Functional Characterization of Temperature-Sensitive Mutants of Simian Virus 40 Large T Antigen

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We investigated the molecular properties of eight temperature-sensitive mutants of simian virus 40 large T antigen (*tsA* mutants). The mutants have single amino acid substitutions that block DNA replication at 39 to 41°C in vivo. In vitro, five of the mutant proteins were highly sensitive to a brief heat shock at 39°C, while the three remaining proteins were only partially sensitive at 41°C. We characterized the five most defective mutant proteins, using a variety of biochemical assays for replication functions of T antigen. Heat shock of purified T antigen with a mutation at amino acid 422 significantly impaired the oligomerization, origin-binding, origin-unwinding, ATPase, and helicase functions of T antigen. In contrast, substitution of amino acid 186, 357, 427, or 438 had more selective, temperature-sensitive effects on T-antigen functions. Our findings are consistent with the conclusion that T antigen functions via a hierarchy of interrelated domains. Only the ATPase activity remained intact in the absence of all other functions. Hexamer formation appears to be necessary for core origin-unwinding and helicase activities; the helicase function also requires ATPase activity. All five *tsA* mutants were impaired in functions important for the initiation of DNA replication, but three mutants retained significant elongation functions.

Simian virus 40 (SV40) large T antigen, the product of the viral A gene, regulates the initiation and elongation stages of SV40 DNA synthesis in viral infection. In the presence of ATP, T antigen assembles into a double-hexamer structure that covers the entire core origin of replication (1, 13, 33, 38) and induces changes in origin DNA that lead to the formation of a replication bubble (2, 11, 37). After release from specific recognition sequences in the origin, T antigen acts as a helicase for the extension of the primary replication bubble in both directions (15, 37, 52). T antigen also interacts with DNA polymerase α to initiate DNA synthesis and to assemble a replication complex (16, 45). Presumably, T antigen coordinates the various functions of the replication machinery as it moves the replication forks around the circular viral DNA. T antigen also interacts with the retinoblastoma (Rb) and p53 proteins, which are suppressors of cellular proliferation (14, 18, 24). The sequestration of Rb and p53 by T antigen is thought to prime infected, permissive cells for viral DNA replication.

Many functions of T antigen have been mapped to specific domains of the protein. Particularly well characterized is the T-antigen domain for specific binding to the origin of viral DNA replication (39, 43, 44, 48, 54). Isolated segments of T antigen extending from amino acids 132 to 246 bind in a site-specific manner to the origin of replication (48). About 50 amino acids away from the DNA binding domain, a single zinc finger motif extends from amino acids 302 to 320 (29, 30). This region is required for the formation of T-antigen hexamers in the presence of ATP. The ATP binding and ATPase domains map toward the C terminus of T antigen. The origin-unwinding and helicase activities of T antigen require the DNA binding, zinc finger, and ATPase domains (4, 30, 54). T antigen also has specific sites for binding to DNA polymerase α , Rb, and p53 (14, 16, 17, 27, 28, 45, 56).

Much of the early information about the replication and

transforming functions of T antigen was obtained by using temperature-sensitive mutants with changes in the A gene (*tsA* mutants). The mutants allow viral replication in permissive cells and transformation of restrictive cells at the permissive temperature of 32°C but fail to do so at higher temperatures. Analysis of several *tsA* mutants led to the recognition of T antigen's role in the initiation of DNA replication but did not identify an elongation function in the replication cycle (6, 49, 51). Temperature shift experiments showed that T antigen was required continuously for the maintenance of the transformation of nonpermissive cells (5, 32, 35, 50). The expression of temperature-sensitive T antigens in transgenic mice has been useful for the conditional immortalization of differentiated cells from a variety of tissues (23, 55). Recently, Loeber et al. (31) sequenced the DNA of all known *tsA* mutants of SV40 large T antigen and identified single amino acid substitutions in each of eight mutated alleles. The temperature-sensitive phenotypes of the mutants strongly suggest that the mutated amino acids play crucial roles in organizing the structure of the protein for one or more functions of T antigen. Our present results describe the biochemical defects of the *tsA* mutant T antigens in viral DNA replication.

MATERIALS AND METHODS

Construction of *tsA* mutants in an isogenic background. Because the *tsA* mutants were isolated from several wild-type (WT) strains of SV40, we constructed all eight mutations in the T-antigen sequence of strain 776 to place the mutations in an isogenic background. Most of the mutants were constructed by using oligonucleotide-directed mutagenesis of the Bluescript vector BS*SV40 as described previously (29). Mutagenesis of 562F-S was carried out by using the vector pSK(-)SVTC, which contains T-antigen cDNA from SV40 strain 776 in the *Bam*HI site of Bluescript (kindly provided by Dan Simmons).

Overexpression of the *tsA* mutants. We chose to express

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the WT and temperature-sensitive mutant T antigens by using a baculovirus expression system for a number of reasons. First, T antigen overexpressed in insect cells is remarkably similar to T antigen isolated from SV40-infected monkey cells in terms of its known biochemical functions (25). Second, insect cells (SF9) in culture are best grown at 27°C. This temperature is ideal for the expression of native *tsA* proteins, which are partially inactivated at temperatures as low as 32°C (53). We transferred restriction fragments containing the DNA mutations from the Bluescript vectors to the pVL941T vector (25), which has baculovirus sequences on either side of the WT T-antigen gene, as described by Loeber et al. (30). The T-antigen genes were then recombined with baculovirus DNA by cotransfection with whole viral DNA, and occlusion-negative plaques were selected. After the viruses were screened for T-antigen expression, recombinant viral stocks were plaque purified until free of WT baculovirus.

Purification of T antigen. WT and mutant T antigens were extracted from SF9 cells infected with recombinant baculoviruses, and T antigen was purified by immunoaffinity purification, using PAb419 as described by Simanis and Lane (42). T antigen was eluted from the column with 20 mM Tris-HCl (pH 8.5)–1 mM EDTA–0.5 M NaCl–10% glycerol–50% ethylene glycol (33). After an overnight dialysis against storage buffer containing 10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 7.0)–5 mM NaCl–0.1 mM EDTA–1 mM dithiothreitol (DTT)–50% glycerol, the purified proteins were stored at –20°C. T antigen was the only polypeptide evident in stained sodium dodecyl sulfate (SDS)-gels of the purified proteins.

Conditions for heat shock. T antigen (1.0 µg in 10 µl of storage buffer) was added to 15 µl of 30 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5)–7 mM MgCl₂–1 mM DTT and heated at 39 or at 41°C. These buffer components were designed for the efficient replication of SV40 DNA in vitro (47). We omitted nucleosides and deoxynucleosides during preheating because Dean et al. (10) have reported that preincubation of T antigen with ATP interferes with ATP-dependent binding, unwinding, and replication of SV40 origin-containing DNA. We omitted protein components required for in vitro DNA replication because these would interfere with some of our assays for T-antigen functions. It is important to note that buffer components affect the rate of heat inactivation of T antigen; glycerol, for example, has a protective effect on protein function. Therefore, all heat shocks were done under identical conditions. We used a transient heat shock under nonpermissive conditions rather than continuous incubations at permissive and nonpermissive temperatures to allow subsequent measurement of various functions with and without preheating under a single set of conditions.

In vitro replication. T antigen (1.0 µg) was incubated at 32°C for 2 h with SV40 origin DNA in plasmids (0.5 µg) and 18 µl of 293 cell S100 extract in 30 mM HEPES (pH 7.5)–7 mM MgCl₂–1 mM DTT–40 mM creatine phosphate–4 mM ATP–0.2 mM each GTP, UTP, and CTP–0.1 mM each dCTP, dGTP, and TTP–0.025 mM dATP and [α-³²P]dATP (800 Ci/mmol; specific activity 1,000 cpm/pmol)–20 µg of creatine phosphokinase per ml–0.1 mg of bovine serum albumin (BSA) per ml–20% glycerol (replication buffer). DNA synthesis was stopped with 20 mM EDTA, and DNA was precipitated with 8% ice-cold trichloroacetic acid containing 1% sodium pyrophosphate. Precipitates were collected on GF/C filter disks, washed extensively, and dried. The amount of DNA synthesis was quantitated by liquid

scintillation counting. Unless noted otherwise, we monitored the biochemical functions of the WT and mutant T antigens under replication conditions to allow a direct comparison of the different properties of T antigen under identical conditions.

KMnO₄ and DNase I footprinting. KMnO₄ and DNase I footprinting were performed as described by Gralla (21), with minor modifications (37). T antigen (1 µg) was incubated for 2 h at 32°C with 0.5 µg of core origin DNA in replication buffer in a 50-µl volume. DNAs were probed at 32°C with 30 mM KMnO₄ for 3 min or with 0.1 U of DNase I for 50 s. KMnO₄ reactions were stopped with 3 µl of β-mercaptoethanol, and DNase I reactions were stopped with 4 µl of 0.2 M EDTA. DNAs were extracted with phenol and were spun through Sephadex G-50 (Pharmacia) to suspend the DNA in distilled H₂O. Isolated DNAs were annealed to a primer 5' end labeled with [³²P]ATP. The primer was extended with 1 U of Klenow polymerase, and labeled products were separated on 8% polyacrylamide–8 M urea sequencing gels at 50°C.

Hexamer formation. T antigen was added to replication buffer, without BSA or creatine phosphokinase, and incubated in a final volume of 50 µl for 2 h at 32°C. The reaction products were cross-linked with 0.1% glutaraldehyde for 20 min at 32°C and analyzed immediately by nondenaturing, gradient gel electrophoresis as described previously (38).

ATPase assay. T antigen was added to replication buffer, without creatine phosphate and creatine phosphokinase, and incubated in a final volume of 50 µl for 2 h at 32°C. ATPase activity was measured by colorimetric quantitation of P_i released from ATP at 32°C as described by Lanzetta et al. (26).

Helicase assay. Helicase substrates were prepared by elongating a primer on M13 single-stranded DNA in the presence of dideoxynucleotides as described by Stahl et al. (46). T antigen was added to replication buffer containing 10 ng of labeled substrate and incubated at 32°C for 2 h in a final volume of 50 µl. Creatine phosphate and creatine phosphokinase were omitted to allow a direct correlation of the helicase assay with the ATPase assay. The reactions were stopped with 5 µl of 3.3% SDS–0.5 M EDTA, and the products were resolved by 6% polyacrylamide gel electrophoresis.

RESULTS

Nomenclature and locations of *tsA* mutations. Loeber et al. (31) sequenced all known *tsA* mutations in T antigen and changed the nomenclature of the mutants to identify the mutated amino acid of each mutant. For example, *tsA3900*, which has an arginine-to-threonine substitution at amino acid position 186, was renamed *tsA186R-T*. Many mutants isolated by different groups have identical amino acid substitutions. Figure 1 shows the sequence alterations of the *tsA* mutants and the locations of the mutations relative to the functional domains of T antigen. The mutation *tsA186R-T* (22) maps in the region of the protein that is sufficient for specific binding to the SV40 origin of DNA replication (48). The region around amino acid 186 is particularly important for DNA binding; mutants with single amino acid changes at positions 185 and 187 in the protein are defective for DNA binding (44). Mutation *tsA357R-K* is located in a region with a distinctive pattern of repeated leucines and arginines that are conserved among all known polyomaviruses (31). Mutations causing the temperature-sensitive defects of *tsA393W-C*, *tsA422W-C*, *tsA427P-L*, *tsA438A-V*, and

FIG. 1. Nomenclature and locations of temperature-sensitive mutations in SV40 large T antigen. Functional domains are shown at the top (17). NLS, nuclear localization signal; Pol, DNA polymerase α . Mutations are shown at the bottom. Mutations are identified by amino acid number, the WT amino acid, and the substituted amino acid. The amino acid numbers correspond to the sequence of T antigen in strain 776. Amino acids are given in the single-letter code. Below these designations are previous, less specific names for the same mutations.

tsA453P-S are located in or near a highly conserved region with structural homology to an ATP binding fold (4). The tsA562F-S mutation maps distal to the ATP binding fold but in the ATPase domain. All of the tsA mutations map within the broad region required for the origin-unwinding and helicase functions of T antigen.

In vitro DNA replication by mutant T antigens. Loeber et al. (31) showed that all tsA mutants except tsA453P-S replicate DNA poorly at 39 to 41°C in vivo. Their assay measured the accumulation of viral DNA during the course of several days at the restrictive temperature, so the replication defects of some of the mutations might have reflected a rapid degradation of the mutant T antigens intracellularly rather than a loss of replication function per se. Therefore, we measured the effects of a short heat shock on purified mutant proteins to determine the intrinsic effects of thermal denaturation on T-antigen function. We made WT and mutant T antigens in insect cells at 27°C by using baculovirus expression vectors. After immunoaffinity purification at 4°C, we stored the purified T antigens at -20°C in 50% glycerol. The purified proteins were exposed to a heat shock in a 39°C water bath for 5 to 10 min. Immediately after heating, the T antigens were added to cellular extracts containing a plasmid with the SV40 origin of replication for 2 h at 32°C (Fig. 2). WT T antigen, exposed to heat shock at 39 to 41°C, replicated the test plasmid DNA nearly as well as unheated WT protein did. Five of the tsA mutants, those with amino acid substitutions at positions 186, 357, 422, 427, and 438, were very sensitive to brief heating at 39°C (Fig. 2A). The remaining three tsA mutants, those with changes at positions 393, 453, and 562, were not sensitive to heating at 39°C but were moderately sensitive to heating at 41°C for 10 min (Fig. 2). We immunoprecipitated T antigen from similar replication mixtures and found that little, if any, of the WT or mutant T antigens was degraded under the conditions of the replication assay (data not shown). We conclude that heat shock leads to the irreversible loss of one or more intrinsic functions of the tsA mutant T antigens. In the remainder of this report, we will describe the effects of heating on individual functions of the five tsA mutants that are highly heat

sensitive. All subsequent assays were carried out under the same conditions as were the replication assays unless indicated otherwise.

Hexamer assembly of mutant T antigens. T antigen binds to the SV40 replication origin as double hexamers (33, 38, 52). After unwinding the origin, the double hexamers appear to act as bidirectional helicases by threading DNA through the binary hexamer complex (52). The loss of hexamer assembly could interfere with the origin-unwinding or helicase function and explain the replication defect of the tsA mutants. We used chemical cross-linking and gradient gel electrophoresis to determine the oligomeric structure of the tsA mutant T antigens (Fig. 3). The proteins were heated for 10 min at 39°C and then incubated in replication buffer for 2 h at 32°C in the absence of other proteins. The T antigens were then cross-linked and analyzed by gradient gel electrophoresis and silver staining. In the absence of ATP, WT T antigen consisted mostly of monomers and dimers. The addition of ATP induced the assembly of most of the smaller forms into hexamers with or without a heat shock. In the presence of ATP, all of the mutant T antigens formed hexamers and, in some cases, double hexamers very efficiently at 32°C. Heat shock drastically reduced the number of 186R-T, 357R-K, and 422W-C mutant hexamers and partially reduced 427P-L and 438A-V hexamers. Because the loss of hexamer forms was not accompanied by a dissociation into smaller oligomeric forms, heating apparently resulted in the formation of protein aggregates that would not enter the gel efficiently. Indeed, a protein smear was present in the stacking gel of lanes in which T-antigen hexamers were reduced by heating (data not shown). We conclude that a brief heat shock has significant effects on the structures of most tsA mutant T antigens.

Origin binding by mutant T antigens. In the absence of ATP, T antigen binds to origin sequences in a site-specific manner at 4°C even though T antigen does not form hexamers under these conditions (1, 13, 33, 34). At temperatures over 30°C, however, T antigen binds the core origin efficiently only in the presence of ATP. Under these conditions, WT T antigen assembles as hexamers on each half of the

FIG. 2. DNA replication in vitro by WT and *tsA* mutant T antigens. Purified T antigens were exposed to a heat shock for 0, 5, or 10 min at either 39°C (A) or 41°C (B). The T antigens were then added to reaction mixtures containing replication buffer, cellular extracts, and plasmid DNA with an SV40 origin of replication. After mixtures were incubated for 2 h at 32°C, replicated DNA was precipitated and quantitated by measuring incorporation of radiolabeled precursors as described in Materials and Methods. Symbols identify the various mutant T antigens.

core origin of replication (33, 38, 52). ATP not only enhances the efficiency of T-antigen binding but also increases the span of origin DNA protected from DNase I. Mutations in the zinc finger region of T antigen block the formation of hexamers but have only minor effects on the efficiency of binding to the pentanucleotide (PEN) domain of the core origin (30); they do, however, reduce DNase protection over the inverted repeat (IR) domain of the origin where T-antigen-induced melting occurs.

We used DNase footprinting to determine the effects of heat shock on the DNA binding properties of the *tsA* mutant proteins in the presence of ATP (Fig. 4). Following heat shock at 39°C, T antigen was incubated with origin-containing plasmids at 32°C under replication conditions. After 2 h, the DNA was cut with DNase I. An end-labeled primer was annealed to the cut DNA and extended to the nick site with the Klenow fragment of *Escherichia coli* DNA polymerase I.

FIG. 3. Hexamer formation by WT and *tsA* mutant T antigens. Purified T antigens (T ag) were either not exposed (–) or exposed (+) to a heat shock, incubated in replication buffer in the presence (+) or absence (–) of 4 mM ATP for 2 h at 32°C, cross-linked with 0.1% glutaraldehyde, and analyzed by native gradient gel electrophoresis as described in Materials and Methods. WT protein was exposed to a 30-min heat shock, and mutant proteins were exposed to 10-min heat shocks. Numbers identify the amino acid positions of the various mutations. The positions of monomers (1-mer) and hexamers (6-mer) are shown at the left.

A primer that anneals to the upper strand and monitors upper-strand scissions was used. The primer-extended DNA was then analyzed on a sequencing gel. WT T antigen protected the entire origin of replication from DNase in the presence or absence of a 39°C heat shock. Heat shock nearly eliminated DNase protection of the core origin by *tsA* mutant 422W-C and caused a significant reduction in DNase protection by mutants 186R-T, 357R-K, 427P-L, and 438A-V

FIG. 4. Origin binding by WT and mutant T antigens. (A) Sequence of the core origin. The IR, PEN, and AT domains are shown above the origin sequence. (B) Footprinting assay. Purified T antigens (T ag) were either not exposed (–) or exposed (+) to a heat shock for 10 min at 39°C, incubated in replication buffer containing plasmids with the SV40 origin of replication for 2 h at 32°C, treated with DNase I, and analyzed as described in Materials and Methods. Numbers identify the amino acid positions of the various mutations. The positions of IR, PEN, and AT domains of the core origin are shown at the left.

FIG. 5. Unwinding of origin DNA by WT and mutant T antigens. Purified T antigens (T ag) were either not exposed (–) or exposed (+) to a heat shock for 10 min at 39°C, incubated in replication buffer containing plasmids with the SV40 origin of replication for 1 h at 32°C, treated with KMnO_4 , and analyzed as described in Materials and Methods. Numbers identify the amino acid positions of the various mutations. The positions of IR, PEN, and AT domains of the core origin are shown at the left.

(Fig. 4). The *tsA* mutant T antigens reduced the efficiency of DNase protection over the entire core origin of replication; in contrast, the zinc finger mutant T antigen 320H-L protected the PEN domain but not the IR domain.

Origin unwinding by mutant T antigens. We and others have shown that in the presence of ATP, T-antigen double hexamers bound to the origin melt about 10 bp in the IR domain and distort about 15 bp in the A+T-rich (AT) domain (2, 12, 37). These alterations in DNA structure can be monitored with KMnO_4 , which oxidizes thymines in denatured or distorted DNA (3). We performed KMnO_4 footprints on all *tsA* mutants that fail to replicate DNA at 39°C (Fig. 5). The footprinting procedure was the same as that described for DNase footprinting except that KMnO_4 was used to modify DNA. KMnO_4 modification blocks the primer extension reaction. WT T antigen induced KMnO_4 modification of thymines in the IR and AT domains of the core origin to similar extents in the presence and absence of a heat shock. In contrast, heat shock nearly eliminated KMnO_4 modifications induced by *tsA* mutants 186R-T, 357R-K, 422W-C, and 438A-V and reduced the function of mutant 427P-L more than 50%. We conclude that the *tsA* mutations affected one or more components of the origin-unwinding function of T antigen.

ATPase function of mutant T antigens. Many of the *tsA* mutations map in or near the putative ATP binding fold in the ATPase domain of T antigen (Fig. 1). A defect in ATP binding or hydrolysis would account for the temperature sensitivity of these *tsA* mutants. We carried out ATPase assays under replication conditions except that the ATP-regenerating components of the reaction mixture were omitted. We used colorimetric assays to measure the release of P_i from ATP so that we could measure ATPase activity in the presence of 4 mM ATP under replication conditions (Table 1). WT T antigen had similar ATPase activities with and without heating. Heat shock of mutants 357R-K and 422W-C caused a 10-fold or greater reduction of ATPase activity. Heating caused a more limited reduction in the ATPase

TABLE 1. Temperature sensitivity of WT and mutant T-antigen ATPase activities^a

T antigen	ATPase, 39/32°C (%)
WT	86
<i>tsA</i> mutants	
186R-T	88
357R-K	9
422W-C	7
427P-L	27
438A-V	52
Zinc finger mutant 320H-L	90

^a Reactions were carried out at 4 mM ATP. WT T antigen (1 μg) released 2 nmol of phosphate in 2 h.

activities of mutants 427P-L and 438A-V and no significant reduction in the ATPase activity of mutant 186R-T. With the possible exception of mutant 357R-K, the effects of mutations on ATPase activity correlated well with the location of the *tsA* mutations relative to the putative ATP binding fold. Mutation of position 320 in the zinc finger region caused no significant loss of ATPase activity after a heat shock.

Helicase activities of mutant T antigens. The helicase activity of T antigen is a complex function that requires a broad span of the protein, and all known *tsA* mutations map within this segment of T antigen (Fig. 1). We compared the helicase activities of WT and *tsA* mutant T antigens with and without a heat shock. The substrate for the helicase assay consisted of M13 single-stranded DNA on which a primer had been extended to various lengths up to several hundred bases. After incubation of T antigen with the substrate for 2 h under replication conditions, displacement of the radiolabeled oligonucleotides was measured by gel electrophoresis (Fig. 6). Heat shock slightly stimulated the helicase activities of WT T antigen and mutants 427P-L and 438A-V. Heating reduced the helicase function of mutant 357R-K about 2-fold and that of mutants 186R-T and 422W-C more than 20-fold, regardless of the length of the substrate oligonucleotides.

FIG. 6. Helicase activities of WT and mutant T antigens. Purified T antigens (T ag) were either not exposed (–) or exposed (+) to a heat shock for 10 min at 39°C and incubated in replication buffer containing partially double stranded M13 DNA (dsDNA) for 2 h at 32°C. Displaced single-stranded DNAs (ssDNA) of various lengths were analyzed by gel electrophoresis as described in Materials and Methods. Numbers identify the amino acid positions of the various mutations. The double-stranded DNA substrate is shown in the leftmost lane; boiled, single-stranded DNA is shown in the rightmost lane.

TABLE 2. Summary of WT and mutant phenotypes

Function	Level of activity ^a					
	WT	<i>tsA</i> mutant at 39°C				
		186	357	422	427	438
Replication	++++	—	—	—	+	—
Hexamer	++++	—	+	—	++	++
Origin binding	++++	+	++	—	++	++
Origin unwinding	++++	—	—	—	++	+
ATPase	++++	++++	+	+	++	++
Helicase	++++	—	++	—	++++	++++

^a Numbers identify the amino acid positions of the mutations. Results for the zinc finger mutant were taken from Loeber et al. (30) and from Table 1. Although zinc finger mutant T antigens protected the PEN domain of the core origin from DNase, they did not protect the IR domain. +++++, +++, ++, +, and — indicate 100 to 75, 75 to 50, 50 to 25, 25 to 5, and 5 to 0% of the level of the WT function.

DISCUSSION

We have investigated the replication functions of all known *tsA* mutants of SV40 large T antigen. The temperature dependence of the mutants strongly suggests that the mutated amino acids play crucial roles in organizing protein structure. We wanted to determine whether the mutations have global effects on many functional domains of T antigen or affect only the domains in which the substituted amino acids reside. If the mutations affect only selected functions, then a comparison of results from a number of mutants might suggest which functions are interdependent. Table 2 summarizes the effects of heat shock on the replication functions of *tsA* mutants and compares these with the functions of zinc finger mutants described by Loeber et al. (30). The data from each assay were quantitated by densitometry, and the ratios of mutant function with and without a heat shock were compared with the ratios of wild-type function with and without a heat shock. The levels of various functions are expressed in quartiles for ease of comparison. The quantitation of functions varied by about $\pm 10\%$ from experiment to experiment. Mutations that consistently reduced function more than 95% are represented by minuses.

Some mutations appear to have global effects on protein functions. Mutant 422W-C affected all of the tested functions of T antigen to a significant extent. It is not clear why this single amino acid substitution has such dramatic effects on protein structure. Tryptophan 422 is located in a predicted beta sheet in a region that has the structural features of an ATP binding fold (4). The substitution of a cysteine for tryptophan 422 would not be expected to change the beta-sheet structure (7) even though this substitution is rare in conserved proteins (36). Apparently, heat shock of the 422W-C mutant protein either causes extensive denaturation of T antigen or interferes with a critical function upon which other functions are dependent. For example, the absence of hexameric structures might contribute to the extent and severity of the defects of this mutant. The *tsA* mutation of amino acid 186 also caused extensive changes in protein function. However, in contrast to mutant 422W-C, mutant 186R-T had no effect on ATPase activity even though all other functions tested were severely impaired. This finding indicates that the ATPase function does not depend on any of the other functions of T antigen that we tested. The different effects of these two mutations on ATPase function can be explained, in part, by their distance from the ATPase domain of the protein (Fig. 1). Amino acid 186 is located far

from the ATPase domain, while amino acid 422 is in the ATPase domain. Our findings are consistent with and extend previous data showing that T antigens with deletions that block DNA binding retain ATPase activity (8, 9).

Mutants 357R-K, 427P-L, and 438A-V have a number of common properties. After a brief heat shock, they are the only mutants that form hexamers in the presence of ATP. The same mutants have some ATPase activity and have significant levels of helicase activity. These correlations are consistent with the idea that hexamers are needed for helicase activity. Recently, Wessel et al. (52) presented electron microscopic evidence that T antigen formed hexamers at active helicase forks. It is not clear why reduced numbers of hexamers and partial ATPase activity are sufficient for significant levels of helicase activity in our assays. The conditions for these assays were the same except that hexamer assembly and ATPase assays were done in the absence of DNA. Perhaps the substrate DNA in the helicase assay enhanced the assembly of T-antigen hexamers and ATPase activity during the course of the 2-h assay at 32°C after the heat shock of T antigen. Indeed, Parsons et al. (38) have presented direct evidence that origin DNA enhances the cooperative assembly of T-antigen hexamers, and Giachero et al. (20) have shown that DNA increases the ATPase activity of T antigen. Mutants 357R-K, 427P-L, and 438A-V also bind reasonably well to core origin DNA but differ in the ability to unwind origin DNA. Clearly, hexamer formation and DNA binding are not sufficient for origin melting. Our studies of 438A-V are in agreement with the results of Reynisdóttir et al. (41), even though different conditions were used for the heat shock of the mutant proteins in their studies. Mutant 438A-V has been reported to be defective in binding to DNA polymerase α (19, 40); this defect may further contribute to its loss of DNA replication.

Our findings together with those of Loeber et al. (30) and Reynisdóttir et al. (41) are consistent with a hierarchy of interrelated T-antigen functions. The ATPase activity is the only autonomous function among those that we tested. The unwinding of origin DNA and helicase activity are consistently associated with hexamer formation, and the helicase function is active only in the presence of ATPase activity. These correlations suggest that hexamers are needed for the origin-unwinding and helicase functions. The relationship of T-antigen quaternary structure to binding of the core origin remains unclear. Interestingly, heat shock reduces nuclease protection by *tsA* mutant T antigens over the entire span of the core origin. In contrast, zinc finger mutant T antigens protect the PEN domain of the core origin even though they fail to protect the IR domain. This difference in DNase protection patterns suggests that the *tsA* mutations affect interactions with both the PEN and IR domains, while the zinc finger mutations target interactions with the IR domain, and is consistent with previous data showing that T antigen can interact independently with isolated PEN and IR domains (34).

Because the helicase function of T antigen appears to depend on both hexamer formation and ATPase function, one might expect that the helicase function would be the replication function most susceptible to mutation. However, our assays clearly indicate that the origin-unwinding function is even more sensitive to mutation. All five *tsA* mutant T antigens lost functions important for the initiation of DNA replication, but three mutants retained elongation functions. Reynisdóttir et al. (41) reported similar conclusions in their study of mutant 438A-V. The specificity of the interaction of T antigen with recognition sequences probably explains why

origin-related functions are more fastidious than subsequent elongation functions. This difference may explain why in vivo studies of mutants like 357R-K, 427P-L, and 438A-V implicated T antigen in the initiation of DNA replication but not in strand elongation (6, 49, 51).

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