Dimerization of Simian Virus 40 T-Antigen Hexamers Activates T-Antigen DNA Helicase Activity

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Chromosomal DNA replication in higher eukaryotes takes place in DNA synthesis factories containing numerous replication forks. We explored the role of replication fork aggregation in vitro, using as a model the simian virus 40 (SV40) large tumor antigen (T antigen), essential for its DNA helicase and origin-binding activities. Previous studies have shown that T antigen binds model DNA replication forks primarily as a hexamer (TAG_H) and to a lesser extent as a double hexamer (TAG_{DH}). We find that DNA unwinding in the presence of ATP or other nucleotides strongly correlates with the formation of TAG_{DH}-DNA fork complexes. TAG_H- and TAG_{DH}-fork complexes were isolated, and the TAG_{DH}-bound fork was denatured at a 15-fold-higher rate during the initial times of unwinding. TAG_{DH} bound preferentially to a DNA substrate containing a 50-nucleotide bubble, indicating the bridging of each single-stranded DNA/duplex DNA junction, and this DNA molecule was also unwound at a high rate. Both the TAG_H- and TAG_{DH}-fork complexes were relatively stable, with the half-life of the TAG_{DH}-fork complex greater than 40 min. Our data therefore indicate that the linking of two viral replication forks serves to activate DNA replication.

A notable event during the onset of S phase in higher eukaryotic cells is the establishment and clustering of DNA replication forks. As chromosomal replication proceeds, tens of replication forks aggregate to generate apparent DNA replication factories or granules which catalyze nascent-strand synthesis in discrete nuclear foci (reviewed in references 2 and 18). These factories appear fixed to the nuclear scaffold with the DNA spooled through the sites of active replication (15). Although the existence of these complexes is clear, the role of replication fork aggregation during eukaryotic replication is unknown.

Numerous aspects of DNA replication in eukaryotes have been productively studied by using simian virus 40 (SV40) (reviewed in references 17 and 35). SV40 DNA replication requires a single viral factor, the large tumor antigen (T antigen) (11), with all other replication factors provided by the host cell. Fractionation of cellular extracts promoting replication in vitro has led to the identification of various host factors required for viral DNA duplication as well as the replication and maintenance of the host chromosomal DNA (21, 36, 39). Moreover, separation of SV40 DNA replication into various subreactions has allowed mechanistic information to be gained concerning the initiation and elongation stages. These studies indicate that T antigen first binds the viral origin of replication (*ori*) in an ATP-dependent reaction (4, 7, 8). This initiation complex contains a double hexamer of T antigen with each hexamer positioned over one half of *ori* (23, 26). In the presence of human replication protein A (hRPA), the origin becomes denatured and the DNA helicase activity of T antigen (13, 32, 33, 41) bidirectionally unwinds the DNA outward (6, 42). The DNA polymerase machinery then binds the exposed single-stranded DNA (ssDNA) to synthesize the nascent strands (38).

Examination of the DNA helicase activity of T antigen in-

icates that hexamers (TAG_H) and to a lesser degree double hexamers (TAG_{DH}) can recognize model DNA replication forks in an ATP-dependent reaction (31, 40). One interpretation of these results is that the *ori*-bound TAG_{DH} separates during replication initiation, leading to a TAG_H unwinding the viral DNA independently at each replication fork. However, electron microscopic analysis of an *ori*-dependent DNA unwinding reaction revealed a sizable fraction of product molecules in which the two forks of the plasmid DNA substrate were joined through a T-antigen bridge (40). In these molecules, ssDNA loops ("rabbit ears") were seen extruding from an apparent TAG_{DH}. Thus, an alternative model is that the TAG_{DH} remains intact during replication initiation and elongation, catalyzing DNA unwinding as part of a minireplication factory.

To explore the biological relevance of TAG_{DH} in DNA unwinding, we examined the role of T-antigen oligomeric state on DNA helicase activity. We find that dimerization of TAG_H on DNA fork or DNA bubble substrates can stimulate DNA unwinding 15-fold. Complexes formed between TAG_{DH} and a model DNA replication fork were relatively stable, with a half-life of greater than 40 min. These results indicate that coupling of replication forks through T antigen leads to the production of a high-activity replication complex.

MATERIALS AND METHODS

DNA substrates. The DNA fork was assembled from two partially complementary oligonucleotides with top- and bottom-strand sequences as described by SenGupta and Borowiec (the two-strand fork) (31). The DNA bubble substrate was assembled by using top- and bottom-strand sequences as follows: top strand (100 nucleotides), 5' TTC TGT GAC TAC CTG GAC GAC CGG GGA CTG ACT GAC TGA CTG ACT GAC TGA CTG ACT GAC TGA CTG ACT GAC TGA GGG CCA GCA GGT CCA TCA GTG TCT T 3'; bottom strand (100 nucleotides), 5' AAG ACA CTG ATG GAC CTG CTG GCC CGA CTG ACT GAC TGA CTG ACT GAC TGA CTG ACT GAC TGA CTG ACT GAC TGA CCC GGT CGT CCA GGT AGT CAC AGA A 3'. For each substrate, the top- or bottom-strand oligonucleotide was first labeled at the 5' end with [γ -³²P]ATP and T4 polynucleotide kinase to a specific activity of $\sim 1 \times 10^6$ to 2×10^6 cpm/pmol. Oligonucleotides were then annealed in reaction mixtures (50 to 100 μ l) containing 5 to 10 pmol each of the top and bottom strands, 50 mM Tris-HCl (pH 8.0), and 10 mM MgCl₂. Reaction mixtures were heated to 95°C and then

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slowly cooled. Complete annealing was verified by native gel electrophoresis and autoradiography of the reaction products.

Gel shift and helicase assays. T antigen was purified from Sf9 insect cells infected with recombinant baculovirus strain Ac941SVT as described previously (3).

Standard DNA binding reaction mixtures (25 μ l) containing 20 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 0.1 mg of bovine serum albumin (BSA) per ml, 2 mM ATP, 0.1 pmol of DNA fork, and T antigen (as indicated) were incubated for 15 min at 37°C. To determine the influence of different nucleotides on T antigen-DNA fork complex formation and DNA helicase activity, 2 mM ATP was replaced with 2 mM of the desired nucleoside triphosphate (NTP) or dNTP. For the detection of T antigen-fork complexes by silver staining, 0.2 pmol of the ³²P-DNA fork was incubated with 1,600 ng of T antigen in the presence of 0.2 mM β , γ -imidoadenosine 5'-triphosphate (AMP-PNP) or 2 mM ATP. For assays comparing T-antigen binding to the DNA fork and DNA bubble, reaction mixtures containing 0.05 pmol of the DNA fork or bubble substrates, 700 ng of T antigen, 0.25 mM AMP-PNP, and 0.9 pmol of a 55-bp duplex DNA competitor were preincubated for 10 min at 37°C. DNA unwinding was stimulated by the addition of ATP to 2.5 mM. In all cases examining T antigen-DNA complex formation or the oligomeric state of T antigen, reaction mixtures were subjected to cross-linking with glutaraldehyde (0.05%, final concentration) and separated by electrophoresis through a nondenaturing 5% polyacrylamide gel (40:0.5, acrylamide/bisacrylamide) and autoradiographed. To detect the oligomeric state of T antigen bound to the fork, T antigen and T antigen-fork complexes were separated by electrophoresis through a nondenaturing polyacrylamide gel containing a 3.5 to 17.5% acrylamide gradient (40:0.5, acrylamide/bisacrylamide) and visualized by silver staining and autoradiography.

Similar conditions were used to assay DNA helicase activity with the exception that reaction mixtures were incubated for 25 min and reactions were quenched by addition of 0.1 volume of 3.3% (wt/vol) sodium dodecyl sulfate–0.5 M EDTA. When the influence of the amount of T antigen-DNA fork complexes on the initial rate of fork denaturation was investigated, the reaction volume was increased to 30 μ l and brought to a constant glycerol concentration (21 to 27%). To quantify the amount of T-antigen binding to the DNA fork or the extent of DNA unwinding, the bands of interest were excised from the gel and the radioactivity was determined.

Glycerol gradient centrifugation. Reaction mixtures (250 μ l) containing 20 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 0.5 mM DTT, 0.25 mM AMP-PNP, 0.1 mg of BSA per ml, 5 pmol of ³²P-labeled DNA fork, and 25 μ g of T antigen were incubated at 37°C for 40 min. The reaction mixture was layered directly onto a 5-ml glycerol gradient (15 to 30%) containing 20 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 0.5 mM DTT, and 0.1 mg of BSA per ml. Gradients were centrifuged (4°C) for 20 h at 29,000 rpm, using a Beckman SW50.1 rotor, and 15 fractions (330 μ l) were collected from the top. To visualize the T antigen-DNA complexes, 25 μ l from each fraction was cross-linked with glutaraldehyde (0.05%, final concentration), separated by electrophoresis through a nondenaturing 5% polyacrylamide gel (40:0.5, acrylamide/bisacrylamide), and autoradiographed. The isolated T antigen-DNA fork complexes were used within 1 week, and no significant dissociation was observed after storage at 4°C during this period.

RESULTS

Activation of the T-antigen DNA helicase correlates with formation of TAG_{DH}. To determine the role of TAG_{DH} during the elongation phase of viral DNA replication, we examined the relationship between T-antigen oligomeric state and DNA helicase activity. Increasing levels of T antigen were incubated with a model DNA replication fork (Fig. 1A) in the presence of ATP and fixed with glutaraldehyde. The reaction products were then separated by nondenaturing gel electrophoresis to detect T antigen-DNA complexes (Fig. 2A). Unbound single-stranded product DNA generated by the DNA helicase activity of T antigen could also be detected, by comparison to a ssDNA control (not shown). The generation of ssDNA was quantitated by DNA helicase assays performed in parallel (Fig. 2B), in the absence of a cross-linking agent.

Addition of low levels of protein (100 to 200 ng) to the ³²P-labeled fork resulted in the formation of a prominent band that was identified as the TAG_H-fork complex by its comigration to T-antigen hexamers on a silver-stained gel (Fig. 2C) (31). A minor level of ssDNA product was generated under these conditions. Further increases in the amount of T antigen caused little change in the amount of bound TAG_H and yet greatly stimulated the formation of a more slowly migrating band (Fig. 2A, lanes 5 and 6). The comigration of this slow

FIG. 1. DNA substrates used to examine T-antigen binding and DNA helicase activity. Partially complementary oligonucleotides were annealed to give rise to the model replication fork (A) and model replication bubble (B). nt, nucleotides.

band with T-antigen double hexamers, the latter visualized by silver staining (Fig. 2C) (31), identifies it as the TAG_{DH}-DNA fork complex. Over this same range of T antigen (400 to 800 ng), a striking increase was observed in the amount of ssDNA product (Fig. 2A, lanes 5 and 6). Direct assay of the DNA helicase activity indicated a similar response to T-antigen concentration (Fig. 2B). Thus, we find that activation of the T-antigen DNA helicase correlates with the binding of TAG_{DH} to the DNA fork.

The effect of T-antigen concentration on both the generation of TAG_H- and TAG_{DH}-fork complexes and DNA helicase activity was quantitated. Plots of both the raw data (Fig. 2D, inset), and the data normalized to facilitate comparison (Fig. 2D, main panel) are shown. Increases in the level of T antigen from 200 to 400 ng stimulated TAG_H-fork complex formation less than twofold and TAG_{DH}-fork complex formation approximately fivefold. Over the same range of T antigen, substrate denaturation increased nearly sevenfold, indicating that TAG_{DH} possesses considerably greater DNA helicase activity than TAG_H and again showing a correlation between TAG_{DH}-fork complex formation and activation of the T-antigen DNA helicase.

Isolated TAG_{DH}-fork complexes display 15-fold-greater DNA helicase activity than TAG_H-fork complexes. To directly demonstrate that formation of TAG_{DH} activates DNA helicase activity, we isolated TAG_H- or TAG_{DH}-DNA fork complexes and measured the helicase activity of each. Reactions were prepared with AMP-PNP, a nonhydrolyzable analog of ATP, to facilitate T-antigen binding to the ³²P-labeled fork and yet prevent premature DNA unwinding. Reaction mixtures were then subjected to glycerol gradient centrifugation to separate DNA forks bound by TAG_H from those bound by TAG_{DH}. Native gel electrophoresis of aliquots from each fraction, subsequent to glutaraldehyde fixation, indicated a significant enrichment of both the TAG_H and TAG_{DH} pools (Fig. 3). This isolation procedure allowed recovery of gradient fractions that contained 78 or 92% of T antigen bound to the DNA fork in the form of TAG_H or TAG_{DH}, respectively. We also tested a

FIG. 2. Correlation of DNA unwinding with TAG_{DH} formation. (A) Effect of T-antigen concentration on the formation of TAG_H and TAG_{DH} oligomers complexed to a model DNA replication fork. Various amounts of T antigen (as indicated) were incubated with a ³²P-labeled DNA fork in the presence of ATP and cross-linked with glutaraldehyde. The complexes were separated by electrophoresis through a 5% native polyacrylamide gel and visualized by autoradiography. The positions of the DNA fork bound by TAG_H or TAG_{DH} are indicated, as are the positions of the unbound DNA fork and ssDNA resulting from the DNA helicase activity of T antigen. (B) Effect of T-antigen concentration on the unwinding of the DNA fork. Reaction mixtures were prepared as described for panel A except that glutaraldehyde was not used. Reactions were quenched as described in Materials and Methods, and the products were separated by electrophoresis through a native 10% polyacrylamide gel and visualized by autoradiography. Lane 7 (Δ) shows the heat-denatured substrate. The locations of the ssDNA product and DNA fork substrate are shown. (C) Determination of the oligomeric state of the T antigen bound to the DNA fork. T antigen (1,600 ng) was incubated in the absence of NTP (lane 1), in the presence

fraction, TAG_{mix}, in which approximately half of the available fork substrate was bound by TAG_H and half was bound by TAG_{DH}.

Striking differences were observed between the DNA helicase activities of the TAG_H (Fig. 4A) and TAG_{DH} (Fig. 4C) pools. During the first minute of incubation, the rate of DNA unwinding by TAG_{DH} was an average of 15-fold greater than that by TAG_H (Fig. 4D). Use of longer reaction times lessened the difference between TAG_{DH} and TAG_H, partly because the reaction plateaued when roughly 50% of the ssDNA substrate was unwound. Note that DNA unwinding was invariably limited to ~50% of the substrate pool (see also Fig. 6 and 7), indicating that approximately half of the T-antigen complexes are inactive in this reaction. The rate of DNA unwinding by the TAG_{mix} fraction was intermediate between that found for the TAG_H and TAG_{DH} pools (Fig. 4B and D). When a similar protocol was used to test a DNA fork containing a longer duplex region (42 versus 25 bp), forks bound by a TAG_{DH} were unwound at a rate 12-fold greater than forks bound by a TAG_H (data not shown). We therefore conclude that formation of TAG_{DH} activates the T-antigen DNA helicase.

As differences in the concentration of T antigen-DNA fork complexes in the individual fractions could conceivably lead to an observed difference in DNA helicase activity, a titration of TAG_H, TAG_{mix}, or TAG_{DH} fractions was performed and the amount of ssDNA generated over the initial 20 s of incubation was determined. For each fraction tested, the rate of DNA unwinding was not significantly affected by changes in the amount of fraction used (Fig. 5). Therefore, the individual protein-DNA complexes appear to act as independent entities, with fork denaturation not appreciably affected by the concentration of T antigen-fork complexes over the tested range.

Preferential formation of the TAG_{DH}-DNA complex by using a model replication bubble. A possible criticism of the experiments described above is that binding of the TAG_{DH} to the DNA fork does not accurately represent the structure of a double hexamer bridging two DNA replication forks. To address this issue, we examined the binding of T antigen to a model replication bubble that contains two ssDNA/double-stranded DNA (dsDNA) junctions (Fig. 1B). T antigen (700 ng) was incubated with the ³²P-labeled replication bubble and, for comparison, the ³²P-labeled model fork. The replication bubble was bound primarily by TAG_{DH} (31% TAG_H and 69% TAG_{DH} [Fig. 6A, lane 4]), while binding to the fork occurred mainly by TAG_H (70% TAG_H and 30% TAG_{DH} [Fig. 6A, lane 2]). Thus, formation of the TAG_{DH} is stimulated by the presence of two ssDNA/dsDNA junctions, indicating that each hexamer binds a junction.

The ability of T antigen to unwind the replication bubble and fork was tested. T antigen (700 ng) was incubated with each of the radiolabeled substrates in the presence of 250 μM AMP-PNP to allow complex formation. ATP (2.5 mM) was then added to stimulate the T-antigen DNA helicase activity. At various times of incubation, the reaction was quenched and

FIG. 3. Isolation of TAG_H- and TAG_{DH}-DNA fork complexes by glycerol gradient centrifugation. T antigen (25 μg) was incubated with 5 pmol of ³²P-labeled DNA fork and 0.25 mM AMP-PNP and then loaded onto a 15 to 30% glycerol gradient and centrifuged for 20 h at 29,000 rpm, using a Beckman SW50.1 rotor. Fifteen gradient fractions were collected from the top. Aliquots of the fractions were subjected to glutaraldehyde fixation and electrophoresed as described in the legend to Fig. 2 to visualize the T antigen-fork complexes. Saturating concentrations of T antigen were used in this experiment, and thus no free DNA was detected after centrifugation. Fractions 6, 10, and 14 were chosen as representative of the TAG_H-, TAG_{mix}-, and TAG_{DH}-DNA fork complexes, respectively. Bound DNA fork in the TAG_H fraction was a mixture of 78% TAG_H- and 22% TAG_{DH}-DNA fork complexes, the TAG_{mix} fraction was a mixture of 46% TAG_H- and 54% TAG_{DH}-DNA fork complexes, and the TAG_{DH} fraction was a mixture of 8% TAG_H- and 92% TAG_{DH}-DNA fork complexes.

the amount of unwound DNA was determined (e.g., Fig. 2B). Quantitation of DNA unwinding indicated that during the first 2.5 min of unwinding, the replication bubble was unwound at a >14-fold-higher rate than the DNA fork (Fig. 6B). Our data therefore indicate that stimulation of the T-antigen DNA helicase activity also occurs when two model replication forks are bridged by the TAG_{DH} complex.

TAG_{DH}-DNA fork complexes have significant stability. The stability of TAG_H- and TAG_{DH}-DNA fork complexes was examined. Glycerol gradient fractions containing the DNA fork bound by TAG_H or TAG_{DH} were prepared as described above (Fig. 3), and aliquots were then incubated at 37°C in the presence of ATP. At various times, reaction mixtures were cross-linked with glutaraldehyde. Following separation by native gel electrophoresis, the amount of TAG_H- and TAG_{DH}-fork complexes was quantitated (Fig. 7). Each complex was found to break down in a generally biphasic pattern. Approximately 10% of the predominant species (i.e., TAG_H or TAG_{DH}) was lost in the first few seconds of incubation, suggesting that a similar fraction of T antigen-fork complexes was only weakly associated. In the case of TAG_H, the remaining complexes were quite stable in that only 5% were lost during the subsequent 40 min of incubation (Fig. 7A). The remaining TAG_{DH}-fork complexes were broken down at a higher rate in that ~20% was displaced over a similar period (Fig. 7B). Dissociation of the TAG_{DH} complex occurred only during the first 10 min of incubation (after loss of the weakly associated complexes) and was mirrored by the generation of TAG_H complexes. The half-life of TAG_{DH}-fork complexes was therefore greater than 40 min,

of ATP (lanes 2, 4, and 6) or AMP-PNP (lanes 3, 5, and 7), and in the absence (lanes 1 to 3) or presence (lanes 4 to 7) of the ³²P-labeled DNA fork and then cross-linked with glutaraldehyde. The reaction products were separated by native polyacrylamide gel electrophoresis, and the products were visualized both by silver staining (lanes 1 to 5) and autoradiography (lanes 6 and 7). The T-antigen oligomeric state is indicated on the left, determined by counting up from the monomeric species (labeled 1). The prominent T antigen-DNA complexes are seen to comigrate with the hexamer (labeled 6) and double-hexamer (labeled 12) forms of T antigen. Although the level of T antigen used in panel C (1,600 ng) is higher than that used in panel A (800 ng), variations in the T-antigen levels over this range did not affect the mobility of the individual T antigen-DNA complexes (data not shown). Note also that as the DNA fork (36 kDa) has much lower mass than either TAG_H (490 kDa) or TAG_{DH} (980 kDa), the bound DNA does not appreciably affect the migration of T-antigen complexes. (D) Quantitation of TAG_H- and TAG_{DH}-DNA fork complex formation and DNA helicase activity as a function of T-antigen concentration. Bands in the panel A gel corresponding to the TAG_H and TAG_{DH} complexes or in the panel B gel corresponding to the ssDNA product were excised from the gels, and the radioactivity was determined. The inset shows the percentage of the DNA substrate that was bound either by TAG_H (circles) or TAG_{DH} (squares) or was unwound by T antigen (triangles). In the main panel, the data were individually normalized with respect to the maximum amount of TAG_H (solid bars) or TAG_{DH} (hatched bars) complex formation or ssDNA produced (stippled bars).

FIG. 4. Activation of the T-antigen DNA helicase activity by TAG_{DH} formation. TAG_H (A)-, TAG_{mix} (B)-, and TAG_{DH} (C)-DNA fork complexes were prepared as described in the legend to Fig. 3 and Materials and Methods. DNA helicase activity was stimulated by the addition of 3 mM ATP and incubation at 37°C for the times (seconds ['] or minutes [']) indicated. Reactions were quenched, and the products were separated by electrophoresis through a native 10% polyacrylamide gel and visualized by autoradiography. Lane 8 (Δ) shows the heat-denatured substrate. The locations of the ssDNA product and DNA fork substrate are shown. (D) The amount of ssDNA product is graphed as a function of incubation time for the TAG_H-, TAG_{mix}-, and TAG_{DH}-DNA fork complexes.

indicating that these complexes have significant stability under conditions that allow DNA unwinding.

Effects of nucleotides on T-antigen oligomeric state and DNA unwinding. The unwinding of DNA helicase substrates by T antigen is ATP dependent, although other nucleotides can substitute to various degrees (13, 33, 41). We therefore tested the effects of various nucleotides on T-antigen DNA helicase activity and on the production of TAG_H- and TAG_{DH}-DNA fork complexes, using two different levels of T antigen (200 and 600 ng) (Table 1).

With the exception of AMP and CTP, all tested nucleotides efficiently supported TAG_H- and TAG_{DH}-fork complex formation. For those nucleotides supporting DNA unwinding, we found that more efficient DNA unwinding paralleled changes in the level of TAG_{DH} rather than TAG_H complexes. In the presence of dITP, for example, an increase in the amount of T antigen from 200 to 600 ng lowered the amount of DNA fork bound by TAG_H from 54 to 41% (Table 1) and yet stimulated both DNA fork binding by TAG_{DH} (from 19 to 41%) and the amount of ssDNA product (from 4 to 17%). Thus, we find a close correlation between DNA helicase activity and TAG_{DH}-complex formation with use of nucleotides other than ATP. For those nucleotides supporting DNA unwinding, the following hierarchy of activity was observed: ATP ≈ dATP ≈ dUTP > dTTP ≈ dITP > dCTP > UTP. Other nucleotides were essentially inactive.

With the nucleotides moderately active in the DNA helicase reaction, we noted that replacement of ribose with deoxyribose significantly increased the effectiveness of the nucleotide. That is, for the ITP-dITP, UTP-dUTP, and CTP-dCTP pairs, presence of the deoxyribose sugar was correlated with a three- to fivefold stimulation of DNA helicase activity. Only the most active nucleotides (ATP and dATP) and the inactive GTP-dGTP pair did not show a significant sugar effect. Phosphate groups were also noted to play a critical role in that ADP efficiently stimulated TAG_{DH}-fork complex formation and yet was unable to support DNA unwinding, while AMP was inactive in either activity. Thus, we find that the nucleotide sugar, base, and phosphate composition has significant effects on T-antigen binding to the fork and on DNA unwinding. More generally, our data indicate that while formation of TAG_{DH} is coupled to activation of the T-antigen DNA helicase, it is not sufficient.

DISCUSSION

The onset of S phase in higher eukaryotic cells involves the formation of nuclear bodies termed replication factories or granules that precede DNA synthesis (15, 16, 25). Even though chromosomal replication in higher eukaryotes appears depen-

FIG. 5. The initial DNA unwinding rate is independent of the concentration of TAG-DNA fork complexes. Various amounts of the glycerol gradient fractions corresponding to the TAG_H-, TAG_{mix}-, and TAG_{DH}-fork pools (isolated as described in the legend to Fig. 3 and Materials and Methods) were incubated at 37°C for 20 s. Reactions were quenched as described in Materials and Methods, and the products were separated by electrophoresis through a native 10% polyacrylamide gel and visualized by autoradiography. The amount of ssDNA generated was quantitated and plotted against the volume of the glycerol gradient fraction tested.

FIG. 6. Preferential binding of the TAg_{DH} to a model replication bubble. (A) The ³²P-DNA fork (lanes 1 and 2) or ³²P-DNA bubble (lanes 3 and 4) was incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 700 ng of T antigen and 250 μ M AMP-PNP for 15 min at 37°C as described in Materials and Methods. Reaction products were cross-linked with glutaraldehyde, separated by electrophoresis through a 5% native polyacrylamide gel, and visualized by autoradiography. The positions of TA_{gH} and TA_{gDH} complexes are indicated, as are the positions of the unbound DNA fork and replication bubble. (B) T antigen (700 ng) was incubated with the ³²P-DNA fork or ³²P-DNA bubble and 250 μ M AMP-PNP for 15 min at 37°C. DNA helicase activity was stimulated by the addition of 2.5 mM ATP, and the reaction mixture was incubated at 37°C for various times. Reactions were quenched, and the amount of ssDNA product was determined, as described in the legend to Fig. 2B, for the T antigen-DNA bubble (open circles) and T antigen-DNA fork (closed circles) complexes.

dent on an intact nuclear structure (19), the *raison d'être* of replication factories is unclear. Exploring this issue with the SV40 T antigen as a model, our data indicate that oligomerization of DNA replication forks can convert elements of the replication machinery into a high-activity form.

Our data shows that the TAg_{DH} has >15-fold-higher DNA helicase activity than a TAg_H. Use of the replication bubble substrate indicates that activation of the T-antigen DNA helicase activity by TAg_{DH} formation can occur with each hexamer bound to a ssDNA/dsDNA junction. Similarly, Wessel et al. have found that the TAg_{DH} joins two forks in an *ori*-dependent DNA unwinding reaction (40). Our results therefore indicate that T-antigen-mediated DNA unwinding is stimulated upon linking of the viral replication forks (Fig. 8). Our data do not address whether each hexamer in a TAg_{DH} complex must be

bound by DNA for DNA helicase activation or can occur in TAg_{DH} complex in which only one hexamer is bound to DNA.

Binding of a TAg_{DH} is required for the initiation of viral DNA replication at *ori* (23, 26). These data suggest that the joining of two hexamers induces conformational changes in T antigen which increase its activity on DNA, both as a replication initiation factor and as a DNA helicase. It is interesting that factors that negatively modulate hexamer-hexamer interaction at *ori*, such as T-antigen phosphorylation, also inhibit the initiation of DNA replication (37). Although changes in phosphorylation do not appear to affect DNA helicase activity (20), it may prove useful to reexamine this question in assays using TAg_{DH}-DNA fork or bubble complexes that more closely resemble replication intermediates. More generally, a reasonable speculation is that the elongation stage of DNA replication can be modulated by influencing the relative equilibrium between TAg_H and TAg_{DH}. As an example, the termination of replication on viral DNA circles has the potential of causing the conversion of TAg_{DH} to TAg_H because of steric constraints involved as two joined forks attempt to unwind the final duplex DNA. Viral replication would therefore proceed efficiently from the initiation of DNA replication until termi-

FIG. 7. Stability of TAg_H- and TAg_{DH}-DNA fork complexes. TAg_H- (A) and TAg_{DH}-DNA fork complexes (B), prepared as described in the legend to Fig. 3 and Materials and Methods, were incubated with 3 mM ATP at 37°C. Reaction mixtures were subjected to glutaraldehyde cross-linking and separated by electrophoresis. The radioactivity of bands corresponding to TAg_H and TAg_{DH} complexes were determined, and the fraction of DNA fork in each complex was plotted against the time of incubation.

TABLE 1. Influence of different nucleotides on formation of TAg_H - and TAg_{DH} -DNA fork complexes and T-antigen DNA helicase activity^a

Nucleotide	Amt of T antigen (ng)	Double hexamer (%)	Hexamer (%)	Unwound DNA (%)
None	200	3.3	5.0	0.8
	600	5.3	13.4	3.0
ATP	200	19.0	37.0	8.6
	600	31.5	33.0	28.5
AMP	200	1.8	5.4	1.0
	600	6.4	13.0	1.6
ADP	200	13.0	36.0	2.2
	600	48.0	35.0	2.0
dATP	200	17.5	46.0	9.0
	600	37.0	44.0	24.0
ITP	200	8.8	28.4	1.5
	600	31.0	47.0	3.6
dITP	200	18.6	54.0	3.5
	600	41.0	41.0	16.5
UTP	200	10.4	27.0	2.5
	600	33.0	45.0	7.0
dUTP	200	21.0	55.0	4.0
	600	42.5	45.0	22.0
CTP	200	0.9	1.8	1.9
	600	6.2	16.0	2.0
dCTP	200	12.0	30.0	1.8
	600	32.0	49.0	10.4
GTP	200	8.8	26.0	1.6
	600	37.0	44.0	2.5
dGTP	200	7.6	23.0	1.1
	600	33.0	52.0	1.8
dTTP	200	18.4	53.0	2.7
	600	42.0	46.0	17.5

^a T antigen (200 and 600 ng) was incubated with a ³²P-labeled DNA fork in the presence of 2 mM nucleotide, and the reaction products were subjected to gel shift and DNA helicase analyses. Quantitation of T-antigen fork complex formation and generation of the ssDNA product were as described in Materials and Methods. Hexamer (%) and double hexamer (%) indicate the percentages of DNA fork substrate bound by TAg_H and TAg_{DH} , respectively.

nation, when disruption of TAg_{DH} causes replication fork movement to greatly slow.

Nearly all helicases are oligomeric, with a number existing primarily in a hexameric state (22). In addition to T antigen (23), these include the bacteriophage T7 gp4 (10), the bacteriophage T4 gp41 (9), and the *Escherichia coli* RuvB (24), DnaB (27), and transcription termination factor Rho (1). The recombination factor RuvB, which provides DNA helicase activity during branch migration, exists also as a double hexamer, particularly at Holliday junctions (34). Hexamer dimerization has also been observed for gp4 (14) and Rho (12), and it will be of interest to determine the effect of higher-order oligomerization of these enzymes on helicase activity.

What is the mechanistic basis for the differences in DNA helicase activity between TAg_H and TAg_{DH} ? One explanation is that the reduced DNA helicase activity of TAg_H is a result of low helicase processivity, such that complete DNA unwinding requires multiple binding events between T antigen and DNA. We believe this unlikely because the TAg_H -fork complexes were relatively stable under our reaction conditions (Fig. 7A). A second possibility is that a rate-limiting step of the DNA unwinding reaction is significantly affected by the higher-order oligomeric state of T antigen. In this view, the TAg_{DH} -fork complex has an increased rate of base pair denaturation relative to the TAg_H complex, and thus the DNA travels more rapidly through the T-antigen complex.

The nucleotide requirements for the T-antigen DNA heli-

case (13, 41) and hexamer formation (28) have been previously examined. Although there is some conflict between these data, particularly with regard to CTP, the basic trend is that ATP and dATP are most active in supporting both of these activities, dTTP, dCTP, and UTP can substitute for ATP to a limited extent, and GTP and dGTP are inactive or only poorly active. We find a similar nucleotide classification for TAg_H - and TAg_{DH} -fork complex formation and DNA helicase activity. Our data also suggests that in general, nucleotides were more efficient at facilitating TAg_H and TAg_{DH} complex formation with the fork than at supporting DNA helicase activity. We take these results to indicate that all active nucleotides can induce structural transitions within the T-antigen structure, but DNA unwinding requires a more extensive or more prolonged conformational change compared to T-antigen oligomerization. Study of the *E. coli* DnaB DNA helicase indicates that nucleotide activity depends in part on the binding affinity of nucleotide and protein (5). The degree to which the nucleotide binding affinity controls nucleotide activity for the T-antigen DNA helicase and oligomerization reactions will require further investigation.

SV40 DNA replication, like chromosomal replication, takes place on the nuclear matrix (29, 30). Although the nuclear matrix is still ill defined, a clearer picture of the structural proteins is emerging (2). Our results suggest that SV40 may be used as a model system to study the role of the nuclear matrix during DNA replication. One can test, for example, the effect of nuclear matrix components on the TAg_H - TAg_{DH} equilibrium and on the DNA unwinding reactions mediated by T antigen. In this way, a replication system more closely resembling the physiological state in nucleos can be developed.

FIG. 8. Model showing that dimerization of the TAg_H -DNA fork complex activates the T-antigen DNA helicase activity.

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