Strand-Specific Recognition of a Synthetic DNA Replication Fork by the SV40 Large Tumor Antigen

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The mechanism by which DNA helicases unwind DNA was tested; an "unwinding complex" between the SV40 large tumor antigen (T antigen) and a DNA molecule designed to resemble a replication fork was probed. In an adenosine triphosphate (ATP)–dependent reaction, T antigen quantitatively recognized this synthetic replication fork and bound the DNA primarily as a hexamer. The T antigen bound only one of the two strands at the fork, an asymmetric interaction consistent with the 3′ → 5′ directionality of the DNA helicase activity of T antigen. Binding to chemically modified DNA substrates indicated that the DNA helicase recognized the DNA primarily through the sugar-phosphate backbone. Ethylation of six top strand phosphates at the junction of single-stranded and double-stranded DNA inhibited the DNA helicase activity of T antigen. Neither a 3′ single-stranded end on the DNA substrate nor ATP hydrolysis was required for T antigen to bind the replication fork. These data suggest that T antigen can directly bind the replication fork through recognition of a fork-specific structure.

The processes of DNA replication, DNA recombination, and DNA repair require the conversion of relatively inert double-stranded DNA to the single-stranded form to activate the DNA into a substrate for DNA polymerases and the DNA recombination machinery. A class of enzymes, the DNA helicases, can provide the required DNA strand-separating activity for these processes by using the energy of nucleoside triphosphate (NTP) hydrolysis to unwind the DNA helix (*1*). The mechanism of DNA helicase action has not been elucidated. We have now examined a DNA helicase bound in a complex with the DNA during unwinding in order to gain understanding of the mechanism of DNA helicase action.

A well-characterized DNA helicase is the SV40 (simian virus 40) large tumor antigen (T antigen), which is an 82-kilodalton (kD) early gene product of SV40 and is the only viral protein required for SV40 replication (*2*). In addition to its role as an adenosine triphosphate (ATP)–dependent DNA helicase (*3*), T antigen has other activities including site-specific binding to the SV40 origin of DNA replication (*ori*) (*4*), unwinding of RNA·RNA duplexes (*5*), ATP-dependent oligomerization (*6–8*), and the ability to transform certain cell lines, probably because it binds to the tumor suppressor proteins p53 (*9*) and the retinoblastoma gene product (*10*). The T antigen acts during SV40 DNA replication by first binding to *ori* and forming an ATP-dependent initiation complex (*6, 7, 11, 12*). This complex contains up to 12 monomers of T antigen arranged in two lobes (*6–8*), in which the protein appears to completely surround the 64-bp core *ori* (*11, 12*). The complex contains two flanking regions of DNA within *ori* that are structurally deformed by T antigen (*13–15*). When human single-stranded DNA binding protein [human SSB; also known as RF-A and RP-A (*16*)] or certain other heterologous SSB proteins are added to the complex, the two lobes of T antigen are thought to separate and individually unwind the DNA outward from *ori* (*17*). Isolation of DNA molecules replicating either in vivo or in vitro reveals the presence of T antigen at DNA regions that are proximal to the DNA replication forks (*18*).

DNA helicases can be characterized by the directionality of their activity, that is, whether their operation proceeds in the 5′ → 3′ or 3′ → 5′ direction on single-stranded DNA (*1*). The T antigen is a 3′ → 5′ DNA helicase, an indication that it is more closely associated with the leading strand during DNA replication (*19, 20*). The nucleic acid structures that T antigen recognizes in order to establish polarity of movement are still incompletely defined.

Complex formation between T antigen and a synthetic replication fork. DNA helicase binding substrates were designed to resemble a DNA replication fork (Fig. 1). The first synthetic fork was constructed of two partially complementary oligonucleotides annealed to form a molecule that is double-stranded along half its length and single-stranded in the remainder of the DNA (the "two-strand" fork); (Fig. 1A). The top strand of this molecule is defined as the strand that contains the 3′ single-stranded end.

Since DNA denaturation is an intrinsic function of T antigen, we determined reaction conditions that would support efficient binding of T antigen to the synthetic replication fork, yet allow only minimal denaturation of this two-strand substrate. The Michaelis constant (K_m) of ATP for T antigen during DNA helicase action is 600 μM (*20*). In that a lower concentration of ATP might reduce helicase activity but satisfied binding requirements for T antigen to the fork, we tested the effect of ATP concentration on the denaturation of the fork (Fig. 2A). Gel electrophoresis and autoradiography indicated that T antigen denatured the synthetic (^{32}P-labeled) replication fork as a function of ATP concentration. Concentrations of ATP under 100 μM resulted in less than 5 percent denaturation.

Similar reaction conditions were used in a gel shift assay of the binding of T antigen to the synthetic fork (Fig. 2B). Whereas binding of T antigen to the synthetic replication fork was scant in the absence of ATP, the addition of 30 μM ATP resulted in the appearance of a slowly migrating complex. Detection of the complex between T antigen and the fork required the cross-linking reagent glutaraldehyde, a reagent also necessary for formation of gel shift complexes between T antigen and the SV40 *ori* (*6*). ATP hydrolysis was not required for complex formation between T antigen and the synthetic replication fork

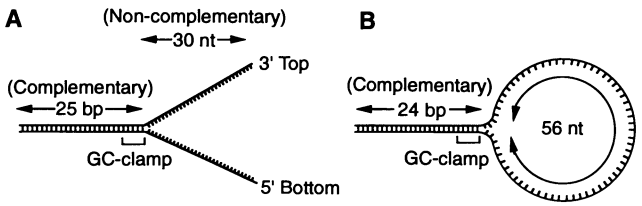

Fig. 1. Synthetic replication forks used to bind T antigen. (**A**) The synthetic fork was constructed from two partially complementary oligonucleotides that, when annealed, form a molecule that is approximately one-half duplex and one-half single-stranded. (**B**) A synthetic replication fork constructed from a single oligonucleotide. The oligonucleotide was designed so that the 5′ and 3′ terminal sequences were able to form a 24-bp duplex region constraining a central 56-nt region of single-stranded DNA. Each of the substrates was designed so that 11 of 14 base pairs in the duplex region closest to the fork were G·C pairs to inhibit movement of T antigen into the double-stranded portion of the molecule (*22*). The molecules do not contain sequence-specific elements (such as 5′-GAGGC-3′ boxes) that promote T antigen binding.

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as both adenosine diphosphate (ADP) and β,γ-imidoadenosine 5′-triphosphate (AMP-PNP), a nonhydrolyzable analog of ATP, promoted complex formation although at higher concentrations (1 mM compared to 30 μM ATP). Therefore, these data show that T antigen binds to the synthetic replication fork in an ATP-dependent reaction.

It was necessary to establish whether the complex formed by T antigen with the DNA was a consequence of the ATP-dependent stimulation of DNA binding by T antigen (*12*), or whether it was per se a specific complex between T antigen and the synthetic replication fork. We therefore enzymatically probed the region of DNA bound by T antigen. After T antigen was incubated with 30 μM ATP (*21*), the T antigen–DNA complex was formed by an addition of the two-strand fork, which had been labeled with $^{32}P_i$ on the 5′ end of the top strand. Complex formation was probed either with the single strand–specific nuclease P1 or with deoxyribonuclease (DNase) I, which prefers double-stranded DNA (Fig. 3A). In the absence of T antigen, nuclease P1 exclusively nicked the single-stranded region of the labeled helicase substrate. There was little change in the cleavage pattern when T antigen was added without ATP.

The addition of ATP resulted in significant protection by T antigen of fork-proximal regions in the top strand. Binding was essentially quantitative with densitometric tracing (of the −4 band of the top strand), indicating that 85 percent of the replication fork was bound. The total protection extended over a ten-nucleotide (nt) region (−1 to −10), although the central five nucleotides were most highly defended against nuclease P1 attack (high and moderate amounts of protection are indicated by a solid and dotted lines, respectively, on the left side of the figure). The slight protection observed 3′ to the −10 position was not always present (Fig. 3C). Gel shift experiments (Fig. 2B) indicated that ATP hydrolysis was not required by T antigen to bind the fork. The presence of either AMP-PNP or ADP allowed T antigen to protect similar regions of the top strand (Fig. 3A). Subtle, but reproducible, changes in the protection pattern were observed and were dependent on the nucleotide cofactor present in the incubation (compare bands −4 and −8 in lanes 3 to 5). These changes are reminiscent of the trio of characteristic structural changes induced in the SV40 *ori* by T antigen in the presence of either ATP, AMP-PNP, or ADP (*13*) and possibly reflect conformational changes induced in T antigen during the cycle of ATP hydrolysis.

DNase I was used to detect interactions between T antigen and the double-stranded portion of the synthetic replication fork (Fig. 3A). There was virtually no change in the enzymatic cleavage pattern if T antigen was incubated with the synthetic replication fork in the absence of nucleotide. The addition of either ATP or AMP-PNP was slightly protective to the four nucleotides of double-stranded DNA proximal to the fork. T antigen did not protect the double-stranded DNA in the presence of ADP. Thus, the predominant interactions between T antigen and the top strand of the replication fork apparently occur in the single-stranded portion of the molecule closest to the fork, although there was also some protection of the double-stranded DNA proximal to the fork.

To determine whether T antigen bound in a quasi-symmetrical manner to the top and bottom strands of the synthetic fork, we probed a substrate labeled on the 3′ end of the bottom strand (Fig. 3B). The T antigen–DNA complex that formed in the presence of ATP showed no significant degree of protection of the single-stranded region of the bottom strand. Substitution of ATP with AMP-PNP or ADP also gave no evidence of binding by T antigen to this strand. Similar assays with DNase I gave no evidence of protection by T antigen of the double-stranded region of the bottom strand; we did observe, however, that the 5′ single-stranded end became somewhat hypersensitive to DNase I cleavage in the presence of ATP and AMP-PNP. Similar hyperreactivity to enzymatic cleavage was also noted in the 3′ flanking region of the top strand (Fig. 3A). The hypersensitivity of these regions demonstrates the lack of protection by T antigen and suggests that the binding of T antigen to the fork causes conformational changes at the single-stranded ends, which results in more cleavage by DNase I and nuclease P1. These nuclease protection studies show that T antigen binds asymmetrically to the replication fork, with detectable interactions limited to the top strand of DNA proximal to the fork.

A possible criticism of these nuclease protection studies is that T antigen does not truly recognize the top strand proximal to the fork but rather is binding to a sequence-specific element situated by chance on the top strand. Therefore, we synthesized two new oligonucleotides in which the sequence of each was specified in reverse orientation (*22*). Annealing these oligonucleotides gave a "reverse-sequence" replication fork in which the region that the T antigen previously recognized on the "forward-sequence" substrate was now found on the bottom strand. The binding of T antigen to this reverse-sequence replication fork was examined by nuclease P1 probing (Fig. 3C). When this substrate was labeled on the 5′ end of the top strand, T antigen again protected the fork-proximal region. Conversely, no protection was afforded by T antigen to the reverse-sequence fork labeled on the bottom strand. Thus, the binding of T antigen to the replication fork did not require the recognition of any sequence-specific element in the DNA.

During the initiation of SV40 DNA replication, T antigen associates with a replication fork on DNA molecules lacking a 3′ single-stranded end. The presence of such a 3′ end on our binding substrates may provide an entry site for T antigen. Nuclease protection assays with a synthetic fork constructed from a single oligonucleotide

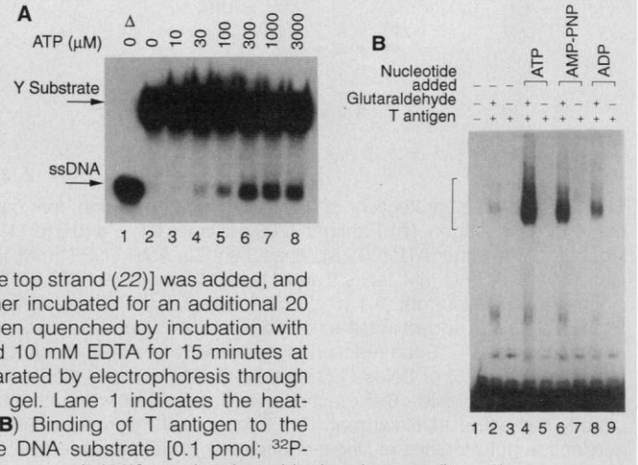

Fig. 2. Binding and unwinding of the synthetic replication fork by T antigen. (**A**) Effect of ATP concentration on denaturation of the synthetic fork by T antigen. T antigen was incubated at the indicated ATP concentration for 15 minutes at 37°C (*21*). The DNA substrate [0.1 pmol; ^{32}P-labeled on the 5′ end of the top strand (*22*)] was added, and the reaction mixture was further incubated for an additional 20 minutes. The reaction was then quenched by incubation with proteinase K (500 μg/ml) and 10 mM EDTA for 15 minutes at 37°C; the products were separated by electrophoresis through an 8 percent polyacrylamide gel. Lane 1 indicates the heat-denatured DNA substrate. (**B**) Binding of T antigen to the synthetic replication fork. The DNA substrate [0.1 pmol; ^{32}P-labeled on the 5′ end of the top strand, (*22*)] was incubated in the absence (lane 1) or presence (lanes 2 to 9) of 200 ng of T antigen for 15 minutes at 37°C (*21*). Nucleotides were added to the following concentrations: ATP, 30 μM (lanes 4 and 5); AMP-PNP, 1 mM (lanes 6 and 7); ADP, 1 mM (lanes 8 and 9). When nucleotides were used, T antigen was first incubated with these nucleotides in the absence of DNA for 15 minutes at 37°C. When the DNA binding reaction was completed, glutaraldehyde was added to a final concentration of 0.1 percent (as indicated), and the mixture was incubated for 15 minutes. The samples were then subjected to electrophoresis through a 4 percent polyacrylamide gel.

that can form a "panhandle" structure (Fig. 1B) were used to establish whether a free 3′ end was needed for T antigen to bind to the fork. Control experiments with nuclease P1 in the presence or absence of T antigen, indicated that more than 95 percent of the flanking DNA was in the duplex state and thus unable to provide a free 3′ end for the loading of T antigen. As nuclease P1 did not give information concerning the double-stranded DNA, the binding of T antigen in the presence of AMP-PNP was probed with DNase I (Fig. 3D). Compared to the control lane lacking T antigen, T antigen protected a fork-proximal region of the oligonucleotide analogous to the site of binding on the top strand of the two-strand fork. Approximately 14 nucleotides of single-stranded DNA and 4 nucleotides of double-stranded DNA were moderately protected. While the sites of T antigen binding to the one- and two-strand forks were quite similar, the extent of binding to the one-strand fork was somewhat less. The addition of greater amounts of T antigen led to its aggregation on the DNA substrate and thus, less specific protection. Nevertheless, a 3′ single-stranded end is not required for the binding of T antigen to the fork. These conclusions are supported by studies showing that unwinding of partially duplex substrates by T antigen can take place in the absence of a free 3′ end (*19*, *20*).

To determine whether T antigen binds the synthetic fork more stably compared to other DNA molecules, we measured the rate of dissociation for T antigen bound individually to three labeled DNA substrates; namely, the two-strand replication fork, the top strand of this fork alone (single-strand DNA), or a nonspecific double-stranded DNA (*21*). After formation of the T antigen–DNA complex in the presence of ATP or AMP-PNP, the complex was challenged with a large molar excess of unlabeled poly(dT). The amount of T antigen-DNA complex remaining at various times after the challenge was determined with a nitrocellulose filter binding assay (Fig. 4). We found that 80 percent of T antigen bound in the presence of ATP to either single-strand DNA or a nonspecific double-stranded DNA was lost within the first 5 minutes after challenge. In contrast, a similar degree of dissociation of T antigen from the synthetic replication fork took more than 60 minutes. The stability of T antigen bound to all three DNA molecules was measurably increased in the presence of AMP-PNP compared to ATP. After the first 2 minutes of poly(dT) challenge, no significant dissociation of T antigen bound to the fork was observed. In contrast, more than 50 percent of the T antigen bound to double-stranded DNA or single-stranded DNA was lost to the competitor after 60 minutes. These results, combined with the effect of T antigen titration on our nuclease

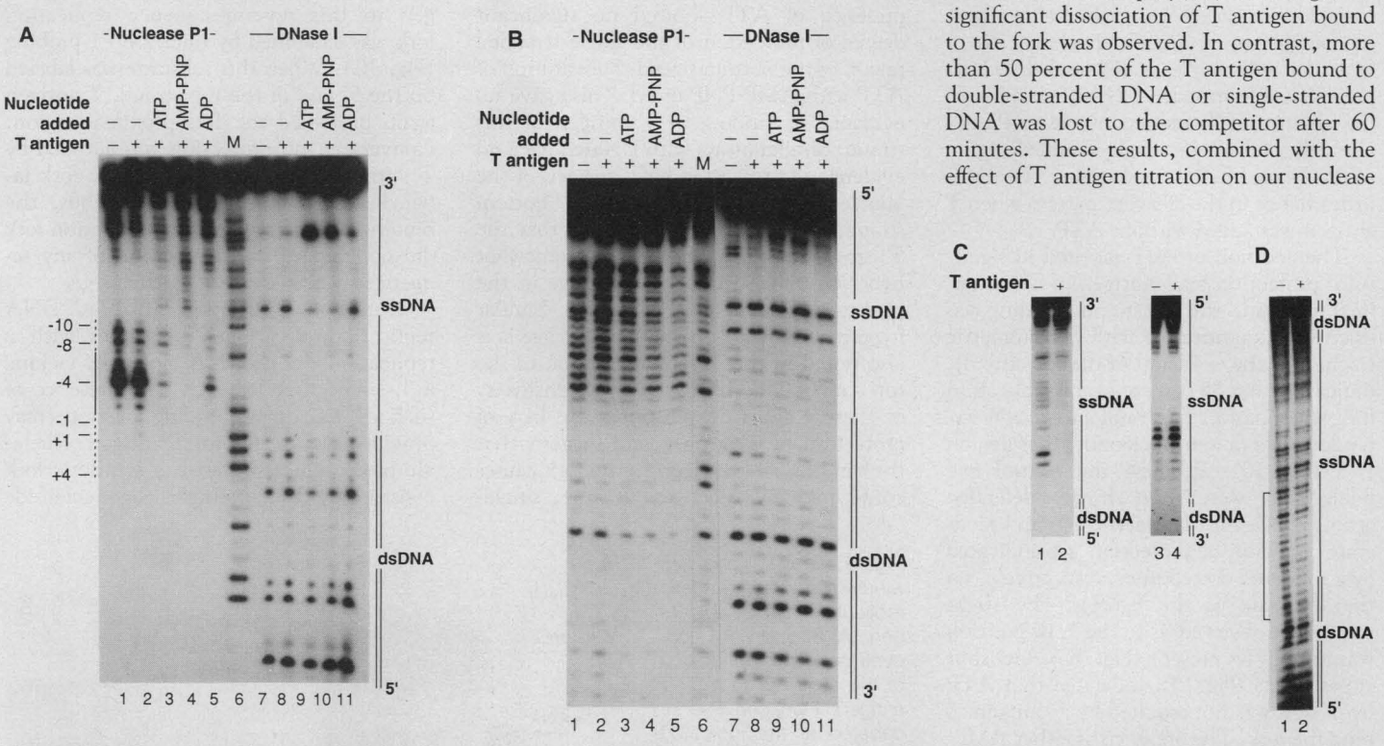

Fig. 3. Nuclease protection of the synthetic replication fork in the presence of T antigen. (**A**) T antigen (200 ng; lanes 2 to 5 and 8 to 11) was incubated with either ATP (30 μM; lanes 3 and 9), AMP-PNP (1 mM; lanes 4 and 10), or ADP (1 mM; lanes 5 and 11) for 15 minutes at 37°C (*21*). The synthetic replication fork [0.1 pmol; ^{32}P-labeled at the 5′ end of the top strand, (*22*)] was added and the reaction was incubated for an additional 15 minutes at 37°C. Each reaction mixture was treated with nuclease P1 (1 μg; lanes 1 to 5) or DNase I (1 unit; lanes 7 to 11), and the DNA was digested for 1 minute. The reactions were extracted with phenol and directly subjected to electrophoresis through a 12 percent denaturing acrylamide gel. Regions of single-stranded or double-stranded DNA are indicated on the right. Negative and positive numbers on the left refer to the nucleotide position of single-stranded DNA and double-stranded DNA, respectively, relative to the fork. Protection (high or moderate) is indicated by solid or dotted lines, respectively, on the left. (**B**) T antigen binding to the synthetic fork labeled with $^{32}P_i$ on the 3′ end of the bottom strand (*22*). As for (A) except that 0.1 μg of nuclease P1 was used to cleave the DNA (lanes 1 to 5), the use of 1 μg of nuclease led to a slight decrease (two times lower) in the amount of uncut bottom strand. (**C**) T antigen binding to the "reverse-sequence" fork. T antigen (200 ng; lanes 2 and 4) was first incubated with AMP-PNP (1 mM) for 15 minutes at 37°C (*21*). The "reverse-sequence" fork [top strand, a ^{32}P label at 5′ end (*22*), lanes 1 and 2; bottom strand, a ^{32}P label at 3′ end (*22*), lanes 3 and 4] was added, and the reaction was incubated for an additional 15 minutes. Nuclease P1 (1 μg) was then added to each reaction, and the DNA was digested for 1 minute. Further treatment was identical to that in (A). (**D**) T antigen binding to the synthetic replication fork formed by a single oligonucleotide (Fig. 1B). T antigen (200 ng; lane 2) was first incubated with AMP-PNP (1 mM) for 15 minutes at 30°C (*21*). The "one-strand" fork [0.1 pmol; ^{32}P-labeled at the 5′ end of the top strand, (*22*)] was added and the reaction was incubated again for 15 minutes. DNase I (1 unit) was then added, and the reaction was incubated for 1 minute and treated as in (A). The binding reactions were performed at 30°C to minimize denaturation of the fork. The protection by T antigen to the fork is indicated by a bracket. The marker lanes (M) in (A) and (B) were produced with the standard "G-only" sequencing reaction (*26*). Glutaraldehyde was not used in these DNA nuclease protection assays because of the resulting protein-DNA cross-links that interfered with the interpretation of the data.

protection assays, led us to estimate that T antigen has a five times higher affinity for the synthetic replication fork compared to single-stranded DNA. Others have measured the effect of competitor DNA molecules on the binding of T antigen to partially duplex DNA, and have also found that T antigen has a higher affinity for double-stranded DNA containing a 3′ overhang than to single-stranded DNA or to blunt-ended double-stranded DNA (*20, 21*).

Binding of T antigen to the sugar-phosphate backbone. We examined the structural features of single-stranded DNA required for T antigen binding in order to deduce the critical contacts during helicase movement along the DNA. Single-stranded DNA was treated with chemical agents that modify selective sites on the purine or pyrimidine bases, or at phosphate residues on the sugar-phosphate backbone. Six different reagents were used; five that modify base positions were dimethyl sulfate (*23*), diethyl sulfate (*24*), potassium permanganate (*25*), diethylpyrocarbonate (*24*), and formic acid (*26*); the sixth was ethylnitrosourea which ethylates phosphate residues (*27*). The destructive nature of treatment with chemicals such as formic acid, which causes depurination of DNA, results in irreversible denaturation of the synthetic replication fork. We therefore took advantage of the ATP-stimulated binding of T antigen to single-stranded DNA. A 15-nt single-stranded DNA molecule was used as the substrate for T antigen binding so that the effect of modifying one or two sites on each DNA molecule could be examined (*28*).

The binding of T antigen to the modified single-stranded DNA molecules was determined by a gel shift assay (Fig. 5). T antigen bound efficiently to the unmodified oligonucleotide in the presence of ATP and glutaraldehyde. Binding of T antigen to DNA methylated with dimethyl sulfate, ethylated with diethyl sulfate, or oxidized with potassium permanganate was not significantly affected by these modifications. DNA modified with diethylpyrocarbonate, a reagent which causes breakage of the six-membered ring of purines by the addition of one or two bulky carbethoxyl adducts, was bound by T antigen slightly less well than the control DNA substrate. The only base-specific modification reproducibly found to affect the binding of T antigen was depurination with formic acid. As measured by this assay, T antigen binding to DNA molecules previously treated with formic acid was reduced from 30 to 50 percent. These results show that modification of the DNA bases with either small or bulky substituent groups only modestly affected the binding of T antigen to the single-stranded DNA. Even destruction of one or two of the bases in the DNA substrate did not prevent T antigen from forming a complex with the DNA.

In contrast to the relatively minor effects of base-specific modifications on the binding of T antigen to single-stranded DNA, modification of the sugar-phosphate backbone had more profound consequences. Ethylnitrosourea treatment of DNA results in the addition of ethyl adducts to the phosphate (*27*). DNA ethylated at approximately one to two sites per DNA molecule was severely affected in its ability to bind T antigen. Binding was more than 90 percent lower, and the residual complexes were found as a smear. These results indicate that the primary interactions between T antigen and the single-stranded DNA are via the sugar-phosphate backbone.

If T antigen forms critical contacts with the sugar-phosphate backbone during DNA helicase movement, ethylation of certain phosphate residues should affect the ability of T antigen to unwind the synthetic replication fork. As a test, the two-strand replication fork, labeled with $^{32}P_i$ on the 5′ end of the top strand, was modified with ethylnitrosourea so that approximately one phosphate was ethylated per strand. The modified fork was used as a substrate for unwinding by T antigen in the presence of 1 mM ATP. The single-stranded product was purified by electrophoresis on a nondenaturing gel and cleaved at the ethylated residues by alkaline hydrolysis. The cleavage products were separated on a DNA sequencing gel and autoradiographed (Fig. 6).

The ethylation pattern of the top strand, isolated from the original pool of nondenatured substrate molecules, was generally uniform with little sequence preference. In contrast, the cleavage pattern from the unwound product molecules showed a significant and reproducible reduction in cleavage products from a distinct region (location indicated on the right side of figure). The affected area spanned six contiguous phosphates found at the fork; two phosphates were located in the double-stranded portion of the molecule while the remaining four were found in the adjacent single strand. Similar studies with the ethylated substrate labeled on the bottom strand showed no evidence of interference with the DNA helicase action of T antigen. Base methylation or base ethylation of the synthetic replication fork also had insignificant effects on DNA unwinding by T antigen. Therefore, these results indicate that phosphate contacts are critical elements for the DNA helicase activity of T antigen. Moreover, these contacts lie on only one of the two strands, the same strand that was protected by T antigen in the nuclease protection studies.

Ethylation of most of the top strand

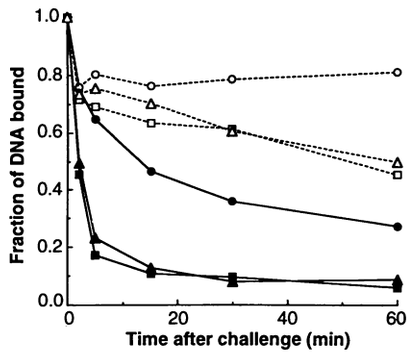

Fig. 4. Time course of dissociation of T antigen bound to various DNA molecules. T antigen (200 ng) was first incubated with ATP (100 μM; solid lines and closed symbols) or AMP-PNP (1 mM; broken lines and open symbols) for 15 minutes at 37°C (50-μl reaction), (*21*). The "two-strand" fork [circles; 0.02 pmol; ^{32}P-labeled at the 5′ end of the top strand, (*22*)], the top strand of the fork [squares; 0.02 pmol; ^{32}P-labeled at the 5′ end, (*22*)], or a nonspecific double-stranded DNA molecule [triangles; 0.02 pmol; ^{32}P-labeled at the 5′ end of one strand, (*22*)] was then added. T antigen was incubated with DNA for 15 minutes at 37°C, and challenged with 0.2 pmol of poly(dT). At intervals, the reaction mixture was filtered through nitrocellulose (*12*). The poly(dT) was approximately 5000 nucleotides in length. The molar ratio of poly(dT) to labeled DNA (in moles of nucleotide), was approximately 1000:1.

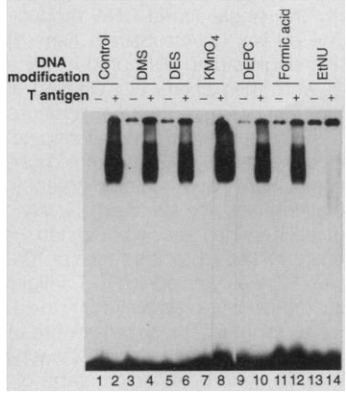

Fig. 5. Effect of chemical modification of single-stranded DNA on T antigen binding. T antigen was first incubated with 100 μM ATP (*21*). To these reactions (even lanes) or reactions lacking T antigen and ATP (odd lanes), was added untreated control DNA (lanes 1 and 2), or DNA treated with dimethyl sulfate (DMS, lanes 3 and 4), diethyl sulfate (DES, lanes 5 and 6), potassium permanganate ($KMnO_4$, lanes 7 and 8), diethyl pyrocarbonate (DEPC, lanes 9 and 10), formic acid (lanes 11 and 12), or ethylnitrosourea (EtNU) (lanes 13 and 14). Reactions were incubated for 20 minutes at 37°C, and the protein-DNA complexes were then treated with glutaraldehyde to 0.1 percent. After 15 minutes at 37°C, the samples were placed directly on a 4 percent acrylamide gel. The DNA used was the standard 15-nt Eco RI DNA sequencing primer for pBR322 (*22*). The chemical modification was as described (*28*).

phosphates did not affect DNA unwinding by T antigen (Fig. 6). The minimal effect of ethylation in 23 bp of double-stranded DNA is surprising since T antigen must traverse this region during unwinding of the forked substrate. It thus appears that the DNA helicase action of T antigen can be separated into an ethylation-sensitive recognition stage and an ethylation-insensitive translocation stage.

T antigen can exist in a variety of oligomeric states ranging from monomer to hexamer and double hexamer (*7, 8, 29*). Using a native gel analysis, we determined the number of T antigen monomers bound to the two-strand replication fork (*8*). The T antigen was incubated in the presence or absence of the synthetic fork labeled with $^{32}P_i$ on the top strand. These reaction mixtures were subjected to glutaraldehyde cross-linking, electrophoresis through a native gradient gel, and silver staining. The T antigen subjected to gel electrophoresis before any incubation (that is, purified T antigen stock) ranged in size from monomer to hexamer and even as higher-order aggregates (Fig. 7). The incubation of T antigen with 100 μM ATP increased the percentage of hexamer, with a corresponding loss of T antigen pentamers. Similar findings have been described by Tegtmeyer and colleagues, although they found a greater percentage of T antigen converted to hexamer (*8*). The incubation of T antigen and ATP with the synthetic fork had little effect on the distribution of T antigen species. Autoradiography of this lane indicated that most of the synthetic fork appeared to be bound by the T antigen hexamer, while a lesser amount comigrated with the double hexamer of T antigen. Essentially no fork binding by T antigen oligomers smaller than hexamer was observed. Similar results were found when single-stranded DNA was used as a binding substrate. We conclude from these experiments, therefore, that T antigen binds the replication fork primarily as a hexamer.

Implications of the replication fork recognition by T antigen. Our data suggest that T antigen, composed of a hexamer of protein, binds to the replication fork primarily through the sugar-phosphate backbone. Our data indicate that only the top strand of the fork participates in T antigen binding, an asymmetric interaction consistent with the 3′ → 5′ directionality of the DNA helicase activity of T antigen. The ability of T antigen to bind the fork in the absence of a free 3′ end suggests that T antigen does not encircle the DNA, but rather binds to the outside of the fork by way of the sugar-phosphate backbone of the top strand.

One way that the T antigen may locate the fork is that T antigen binds randomly to a region of single-stranded DNA, and then migrates along the DNA in the 3′ → 5′ direction until the movement of T antigen is blocked by the fork. However, T antigen can bind the fork in the presence of nonhydrolyzable analogs of ATP (AMP-PNP, ADP) and the binding of DNA containing a fork structure was more stable than to single-stranded DNA alone. We therefore believe that T antigen translocation is not required to bind the fork because the movement of T antigen would have to proceed in an energy-independent, unidirectional manner. Our favored hypothesis is that T antigen has the intrinsic ability to recognize structural features of the DNA comprising the fork. This binding would have no requirement for ATP hydrolysis, and would explain the slower dissociation of T antigen from the synthetic replication fork compared to single-stranded DNA. Moreover, the inhibitory effects on DNA unwinding of ethylation at six top strand phosphates suggests the recognition element for T antigen binding. We postulate that the "fork-specific" structure recognized by T antigen is an individual strand of DNA with two distinct conformations; a region of DNA in a single-stranded conformation contiguous in the 3′ → 5′ direction to a region of DNA in a double-stranded conformation.

Our probing experiments suggest two simple mechanisms by which T antigen could convert the energy of nucleotide hydrolysis into DNA unwinding. First, the T antigen hexamer could "roll" along the DNA, with each of the six monomeric units contacting and unwinding the DNA in sequence during movement. This model is supported by the results of Zhu and Cole (*30*), who have shown that nonfunctional mutants of T antigen had a dominant effect in trans on the replication activity of wild-type T antigen in vivo in co-transfection assays. Similarly, T antigen complexed with substoichiometric amounts of wild-type murine p53 (containing one molecule of p53 per four to ten molecules of T antigen) is greatly inhibited compared to free T antigen in the replication of *ori* DNA in vitro (*31*). Such studies suggest that each monomer of T antigen must be active for the DNA helicase to function. In the second model, only one or, possibly, a small number of T antigen monomers within the hexameric complex actively unwind the DNA. Gefter and co-workers have suggested that the *Escherichia coli rep* DNA helicase unwinds DNA by transposition of DNA phosphates into distinct phosphate-binding sites on *rep* (*32*). Similarly, conformational changes within T antigen driven by nucleotide hydrolysis could allow a single monomer to "creep" along the DNA. In this second model, the T antigen monomers that are not directly interacting with the DNA serve either as passive partners or to

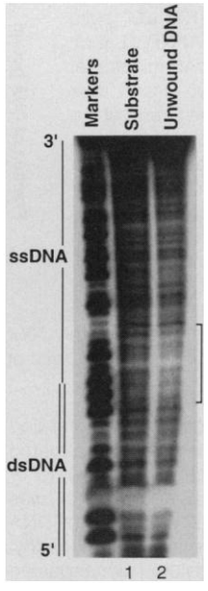

Fig. 6 (left). Ethylation interference of the T antigen–mediated denaturation of the synthetic replication fork. The synthetic replication fork labeled with $^{32}P_i$ on the top strand was subjected to ethylation at phosphates by ethylnitrosourea (*27*). The duplex product molecule was purified by nondenaturing gel electrophoresis (8 percent polyacrylamide) and used as a substrate for DNA unwinding by T antigen. The DNA (5 pmol) was incubated with 400 ng of T antigen in the presence of 1 mM ATP for 20 minutes at 37°C (*21*). The samples were then placed directly on an 8 percent nondenaturing polyacrylamide gel to purify the single-strand DNA product. The duplex DNA substrate (lane 1) and single strand DNA product (lane 2) were then chemically cleaved at the ethylated phosphate positions (*28*) and subjected to denaturing gel electrophoresis (12 percent polyacrylamide) and autoradiography. Marker lanes, the standard "G-only" sequencing reaction (*26*).

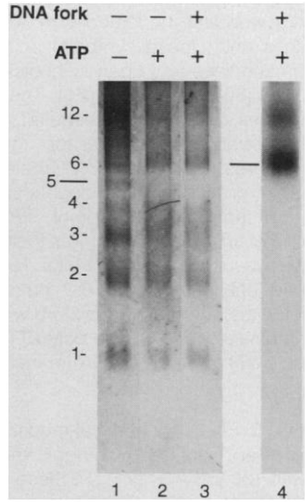

Fig. 7 (right). Oligomeric state of the T antigen species bound to the synthetic replication fork. T antigen was first incubated in the absence (lane 1) or presence of 100 μM ATP (lanes 2 and 3). The synthetic replication fork labeled with $^{32}P_i$ was then added to T antigen (lane 3). After incubating the samples for 20 minutes at 37°C, the protein was cross-linked by the addition of glutaraldehyde to 0.1 percent and incubation at 37°C for 15 minutes. The samples were then subjected to gradient gel electrophoresis (4 to 20 percent acrylamide; 90:1 acrylamide:bisacrylamide). T antigen was then visualized by silver staining (lanes 1 to 3). Lane 4, Autoradiograph of lane 3 (free probe not shown). The positions of monomeric through double hexameric T antigen oligomers are indicated on the left.

contact other replication factors at the fork.

The binding of T antigen to the SV40 *ori* and subsequent formation of a bifurcated complex containing a double hexamer of protein (6–8) has led to the proposal that the two hexameric lobes separate during the initiation of replication to bidirectionally unwind the DNA outward from *ori* (*7, 8, 29*). The binding of hexamers of T antigen to the synthetic fork is consistent with this hypothesis. Our results indicate that oligomeric species of T antigen smaller than hexamer are unable to stably bind the synthetic replication fork, and therefore it may be that the formation of a hexamer of T antigen "activates" the protein to both recognize the DNA fork and unwind the DNA.

The establishment of polarity during DNA helicase movement indicates that T antigen obtains information on direction, 3′ to 5′ or 5′ to 3′, from the DNA on which it is bound. The critical nature of the sugar-phosphate residues for both T antigen binding and DNA helicase activity suggests that the main cues for polarity lie in the sugar-phosphate backbone. The importance of the sugar-phosphate backbone for DNA helicase binding has also been suggested for the *E. coli rep* DNA helicase from comparison of binding properties with the *E. coli* single-stranded DNA binding protein (*32*). The recognition of polarity through the sugar-phosphate backbone predicts that T antigen has the ability to recognize the inherent asymmetry of the deoxyribose ring associated with the 3′ and 5′ ribose linkages to the phosphate residues.

The description of the interaction of T antigen with a synthetic replication fork may be an example of how other DNA helicases unwind DNA. The hexameric structure of the T antigen DNA helicase is similar to that of two *E. coli* helicases, the replicative helicase *dnaB* (*33*), as well as the termination factor for transcription, *rho*, an RNA helicase that can unwind both RNA·DNA and RNA·RNA duplexes (*34*). However, many other DNA helicases do not appear to share this hexameric structure (*1*). Similar investigation of these nonhexameric DNA helicases should indicate whether the mechanisms are general, or whether the conversion of double-stranded DNA into two single strands can occur in multiple ways.

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21. T antigen was purified from Sf9 insect cells infected with recombinant baculovirus [as described; (*15*)]. Binding reactions (generally 20 μl) between T antigen and DNA took place in 40 mM creatine-phosphate (pH 7.8), 7 mM $MgCl_2$, and 0.5 mM dithiothreitol (DTT) at 37°C. For experiments testing the binding of T antigen to DNA, T antigen was incubated for 15 minutes at 37°C in the presence of 30 to 100 μM ATP, 1 mM AMP-PNP, or 1 mM ADP. Prior incubation of T antigen with ATP improved the nuclease protection pattern of T antigen binding to the synthetic replication forks probably because of increased formation of T antigen hexamers which were shown to be active in binding to the synthetic replication fork (Fig. 7). Although we did not find any significant binding to the bottom strand in our nuclease P1 protection experiments, T antigen can bind single-strand DNA with a significant affinity in the presence of ATP (Fig. 5). The molar ratio of T antigen to DNA in our nuclease protection assays was approximately 25 to 1, or a 4 to 1 ratio of T antigen hexamers over DNA (Fig. 7). Since we estimate the affinity of T antigen for replication fork DNA to be roughly five times higher compared to the affinity for single-stranded DNA, significant binding of T antigen to the bottom strand would not be expected in our footprinting assays. As Auborn *et al.* (*35*) did not examine the effect of ATP, these data cannot be used to compare the binding of T antigen to *ori* and the synthetic fork.
22. The sequence of the top strand of the synthetic replication fork was 5′ *TTCTG TGACT ACCTG GACGA CCGGG* TGACT AGCTG CGACG AGATG GGTGC ACTGC 3′. The sequence of the bottom strand was 5′ GTTCT AGCAC TTCGA GTCAA CATCG TCGTT *CCCGG TCGTC CAGGT AGTCA CAGA* 3′. Italicized sequences refer to regions which are duplex in the synthetic fork. To prepare the synthetic fork, the two oligonucleotides (5 pmol each) were mixed in the presence of 50 mM tris-HCl (pH 8.0) and 10 $MgCl_2$ (50 μl, reaction volume) and heated to 90°C. The oligonucleotides were annealed by slowly cooling the mixture to room temperature for 2 hours. The top strand of the fork was labeled at the 5′ end with polynucleotide kinase (*26*) before the annealing reaction. The bottom strand was labeled at the 3′ end by incubation of the annealed DNA fork with the Klenow fragment of DNA polymerase I (Boehringer Mannheim) or Sequenase (USB) and α-$^{32}P_i$-dATP (*26*). To construct the "reverse sequence" replication fork (Fig. 3C), the sequence of each of oligonucleotide was synthesized in reverse orientation (that is, the sequence of the "reverse" bottom strand is 5′ CGTCAC . . . 3′). These oligonucleotides were annealed and labeled by identical procedures as those used for the "forward-sequence" oligonucleotides. The sequence of the "one-strand" fork was 5′ *TCTG TGACT ACCTG GACGA CCGGG* $(GACT)_{14}$ GT *CCCGG TCGTC CAGGT AGTCA CAG* 3′. Italicized sequences refer to regions which are duplex in the synthetic replication fork. The duplex forming regions were nearly identical in sequence to similar sequences in the "two-strand" fork. The sequence of the central single-stranded region was modified to further prevent the formation of secondary structure. The nonspecific double-stranded DNA used in Fig. 4 was a blunt-ended (51 bp) sequence found in the bovine papilloma virus upstream regulatory region. The single-stranded DNA used in Fig. 5 was the standard pBR322 Eco RI sequencing primer (5′ GTATC ACGAG GCCCT 3′).
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28. Chemical modifications were as follows: Dimethyl sulfate and potassium permanganate modification was performed as described (*13*). Diethyl pyrocarbonate and diethyl sulfate modification was performed as described (*24*). Formic acid treatment was as described (*26*). Each DNA was checked for structural integrity and extent of modification subsequent to chemical treatment. Samples of the modified DNA were subjected to electrophoresis on denaturing gels (*26*); the dried gels were subjected to autoradiography. Little or no degradation of the DNA resulted from the modification reaction. The extent of chemical modification was assessed by cleavage of each DNA sample at the modified residues as follows: With ethylnitrosourea treatment, the ethylated DNA was subjected to alkaline hydrolysis in 17.5 μl of 10 mM sodium phosphate and 150 mM NaOH for 30 minutes at 90°. With all base-specific treatments, the modified DNA samples were heated at 90°C for 60 minutes in the presence of 1 M piperidine. After chemical cleavage, the DNA samples were electrophoresed under denaturing conditions (*26*) and the dried gels were subjected to autoradiography. These experiments indicated that each chemical modification procedure proceeded to approximately the same extent and that each DNA molecule contained one to two modifications per molecule.
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