J. Virol. **61:**411–418.
66. **Wiekowski, M., M. W. Schwarz, and H. Stahl.** 1988. Simian virus 40 large T antigen DNA helicase. Characterization of the ATPase-dependent DNA unwinding activity and its substrate requirements. J. Biol. Chem. **263:**436–442.
67. **Wobbe, C. R., L. Weissbach, J. H. Borowiec, F. Dean, and J. Hurwitz.** 1985. *In vitro* replication of duplex circular DNA containing the simian virus 40 DNA origin site. Proc. Natl. Acad. Sci. USA **82:**5710–5714.
68. **Wold, M. S., J. J. Li, and T. J. Kelly.** 1987. Initiation of simian virus 40 DNA replication in vitro: large-tumor-antigen- and origin-dependent unwinding of the template. Proc. Natl. Acad. Sci. USA **84:**3643–3647.
69. **Wun-Kim, K., and D. T. Simmons.** 1990. Mapping of helicase and helicase substrate binding domains on simian virus 40 large T antigen. J. Virol. **64:**2014–2020.
70. **Wun-Kim, K., R. H. Upson, W. Young, and D. T. Simmons.** 1993. The DNA binding domain of simian virus 40 large T antigen has multiple functions. J. Virol. **67:**7608–7611.