JOURNAL OF VIROLOGY, June 1991, p. 3167–3174
0022-538X/91/063167-08$02.00/0

Vol. 65, No. 6

The Zinc Finger Region of Simian Virus 40 Large T Antigen Is Needed for Hexamer Assembly and Origin Melting

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Received 25 January 1991/Accepted 20 March 1991

Simian virus 40 large T antigen contains a single sequence element with an arrangement of cysteines and histidines that is characteristic of a zinc finger motif. The finger region maps from amino acids 302 through 320 and has the sequence C-302 L K C-305 I K K E Q P S H Y K Y H-317 E K H-320. Previous genetic analysis has shown that the cysteine and histidine sequences and the contiguous S H Y K Y region in the finger are important for DNA replication in vivo. We show here that representative mutations in either of these elements of the finger prevent the assembly of large T antigen into stable hexamers in vitro. These same mutations have a characteristic effect on the interaction of T antigen with the simian virus 40 core origin of replication. The mutant T antigens bind to the central pentanucleotide domain of the core origin but fail to melt the adjacent inverted repeat domain and to untwist the adenine-thymine domain. These defects would prevent the formation of a replication bubble and the initiation of DNA replication. Finger mutations have lesser effects on the helicase function of T antigen and no observable effect on binding of T antigen to the mouse p53 protein. We propose that the zinc finger region contributes to protein-protein interactions essential for the assembly of stable T-antigen hexamers at the origin of replication and that hexamers are needed for subsequent alterations in the structure of origin DNA. We cannot exclude the possibility that the zinc finger region also makes specific contacts with components of origin DNA.

Simian virus 40 (SV40) large tumor antigen (T antigen) regulates the initiation and elongation stages of SV40 DNA synthesis in productive infection. T antigen binds to a central pentanucleotide domain and, in the presence of ATP, assembles into double hexamers that cover the entire core origin of replication (12, 26, 32, 44). Bound T antigen melts an inverted repeat element on the early side of a central binding domain and untwists DNA in an adenine-thymine (AT) domain on the late side of the origin (5, 31). These structural changes appear to release T antigen bound to the central domain to act as a helicase for the extension of the primary replication bubble in both directions (9, 39, 47, 48). T antigen interacts with DNA polymerase α and perhaps other cellular proteins to assemble a replication complex for the synthesis of RNA primers and DNA (15, 38). Presumably, T antigen coordinates the various functions of the replication machinery as it drives the replication forks around the circular viral DNA. T antigen also interacts with the retinoblastoma and p53 suppressors of cellular proliferation (11, 23). Under certain conditions, p53 can suppress the replication function of T antigen in vitro, but it is not clear that p53 modulates replication in vivo by similar mechanisms (43, 46).

Some functions of T antigen have been mapped to domains of the protein, but the domain boundaries for many of these functions are still vague (13). Particularly well characterized is the T-antigen domain for specific binding to target sites in the origin of viral DNA replication (1, 20, 33, 36, 41), which itself is organized into a subdomain structure (37). Isolated segments of T antigen extending from amino acids 131 to 246 bind in a site-specific manner to the origin of replication but do not induce structural changes in the core origin region (41). About 50 amino acids away from the DNA binding domain, a single zinc finger motif (21) extends from amino acids 302 to 320 (45). This region is highly conserved among the polyomaviruses. Zinc finger elements are often found in DNA-binding proteins. In many cases, such as that of TFIIIA (21, 27), multiple fingers interact with specific sequences repeated in the major groove of DNA. A single zinc finger, however, can exist as an independent structure sufficient for zinc-dependent DNA binding (30). There are also examples of metal binding domains that contribute to overall protein structure or protein-protein interactions (2, 3, 16, 17, 19, 42).

The zinc finger motif in T antigen is located outside the minimal region necessary for specific binding to the recognition pentanucleotides in the center of the core origin of replication. This element, therefore, may play a different role in the interaction of T antigen with origin DNA. We have shown previously that two classes of mutations in the zinc finger region block SV40 replication in vivo (25). Mutations that alter any of the cysteine or histidine residues of the finger completely block replication. This result supports the idea that these residues have a common, zinc binding role in finger function. In addition, mutations in a contiguous block of five amino acids in the central portion of the finger reduce replication significantly. These findings suggest that this segment is an active site within the finger. To characterize the effects of these two kinds of mutations on the biochemical properties of T antigen, we have expressed wild-type and mutant proteins in a baculovirus-derived expression system for the purification and characterization of T antigen (24). We find that normal zinc finger function is crucial for hexamer assembly and for the melting of origin DNA.

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MATERIALS AND METHODS

Expression of T antigen in baculovirus-infected cells. All mutants were constructed by using oligonucleotide-directed mutagenesis in the plasmid BS*SV40 as described previously (25). The restriction fragment between the *Pfl*MI (nucleotide 4554) and *Pst*I (nucleotide 3204) sites was then excised and religated into the appropriately prepared vector pVL941T (24) lacking the wild-type sequences between those restriction sites. pVL941T contains a functional cDNA copy of T antigen under the control of the baculovirus polyhedrin gene promoter. The recombinant plasmid carrying the mutation in the T-antigen gene and intact baculovirus DNA were cotransfected into Sf9 cells, and occlusion-negative plaques were selected. After the viruses were screened for T-antigen expression, recombinant viral stocks were plaque purified at least three times before they were used for protein expression.

T-antigen purification. Mutant and wild-type T antigens were extracted from Sf9 cells infected with recombinant baculoviruses (35). T antigen was purified by a standard immunoaffinity purification (35) using either PAb419 or PAb101. After elution, the purified proteins were dialyzed overnight against a buffer containing 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 5 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 50% glycerol (pH 7.0). Purified proteins were stored at −20°C.

Replication in vitro. Origin DNA in plasmids was incubated at 37°C in the presence of 0.2 to 1.2 μg of T antigen, 18 μl of 293 cell S100 extract, and replication buffer containing [α-^{32}P]dATP (800 Ci/mmol) in a final volume of 50 μl (40). DNA synthesis was stopped after 2 h with EDTA. Synthesized DNA was precipitated with trichloroacetic acid in the presence of pyrophosphate. Precipitates were collected on glass fiber filters, washed extensively, and dried. The amount of label incorporated into DNA was determined by scintillation counting.

p53 binding assays. Sf9 cells (80% confluent) were coinfected with stocks of a recombinant baculovirus expressing murine p53 and recombinant plasmids expressing either wild-type or mutant T antigens (29) at multiplicities of infection of about 100:1. After 48 h of infection, the cells were labeled with [^{35}S]methionine for 2 h. Subsequently, the cells were lysed, and the T antigen-p53 complexes were immunoprecipitated by using PAb419 bound to protein A-Sepharose. T antigen and p53 were separated and identified in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Helicase assay. The helicase substrate consisted of a ^{32}P-labeled, 23-base oligonucleotide annealed to single-stranded M13 DNA prepared according to Stahl et al. (39). Ten nanograms of labeled substrate was incubated with various amounts of purified T antigen for 30 min at 37°C in 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 2 mM ATP in a total volume of 50 μl. The reaction was stopped by addition of 5 μl of 3% SDS–0.5 M EDTA, and the products were separated in a 10% polyacrylamide gel.

DNase I footprinting. A thorough discussion of the DNase I footprinting protocol can be found in reference 31. It is based on the method of Gralla (18). In 30 μl, T antigen and 0.5 μg of supercoiled plasmid were incubated for 45 min at 37°C in 30 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5)–7 mM $MgCl_2$–40 mM creatine phosphate (pH 7.5)–1 mM dithiothreitol–0.1 mg of bovine serum albumin per ml–20% glycerol (replication buffer) in the presence 4 mM ATP and 20 μg of creatine phosphokinase per ml. Then 0.1 U of DNase I (Worthington Diagnostics) in 2 μl was added to the reaction mixture for 50 s at 37°C. The reaction was stopped with 4 μl of 0.2 M EDTA and 35 μl of phenol, and the samples were placed on ice for 2 min. Then the samples were heated to 80°C for 2 min and placed on ice again. The aqueous phase was run through a 1-ml spin column of Sephadex G-50-fine (Pharmacia) in distilled water. The column eluate was annealed to a primer labeled with [γ-^{32}P]ATP. The primer was extended with 1 U of Klenow polymerase for 10 min at 52°C. Extension products were separated on 8% acrylamide–8 M urea sequencing gels equilibrated at 50°C in a Hoefer Scientific Poker Face gel rig.

$KMnO_4$ sensitivity. A complete description of the $KMnO_4$ sensitivity assay can be found in reference 31. The reaction mixtures and incubation were as described above except that $KMnO_4$ was used in place of DNase I. After a 45-min incubation, 0.3 M $KMnO_4$ was added to a final concentration of 30 mM, and the reaction mixture was incubated for a further 4 min at 37°C. The reaction was stopped with β-mercaptoethanol. The samples were brought to 80 μl and to a final concentration of 0.3% SDS and 20 mM EDTA. The samples were extracted with an equal volume of phenol and chloroform. DNA in the aqueous phase was run through a spin column to exchange the aqueous phase for distilled water. Modified thymines were mapped by primer extension and gel electrophoresis as described above.

Nondenaturing gradient gel electrophoresis. T antigen was incubated in replication buffer as described above except that bovine serum albumin was omitted and the concentration of creatine phosphokinase was reduced 10-fold. Reactions were performed for 20 to 90 min at 37°C as indicated in the figure legends. The protein was cross-linked for 15 min at 37°C with 0.1% glutaraldehyde. Samples were diluted twofold in sample buffer containing 1.52% (wt/vol) Tris base, 20% glycerol, and 0.001% bromophenol blue at pH 6.8. T-antigen oligomers were resolved on a 4 to 25% native gradient gel. We used the Tris-glycine buffer system described by Laemmli without SDS (22). The separating gel had an acrylamide-to-bisacrylamide ratio of 33.5:1 and was polymerized with 0.016% (top) or 0.007% (bottom) ammonium persulfate. A 2.6% acrylamide–0.03% ammonium persulfate stacking gel was made from a stock solution of acrylamide containing acrylamide and bisacrylamide at a ratio of 41:1. Samples were electrophoresed at 35 mA for 20 h submerged in a 4°C bath in an SE 600 Hoefer Scientific apparatus. The gel was fixed and silver stained (28).

RESULTS

Purification of mutant T antigens overproduced in insect cells. We previously identified two classes of zinc finger mutants that fail to replicate in vivo (25) (Fig. 1). T-antigen mutants with substitutions of single cysteines or histidines in the zinc finger region fail completely to replicate. In contrast, single-amino-acid substitutions at amino acid positions 312 to 316 reduce replication 2- to 50-fold. The contiguous location of mutants with this phenotype suggests that they represent the active site of the finger domain. We constructed a new double mutant (314G/315E) with amino acid substitutions at positions 314 and 315 to ensure that DNA replication was completely restricted before proceeding with our biochemical studies. The mutants are identified by amino acid number (45) and the single-letter code identifying the new amino acid at that position. We recombined representative DNAs of both mutant classes with baculovirus

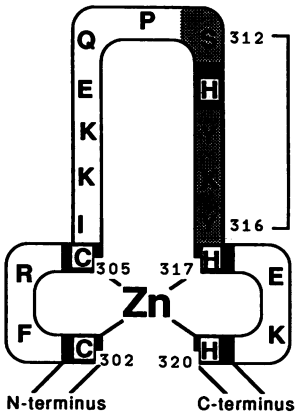

FIG. 1. Zinc finger region of SV40 T antigen. The amino acids of the zinc finger region (positions 302 to 320) are shown in single-letter code. Mutants with single substitutions at most positions have a wild-type phenotype. Shaded areas indicate a contiguous area (312 to 316) of the finger in which mutations reduce viral DNA replication partially. Solid areas (302, 305, 313, 317, and 320) identify amino acids that cannot be mutated without a complete loss of DNA replication.

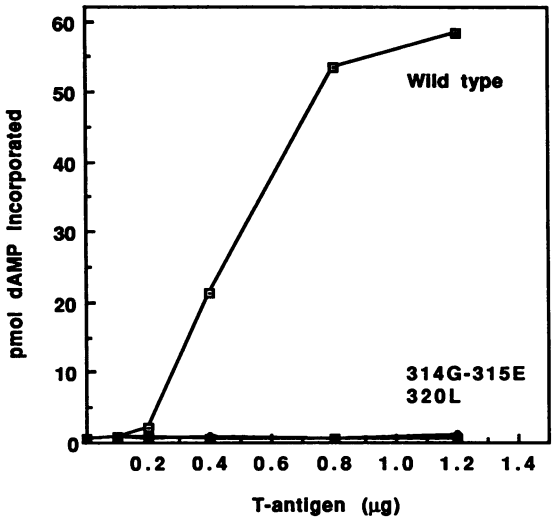

FIG. 2. T-antigen-induced DNA replication in vitro. Wild-type or mutant T antigens were incubated at 37°C with origin-containing plasmid DNA, 293 cell extract, and replication buffer containing [α-^{32}P]dATP. DNA synthesis was determined by quantitating the incorporation of radiolabel as described in Materials and Methods.

vectors and purified proteins from infected insect cells. Pulse-labeling with [^{35}S]methionine (shown in subsequent experiments) indicated that all of the mutants synthesized full-length T antigen at rates similar to those of the wild-type T antigen. However, mutants 302S, 305S, 313L, 317Q, and 317L/320L failed to accumulate sufficient amounts of stable T antigen for a complete biochemical characterization. Fortunately, mutants 314G/315E and 320L produced 0.1 to 0.5 mg of soluble protein per 175-cm^2 tissue culture flask of Sf9 cells. Therefore, we had sufficient protein from one mutant representing each class of zinc finger mutant for all of our biochemical assays.

Zinc finger mutants fail to replicate in vitro. We wanted to confirm that the mutant T antigens were unable to replicate plasmid DNA containing the SV40 origin of replication in vitro (Fig. 2). We incubated wild-type or mutant proteins with the plasmid DNA and 293 cell extracts for 2 h at 37°C under conditions suitable for DNA replication. After replication, incorporation of radiolabel into DNA was quantitated. The wild-type T antigen supported replication of more than 50 pmol of DNA in a 2-h incubation. Neither mutant 314G/315E nor mutant 320L supported replication of the plasmid DNA to a detectable level. Thus, both mutations lead to the same general phenotype in cell extracts and in infected cells.

Zinc finger mutant T antigens bind the cellular protein p53. We tested the ability of the mutant T antigens to bind p53 by coinfecting Sf9 cells with a recombinant baculovirus expressing one of the mutant proteins and another recombinant baculovirus expressing murine p53. In a previous study, O'Reilly and Miller (29) have shown that murine p53 produced in insect cells has T-antigen-binding activities equivalent to the activities of p53 from rodent cells. After coinfection, the cells were labeled with [^{35}S]methionine for 2 h, the T antigen-p53 complexes were immunoprecipitated from the crude lysates with PAb419, and the proteins in the complexes were separated by electrophoresis through a 10% SDS-polyacrylamide gel. Figure 3 shows that all mutant T antigens tested were able to bind radiolabeled p53 as well as wild-type T antigen did. Because the p53 binding region of T antigen extends over a large area of the protein (34), a stable association of T antigen with p53 argues that the general structure of the mutant proteins is intact in this segment of the protein. We also conclude that the transformation defect of some of these mutants (25) cannot be accounted for by a reduced stability of the T antigen-p53 interaction.

Zinc finger mutant T antigens have reduced but significant levels of helicase activity. A complete loss of helicase activity of the mutant T antigens would account for their inability to support replication of viral DNA. We compared the helicase activities of wild-type and mutant T antigens over a range of protein concentrations (Fig. 4). In this set of experiments, 0.05 μg of wild-type T antigen was sufficient to unwind more than half of the 23-bp oligonucleotides from M13 DNA. In contrast, 0.5 μg of T antigen encoded by the zinc finger mutant 314G/315E was needed to unwind approximately half of the same substrate. Mutant 320L had an even greater loss of activity than mutant 314G/315E did. Therefore, both

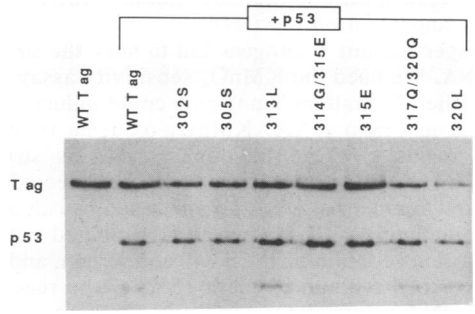

FIG. 3. Binding of wild-type and mutant T antigens to murine p53. Sf9 cells were coinfected with stocks of a recombinant baculovirus expressing murine p53 and recombinants expressing wild-type (WT) or mutant T antigens (T ag) as indicated. After 48 h of infection, the cells were labeled with [^{35}S]methionine for 2 h and subsequently lysed, and the T antigen-p53 complexes were immunoprecipitated with PAb419. T antigen and p53 were separated and identified in 10% polyacrylamide gels.

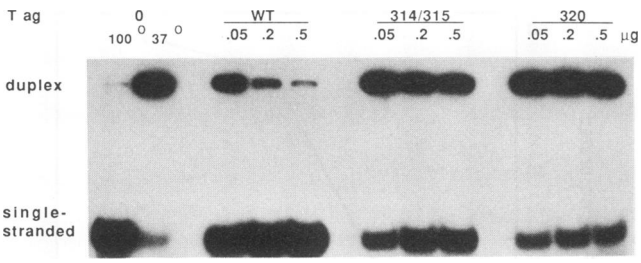

FIG. 4. Helicase activity of wild-type (WT) and mutant T antigens (T ag). The helicase substrate consisted of a ^{32}P-3′-end-labeled, 23-base oligonucleotide annealed to single-stranded, circular M13 DNA. Labeled substrate was incubated with various amounts of purified T antigen for 30 min at 37°C in 50 μl of buffer. The reaction products were separated in a 10% polyacrylamide gel.

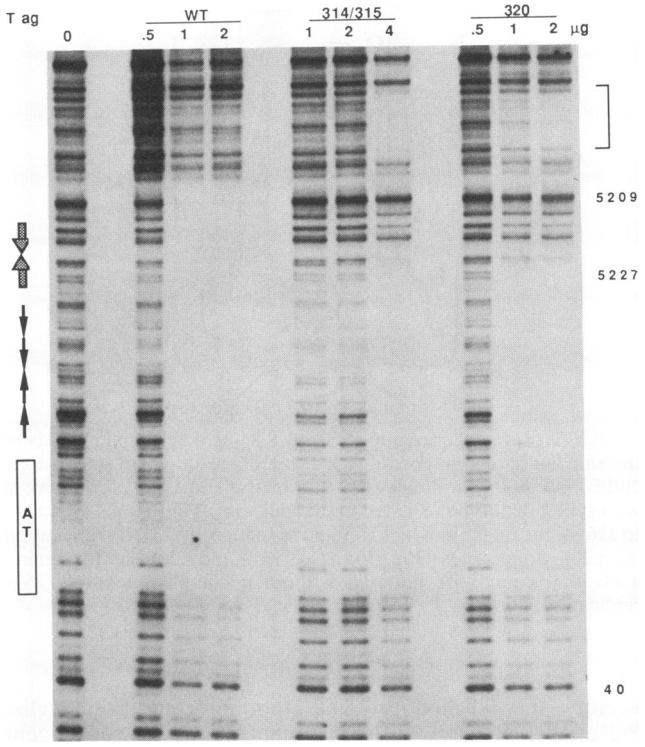

FIG. 5. DNase I footprints of wild-type (WT) and mutant T antigens (T ag) bound to the SV40 core origin of replication. The indicated amounts of T antigen were incubated with 0.5 μg of supercoiled plasmid DNA containing core origin DNA for 45 min and probed with DNase I. Footprints were analyzed as described in Materials and Methods. Core origin domains are identified at the left. The shaded arrows locate the inverted repeat domain, the solid arrows identify the pentanucleotide repeats in the central domain, and the open rectangle shows the AT-rich domain. Nucleotide numbers are shown on the right. The bracket on the right identifies a new area of DNase I protection by the zinc finger mutants of T antigen.

mutants retained helicase activity, but the levels of activity were more than 10-fold lower than the levels of wild-type T antigen. We conclude that the reduced helicase activity of the mutant T antigens may well contribute to the overall defect in DNA replication but would be unlikely to explain a total loss of replication function.

Zinc finger mutant T antigens fail to protect the inverted repeat domain of the core origin from DNase I. Since the zinc finger motifs of some proteins interact directly with DNA, we used DNase I footprinting to examine the interaction of the mutant T antigens with the core origin of replication (Fig. 5). We compared the DNA binding properties of wild-type and mutant T antigens over a range of protein concentrations. Under conditions that allow DNA replication in vitro, 1 μg of wild-type T antigen strongly protected the entire origin of replication from DNase I. Both classes of zinc finger mutant proteins protected the pentanucleotide domain. Mutant 320L T antigen was as efficient as wild-type protein, but mutant 314G/315E was slightly less efficient. Both mutant proteins, however, failed to protect the inverted repeat region from DNase I and instead protected an adjacent segment of plasmid DNA that was not protected by the wild-type protein. Both mutant T antigens protected part of the AT domain, but we could not determine whether the mutant proteins protected the entire AT domain because much of that domain is not susceptible to DNase even in the absence of bound protein. These findings strongly suggest that these zinc finger mutant T antigens bind well to the viral origin of replication but that the mutant proteins have an altered arrangement on the DNA.

Zinc finger mutant T antigens fail to alter the structure of origin DNA. We used the $KMnO_4$ sensitivity assay to determine whether the mutant T antigens could induce structural alterations in origin DNA. $KMnO_4$ oxidizes thymine and cytosine residues when the duplex DNA is structurally distorted (6). $KMnO_4$ modifications can be detected because the Klenow fragment of DNA polymerase I pauses at or near modified nucleotides. T antigen was incubated with supercoiled plasmids containing the SV40 core origin, and $KMnO_4$ was added to the binding reaction. After the reaction was stopped, DNA was isolated, and primers were annealed to the DNA and extended by Klenow polymerase. Borowiec and Hurwitz (5) have shown that T-antigen-induced $KMnO_4$ modifications in the inverted repeat domain are associated with melting of duplex DNA and that modifications in the AT domain represent untwisting of DNA with little melting.

Figure 6 shows that wild-type T antigen (1 μg) induced very strong $KMnO_4$ modification of a thymine residue at position 5217 and prominent modifications of other thymines in the inverted repeat domain and in the AT domain. Mutants 314G/315E (4 μg) and 320L (2 μg) failed to induce any structural alterations in the origin at protein concentrations sufficient to protect the central pentanucleotide domain of the origins from DNase I under the same conditions. We conclude that both mutant T antigens bind to origin pentanucleotides but are completely unable to alter the structure of DNA in subsequent steps.

Zinc finger mutant T antigens fail to form stable hexamers. Mastrangelo et al. (26) and Parsons et al. (32) have shown that T antigen assembles as two hexamers, one on each half of the origin of replication. Therefore, the altered arrangement of the mutant T antigens at the origin of replication and their loss of function could reflect altered protein-protein interactions. To examine the quaternary structures of the mutant proteins, we used nondenaturing, gradient gel electrophoresis (32). T antigen purified by immunoaffinity chromatography was incubated in the same buffer that was used for DNase I and $KMnO_4$ footprinting in the absence and presence of 4 mM ATP. After 20 min at 37°C, we cross-linked complexes with glutaraldehyde and analyzed the samples by electrophoresis (Fig. 7). In the absence of added ATP, wild-type and mutant T antigens behaved mostly as a

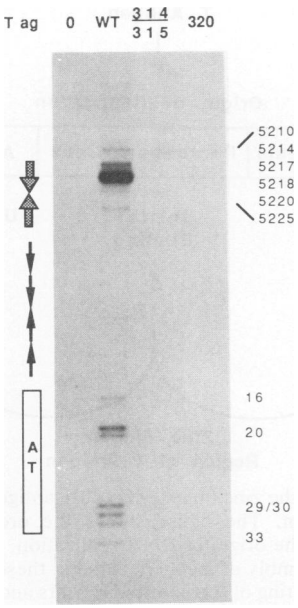

FIG. 6. $KMnO_4$ sensitivity of wild-type and mutant origins. Wild-type (WT; 1 μg) or mutant (4 μg) T antigens (T ag) were incubated with 0.5 μg of supercoiled plasmid DNA containing the core origin for 45 min at 37°C. The protein-DNA complexes were probed with 30 mM $KMnO_4$ for 4 min. Footprints were analyzed as described in Materials and Methods. The 0 indicates that no T antigen was added to the plasmid analyzed in the first well of the gel. Core origin domains are identified at the left. The shaded arrows locate the inverted repeat domain, the solid arrows identify the pentanucleotide repeats in the central domain, and the open rectangle shows the AT-rich domain. Nucleotide numbers are shown on the right.

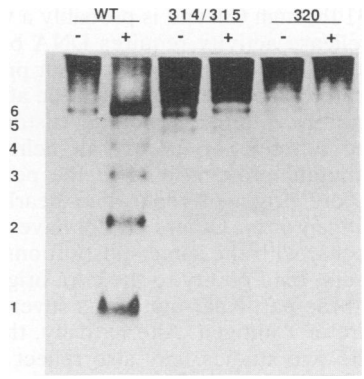

FIG. 7. Analysis of wild-type (WT) and mutant T-antigen oligomers on a nondenaturing gradient gel. T antigen (5 μg) was incubated for 90 min at 37°C in replication buffer in the presence (+) or absence (−) of 4 mM ATP. The final glycerol concentration was 50%. The samples were electrophoresed in a nondenaturing gradient gel for 20 h at 4°C as described in Materials and Methods. The gel was fixed and silver stained. The number of subunits in each oligomer is indicated on the left.

continuous spectrum of large oligomeric forms near the top of the gel. In the presence of ATP, wild-type T antigen was converted to smaller forms ranging from monomers through hexamers; the majority of the protein formed hexamers. We have shown elsewhere, using ATP titrations, that ATP binding converts large oligomers of T antigen first into monomers and then into stable hexameric structures (32). This result is in marked contrast to the behavior of the mutant T antigens. Like wild-type T antigen, both zinc finger mutant T antigens formed large oligomers near the top of the gel in the absence of added ATP. In contrast to wild-type T antigen, the zinc finger mutant T antigens failed completely to respond to ATP; there was no conversion of the large oligomers to smaller forms or into a predominant, hexameric form. Therefore, both mutant T antigens are able to oligomerize but they are not able to form stable hexamers in response to the binding of ATP. We conclude that the zinc finger region of T antigen is important in protein-protein interactions that lead to stable hexamer formation.

DISCUSSION

We previously identified two classes of zinc finger mutants that fail to replicate viral DNA in vivo (25). The first class of mutations, with changes in cysteine and histidine residues of the finger region, blocks viral DNA replication completely and also reduces the size of transformed foci. The common phenotype of these mutants supports the idea that these amino acids have similar functions. We presume that a zinc molecule organizes the folding of the finger region, although zinc binding has not been demonstrated directly. The second class of mutations, localized to a contiguous segment of the finger, reduces viral replication but has no effect on transformation functions. A double mutation in this segment blocks replication completely. Our present studies indicate that representatives from both classes of mutants have the same biochemical defects. We conclude that the finger region has a single set of functions in replication and that the differences between the two classes of mutants in vivo most likely reflect quantitative differences in protein functions.

A single-amino-acid substitution in the zinc finger region could have two different kinds of effects. The mutation could affect only local domains, or it could alter the overall structure of the protein and thereby disrupt functions in distant regions of the protein or affect the stability of the protein in vivo (14). Some of our mutations (302S, 305S, 313L, 317Q, and 317L/320L) appear to cause instability of the altered protein in cells. Pulse-labeling shows that the mutant proteins are expressed at levels similar to those of the wild-type protein, but the mutant proteins fail to accumulate in insect cells. This instability could well be the result of major structural changes in these particular mutant proteins. Such changes have been documented in another study. Zhu and Cole (49) reported that a two-amino-acid insertion in the zinc finger at position 303 affected protein stability and rendered the mutant transformation defective. In contrast, the 314G/315E and 320L mutations encode proteins that are only slightly less stable than wild-type T antigen and can be isolated in significant amounts from insect cells. These are the mutant proteins that would provide the most reliable clues to the specific functions of the finger region.

Purified T antigens encoded by mutants 314G/315E and 320L are completely unable to support DNA replication in vitro. Nevertheless, the mutant proteins retain a number of important molecular functions. The mutant proteins bind to mouse p53 with an efficiency similar to that of the wild-type T antigen. The mutant proteins have reduced but significant levels of helicase activity. The helicase domain maps from

amino acids 131 through 680 and is probably a very complex region (37). Helicase activity requires DNA binding, DNA unwinding, and ATPase functions. From our present data, it is hard to interpret which activities might be affected by the mutations. Possibly, limited structural distortions in the protein lead to a reduction in overall helicase activity. Finally, both mutant proteins bind to the pentanucleotide region of the core origin of replication nearly as well as wild-type T antigen does. Others (1, 20) have reported that mutant T antigens, with the same substitutions as our 302S and 305S mutants, bind poorly to the core origin of replication. Perhaps these particular mutations severely affect the overall structure of T antigen. Alternatively, the differences in results in the two studies may also reflect variations in modifications of T antigen produced in *Escherichia coli* (1, 20) and insect cells or in the conditions of the binding assays. We conclude that the 314G/315E and 320L mutant proteins have a conformation that is adequate to accomplish some of T antigen's complex functions. The 10-fold reduction in helicase functions of the proteins would not account for a complete loss of DNA replication.

The 314G/315E and 320L proteins share a number of more severe defects that would explain their total loss of replication function. Both mutant proteins fail to protect the inverted repeat domain of the core origin of replication from DNase even though they completely protect the pentanucleotide domain in the same footprints. It is difficult to determine whether or not the mutant proteins cover the entire AT domain because much of that domain is not susceptible to DNase even in the absence of bound protein. Hoss et al. (20) have shown that truncated T antigens that have a DNA binding domain but lack a zinc finger region also fail to protect the inverted repeat region of the origin from DNase. They attributed the altered footprints to the truncation of T antigen. Our results, however, show that zinc finger mutant T antigens have this altered DNA binding pattern even when the protein is full length. The 314G/315E and 320L mutant proteins also completely fail to induce structural changes in the inverted repeat and AT domains, as judged by $KMnO_4$ footprinting. Based on our previous findings (32) and those of others (26), we interpret these results to mean that the mutant T antigens fail both to form normal nucleoprotein complexes with origin sequences and to melt origin DNA. These defects would be sufficient to block the initiation of DNA replication and could account for the complete replication defect of the mutants.

We show here that the zinc finger region is necessary for the formation of stable hexamers of T antigen in response to the addition of ATP. Figure 8 indicates the possible roles of hexamers in the initiation of viral DNA replication. Previous studies (4, 10) have shown that wild-type T antigen binds to the pentanucleotide region of the replication origin in the absence of ATP. Because ATP is required for hexamer formation, we conclude that stable hexamers are not needed for pentanucleotide binding. In the presence of ATP, T antigen assembles as stable hexamers on each half of the origin and protects the entire origin from DNase I (26, 32). The failure of the zinc finger mutant T antigens to form hexamers in response to ATP may account for defects in subsequent steps in the initiation of DNA replication; we propose that hexamer assembly of T antigen is required for the unwinding of the inverted repeat domain and the untwisting of the AT domain of the origin of replication. If this is the case, the defect in hexamer formation would be sufficient to explain the biological and the biochemical properties of the finger mutants. We cannot, however, exclude the possibility

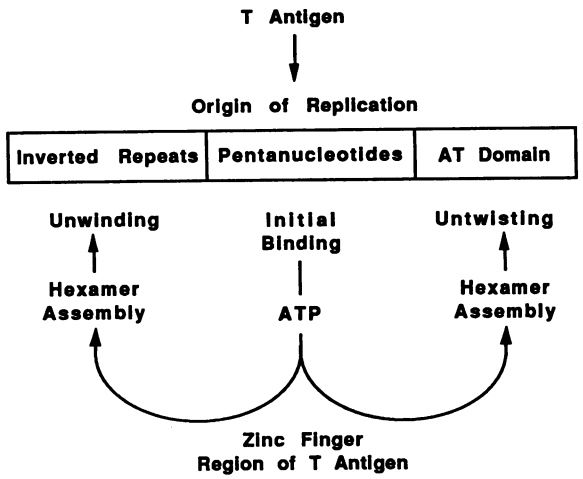

FIG. 8. Role of the zinc finger of SV40 T antigen in the initiation of DNA replication. The figure shows the order of T-antigen-induced events at the origin of DNA replication. The zinc finger is needed for the assembly of stable hexamers; these, in turn, may be required for the melting of the inverted repeats and the untwisting of the AT domain of the origin.

that the zinc finger has additional functions in the melting of the inverted repeat domain and the unwinding of the AT domain. A 10-fold reduction in the helicase activity of the mutant T antigens would slow the elongation of any nascent DNA strands in molecules that might escape the severe block in the initiation of replication.

We do not know the mechanism by which alterations in the zinc finger region block assembly of T-antigen hexamers in response to the addition of ATP. The mutant proteins form a variety of oligomeric structures. Unless these oligomers assemble by unusual mechanisms, we assume that the mutant subunits have at least one donor and one acceptor site for rudimentary protein-protein interactions. Possibly, the zinc finger region could serve as a second, direct-contact site that is necessary for the proper alignment of T-antigen subunits in the formation of stable hexamers. Alternatively, the zinc finger may have a role in organizing the conformation of the T-antigen subunits for hexamer formation in response to ATP. We (32) have proposed that, in the absence of ATP, T-antigen monomers may have a conformation that leads to a continuum of interactions without an intrinsic structural limit on the extent of oligomerization and that ATP induces changes in the conformation of the monomers that would limit oligomerization after the assembly of six subunits. For example, spherical or cylindrical hexamers might form closed structures that could not be extended. The zinc finger region may be required for the appropriate change in tertiary structure in response to the binding of ATP (7, 8) so that oligomerization is limited to hexamer assembly.

ACKNOWLEDGMENTS

This investigation was supported by PHS grants CA-18808, CA-28146, and 5-T32 CA-09176 awarded by the National Cancer Institute and by a postdoctoral fellowship to G.L. from the Deutsche Forschungsgemeinschaft.

We thank Kris Mann for her valuable comments on the manuscript.

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