Two Conditional *tsA* Mutant Simian Virus 40 T Antigens Display Marked Differences in Thermal Inactivation

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We have characterized the simian virus 40 (SV40) origin-containing DNA (ori-DNA) replication functions of two SV40 conditional mutant T antigens: *tsA438* A-V (*tsA58*) and *tsA357* R-K (*tsA30*). Both *tsA* mutant T antigens, immunopurified from recombinant baculovirus-infected insect cells, mediated replication of SV40 ori-DNA in vitro to similar extents as did wild-type T antigen in reactions at 33°C. However, at 41°C, the restrictive temperature, while *tsA438* T antigen still generated substantial levels of replication products, *tsA357* T antigen did not support any detectable DNA synthesis. Furthermore, preincubation for approximately fourfold-longer time periods at 41°C was required to heat inactivate *tsA438* T antigen than to heat inactivate *tsA357* T antigen. Unexpectedly, results of analyses of the various DNA replication activities of the two mutant T antigens did not correlate with results from ori-DNA replication reactions. In particular, although *tsA357* T antigen was incapable of mediating replication at 41°C at all protein concentrations examined, it displayed either wild-type levels or only partial reductions of the several T-antigen replication-associated activities. These data suggest either that *tsA357* T antigen is defective in an as yet unidentified replication function of T antigen or that the combination of its partial defects result in a protein that is unable to support replication. The data also show that two conditional mutant T antigens can be markedly different with respect to thermal sensitivity.

Analysis of conditional temperature-sensitive (*tsA*) simian virus 40 (SV40) mutants revealed that the product of the viral A gene, SV40 T antigen, has a role in the initiation of viral DNA replication in infected monkey cells (9, 63). Since the original isolation and characterization of these mutants, the availability of methods to overproduce T antigen and the development of experimental procedures to analyze SV40 origin-containing DNA (ori-DNA) replication in vitro have led to the identification of several biochemical activities of T antigen that pertain to its role in viral DNA synthesis (for reviews, see references 3, 20, and 37 and references therein). T antigen is a sequence-specific DNA-binding protein that binds to regions within and adjacent to the viral core replication origin (17, 19, 36, 46). In addition to its well-characterized interactions with the viral core origin, T antigen was shown to bind to and hydrolyze nucleotides such as ATP (11, 28, 67) and to be a DNA helicase, translocating along single-stranded DNA in the 3'-to-5' direction (13, 60, 71). The interactions of T antigen with ATP and other nucleotides cause the protein to assemble into hexamers (41). In the presence of ATP or ATP analogs, T antigen displays altered and increased binding to the SV40 origin (5, 18), resulting in the formation of a nucleoprotein complex that contains two T-antigen hexamers (14, 16, 41, 50, 52). This complex induces melting of specific regions within the origin (4, 6, 49). Extensive unwinding of the origin by utilizing the T-antigen DNA helicase is subsequently achieved by a process that requires hydrolysis of ATP and single-stranded-DNA-binding protein (SSB) (13, 15, 22, 73). T antigen also binds to DNA polymerase α (23, 26, 59), and this association brings polymerase α -primase and the replication machinery to the unwound SV40 origin to initiate DNA synthesis. The ATPase and DNA helicase activities of T antigen suggest that T antigen also has a role in DNA elongation as well as initiation. Evidence supporting a role

for T antigen in elongation is derived from studies indicating that T-antigen-specific monoclonal antibodies block chain elongation from SV40 minichromosome templates in vitro (61, 70). T antigen also associates with preformed replication forks, as shown by electron microscopy and DNase I footprints (2, 69).

Insight into the roles played by T antigen in viral DNA replication has been gained by the analysis of mutant forms of this protein. While many studies of the properties and defects of nonconditional T-antigen mutants were reported (reviewed in reference 20), less is known about the functional defects of conditional mutants. Loeber et al. (38) have identified the positions of the mutations of a number of the *tsA* T antigens and shown that many of these mutations are clustered within or close to the region of the protein involved in nucleotide binding and hydrolysis.

The recombinant baculovirus expression system is uniquely suited to the analysis of conditional heat-sensitive mutant proteins because insect cells grow optimally at temperatures below 30°C and proteins can be extracted and purified in their wild-type forms and then tested for their responses to increasing temperatures. We previously reported the partial characterization of the SV40 temperature-sensitive mutant T antigen *tsA58* (Ala-438 $\rightarrow$ Val) that had been cloned into a recombinant baculovirus expression vector (53). After immunopurification from baculovirus-infected insect cells, this protein was able to mediate replication of SV40 ori-DNA to the same extent as did wild-type T antigen in reactions at 33°C but was somewhat defective (25 to 50% of the level of wild-type T antigen) when added to reactions at 41°C. However, when *tsA438* T antigen was first preheated at the nonpermissive temperature for approximately 15 min, the mutant protein could not initiate viral DNA synthesis in an in vitro replication assay, presumably because it no longer bound to the origin of replication (53). To determine whether additional *tsA* mutants displayed similar properties, we constructed another conditional mutant, *tsA357*. The position of this mutation appears to be a

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hot spot. Six of seventeen independently isolated conditional *tsA* mutants in SV40 T antigen, *tsA30*, *tsA40*, *tsA47*, *tsA57*, *tsA1609*, and *tsA1637*, map to that position (38). In contrast to many of the other *tsA* T-antigen mutations, i.e., those located in the region of the designated ATP binding fold (7), amino acid 357 is positioned somewhat further upstream at a position where functional domains of T antigen overlap.

MATERIALS AND METHODS

Recombinant baculoviruses. pEV55SVT contains the DNA sequence coding for SV40 large T antigen downstream from the polyhedrin promoter (47). *tsA30*-pBR322, which was kindly provided by J. Pipas, has the whole SV40 *tsA30* genome (5,243 bp) inserted into the *Bam*HI site of pBR322 (4,363 bp). The *tsA357* mutation changes amino acid 357 of SV40 large T antigen from arginine to lysine (nucleotide [nt] 3748 from G to A). pEV55SVTsA30 was constructed by exchanging the *Pst*MI (nt 4558)-*Bcl*II (nt 2770) restriction fragment of pEV55SVT with the same fragment from *tsA30*-pBR322. The recombinant virus, vEV55SVTsA30, was constructed by cotransfecting insect cells with pEV55SVTsA30 and purified wild-type baculovirus DNA; this procedure was followed by identification of an occlusion body-negative plaque, from which the recombinant virus was purified (42). Recombinant baculoviruses vEV55SVT (47) and vEV55SVTsA58 (53) express wild-type and *tsA438* (*tsA58*) T antigen, respectively. *tsA438* T antigen has a base change at nt 3505 (C to T) that changes an alanine to valine. The base changes in the mutants were confirmed by sequencing of their DNAs, and comparison with the wild-type sequence.

Purification of T antigen. Sf21 insect cells were infected with recombinant baculoviruses vEV55SVT (47), vEV55SVTsA30, and vEV55SVTsA58 (53). Cells were lysed after 48 h of infection, and extracts were passed over T-antigen-specific cross-linked monoclonal antibody PAb419 (31)-protein A-Sepharose columns, and bound proteins were eluted, pooled, and dialyzed according to published procedures (reference 53 and references therein).

In vitro DNA replication. Replication reaction mixtures (50 μ l) contained wild-type or mutant T antigens at the indicated concentrations, 0.3 μ g of pSVS (34) containing the SV40 replication origin (ori-DNA), HeLa cell extract (300 to 400 μ g of protein), [32 P]dTTP, and other components as previously described (reference 53 and references therein). After incubation at the indicated temperature, 5- μ l aliquots of reaction mixtures were subjected either to acid precipitation and scintillation counting or to product analysis in which DNA was purified from reaction mixtures, linearized with *Bst*XI in the presence or absence of *Dpn*I, and subjected to 0.8% agarose gel electrophoresis for 1 h at 200 V and autoradiography.

Thermolysin digestion. Reaction conditions were as described previously (32) except that Tris-HCl was used at pH 6.5 instead of pH 8.0 to facilitate thermal denaturation. One microgram of each of the T antigens was digested at 41°C with increasing concentrations of thermolysin for 80 min. Protein products were then analyzed on sodium dodecyl sulfate (SDS)-10% polyacrylamide gels and silver stained.

DNase I footprinting. Reaction mixtures containing 4 mM ATP and 7 mM MgCl₂ were incubated for 5 min at 41°C. Wild-type or mutant T antigens were added, and incubation continued at 41°C for 15 min. The reaction conditions were those previously described (53) except that the SV40 origin DNA fragment (nt 5171 to 294) was digested for 2 min with 2.5 ng of DNase I per reaction. The DNase I was diluted in

20 mM CaCl₂. Following digestion, reactions were terminated with stop solution (2 M ammonium acetate, 100 mM EDTA, 0.2% SDS, 100 μ g of sheared salmon sperm DNA); the DNA was purified and then subjected to urea-8% polyacrylamide gel electrophoresis (PAGE) and autoradiography.

T-antigen oligomers. T antigen (0.6 μ g) was incubated at 41°C with 40 mM creatine phosphate and 0.5 mM dithiothreitol (DTT) in the presence or absence of 4 mM ATP and 7 mM MgCl₂. The formation of oligomers was analyzed on either 4.4 to 20% (reactions without added DNA) or 4.4 to 10% (reactions with DNA) gradient gels containing acrylamide-*N,N*-bisacrylamide (80:1) in TG buffer (50 mM Tris-HCl, 50 mM glycine [pH 8.8]). Gels were run for 2 to 4 h at 20 V/cm, electrotransferred to nitrocellulose, and probed with monoclonal antibody PAb416 (31); this procedure was followed by addition of a horseradish peroxidase-conjugated second antibody and development with 4-chloro-1-naphthol and hydrogen peroxide. The positions of native molecular weight protein standards were determined by prestaining of the nitrocellulose with Ponceau S. Where indicated, the DNA used to monitor double-hexamer formation was a 93-bp *Sst*I fragment from the SV40 origin (nt 5187 to 37). The position of the DNA was determined by autoradiography.

KMnO₄ modification. Modification reactions were carried out essentially as described previously (6) except that incubations were carried out at 33 or 41°C. Reaction mixtures were preheated for 5 min at 33 or 41°C before addition of T antigens, and then incubations continued at the respective temperatures for 30 min. Reaction mixtures (30 μ l) included 40 mM creatine phosphate, 7 mM MgCl₂, 4 mM ATP, 0.5 mM DTT, 500 ng of pATSVO DNA (SV40 origin from nt 5171 to 294 inserted between the *Hind*III and *Eco*RI sites of pAT153), and wild-type or mutant T antigen at the specified concentration. DNA (200 ng) purified from KMnO₄ modification reactions was annealed to 3.3 ng of 32 P-end-labeled 16-nt pBR322 *Hind*III primer (counterclockwise) and then extended by DNA polymerase I (Klenow). Products were analyzed on 6% polyacrylamide-urea gels.

DNA fragment unwinding assay. Reaction mixtures (10 μ l) contained 7 mM MgCl₂, 4 mM ATP, 0.5 mM DTT, 40 mM creatine phosphate, 100 μ g of creatine kinase per ml, 2.4 ng of 32 P-labeled ori-DNA (nt 5171 to 294 from the SV40 origin), and 0.3 μ g of *Escherichia coli* SSB. The mixture was incubated for 5 min at 41°C, T antigens were added, and incubation continued for 30 min at 41°C before being terminated by addition of proteinase K to 0.2 mg/ml, SDS to 2%, and EDTA to 50 mM. After incubation for an additional 30 min at 37°C, the samples were heated at 65°C for 5 min and subjected to PAGE (6% polyacrylamide gel) and autoradiography.

Materials. Sources were the following: radioisotopes, NEN Research Products; DNase I, Worthington; *E. coli* SSB, Pharmacia; DNA polymerase I large fragment (Klenow) and *Bst*XI, New England Biolabs; *Dpn*I and thermolysin, Boehringer Mannheim; horseradish peroxidase-conjugated second antibody, Cappel; 4-chloro-1-naphthol, Sigma; hydrogen peroxide, Fisher; and 16-nt pBR322 *Hind*III primer (counterclockwise), Promega.

RESULTS

The *tsA357* T antigen is markedly more thermosensitive than is *tsA438* or wild-type T antigen. Whereas *tsA* mutant SV40 viruses replicate viral DNA and produce infectious virus in monkey cells to the same extent as does wild-type

FIG. 1. Evidence that *tsA357* T antigen (T Ag) does not support replication at 41°C. (A) Complete replication reaction mixtures without DNA and T antigens were incubated for 10 min at 33°C; then 0.3 μg of pSVS and 0.6 μg of T antigens were added, and incubation continued at the indicated temperature for 3 h. One hundred percent replication was set as picomoles of dTMP incorporated at 33°C (T, 43 pmol; *tsA357*, 30 pmol; *tsA438*, 26 pmol). (B) Increasing concentrations of T antigens were incubated at 41°C with 0.3 μg of pSVS for 3 h. Reactions were analyzed by acid precipitation of 5-μl aliquots and scintillation counting.

SV40 at permissive temperatures in the range of 33 to 34°C, *tsA* viruses fail to synthesize viral DNA at restrictive temperatures in the range of 38 to 41°C (9, 63). To analyze and compare the DNA replication-associated properties of SV40 *tsA* mutant T antigens, recombinant baculoviruses vEV55S VT, vEV55*tsA58*, and vEV55*tsA30*, expressing wild-type, *tsA438*, and *tsA357* T antigens, respectively, were constructed. Insect cells infected with these viruses yielded comparable quantities of immunopurified T-antigen proteins, whose concentrations were determined by both colorimetric analysis and visualization on silver-stained polyacrylamide gels. Equivalent quantities of wild-type and mutant proteins (approximately 80% pure) were used for all experiments. To ensure reproducibility of the data obtained, several (more than three) preparations of each of the immunopurified wild-type or mutant T antigens were made, and all assays described below were conducted at least twice (in most cases, multiple times).

The abilities of the three T antigens to mediate SV40 ori-DNA synthesis at different temperatures were compared in vitro (Fig. 1A). Reaction mixtures were incubated for 10 min at 33°C in the absence of DNA and the T antigens. Upon addition of the proteins and DNA, incubation continued for 3 h at 33, 37, 39, or 41°C. Newly replicated DNA was measured by determining the amount of acid-insoluble radioactive products. Wild-type T antigen showed only minor differences in DNA synthesis at temperatures ranging from 33 to 39°C. At 41°C, however, there were relatively lesser

amounts of replication products than in reactions at lower temperatures. This reduction of replication, which was less evident in this experiment than in a study that we previously published, is likely to be the result of differences in preparations of T antigen rather than differences in the HeLa extracts, the latter of which we determined to be similar at all temperatures examined (53). This difference may also be related to the fact that in our prior published experiments (53), the extract and reaction components were not preincubated at 33°C before addition of T antigen and DNA. Replication of ori-DNA mediated by the *tsA438* T antigen was significantly more sensitive to increasing temperatures than was that mediated by wild-type T antigen, confirming our previous findings (53). At 41°C, levels of products of *tsA438* T antigen were usually in the range of 25 to 50% of those obtained with wild-type T antigen in the same experiment and were similarly in the range of 25 to 50% of those from reactions incubated at 33°C. Thus, wild-type and *tsA438* T antigens were capable of mediating detectable levels of replication products at all temperatures.

By contrast, *tsA357* T antigen was extremely thermo-labile. While capable of generating levels of replication products equivalent to those seen with wild-type or *tsA438* T antigen at 33°C, the amount of ori-DNA synthesized by *tsA357* T antigen dropped sharply at 37°C and was barely detectable at 39°C, and no products whatever were detected at 41°C. The abilities of increasing concentrations of the three T antigens to mediate replication at 41°C were compared (Fig. 1B). While *tsA438* T antigen displayed reduced thermosensitivity at higher protein concentrations, *tsA357* T antigen, at all concentrations tested, was unable to mediate ori-DNA replication. These results obtained with acid precipitation of radioactive replication products were confirmed with direct gel analysis of the DNA products. When the DNA from these reactions was cut with *DpnI*, a restriction enzyme that digests only fully methylated DNA, and analyzed on 0.8% agarose gels, no full-length DNA products generated by *tsA357* T antigen were observed (data not shown).

Although *tsA438* T antigen is capable of mediating the synthesis of substantial replication products when added to reactions maintained at 41°C, we previously showed that the ability of this mutant T antigen to support replication at 33°C could be completely inhibited by preincubating the protein at the higher temperature for time periods of 15 min or longer (53). We similarly determined the time period required to heat inactivate *tsA357* T antigen such that it could no longer mediate replication at 33°C. Wild-type and *tsA* mutant T antigens were incubated at 41°C in reaction mixtures lacking SV40 ori-DNA for the indicated times (Fig. 2). Upon addition of ori-DNA, incubation continued at 33°C for 3 h. Although there was no decrease in the ability of this preparation of wild-type T antigen to support replication after preheating for the times indicated, some preparations of wild-type T antigen did lose a slight amount of activity when preheated for longer periods of time. The two *tsA* mutants varied greatly in the time required for thermal inactivation. There was a marked decrease in *tsA357*-mediated ori-DNA synthesis within 2 to 3 min at 41°C, as determined both by acid precipitation of replication mixtures (Fig. 2A) and by analysis of the amount of *DpnI*-insensitive linearized DNA products (Fig. 2B). Only 4 to 5 min at 41°C was required to completely and irreversibly heat denature *tsA357* T antigen (Fig. 2A). Confirming our previous study, at least 15 min at 41°C was needed to inactivate *tsA438* T antigen. Thus, our

FIG. 2. Evidence that *tsA357* T antigen is more thermolabile than *tsA438* T antigen. Reaction mixtures containing HeLa extract, T antigens (T Ag), and the other replication reaction components were preheated at 41°C in the absence of SV40 ori-DNA for the indicated times. Incubation continued at 33°C for 3 h upon addition of 0.3 µg of pSVS. Aliquots (5 µl) were analyzed by acid precipitation (A), or the DNA was purified, linearized, and digested with *DpnI* (B). The digested DNA was analyzed on 0.8% agarose gels and subjected to autoradiography.

data show that two temperature-sensitive mutant T antigens display significant differences in thermosensitivity.

Wild-type and *tsA* mutant T antigens do not reveal striking alterations in stability or structure after incubation at 41°C. It has been reported that *tsA* mutants are unstable at the restrictive temperature in vivo (1, 24, 65). To ensure that the differences that we noted with the *tsA* mutant T antigens were not due to variation in the stability of these proteins, the T antigens were immunoprecipitated with PAb419 at the end of the reaction periods at 33 and 41°C. Both *tsA438* (53) and *tsA357* T antigens (data not shown) were intact and present in similar quantities to wild-type T antigen after incubation in replication extracts at 33 and 41°C. Even when the in vitro replication assays were preceded by a 30-min preheating period at 41°C, there was no difference in the amounts of wild-type and mutant proteins detected (53; data not shown).

Given the marked differences in thermosensitivity of the three proteins in ori-DNA replication reactions, we wished to determine whether heat-induced differential changes in structure could be analyzed. Thermolysin, a protease which recognizes and cleaves at internal hydrophobic amino acids, has been used to show that changes in bovine pancreatic trypsin inhibitor occur after thermolysin treatment (12). Conversely, Prelich and Stillman (51) showed that an adenovirus-encoded temperature-sensitive mutant, Ad+ND1*ts* 23 DNA-binding protein, did not display changes in structure after heat inactivation. Our first experiments using concen-

FIG. 3. Evidence that thermolysin digestion reveals no gross structural alterations in the *tsA* proteins. Reaction mixtures containing 1.0 µg of the T antigens (T Ag) were incubated at 41°C for 80 min without (lanes 1) or with (lanes 2 to 5) increasing concentrations of thermolysin. Ratios of the T antigens to thermolysin were 500:1, 250:1, 100:1, and 50:1 in lanes 2 to 5, respectively. Molecular masses of marker proteins are indicated in kilodaltons at the left.

trations and conditions of thermolysin reported previously (32, 51) revealed little or no digestion of either the normal or mutant proteins (data not shown). To determine whether there were gross structural changes in the *tsA* mutants upon preheating at 41°C, we compared the digestion patterns obtained with wild-type and *tsA* mutants, using an extensive range of concentrations of this protease (Fig. 3). Furthermore, we took advantage of the fact that more acidic conditions (i.e., pH 6.5) facilitate thermal denaturation (33). Under these conditions, no striking differences between wild-type and mutant T antigens were observed. However, we reproducibly observed that the distribution of proteolytic products in the size range of 68 to 92.5 kDa varied between the wild-type and either of the two mutant proteins, suggesting more subtle differences in their respective structures. To stimulate the conditions of replication reactions, the T antigens were also treated with thermolysin under conditions such as those described for Fig. 5 (see below) with and without preincubation of the proteins for 15 min at 41°C. Again, no major differences other than those mentioned above were observed. Preheating of the proteins before digestion or digestion at higher temperatures also gave the same digestion patterns for all three proteins (data not shown). Because thermolysin digestion revealed no marked differences between the wild-type and *tsA* T antigens, we conclude that there are no gross structural changes in these proteins.

***tsA357* T antigen binds to the origin of replication at the restrictive temperature.** Since *tsA357* T antigen was unable to support DNA replication at the nonpermissive temperature, we wanted to determine which of the biochemical functions of the mutant were thermolabile. We first compared the abilities of the T antigens to bind to the viral replication origin, using a DNase I protection assay. A ³²P-end-labeled DNA fragment that contains the whole SV40 origin of replication (nt 5171 to 294) was used. We chose conditions that led to protection of both site I, the high-affinity site adjacent to the early border of the core origin that is involved in autoregulation of T antigen (21, 54), and site II, the binding site within the core origin that is necessary for replication (17, 19, 36, 46). At 33°C, *tsA357* T antigen and *tsA438* T antigen produced the same protection patterns as did wild-type T antigen (data not shown). In binding reactions at 41°C in the presence of ATP and MgCl₂,

FIG. 4. Binding of *tsA357* T antigen to the origin at 41°C. Reaction mixtures including 4 mM ATP, 7 mM MgCl₂, and 1.0 ng of ³²P-end-labeled DNA fragment containing the SV40 origin were incubated for 5 min at 41°C. As indicated, increasing concentrations of the T antigens (T Ag) were added, and incubation continued at 41°C for 15 min.

whereas both mutant T antigens and wild-type T antigen protected the DNA, lesser concentrations of the wild-type protein effectively protected the core origin (Fig. 4). However, with increasing protein concentrations, more protection by both mutant T antigens was observed, and at the higher levels of all three T antigens, protection extended into the region bordering the late side of the core origin, i.e. the 21-bp repeat region. This additional protection on the late side of the origin was far more extensive with *tsA438* T

antigen than with either wild-type or *tsA357* T antigen. Since *tsA357* T antigen loses its ability to support replication at 33°C after preincubation at 41°C, we also determined whether preheated *tsA357* T antigen would protect the origin in a DNase I footprinting assay after such preincubation. As we had demonstrated for *tsA438* T antigen (53), preheated *tsA357* T antigen was unable to bind to the origin (data not shown). This finding offers an explanation for why neither mutant supported replication after the preincubation at 41°C. However, since *tsA357* T antigen, which could not mediate replication when directly added to reactions at 41°C, displayed only a partial deficiency in origin binding at 41°C, we examined whether another function of the *tsA357* T antigen was more defective in replication at the nonpermissive temperature.

***tsA357* and *tsA438* T antigens form hexamers and DNA-dependent double hexamers at 41°C.** To initiate DNA synthesis, T antigen assembles onto the origin as a nucleoprotein structure containing two hexamers. T-antigen hexamer formation, which can also occur in the absence of DNA, requires ATP and MgCl₂. It was of interest to determine whether the mutant T antigens were capable of forming such structures. Detection of the various forms of T antigen (monomers through oligomers as large as dodecamers) has been facilitated by the adaption of nondenaturing PAGE techniques (50, 52). Nondenaturing gels were also used to detect T-antigen DNA-containing double hexamers (50). We first examined hexamer formation in the absence of DNA (Fig. 5A). After preincubation of the T antigens at 33°C, we observed that hexamer formation by the *tsA* mutants was indistinguishable from that of wild-type T antigen (data not shown). Both wild-type and mutant T-antigen preparations, when added to gels after incubation at 41°C in the absence of ATP and MgCl₂, displayed a series of forms. Whereas with wild-type T antigen the predominant form was monomer, both mutant T-antigen preparations contained little if any monomers and a higher proportion of larger multimers (Fig. 5A; compare lanes 1), indicating a tendency of the mutants to aggregate under these conditions. Incubation of all three T antigens in the presence of ATP and MgCl₂ at 41°C led to the appearance of hexamers. The efficiency of hexamer formation was greater with wild-type T antigen than with either *tsA* mutant. Although both mutants formed hexamers at 41°C, they also displayed more intermediate species, i.e., dimers, trimers, tetramers, and pentamers, than did wild-type T antigen (Fig. 5A; compare lanes 2 and 3 for the three T antigens). If the proteins were first preincubated at 41°C for 5 min in the absence of ATP and MgCl₂ and then ATP and MgCl₂ were added, only the wild-type protein was able to form hexamers, and even it did so less efficiently than without this preincubation step (Fig. 5A, lanes 4 and 5). Preincubation of the mutants, particularly *tsA357*, appeared to induce a net loss of protein. We believe that this finding is due to aggregation to forms that cannot enter the gel rather than degradation, because denaturing SDS-gel analysis of the preincubated proteins did not reveal any significant reduction of total protein in each case (data not shown). Thus, ATP not only induces T-antigen hexamer formation but most likely also stabilizes the protein's conformation.

We then compared the abilities of the T antigens to form DNA-bound single and double hexamers by incubating the proteins with a ³²P-labeled SV40 ori-DNA fragment (Fig. 5B). In these experiments, a ratio of T-antigen monomer to DNA of 24:1 was used. Addition of ori-DNA induced the formation of double hexamers, with a concomitant reduction of monomers, dimers, and trimers (Fig. 5B; compare lanes 1

FIG. 5. Evidence that *tsA357* T antigen (T Ag) and *tsA438* T antigen form single and double hexamers on the SV40 origin at the restrictive temperature. (A) T antigens (0.6 μ g) were incubated without (lanes 1) or with (lanes 2 and 3) ATP and $MgCl_2$ at 41°C for 10 min. Lanes 4 and 5 represent reactions in which T antigens were preheated at 41°C in the absence of ATP and $MgCl_2$ for 5 min. ATP and $MgCl_2$ were then added, and incubation continued for 10 min. (B) T antigens (0.4 μ g [lanes 3] and 0.8 μ g [lanes 1, 2, and 4]) were incubated in the presence of ATP and $MgCl_2$ for 5 min at 41°C. Glutaraldehyde was added to 0.1%, and incubation continued for 5 min. A 93-bp ^{32}P -end-labeled pATSVO fragment was included in reactions shown in lanes 2 to 4. The products were analyzed on either 4.4 to 20% (A) or 4.4 to 10% (B) native gradient gels, transferred to nitrocellulose, and probed with T-antigen-specific monoclonal antibody PAb416. Molecular masses of marker proteins run in parallel lanes are indicated in kilodaltons at the left. Autorad, autoradiography.

and 2). This result suggested that the majority of wild-type T antigen was bound to DNA under these conditions. Both wild-type and mutant T antigens were capable of double-hexamer formation at 41°C, as determined by the amount of these forms detected by Western immunoblotting (Fig. 5B). However, somewhat less ori-DNA fragment was bound to the mutants, especially *tsA438* (Fig. 5B). There was a greater relative amount of *tsA438* monomers than of either wild-type or *tsA357* T antigen, suggesting that formation of dodecamer DNA was clearly more inefficient with this mutant than with the other T antigens. Additionally, with *tsA438* T antigen, a higher proportion of DNA was detected at the origin of the gel, suggesting a greater tendency to aggregate. This finding may explain the extended protection pattern seen in the DNase I footprints with this mutant T antigen. Thus, the mutants, especially *tsA438*, are partially defective in the ability of T antigen to form DNA-bound multimers. Again, the difference between the T antigens detected by this assay does not reflect the marked variation in thermal sensitivity of the two mutants for mediating ori-DNA replication.

Both *tsA* mutant T antigens display reduced untwisting of DNA at the replication origin. The formation of a double hexamer on the SV40 origin induces structural changes in the DNA. $KMnO_4$ modification experiments have revealed that specific regions within both the early palindrome and the A+T-rich regions that flank the central palindrome of the SV40 core origin are melted by T antigen in a reaction that depends on ATP or ATP analogs (4, 6, 49). It has been demonstrated that melting of the early palindrome is particularly important for DNA replication (49). The untwisting of viral DNA by *tsA357* and *tsA438* T antigens was compared with that by wild-type T antigen at both permissive and nonpermissive temperatures (Fig. 6). The substrate used was pATSVO, which contains the origin of SV40 (nt 5171 to 294). Even though both *tsA* mutants melted regions within the origin at 33°C, they did not untwist the early palindrome to the same extent as did wild-type T antigen at the lower concentrations examined. Also, the opening of the A+T-rich tract by *tsA438* T antigen was somewhat less than that displayed by wild-type or *tsA357* T antigen. However, at the

FIG. 6. Evidence that *tsA357* T antigen and *tsA438* T antigen (T Ag) cause reduced structural changes in the SV40 origin at 41°C. Reaction mixtures were incubated for 5 min at the indicated temperature before addition of the proteins. T antigens (0.2 µg [lanes 1], 0.4 µg [lanes 2], and 0.8 µg [lanes 3]) were incubated for 30 min in the presence of 0.5 µg of pATSVO at 33 or 41°C; then the DNA was modified with KMnO_4 . A ^{32}P -end-labeled primer was annealed to the DNA and extended with deoxynucleotides and DNA polymerase. EP, early palindrome.

highest concentration (an 80:1 ratio of T antigen to DNA), which approximates the conditions used in replication reactions, the *tsA* mutants were comparable to wild-type T antigen. At 41°C, however, the untwisting of the early palindrome by the *tsA* mutants was poor at all concentrations tested compared with that by wild-type T antigen. Surprisingly, *tsA357* T antigen was far more effective in untwisting the A+T-rich region than was the less thermosensitive *tsA438* T antigen. If this result is a true representation of what happens in replication reactions, then the minimal untwisting by *tsA438* T antigen must be sufficient to allow for replication to initiate. Of course, this would also hold true for *tsA357* T antigen, and therefore this feature is not likely to be the defect that is responsible for the inability of *tsA357* T antigen to support replication at 41°C.

***tsA357* T antigen is defective in unwinding ori-DNA at the restrictive temperature.** Following the localized melting of sequences in the early palindrome and A+T-rich region, T antigen induces more extensive bidirectional unwinding of the origin. An assay that tests the binding, melting, and unwinding of DNA containing the replication origin involves the incubation of T antigen with a ^{32}P -end-labeled double-stranded ori-DNA fragment in the presence of SSB and ATP (13, 15, 22, 55, 73). This process leads to the generation of separated single strands that can be detected on polyacryl-

amide gels (29, 56, 68). It was of interest to determine whether *tsA357* T antigen might be completely deficient in this activity, reflecting its inability to support ori-DNA replication at 41°C. After performing the duplex DNA unwinding assay described above, we observed that both wild-type and *tsA438* T antigens were approximately three times more efficient in unwinding than was *tsA357* T antigen at the lowest concentration (Fig. 7). However, at the higher concentration of *tsA357* T antigen, this mutant unwound ori-DNA to a similar extent as did wild-type T antigen. Although we might conclude that here, too, *tsA357* T antigen is only partially defective in unwinding ori-DNA, it should be mentioned that in order to detect generation of single strands in this assay, a ratio higher than that used in replication assays was used. The lowest concentration used in this assay represents a ratio of 200:1 to 250:1 of T antigen monomers to a single SV40 origin, whereas the ratio of T antigens to DNA in our replication assays is 100:1 to 133:1. This difference might suggest that the greatly reduced unwinding activity of *tsA357* T antigen solely explains its replication defect. However, even at a higher ratio of *tsA357* T antigen to DNA (270:1) in replication reactions, no DNA products were detectable (Fig. 1B). Hence, we conclude that while the defect in unwinding might contribute significantly to the *tsA357* T antigen phenotype observed at 41°C, it does

FIG. 7. Impairment of the unwinding activity of *tsA357* T antigen at 41°C. Reaction mixtures containing 32 P-labeled ori-DNA fragment, *E. coli* SSB, and other components (see Materials and Methods) were incubated for 5 min at 41°C; then increasing concentrations of T antigens (lane 0, no T antigen; lanes 1, 0.25 μ g; lanes 2, 0.5 μ g; lanes 3, 1.25 μ g) were added, and incubations continued for 30 min at 41°C. After digestion with proteinase K, the DNA was analyzed on a 6% polyacrylamide gel. The reactions without T antigen (lane 0) and heat-denatured ori-DNA (lane D) identify positions of double-stranded (ds) and single-stranded (ss) DNA products, respectively. SVT Ag, SV40 T antigen.

not entirely explain the complete inability of the mutant to support DNA replication at the nonpermissive temperature.

We have also compared the DNA helicase and ATPase activities of the three T antigens at both permissive and nonpermissive temperatures (data not shown). Both *tsA* mutants displayed wild-type helicase activities in reactions at 41°C (53; data not shown). The ATPase activity of *tsA357* T antigen at 41°C was 20 to 30% of that displayed by wild-type T antigen, and the activity of *tsA438* T antigen was 50 to 70% of that of wild-type T antigen (data not shown). In the presence of poly(dT), the ATPase activities of all of the T antigens were markedly stimulated. While the ATPase activity of *tsA357* T antigen was impaired at 41°C, it was markedly stabilized and enhanced by the presence of DNA. Thus, *tsA357* T antigen again displayed minimal or partial deficiencies in assays, reflecting DNA replication functions that could not account for its absolute loss of ability to support ori-DNA replication at 41°C.

DISCUSSION

Several studies in which either or both *tsA438* or *tsA357* T antigens were characterized in vivo (9, 63, 66) and in vitro (10, 25, 30, 35, 44, 57) have been published. Monkey cells infected with either *tsA* mutant neither replicate viral DNA at the restrictive temperature nor initiate new rounds of replication in temperature shift-up experiments (9, 63). Also, neither mutant T antigen oncogenically transforms cultured cells maintained at the nonpermissive temperature (8, 40, 48, 64). Considerably more studies describing the properties of *tsA438* T antigen than those of *tsA357* T antigen have been reported. It has been demonstrated that at the restrictive temperature, *tsA438* T antigen does not form oligomers (24, 43), bind to p53 (43, 45), or associate with the nuclear matrix or chromatin (35, 57). Also, *tsA438* T antigen does not associate with polymerase α at the restrictive temperature (27). Both mutant T antigens were reported to be defective in binding to the origin of replication at the restrictive temperature (*tsA357* and *tsA438* [70]; only *tsA438* [35, 58, 62]). However, these mutants (35, 58, 62, 72) either had been preheated or were from cells that were grown at the nonpermissive temperature. We confirmed these results by using purified T antigens that were preincubated at 41°C before assaying for origin binding (53; data not shown). In the experiments described in this study, T antigens were added directly to the reactions at 41°C. It was not surprising that

tsA438 T antigen bound the origin under these conditions because it supports replication at the higher temperature. Even though *tsA357* T antigen does not synthesize DNA at 41°C, we have observed in nitrocellulose filter binding assays that T antigen binds maximally to the origin within 1 min (39). The approximately 4 min required to heat inactivate the mutant should provide ample time for the mutant to bind to DNA.

When *tsA357* T antigen from monkey or hamster cells was incubated at the nonpermissive temperature prior to assay, its ATPase activity was reported to be thermolabile (10, 30). Confirming those results, we observed that preincubation of purified *tsA357* T antigen eliminated its ATPase activity. However, in contrast to a report by Clark et al. (10), who reported that this *tsA* mutant had activity equal to that of wild-type T antigen even after preincubation at the nonpermissive temperature, we observed that preincubation of *tsA438* T antigen eliminated its ATPase activity at the nonpermissive temperature. Our assays were performed at 41°C and theirs were performed at 39°C, which may account for this discrepancy in the ATPase activity of preheated *tsA438* T antigen. Also, the T-antigen-specific antibody PAb205 that was present in their assays (10) may have stabilized the protein.

Fanning et al. (25) and Montenarh et al. (44) reported that *tsA438* T antigen does not form oligomers at the restrictive temperature, as determined by sucrose gradient centrifugation studies. We, on the other hand, observed hexamer formation by both *tsA* mutants at 41°C, but no hexamers were seen after preincubation at the restrictive temperature. Our results do not contradict those studies because in these cases, T antigen was from monkey cells maintained at the restrictive temperature, which is analogous to purified T antigen that has been preincubated at 41°C.

To our knowledge, no studies showing that there are as dramatic differences in thermosensitivity between these *tsA* mutant T antigens as we have identified in this study have been published. However, most experiments involving analysis of DNA replication in infected or transfected cells measure either the end point of DNA synthesis in cells growing at 41°C or DNA synthesis after a shift from one temperature to another, neither of which is likely to reveal such differences. Temperature shift experiments originally yielded results that indicated that T antigen might be a slow-stop mutant (i.e., replication continued up until but not past the next round of synthesis), leading to the conclusion that T antigen functions only in the initial stages of replication. However, while its role in elongation has not yet been proven, several experiments that indicate that it functions as the helicase that moves ahead of the replication fork throughout each round of synthesis have been performed. Our data for both *tsA438* and *tsA357* T antigens can reconcile possible discrepancies between these different experimental approaches, because in DNA helicase reactions (which would be predicted to most closely resemble the function of T antigen during elongation), we have noted that once initiated, both mutants are capable of continuing to separate the strands of duplex DNA at the levels of wild-type T antigen (53; data not shown). Thus, it is likely that in shift-up experiments, once either wild-type or mutant T antigens are part of an assembled replication complex, they remain stable and can function as a helicase until the daughter DNA molecules dissociate and a new round of DNA synthesis must be initiated.

When we attempted to analyze the different replication-associated activities of the T antigens, we came to the

somewhat perplexing realization that the results obtained for either T antigen did not reflect their overall performance in replication reactions. Thus, addition of either *tsA* T antigen to ori-DNA-containing HeLa extracts led to dramatically different results: *tsA438* T antigen was only partially defective, while *tsA357* T antigen was completely unable to support the synthesis of progeny DNA. However, examination of different activities revealed that *tsA438* T antigen was actually less efficient in some cases than was *tsA357* T antigen, particularly in ori-DNA melting as determined by KMnO_4 modification analysis. Indeed, in this assay, *tsA438* T antigen displayed less than 10% of the activity of wild-type T antigen and thus significantly less than its overall reduction of 50% seen in the DNA replication reaction. We considered that this poor display of DNA melting by *tsA438* T antigen might not reflect its actual performance in the HeLa replication extract. However, we determined that in the presence of the HeLa extract, *tsA438* T antigen's ability to melt sequences within the origin is not improved (data not shown). It is therefore more likely, however surprising, that the small amount of origin melting (i.e., <10% of the wild-type level) that *tsA438* T antigen is capable of is sufficient to generate the proportionally greater amount of total replication reaction products detected (approximately 50% of the wild-type level). By contrast, while *tsA357* T antigen appeared to show partial defects in nearly all of the separate activities tested, none of these appeared to fall below about 20% of the wild-type levels and many were much more efficient than that. One conclusion is that all of these partial defects in *tsA357* T antigen somehow are multiplicative such that they do not allow the generation of any replication products. This idea, while not impossible, is unattractive given our results for *tsA438* T antigen. More appealing perhaps, because of the element of mystery associated with it, is the suggestion that *tsA357* T antigen might be completely defective in yet another activity of T antigen. Such an activity might be related to the ability of T antigen to associate specifically with DNA polymerase α or might reflect a replication-associated activity that has not yet been identified. Although the thermolysin assays did not reveal any dramatic conformational changes in the *tsA* mutants that are different from that of wild-type T antigen, it is possible that the more subtle structural changes that we detected at the nonpermissive temperature are related to the inability of heat-treated mutant T antigens to mediate DNA replication. We are currently carrying out experiments to test this possibility.

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