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Thermally Inactivated Simian Virus 40 *ts*A58 Mutant T Antigen Cannot Initiate Viral DNA Replication In Vitro

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The mutation in the temperature-sensitive *ts*A58 mutant T antigen (Ala-438→Val) lies within the presumptive ATP-binding fold. We have constructed a recombinant baculovirus that expresses large quantities of the *ts*A58 T antigen in infected insect cells. The mutant T antigen mediated simian virus 40 origin-containing DNA (ori-DNA) synthesis in vitro to nearly the same extent as similar quantities of wild-type T antigen at 33°C. However, if wild-type and *ts*A58 T antigens were heated at 41°C in replication extracts prior to addition of template DNA, the *ts*A58 T antigen but not the wild type was completely inactivated. The mutant protein displayed greater thermosensitivity for many of the DNA replication activities of T antigen than did the wild-type protein. Some of the replication functions of *ts*A58 T antigen were differentially affected depending on the presence or absence of ATP during the preheating period. When *ts*A58 T antigen was preheated in the presence of ATP at 41°C for a time sufficient to completely inactivate its ability to replicate ori-DNA in vitro, it displayed substantial ATPase and normal DNA helicase activities. Conversely, when preheated in the absence of nucleotide, it completely lost both ATPase and helicase activities. Preheating *ts*A58 T antigen, even in the presence of ATP, led to drastic reductions in its ability to bind to and unwind DNA containing the replication origin. The mutant T antigen also displayed thermosensitivity for binding to and unwinding nonspecific double-stranded DNA in the presence of ATP. Our results suggest that the interactions of T antigen with ATP that are involved in T-antigen DNA binding and DNA helicase activities are different. Moreover, we conclude, consistent with its phenotype in vivo, that the *ts*A58 T antigen is defective in the initiation but not in the putative elongation functions of T antigen in vitro.

Temperature-sensitive mutants (*ts*A) of simian virus 40 (SV40) T antigen were used to demonstrate the role of T antigen in viral DNA replication in infected cells (11, 67). These conditional mutants resembled "slow-stop" mutants: in temperature shift experiments, DNA synthesis continued at the restrictive temperature until, but not past, the next round of replication. Therefore, it was initially concluded that the function of T antigen pertains solely to the initiation but not to the elongation stages of viral DNA replication (67). T antigen displays many separate activities that are related to its role in mediating viral DNA replication (for reviews, see references 5, 19, 23, and 62). The binding of T antigen to sequences within the replication origin is enhanced by the presence of ATP and ATP analogs (7, 15, 17). This leads to alterations in the structure of the DNA at the origin due to an untwisting function of T antigen (6), which is a prerequisite for the more extensive unwinding of sequences at the origin by the viral protein, a reaction that requires ATP hydrolysis and single-stranded binding protein (14, 27, 50, 51, 74). The association of T antigen with DNA polymerase α (24, 59) may also be interpreted as an initiation-related function, serving to bring in the DNA polymerase-primase complex in order to initiate the replication of the template strand. Thus these functions of T antigen could conceivably account for the total set of activities of the protein that are required for its role in viral DNA replication and would be consistent with the DNA initiation phenotype deduced from studies with the *ts*A mutant T antigens.

However, the results of other experiments have suggested that T antigen is required for chain elongation as well as initiation. The most compelling of these results is the observation that some T-antigen-specific monoclonal antibodies block elongation from SV40 minichromosome templates in vitro (61, 76). Furthermore, the ATPase (13, 26, 71) and DNA helicase (14, 60) activities of T antigen are both consistent with a role in elongation. Indeed, the binding of T antigen to DNA polymerase α could alternatively be interpreted as the participation of both proteins in a multiprotein complex containing several factors and enzymes at the replication fork such as has been postulated elsewhere (72).

How is it possible to reconcile the temperature shift experiments performed with *ts*A T antigens in infected cells with a possible elongation function of T antigen? At least two explanations could be proffered to explain the discrepancy. First, *ts*A mutants may be defective in functions associated with initiation but not elongation such as DNA binding and unwinding but not in ATPase and DNA helicase activities. Second, such mutants may require relatively long periods to become heat inactivated, i.e., on the order of a round of replication. However, an alternative possibility may exist: T antigen may function only in initiation. *ts*A proteins may be intrinsically unstable such that they are rapidly degraded in infected cells and in cell replication extracts at higher temperatures. In this case T antigen would be shown not to function in elongation. To further understand the function of *ts*A T antigens in initiation and elongation, we have constructed a recombinant baculovirus that expresses the conditional mutant T antigen *ts*A58.

*ts*A58 T antigen was first identified as temperature sensitive in lytically infected cells, and it has been among the most extensively studied of the *ts*A58 class because it is the least "leaky" of these mutants (69). It shows the same characteristics as other SV40 *ts*A mutants in that it is

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defective in viral DNA replication at the restrictive temperature and fails to initiate new rounds of replication in temperature shift experiments (11, 67). Furthermore, *ts*A58 does not oncogenically transform cultured cells maintained at the nonpermissive temperature (10, 36, 68). Additionally, *ts*A58 does not form oligomers (22, 40), bind to p53 (39, 41), or associate with the nuclear matrix or chromatin at the restrictive temperature (29, 52). It was also reported that this mutant T antigen does not bind to the viral origin of replication after having been heated (29, 54, 64, 78). Recently, evidence was provided that *ts*A58 T antigen does not bind to DNA polymerase α at the restrictive temperature (25).

The development of an experimental approach to study the replication of SV40 origin-containing DNA (ori-DNA) in cell extracts has made it possible to characterize the replication function of wild-type and mutant forms of T antigen (33, 65, 79). Our studies on the replication activities of *ts*A58 T antigen have provided potential explanations for the discrepancy between experiments that suggest a role for T antigen solely in initiation or dual roles in initiation and elongation.

MATERIALS AND METHODS

Cells and viruses. Recombinant baculoviruses vEV55SVT (45) and vEV55SVtsA58 express SV40 wild-type and *ts*A58 T antigens, respectively. *Spodoptera frugiperda* insect cells (Sf27 cells) were grown at 27°C in TC-100 medium (GIBCO) containing 10% heat-inactivated fetal calf serum and 0.25% tryptose broth.

Construction of pEV55SVtsA58 and vEV55SVtsA58. pEV55SVT contains the DNA sequence coding for SV40 large T antigen downstream from the polyhedrin promoter (45). pMK16-6tsA58ori− was kindly provided by H. Ozer. pMK16-6tsA58ori− has the whole SV40 *ts*A58 genome inserted into the *Eco*RI site of pMK16-6 (pMK16 [31]; pMK16-6 has *tet* sequences deleted). The *ts*A58 mutation changes amino acid 438 of SV40 large T antigen from alanine to valine (nucleotide [nt] 3505 from C to T). pEV55SVtsA58 was constructed by exchanging the *Pfl*MI (nt 4558)-*Eco*RI (nt 1782) restriction fragment of pEV55SVT with the same fragment from pMK16-6tsA58ori−. The recombinant virus, vEV55SVtsA58, was constructed by cotransfecting insect cells with pEV55SVtsA58 and purified wild-type baculoviral DNA and then identifying an occlusion body-negative plaque from which the recombinant virus was purified (37, 38).

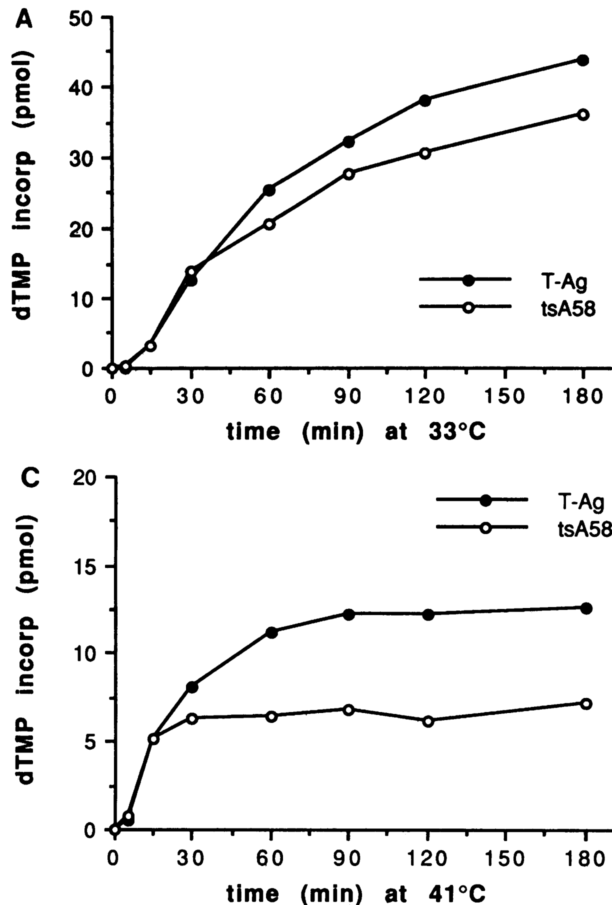
Purification of T antigen. Sf27 cells (2.5×10^7/150-mm dish) were infected with recombinant baculovirus and harvested 48 h postinfection. The cells were washed twice in cold phosphate-buffered saline (1 mM Na_2HPO_4, 10.5 mM KH_2PO_4, 140 mM NaCl, 14 mM KCl [pH 6.2]), extracted with 1.6 ml of lysis buffer (50 mM Tris hydrochloride [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.1% aprotinin, 10 μM benzimidine, 30 μg of leupeptin per ml, 1 μg of bacitracin per ml, 10 μg of α-2-macroglobulin per ml, 1 mM dithiothreitol [DTT], 0.35 mM phenylmethyl-sulfonyl fluoride) per plate, and incubated on ice for 30 min. Lysates were centrifuged at 200 rpm (IEC CRU-5000 centrifuge) for 15 min and then at 20,000 rpm for 30 min. The clarified supernatant was loaded onto a monoclonal antibody column (PAb 419 cross-linked to protein A-Sepharose) and purified by immunoaffinity chromatography (55), with modifications as described by Murakami et al. (42). Peak fractions, as determined by silver staining, were pooled and dialyzed against buffer D (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5], 5 mM NaCl, 0.1 mM EDTA, 50% glycerol, 1 mM DTT).

In vitro DNA replication. HeLa extracts were prepared according to published procedures (33, 79). Standard reaction mixtures contained 40 mM creatine phosphate (pH 7.7; di-Tris salt); 7 mM $MgCl_2$; 0.5 mM DTT; 4 mM ATP; 200 μM each CTP, UTP, and GTP; 100 μM each dATP, dGTP, and dCTP; 20 μM [α-^{32}P]dTTP (2×10^4 cpm/pmol); and 100 μg of creatine kinase per ml (36). For SV40 ori-DNA replication, 300 ng of pAT153SVO DNA (SV40 origin from nt 5171 to 294 inserted between the *Hin*dIII and *Eco*RI sites [4] of pAT153 [73]), HeLa extract (300 to 400 μg of protein), and either 1.0 μg of SV40 T antigen or 1.0 μg of *ts*A58 T antigen were added to the reaction, and the incubation was carried out at 33, 39.5, or 41°C for 3 h unless otherwise indicated. Incorporation of dTMP was determined by acid precipitation of reaction mixtures. To analyze the products, the reactions were terminated by the addition of sodium dodecyl sulfate to 0.2% and EDTA to 15 mM. These additions were followed by proteinase K digestion (0.2 mg/ml) for 1 h at 37°C. The DNA was purified, digested with *Pst*I, in the presence or absence of *Dpn*I, and subjected to 0.7% agarose gel electrophoresis for 1 h at 200 V followed by autoradiography.

DNA filter binding assay. Reaction mixtures (50 μl) containing 40 mM creatine phosphate (di-Tris salt, pH 7.7), 7 mM $MgCl_2$, 0.5 mM DTT, 0.2 mg of bovine serum albumin per ml, 4 mM ATP, 0.3 μg of pAT153, 1.65 ng of ^{32}P-5′-end-labeled DNA fragment (containing site I or site II), and 0.1 μg of either SV40 T antigen or *ts*A58 T antigen were incubated for 15 min at 41°C. Reaction mixtures were filtered through 0.45-μm-pore-size nitrocellulose filters presoaked in wash buffer (100 mM $NaPO_4$ [pH 7.0], 0.5 mM EDTA, 0.5 mM DTT), washed twice with the same buffer, dried, and counted by scintillation. ^{32}P-5′-end labeled *Bam*HI-*Hin*dIII fragment from pAT153SVO (2 ng) was used for nonspecific binding.

DNase I footprinting. Reaction mixtures (50 μl) containing 40 mM creatine phosphate, 4 mM ATP, 7 mM $MgCl_2$, 0.2 mg of bovine serum albumin per ml, 0.5 mM DTT, 10 ng of pAT153, and 0.4 μg of either T antigen or *ts*A58 T antigen were preincubated at 41°C for various periods. ^{32}P-labeled SVO fragment that contains the whole SV40 origin (from nt 5171 to 294) (1 ng) was then added, and incubation proceeded for 15 min. Digestion with 20 ng of DNAse I per ml, in 20 mM $CaCl_2$–200 mM $MgCl_2$–1.0 M NaCl–10 μg of sheared salmon sperm DNA was for 2 min at 41°C, following which DNase stop solution (2 M ammonium acetate, 100 mM EDTA, 0.2% sodium dodecyl sulfate, 100 μg of sheared salmon sperm DNA) was added and the DNA was extracted and ethanol precipitated. The DNA was then subjected to urea–8% polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Duplex DNA fragment unwinding assay. Duplex DNA unwinding was essentially as described by Virshup and Kelly (74). pAT153SVO was cut with *Eco*RI and *Hin*dIII to generate a 366-bp fragment containing the origin of replication (nt 5171 to 294) or with *Bam*HI and *Hin*dIII to generate a 346-bp nonspecific fragment. The ends of the DNA fragments were labeled by filling them in with the large fragment of DNA polymerase I, [α-^{32}P]dATP, and unlabeled deoxynucleotide triphosphates. Reaction mixtures (10 μl) contained 30 mM HEPES (pH 7.5), 15 mM potassium phosphate (pH 7.5), 7 mM $MgCl_2$, 4 mM ATP, 0.05% Nonidet P-40, 0.5 mM DTT, 40 mM creatine phosphate, and 100 μg of creatine kinase per ml. Either 0.12 μg of SV40 T antigen or 0.12 μg of



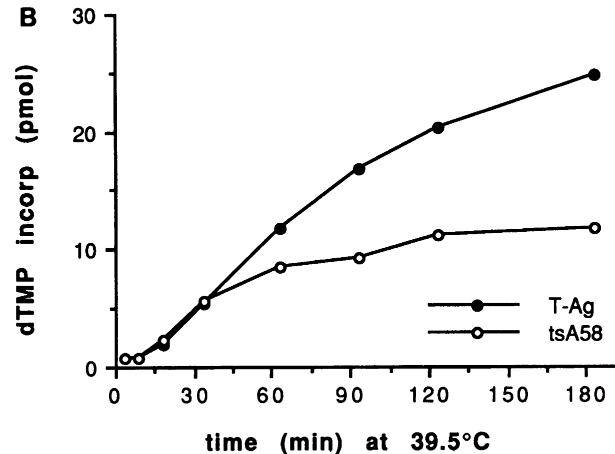


FIG. 1. Time course of SV40 ori-DNA replication. Reactions containing SV40 ori-DNA, HeLa extract, SV40 T antigen (T-Ag), or *ts*A58 T antigen were incubated at 33 (A), 39.5 (B), or 41°C (C). The amounts of acid-insoluble radioactivity in 5-μl aliquots were determined at the indicated times. Levels of dTMP incorporated did not exceed 1 pmol at the 3-h point in reactions lacking T antigen. Note that different scales are used for dTMP incorporation at the three temperatures.

*ts*A58 T antigen was added to the unwinding reaction along with 0.4 ng of ^{32}P-labeled DNA and 0.3 μg of *Escherichia coli* single-stranded binding protein (SSB). Reaction mixtures were incubated for 30 min at 41°C, and reactions were terminated by the addition of 10 μl of 0.2-mg/ml proteinase K, 2% sodium dodecyl sulfate, and 50 mM EDTA. After incubation for an additional 30 min at 37°C, the samples were heated at 65°C for 10 min and subjected to 6% PAGE in 1× TBE running buffer (0.09 M Tris-borate, 0.002 M EDTA [pH 8.0]) and autoradiography.

ATPase assay. Assays were performed essentially as described by Giacherio and Hager (26). Reaction mixtures (15 μl) contained 25 mM Tris hydrochloride (pH 8.0), 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 10 μg of bovine serum albumin per ml, [α-^{32}P]dATP, and 0.8 μg of either SV40 T antigen or *ts*A58 T antigen. Incubation was for 60 min at 41°C. Each reaction mixture (1 μl) was spotted onto polyethyleneimine-cellulose plates and developed in 1 M formic acid and 0.5M LiCl at room temperature for approximately 1 h. The plates were dried and exposed.

Helicase assay. Reaction mixtures (15 μl) containing 25 mM Tris hydrochloride (pH 7.8), 5 mM DTT, 5 mM ATP, 10 mM $MgCl_2$, 15 ng of ^{32}P-heteroduplex helicase substrate, and 0.8 μg of either SV40 T antigen or *ts*A58 T antigen were incubated for 60 min at 41°C. The heteroduplex consisted of single-stranded M13mp9 to which a ^{32}P-labeled 50-mer (complementary to nt 6271 to 6321 in M13mp9) that does not contain specific binding sites for SV40 T antigen was annealed (3). An alternative helicase substrate consisted of single-stranded M13mp18 DNA to which a heterogeneous range of ^{32}P-labeled complementary DNA fragments were annealed. This substrate was generated by hybridizing the 15-nt universal primer (no. 1200 from New England BioLabs) to M13mp18 and elongating it by using [α-^{32}P]dATP, the other deoxynucleotide triphosphates, and the large fragment of DNA polymerase I according to procedures described by Wiekowski et al. (77). Samples were subjected to 6 or 10% PAGE and autoradiography.

Materials. Radioisotopes were purchased from New England Nuclear. DNAse I and *E. coli* SSB were from Worthington and Pharmacia, respectively. Klenow (polymerase I large fragment) and restriction enzymes were from New England BioLabs except for *Dpn*I, which was from Boehringer Mannheim.

RESULTS

***ts*A58 and wild-type T antigens differ in ability to mediate SV40 ori-DNA replication at permissive and restrictive temperatures.** Monkey cell lines infected with *ts*A viruses at the permissive temperature produce amounts of infectious virions and viral DNA similar to the amounts produced by the wild-type virus (11, 67). However, when *ts*A58-infected cells are incubated at the nonpermissive temperature, they no longer produce viral DNA. Our experiments were undertaken to determine whether a *ts*A58 mutant would display similar behavior in vitro. We confirmed that the parent plasmid encoding *ts*A58, from which the *ts*A58 baculovirus was derived, functioned conditionally in SV40 ori-DNA replication in transfected monkey cells (data not shown). Thus, origin-defective plasmids encoding wild-type and *ts*A58 T antigens were similarly competent in replicating pATSVO, a plasmid that contains the SV40 origin of replication (nt 5171 to 294) at 33°C. At 41°C, however, cells expressing the wild-type plasmid, but not those expressing the *ts*A58 plasmid, replicated pATSVO. Baculovirus vectors vEV55SVT (45) and vEV55SVtsA58 were used to direct synthesis of SV40 large T antigen and *ts*A58 T antigen in infected insect cells. Generally the two viruses yielded

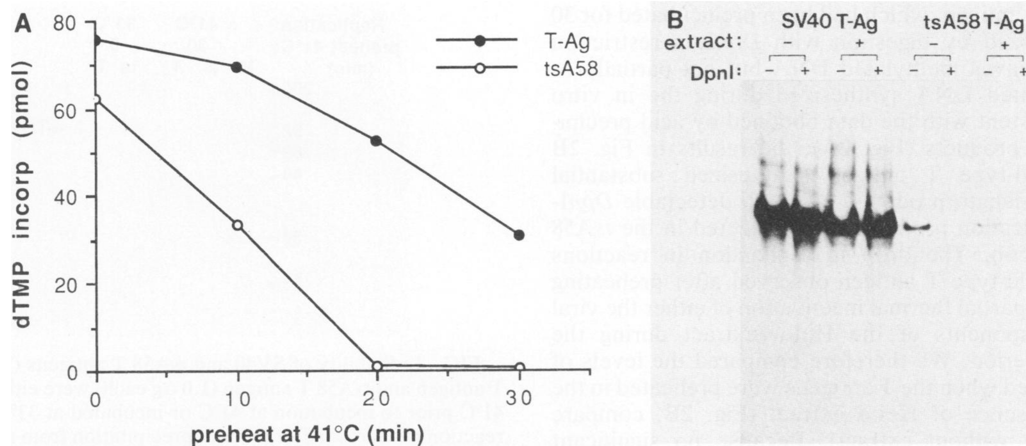


FIG. 2. *ts*A58 T antigen does not support SV40 ori-DNA replication at 33°C after being preheated at 41°C. (A) Reaction mixtures containing HeLa extract, SV40 T antigen (T-Ag), or *ts*A58 T antigen and other reaction components were preheated at 41°C in the absence of SV40 ori-DNA for the indicated times. Incubation continued at 33°C upon addition of ori-DNA. After 3 h of incubation, 5-μl aliquots were analyzed by acid precipitation. (B) Same conditions as in panel A except that HeLa extract was either present (+) or absent (−) during the 30-min preheating period. Incubation was continued at 41°C when HeLa extract and ori-DNA were added. Linearized DNA was digested with (+) or without (−) *Dpn*I.

comparable quantities of T antigen in infected insect cells (data not shown). The T antigens were immunopurified with monoclonal antibody PAb 419, and, as determined by silver staining, equivalent amounts of mutant and wild-type proteins were used in all experiments. SV40 T antigen and *ts*A58 T antigen were compared in the in vitro SV40 ori-DNA replication assay at the permissive and nonpermissive temperatures.

The highest levels of wild-type T-antigen-mediated ori-DNA synthesis were seen at 33°C, and the lowest were seen at 41°C (Fig. 1). In general, quantities of DNA synthesized at 33°C were similar to those at 37°C, the temperature at which most SV40 ori-DNA replication reactions are performed (33, 65, 79), but they decreased significantly at temperatures above 38°C (data not shown). However, the initial rate of incorporation was consistently less at 33 and 39.5°C than at 41°C. The rates of synthesis between 15 and 30 min at the two temperatures then reversed such that by 60 min, incorporation at 33 and 39.5°C continued to increase, while at 41°C it had slowed considerably. Reactions with *ts*A58 T antigen showed the same initial rate as those containing wild-type T antigen at all temperatures up to the 15-min point. At 33 and 39.5°C, but not at 41°C, reactions containing both T antigens were parallel up to the 30-min point. But by the 60-min point at all temperatures, wild-type T-antigen reactions synthesized more DNA than those with *ts*A58 T antigen. The extent to which the *ts*A58 and wild-type proteins differed was a function of temperature, such that at 33°C the mutant slowed down to a rate of 80 to 85% of that of wild type. At 39.5°C, after the 60-min point, the *ts*A58 T-antigen rate was much less than that of wild type, increasing only from 8 to 10 pmol of dTMP incorporated, while the wild-type T-antigen reaction increased from 11 to 25 pmol of dTMP incorporated (Fig. 1B). At 41°C, the wild-type T-antigen reaction rate increased up to the 60-min point, but then it slowed markedly (Fig. 1C). At this highest temperature, *ts*A58 T-antigen reactions showed very little increase from 15 to 30 min, and then virtually no significant new incorporation was observed after the 30-min point. Generally, the relative differences between mutant and wild-type T antigens in rates of SV40 ori-DNA replication observed at different temperatures were consistent over a wide range of T-antigen concentrations (data not shown).

Although the quantities of ori-DNA synthesized in the presence of *ts*A58 T antigen at 39.5 or 41°C were markedly lower than those with wild-type T antigen, nevertheless, substantial levels of DNA products were generated by *ts*A58 T antigen even at 41°C. Explanations for this might be that *ts*A58 T antigen is only partially defective even at high temperatures in vitro or, alternatively, that *ts*A58 T antigen is defective solely in its initiation function but initiation can be accomplished before the protein is effectively heat denatured. Thus, should the initiation-related activity require less time than that necessary to completely heat denature the protein, the initial, but not the subsequent, rates of synthesis mediated by *ts*A58 and wild-type T antigens should be similar under the conditions used. If this were the case, then preincubating the *ts*A58 T antigen at 41°C for various periods prior to adding to the replication reaction should provide a greater difference between the relative abilities of wild-type and *ts*A58 T antigens to function.

Preheating *ts*A58 T antigen inactivates its replication function. T antigen and *ts*A58 T antigen were preincubated in the replication mixtures at 41°C in the absence of SV40 ori-DNA for periods of up to 30 min. DNA was omitted because it has been observed in our laboratory that SV40 T antigen binds efficiently to the SV40 origin of replication within 1 min (H. Lorimer and C. Prives, unpublished data), and the *ts*A58 mutant might, therefore, be stabilized by the ori-DNA. Upon addition of the DNA substrate, the reactions were incubated at 33°C for 3 h. Preincubation for 10 min caused an approximately 50% drop in the amount of DNA synthesized by *ts*A58 T antigen relative to wild-type T antigen (Fig. 2A). By 20 min, however, while wild-type T antigen had lost less than 30% of its replication activity, the ability of *ts*A58 T antigen to mediate ori-DNA synthesis was completely abolished. Therefore, between 10 and 20 min were required to heat inactivate the *ts*A58 T antigen. Preincubation of wild-type T antigen for 30 min was also fairly deleterious to its replication function, as only 40% of the levels generated by the nonpreheated T antigen were observed.

In a separate experiment, the DNA generated by *ts*A58

and wild-type T antigen, which had been preincubated for 30 min, was analyzed by digestion with *Dpn*I, a restriction enzyme that cleaves methylated DNA but not partially or fully unmethylated DNA synthesized during the in vitro reaction. Consistent with the data obtained by acid precipitation of DNA products (Fig. 2A), the results in Fig. 2B show that wild-type T antigen synthesized substantial amounts of full-length products, while no detectable *Dpn*I-insensitive replication products were detected in the *ts*A58 T-antigen reaction. The drop in replication in reactions mediated by wild-type T antigen observed after preheating might be due to partial thermal inactivation of either the viral protein or components of the HeLa extract during the preincubation period. We therefore compared the levels of products obtained when the T antigens were preheated in the presence or absence of HeLa extract (Fig. 2B, compare lanes with and without extract). Because no significant differences were observed when the extracts were present from the start of the reaction or added after the 30-min preheating period at 41°C, we conclude that the HeLa extract itself does not lose significant activity during the preincubation period. These results clearly demonstrate that *ts*A58 T antigen is thermosensitive in vitro, and they also suggest that wild-type T antigen is somewhat thermolabile when heated for sufficient periods.

Wild-type and *ts*A58 T antigens are similarly stable during replication reactions at 33 and 41°C. Although *ts*A58 T antigen is synthesized at higher rates in infected cells, the mutant protein was shown to be unstable at the restrictive temperature (21, 70). We were concerned that *ts*A58 T antigen might be thermolabile in vitro because of its relative instability during the reaction. If there were substantially less *ts*A58 T antigen than wild-type T antigen recovered after the reaction at 41°C, thermolability would provide an explanation for the observed differences. Unfortunately such result would be difficult to pursue. In order to determine the stability of *ts*A58 T antigen at permissive and nonpermissive temperatures, the T antigens were immunoprecipitated from replication assays with PAb 416 (28). The replication assays included a preincubation stage at the nonpermissive temperature for which the T antigens were preheated in the reaction mixtures without ori-DNA for 30 min. Then ori-DNA was added, and incubation continued at 41°C for 3 h. Alternatively, the T antigens were incubated in a replication assay at 33°C, at which temperature we had shown that wild-type T antigen and also *ts*A58 T antigen both supported replication. The T antigens were similarly stable at the end of the preincubation period, irrespective of temperature, and, therefore, *ts*A58 T antigen is not degraded to greater extents than the wild-type protein during incubation at the nonpermissive temperature (Fig. 3). Taken together, these data suggest that *ts*A58 T antigen can be completely heat inactivated, but this requires 10 to 20 min in vitro. Furthermore, they indicate that the initiation functions of T antigen take place well within that time frame.

Preheating *ts*A58 T antigen at restrictive temperature impairs its binding to viral replication origin. Since our in vitro results suggested that *ts*A58 T antigen is defective in initiation of replication, we attempted to characterize the *ts*A58 mutant further and to identify its biochemical defect(s). The specific binding of T antigen to sequences within the viral replication origin is one of the initiation functions required for replication. *ts*A58 T antigen from mammalian cells was reported to exhibit wild-type binding at the permissive temperature when assayed by DNA footprinting or filter binding (29, 54, 64, 78). At the nonpermissive temperature,

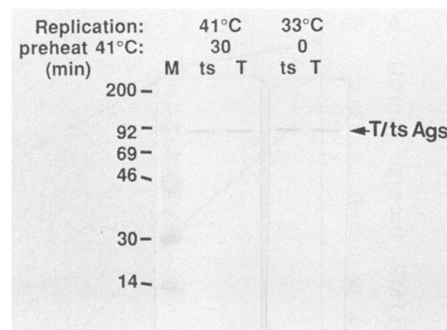
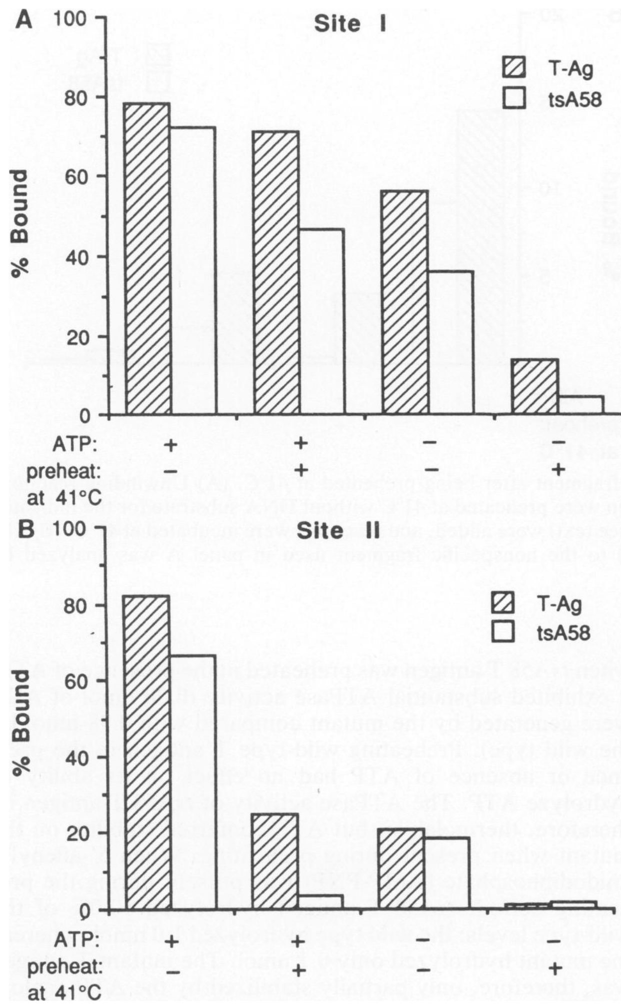


FIG. 3. Stability of SV40 and *ts*A58 T antigens (T/ts Ags). SV40 T antigen and *ts*A58 T antigen (1.0 μg each) were either preheated at 41°C prior to incubation at 41°C or incubated at 33°C in replication reaction mixtures. After immunoprecipitation from replication reactions with monoclonal PAb 416, the T antigens were analyzed by 10% sodium dodecyl sulfate-PAGE or Western blotting (immunoblotting) and probed with PAb 416. Molecular sizes of marker proteins are indicated in kilodaltons (lane M).

however, *ts*A58 T antigen was shown to bind poorly to fragments containing both site I and site II. Accordingly, binding of baculovirus-expressed *ts*A58 T antigen to DNA fragments containing either site I, which is involved in autoregulation by T antigen (20, 49), or site II, which is located within the core replication origin (16, 18, 30, 44), was examined at the permissive and nonpermissive temperatures in the presence and absence of ATP.

Using a filter binding assay, it was observed that the *ts*A58 mutant bound to site I, the high-affinity binding site, to the same extent as wild-type T antigen when ATP was present (Fig. 4A). In the absence of ATP, both wild-type and *ts*A58 T antigens exhibited less binding, but *ts*A58 T antigen binding was relatively more impaired. Both T antigens displayed considerably more heat sensitivity for site I binding in the absence than in the presence of ATP. Site II binding by *ts*A58 T antigen was 80% of that observed with wild-type T antigen when ATP was present. When site II binding was examined in the absence of ATP, both T antigens bound poorly and both lost site II binding completely after preheating. Adding ATP after preincubation at the start of the DNA-binding period gave results similar to those seen in the absence of ATP (data not shown). The most marked difference between the two T antigens was site II binding in the presence of ATP after preheating. While ATP enhanced site II binding by preheated wild-type T antigen (albeit to levels only 22% of nonpreheated wild-type T antigen), binding of *ts*A58 T antigen to this fragment was virtually eliminated. This is consistent with our observations that the *ts*A58 mutant was unable to support replication after preheating. Thus, according to the assay conditions used, the *ts*A58 mutant still exhibited significant binding to site I after preheating and was, therefore, considerably less temperature sensitive for site I binding than for site II binding.

To further examine the DNA binding properties of the two T antigens, DNase I footprinting experiments were performed. Conditions were those which we had determined led to protection of both sites I and II by wild-type T antigen. The abilities of equal quantities of unheated or heated wild-type and *ts*A58 T antigens to bind to a ^{32}P-end-labeled ori-DNA fragment (nt 5171 to 294) in the presence of ATP were then determined (Fig. 4B). As with the filter binding assays, differences between wild-type and *ts*A58 T antigens were observed. However, in contrast to what had been



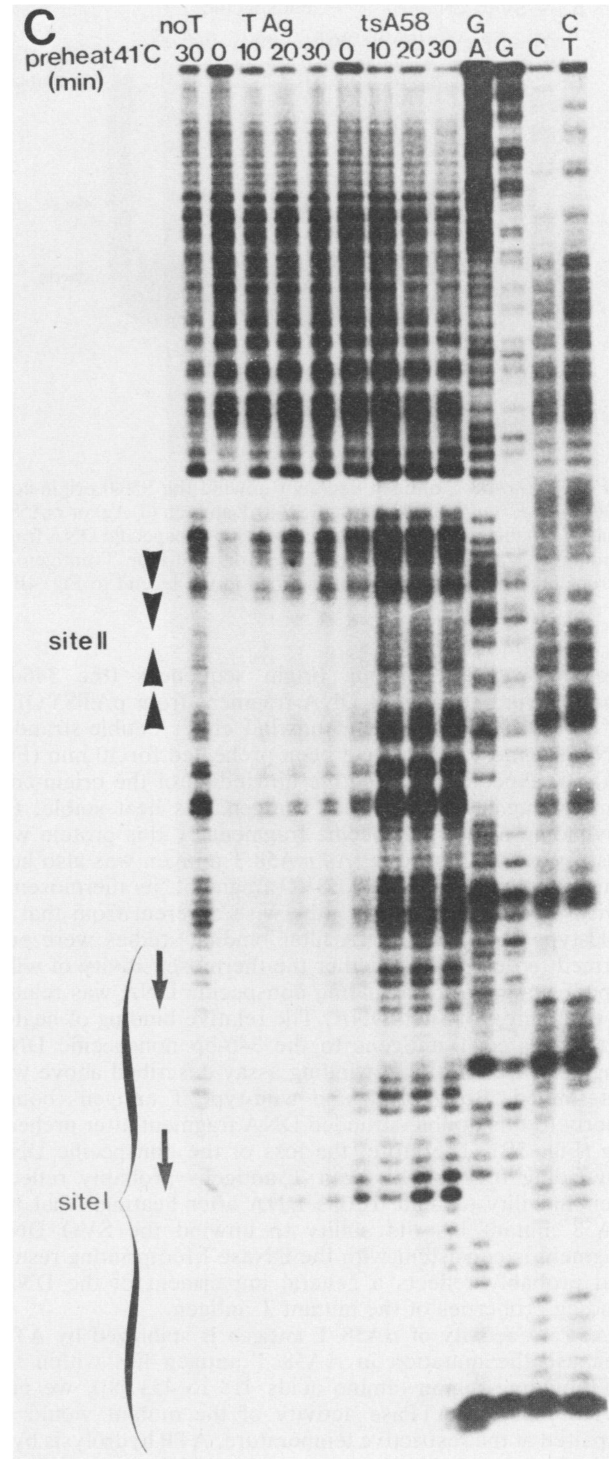


FIG. 4. Binding of *ts*A58 T antigen to site II is greatly impaired when the protein is preheated at 41°C. Filter binding reactions contained SV40 T antigen (T-Ag) or *ts*A58 T antigen and ^{32}P-end-labeled DNA fragments containing either site I (A) or site II (B) (66). ATP (4 mM) was either present or absent throughout the reaction. (C) DNase I footprint reactions contained SV40 T antigen or *ts*A58 T antigen and 1.0 ng of ^{32}P-labeled DNA fragment containing the SV40 origin (sites I and II). Reaction mixtures were preheated without the DNA fragment at 41°C either for 30 min (A and B) or for the indicated times (C). When the DNA was added, incubation was continued at 41°C.

observed in the filter binding assay, *ts*A58 protection of both sites I and II was reduced similarly. Also, unheated *ts*A58 T antigen displayed significantly less protection of either site I or site II than did either unheated or preheated wild-type T antigen. Even after wild-type T antigen was preheated for 30 min, protection of sites I and II was still apparent, while preheating *ts*A58 T antigen for as little as 10 min led to complete loss of protection of both sites I and II. Thus, *ts*A58 T antigen bound more poorly to the origin-containing fragment, and site I sequences were not as well protected by *ts*A58 T antigen as the filter binding assay indicated. This is unlikely to be related to the fact that the fragments used in the filter binding assay each contained only one binding site, because preheated *ts*A58 T antigen displayed significant binding to a fragment that contained both sites I and II in the same assay (data not shown).

Preheating *ts*A58 T antigen destroys its unwinding activity. After binding to the origin of replication, T antigen separates the double strands in a reaction that requires ATP and SSB (14, 27, 51, 74). The ability of *ts*A58 T antigen to unwind an SV40 origin-containing fragment was analyzed by determining the quantities of single-stranded DNA generated after unwinding reactions were performed. *ts*A58 T antigen unwound both an SV40 origin-containing fragment and a non-

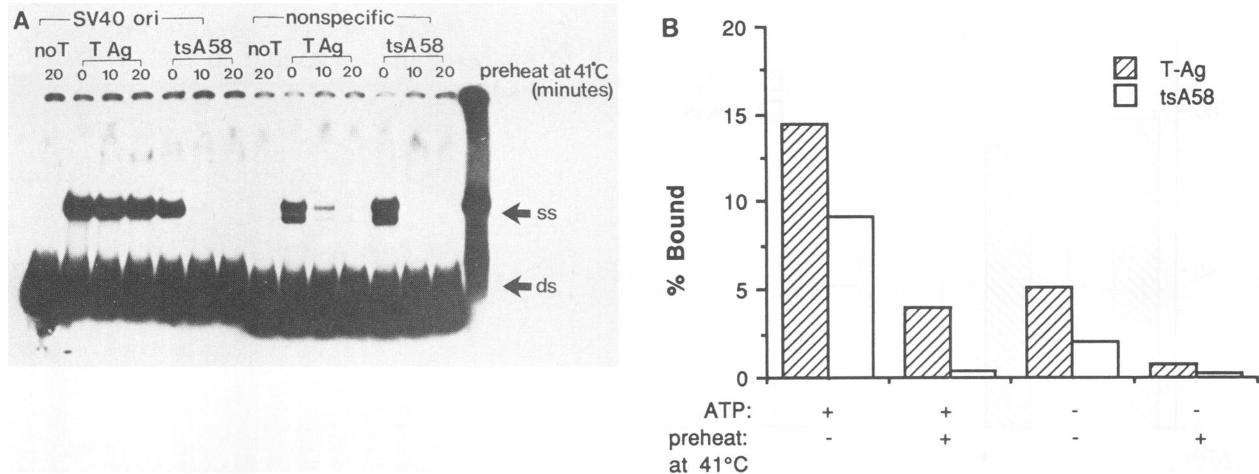


FIG. 5. *ts*A58 T antigen does not unwind the SV40 origin-containing fragment after being preheated at 41°C. (A) Unwinding reactions containing *E. coli* SSB and either SV40 T antigen (T Ag) or *ts*A58 T antigen were preheated at 41°C without DNA substrate for the indicated times. ^{32}P-labeled SV40 origin-containing or nonspecific DNA fragments (see text) were added, and reactions were incubated at 41°C. (B) The binding of *ts*A58 T antigen (0.12 (μg) or wild-type T antigen (0.12 μg) to the nonspecific fragment used in panel A was analyzed by nitrocellulose filter binding as described in the legend to Fig. 4B.

specific fragment lacking origin sequences (the 346-bp *Bam*HI-*Hin*dIII plasmid DNA fragment from pATSVO) at 41°C, but it was unable to unwind either double-stranded DNA fragment after it had been preheated for 10 min (Fig. 5A). Unexpectedly, while the unwinding of the origin-containing fragment by SV40 T antigen was heat stable, the unwinding of the nonspecific fragment by this protein was destroyed by preheating. As *ts*A58 T antigen was also heat sensitive for unwinding the SVO fragment, its thermosensitivity was greater than or otherwise different from that of wild-type T antigen. DNA filter binding studies were performed to determine whether the thermosensitivity of wild-type T antigen for unwinding nonspecific DNA was related to its affinity for such DNA. The relative binding of heated and unheated T antigens to the 346-bp nonspecific DNA fragment used in the unwinding assay described above was determined. Both *ts*A58 and wild-type T antigens bound poorly to this double-stranded DNA fragment after preheating (Fig. 5B). Therefore, the loss of the nonspecific DNA unwinding function by both T antigens probably reflects their inability to bind to this DNA after heating. That the *ts*A58 mutant lost its ability to unwind the SVO DNA fragment is consistent with the DNase I footprinting results and probably reflects a general impairment of the DNA-binding properties of the mutant T antigen.

ATPase activity of *ts*A58 T antigen is stabilized by ATP. Because the mutation in *ts*A58 T antigen lies within the ATP-binding region (amino acids 418 to 453 [8]), we predicted that the ATPase activity of the mutant would be impaired at the restrictive temperature. ATP hydrolysis by T antigen was examined in an assay which measured the release of ADP. At the permissive temperature, the ATPase activity of *ts*A58 T antigen was somewhat lower than that of wild-type T antigen: *ts*A58 T antigen hydrolyzed 70% of the ATP hydrolyzed by the wild-type protein. At 41°C, *ts*A58 T antigen hydrolyzed 42% of that of the wild type (data not shown), indicating that the ATPase activity was impaired but not abolished at the nonpermissive temperature. However, *ts*A58 T antigen preheated in the absence of ATP was subsequently unable to hydrolyze ATP: no release of ADP was observed after this treatment (Fig. 6). Surprisingly, when *ts*A58 T antigen was preheated in the presence of ATP, it exhibited substantial ATPase activity (0.38 nmol of ADP were generated by the mutant compared with 0.98 nmol by the wild type). Preheating wild-type T antigen in the presence or absence of ATP had no effect on its ability to hydrolyze ATP. The ATPase activity of *ts*A58 T antigen is, therefore, thermolabile, but ATP conferred stability on the mutant when present during preheating. When 5′-adenylyl imidodiphosphate (AMP-PNP) was present during the preheating period, *ts*A58 T antigen hydrolyzed 10.0% of the wild-type levels: the wild type hydrolyzed 1.0 nmol, whereas the mutant hydrolyzed only 0.1 nmol. The mutant T antigen was, therefore, only partially stabilized by the ATP analog. It should be noted here that, consistent with results reported by others (13, 26, 51, 71), we found that the ATPase activities of both mutant and wild-type T antigens were markedly stimulated by polydeoxynucleotides such as poly(dT) (data not shown).

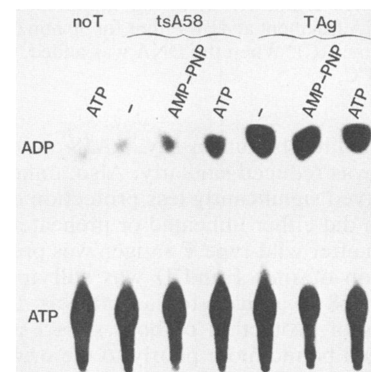


FIG. 6. ATP stabilizes the ATPase activity of *ts*A58 T antigen at the restrictive temperature. Reactions containing SV40 T antigen (T-Ag) or *ts*A58 T antigen were preheated for 30 min at 41°C in the absence of ATP, in the presence of 2.5 mM AMP-PNP, or in the presence of 2.5 mM ATP under helicase assay conditions. [α-^{32}P]dATP (7 × 10^3 cpm/nmol) was added to a final concentration of 5.0 mM, and incubation continued at 41°C.

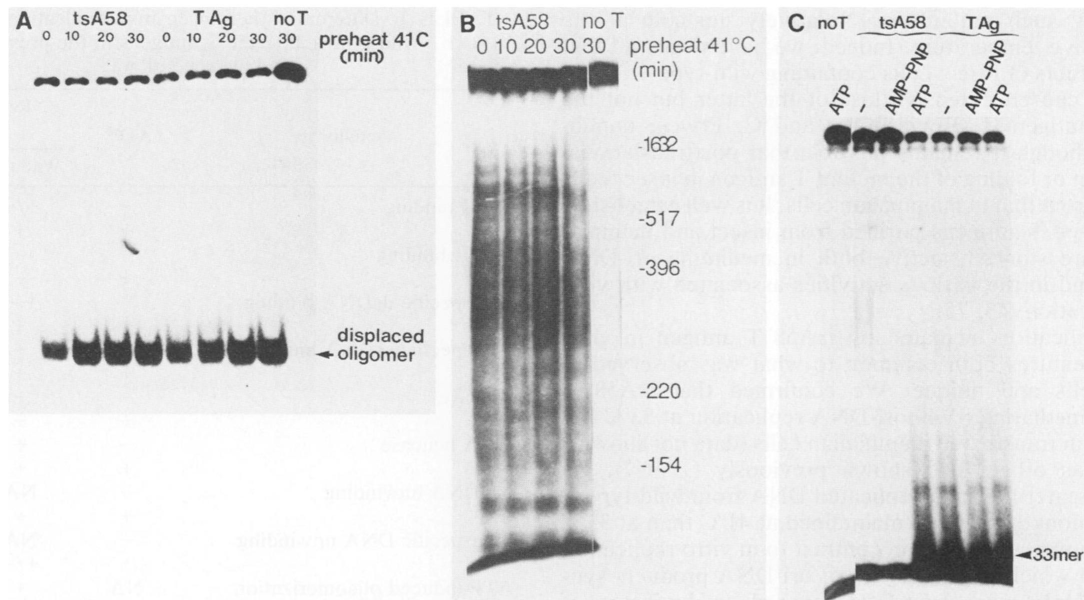


FIG. 7. Helicase activity of *ts*A58 T antigen is stabilized by ATP at 41°C. SV40 T antigen (TAg) or *ts*A58 T antigen was preheated in the absence of helicase substrate at 41°C for the indicated times. The incubation was continued at 41°C upon the addition of substrate, which was either a 50-nt oligomer (A) or heterogeneous DNA fragments annealed to complementary single-stranded DNA (B). (C) Conditions were the same as for panels A and B except that preheating at 41°C for 30 min was without ATP but with 2.5 mM AMP-PNP or 2.5 mM ATP as indicated. Then 2.5 mM ATP was added (5.0 mM to the no-ATP preheating reaction) along with the DNA substrate, which was the same as in panel B except that it had been digested with the restriction endonuclease *Hin*dIII, yielding a ^{32}P-labeled 33-nt oligomer annealed to M13.

tsA58 T antigen is a helicase after preheating. The DNA helicase activity of T antigen is likely to be involved in its putative elongation function. This activity was examined by incubating the T antigen with a labeled heteroduplex consisting of a nonspecific ^{32}P-end-labeled 50-nt oligomer annealed to a complementary circular single-stranded M13mp9 DNA in the presence of ATP. At the nonpermissive temperature, *ts*A58 T antigen displaced the same amount of oligomer as the wild-type protein even after being heated at 41°C for 30 min: in each case, approximately 60% of the oligomer that was bound to M13 was released (Fig. 7A). Thus, under conditions that completely inactivated its ability to mediate DNA synthesis in vitro, *ts*A58 displayed wild-type levels of helicase activity. Neither T antigen displaced any oligomer when reactions were carried out in the absence of ATP (data not shown). To determine whether *ts*A58 T antigen could also displace longer fragments, another helicase substrate was used: a circular single-stranded M13mp18 to which complementary oligomers of various lengths (16 to 1,600 nt) were annealed (see Materials and Methods). Preheated *ts*A58 T antigen displaced DNA fragments up to 1,600 nt long (Fig. 7B). No differences in the reaction products of preheated wild-type and mutant T antigens were detected (data not shown). Both unheated and preheated mutant and wild-type T antigens consistently displayed equivalent DNA helicase activities over a wide range of protein concentrations, including those at which only partial displacement of oligonucleotides occurred (data not shown). This demonstrates that *ts*A58 T antigen is an ATP-dependent helicase at the restrictive temperature and thus that this activity is thermostable. Our ATPase results suggested that the thermostability was dependent on the presence of ATP during the preheating period. Since the ATPase activity of T antigen is required for its helicase function, we tested the ability of *ts*A58 T antigen to displace a 33-nt oligomer after being preheated in the absence of ATP. Preheating *ts*A58 T antigen in the absence of ATP rendered it virtually unable to function as a helicase: the mutant displaced 5% of the helicase substrate, whereas the wild type displaced 63% (Fig. 7C). Similarly, the nonhydrolyzable analog of ATP could not stabilize the mutant T antigen (3 and 53% displaced by *ts*A58 and SV40 T antigens, respectively). The presence of ATP presumably conferred stability on heat denaturation, so that the *ts*A58 T antigen was able to function as a helicase. Here, both T antigens displaced approximately 65% of the helicase substrate. Preheating wild-type T antigen had no effect on its DNA helicase activity, whether or not there was ATP present. We therefore conclude that the ATPase and helicase functions of *ts*A58 T antigen are thermolabile when ATP is absent during the heating period. *ts*A58 T antigen exhibited wild-type levels of binding to the helicase substrate even after being preheated for 30 min both in the presence and absence of ATP (data not shown). This suggests that it is the ATPase activity that is primarily affected by preheating in the absence of nucleotide. Presumably this is why the mutant *ts*A58 T antigen cannot function as a helicase after such treatment.

DISCUSSION

The use of the baculovirus expression system should prove to be very useful for studying thermosensitive mutants. Experiments to characterize such conditional mutants have been hampered by the fact that mammalian cells frequently grow slowly and synthesize relatively small quantities of protein at temperatures in the 32 to 34°C range. At 37°C, many SV40 *ts*A58 mutants were shown to be at least partly defective, and at 41°C, they were completely inactivated. Thus, isolation of a partially denatured protein might not provide clear results about the conditional nature of the mutant. The problem of isolating temperature-sensitive mutant T antigens from mammalian cells is exacerbated be-

cause many such mutants are relatively unstable at the nonpermissive temperature. Indeed, we have observed that heating extracts of insect cells containing wild-type or *ts*A58 T antigens caused extensive loss of the latter but not the former T antigen (I. Reynisdóttir and C. Prives, unpublished). Although it remains possible that posttranslational modification or folding of the mutant T antigen in insect cells may differ from that in mammalian cells, it is well established that wild-type T antigens purified from insect and mammalian cells are similarly active both in mediating ori-DNA synthesis and in the various activities associated with viral DNA replication (43, 75).

DNA replication mediated by *ts*A58 T antigen in vitro displayed features both common to what was observed in infected cells and unique. We confirmed that *ts*A58 is capable of mediating SV40 ori-DNA replication at 33°C but not at 41°C in transfected mammalian cells (data not shown). However, as others had shown previously (11, 67), we observed greater levels of replicated DNA from wild-type T antigen in monkey cultures maintained at 41°C than at 33°C (data not shown). This was in contrast to in vitro replication reactions in which the quantities of ori-DNA products synthesized in HeLa extracts at 41°C were reduced by factors of 3 to 5 compared with those after incubation of extracts at 33°C. The reasons for the relative differences in quantities of DNA replicated at different temperatures in vivo and in vitro are not clear. It can be speculated that heat shock response systems that function in intact cells may allow more efficient DNA synthesis at high temperatures. Our experiments indicate that between 10 and 20 min are required to completely thermally inactivate *ts*A58 T antigen. This alone might explain the slow-stop phenotype characteristic of this mutant. However, the general discrepancies we observed between the relative efficiencies of replication at low and high temperatures would caution against assuming that rates of denaturation in vivo and in vitro are similar.

Our experiments showed that preheating wild-type and mutant T antigens had different effects on the two proteins. The wild-type T antigen was, however, not completely unaffected by preincubation at high temperatures. Wild-type T antigen showed thermosensitive properties that were stabilized by the presence of ATP, namely, binding to nonspecific and site II-containing DNA fragments. But its ability to unwind a nonspecific DNA fragment was drastically impaired even after preheating in the presence of the nucleotide. Table 1 compares the relative effects of preheating the wild-type and mutant T antigens on the different biochemical functions that were tested. These include two activities, single-stranded DNA binding and ATP-induced oligomerization, for which the data were not included because of space considerations. By combining the results obtained for both wild-type and mutant T antigens, a hierarchy of replication properties that vary in thermosensitivity and response to ATP stabilization can be proposed. Following the order of the most to the least thermostable property, it seems that single-stranded DNA binding > ATPase, DNA helicase, and ATP oligomerization > site I binding and origin DNA unwinding > site II binding and nonspecific DNA binding > nonspecific DNA unwinding. It would be of interest to determine whether other agents that affect protein structure, such as salt and pH, display a similar range of differential sensitivities. That the proteins displayed their relatively greatest thermostability for binding to single-stranded DNA is consistent with the data obtained by Wiekowski et al. (77), who showed that, once bound to helicase substrates, T antigen is stable to high concentrations of salt or to competing high quantities of double-stranded DNA but not to single-stranded DNA.

TABLE 1. Differential thermosensitive replication activities of wild-type and *ts*A58 T antigens in the presence and absence of ATP

Activity[a]	ATP[b]	Results[c] with T antigen:	
		Wild type	*ts*A58
Site I binding	−	+/−	−
	+	+	+
Site II binding	−	−	−
	+	+	−
Nonspecific dsDNA binding	−	+/−	−
	+	+	−
Nonspecific ssDNA binding	−	+	+
	+	+	+
ATPase	−	+	−
	+	+	+
DNA helicase	−	+	−
	+	+	+
ori-DNA unwinding	−	NA	NA
	+	+	−
Nonspecific DNA unwinding	−	NA	NA
	+	+/−	−
ATP-induced oligomerization	NA	+	+

[a] dsDNA, Double-stranded DNA; ssDNA, single-stranded DNA.

[b] T antigens were preheated at 41°C in reactions with (+) or without (−) 4 mM ATP under replication conditions as described in the text.

[c] +/−, ~10% of control; −, ≤1% of control; +, >10% of control; NA, not applicable.

The origin-binding domain of SV40 T antigen has been extensively characterized (2, 12, 46, 47, 56–58, 66). It is clear that the vast majority of mutations that affect origin binding map within the defined origin-binding domain and that this region alone is sufficient for binding specifically to sites I and II. However, a small number of mutations that map well outside of the origin binding domain but still affect origin-specific binding have been identified. One such mutant, Ser-677→Ala, when tested in McKay assays using extracts of transformed cells, showed defective binding to the whole origin or site I but normal binding to site II (53). Another mutant, in which amino acids 17 to 27 were deleted from the extreme NH_2 terminus of T antigen, was also defective in origin-specific binding despite the fact that the position of the deletion was not within the limits of the sequence involved in specific DNA binding (12). Our data show that heat-denatured *ts*A58 T antigen falls into the small group of mutants that are defective in DNA binding but whose mutations lie well outside of the origin-binding domain. Such mutants should eventually provide insight into the communication between different functional domains of T antigen.

There was a somewhat puzzling discrepancy between the DNA-binding data obtained when different assays were used. Binding to a site I-containing fragment measured by retention on nitrocellulose filters appeared to be relatively stable when the *ts*A58 protein was preheated in the presence of ATP, while site II fragment binding was abolished after similar treatment. This result confirms and extends previous experiments that were performed in the absence of ATP and $MgCl_2$ showing that the *ts*A58 T antigen has a markedly reduced ability to bind the SV40 origin (78) and that in extracts of infected cells, *ts*A58 T antigen binds more poorly to site II- than to site I-containing DNA fragments (63, 64). However, when the DNase I footprinting assay was used, no difference in the relative loss of protection of sites I and II

after similar preheating of purified *ts*A58 T antigen was noted; both sites were totally unprotected. Indeed, even the unheated *ts*A58 T antigen protected these sites less well than wild-type T antigen that had been extensively preheated. Thus, *ts*A58 T antigen displayed both far greater thermosensitivity for DNA binding and less difference between sites I and II by DNase I footprinting than by filter binding. Our DNase I footprinting data are consistent with the observation that *ts*A58 T antigen fails to autoregulate transcription from the early promoter (1, 48); hence this method may more accurately reflect the impairment of DNA binding by the mutant protein.

Interestingly, a number of other lesions in the function of the protein that were more appropriate for the location of its mutation, that is, within the presumptive ATP-binding fold of the protein, were noted. For instance, *ts*A58, when heated in the absence of ATP, was unable to hydrolyze ATP and to function as a helicase. Somewhat unexpectedly, however, we observed that when the *ts*A58 T antigen was preheated in the presence of the nucleotide, it displayed only partially defective ATPase activity and levels of helicase activity comparable to those of wild-type T antigen. Our data thus show that under conditions that presumably approximate the more physiological state for T antigen, namely in the presence of nucleotides, the major defects in the *ts*A58 T antigen are its inability to bind to and to unwind nonspecific or site II-containing double-stranded DNA. Why preheating *ts*A58 T antigen in the presence of ATP stabilized its ATPase and helicase but not its DNA-binding properties is not understood. Further structural analysis of the effects of thermal denaturation on the mutant and wild-type proteins may provide insight into the regions of T antigen that are altered after this treatment. However, our results imply that ATP can differentially affect the DNA helicase and DNA-binding properties of T antigen.

Filter binding assays revealed that *ts*A58 T antigen is more thermosensitive for binding to a nonspecific fragment than is wild-type T antigen, exhibiting virtually no binding to the DNA fragment even after being preheated in the presence of ATP. This closely resembled the results with the site II fragment and suggests that possibly the primary defect in the denatured *ts*A58 T antigen is, in fact, its ability to remain stably bound to DNA. Simmons et al. (58) have recently identified a region of T antigen within the origin-binding domain, spanning residues 183 to 187, that is likely to be involved in general DNA interactions rather than specific contacts with sequences in the origin. It is possible to speculate that the structure within this small region is specifically disrupted after heat denaturation of *ts*A58. However, an alternative interpretation may be offered. Even though the relatively small origin-binding domain can be isolated as discrete fragments, it was shown earlier, in DNA-cellulose binding experiments (47), that another portion of the T-antigen molecule mapping approximately between amino acids 237 and 325 confers retention of the protein on DNA-cellulose. This region contains the zinc finger motif that has been analyzed by mutagenesis more recently (34). This may indeed represent a second discrete region involved in nonspecific binding by T antigen. As this region is located somewhat closer to the position of the *ts*A58 mutation, it is possible that this is the primary site for disruption of the DNA-binding properties of the T-antigen mutant.

When the viral A gene product was first identified by using the *ts*A series of conditional mutants, studies on the kinetics of DNA synthesis arrest strongly indicated that T antigen functions solely in the initiation of viral DNA synthesis. The analogy between the DNA binding and unwinding functions of T antigen and those of the DnaA, DnaB, and DnaC proteins in initiating DNA replication at *E. coli* OriC (reviewed in reference 9) and the fact that DnaB plays roles both in unwinding DNA and in functioning as a replication fork helicase (32) provides a precedent for similar dual roles for T antigen. Our finding that *ts*A58 is defective in functions pertaining to initiation, i.e., binding to and unwinding DNA containing the SV40 replication origin, but not in those that might also be involved in elongation such as ATPase, single-stranded binding, and DNA helicase provides an explanation for the discrepancy between experiments suggesting one or two discrete roles for T antigen. Isolating and characterizing quick-stop mutants of T antigen would provide a more clearly defined idea as to if and how T antigen functions in elongation. Isolation of such mutants, however, might prove difficult it not impossible: those functions that T antigen might employ for its replication fork helicase role may well be a subset of those it requires for its function in initiation. It remains to be determined whether other *ts*A58 T-antigen mutants, many of which contain amino acid substitutions within or near the ATP-binding region (35), display phenotypes analogous to that of *ts*A58 when they are similarly analyzed in vitro. Such experiments are now in progress.

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