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Simian virus 40 (SV40) large tumor antigen causes stepwise changes in SV40 origin structure during initiation of DNA replication

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ABSTRACT **We have studied structural changes in the simian virus 40 (SV40) replication origin induced by SV40 large tumor antigen (T antigen). T-antigen-induced changes in origin DNA conformation can be visualized as specific and discrete topologic changes in origin DNA minicircles. We discovered three origin–T-antigen complexes defined by changes in DNA linking number. These complexes probably reflected essential early steps in the initiation of DNA replication since their formation required DNA sequences that are necessary for DNA replication but do not affect T-antigen binding. There are striking parallels between the T antigen–origin interactions uncovered by this assay and the interactions between the DnaA, -B, and -C proteins and the *Escherichia coli* replication origin, suggesting a significant evolutionary conservation in the mechanisms that initiate DNA replication.**

The functional activity of DNA binding proteins can involve modulation of DNA structure. For example, wrapping of DNA about a protein core is an important step in the initiation of DNA replication for bacteriophage λ (1, 2) and for *Escherichia coli* (3). RNA polymerase induces a local unwinding of the DNA helix during transcription initiation and elongation (4, 5). DNA looping, mediated by protein–protein interactions, may regulate transcription in phage λ (6), at the *gal* (7) and *araBAD* operons (7–9), and at the simian virus 40 (SV40) promoter (10). Recombination in a number of systems involves the assembly of specialized nucleoprotein complexes in which the template DNA is constrained to a particular path (2, 11, 12).

We have used a topology shift assay to study changes in DNA conformation induced by interactions between SV40 large tumor antigen (T antigen) and the SV40 replication origin. In this assay, T antigen is bound to a small [<650 base pairs (bp)] DNA circle containing the replication origin, in the presence of topoisomerase I. Protein–DNA interactions that undertwist, overtwist, or unwind DNA, as well as interactions that confine the DNA helix to a specific pathway, such as wrapping, bending, or looping, can all be detected as changes in the linking number of the template DNA circle. Small changes in linking number are measured easily and precisely with small DNA circles, primarily because the equilibrium distribution generated by topoisomerase I alone usually consists mostly of just one topoisomer (i.e., the 0, +1, or −1 topoisomer) and because individual topoisomers can be resolved clearly by electrophoresis through low percentage polyacrylamide gels. The linking difference provides a quantitative determination of the change in DNA conformation; also, if multiple different or sequential interactions occur then each can be associated with a unique topoisomer.

We have used this assay to analyze the events that occur during the initiation of DNA replication. Binding of SV40 T antigen to the replication origin is necessary to initiate DNA replication (13–15). In addition to a DNA binding domain, T antigen has an ATP-dependent DNA helicase activity (16). After origin binding, the T-antigen helicase can extensively unwind the template DNA (17–19). Initiation requires origin DNA unwinding (20–22). Using the topology shift assay, we have discovered three specific origin–T-antigen complexes. Formation of each of these complexes required DNA sequences that are necessary for origin function in addition to those necessary for T-antigen binding. Therefore, these complexes probably represent functional interactions that occur during the initiation of replication at the SV40 origin.

METHODS

Reagents. SV40 T antigen was prepared by immunoaffinity isolation from extracts of HeLa cells infected with a mixture of wild-type adenovirus (Ad) type 5 and the T antigen expressing Ad–SV40 hybrid Ad5SVR112. Calf thymus topoisomerase I was from BRL. Ribo- and deoxyribonucleoside triphosphates and adenosine 5′-*O*-(3-thiotriphosphate) were from Pharmacia. Phosphocreatine and creatine phosphokinase were from Sigma. *E. coli* SSB was from United States Biochemical. *Proteus vulgaris* topoisomerase I was a gift from J. Champoux (Univ. of Washington).

DNA. The *Hin*dIII/*Hpa* I fragment of the SV40 genome (complete origin, nucleotides 5171–499) was cloned into the polylinker of M13mp8, excised as a *Hin*dIII/*Eco*RI fragment, and recloned into pAT153 to create the plasmid pSV-Ori. To construct the complete origin DNA circle the 572-bp *Hin*dIII/*Eco*RI fragment of pSV-Ori was purified, blunt ended with the large fragment of *E. coli* DNA polymerase I in the presence of [α-^{32}P]dCTP and [α-^{32}P]dTTP plus unlabeled dATP and dGTP, recircularized with T4 DNA ligase under dilute conditions, extracted with phenol/chloroform, precipitated with ethanol, and electrophoresed through a 3.5% polyacrylamide gel (acrylamide/bisacrylamide, 10:1) at 7 V/cm for 12 hr. The gel was covered with plastic wrap and exposed to x-ray film for ≈15 min to locate the circular DNA. Usually 50–70% of the labeled DNA ligated to form a relaxed DNA circle. The gel was cut with a razor blade and the DNA eluted from the gel slice by incubation in 2–3 vol of 0.5 M NH_4OAc/50 mM EDTA/0.1% SDS at 37°C for 12 hr on a rotating wheel. The eluate was then spun through a 0.22-μM cellulose acetate filter in a Costar Spin-X microcentrifuge tube, extracted with phenol/chloroform, and precipitated with ethanol.

The control 622-bp DNA circle contained DNA sequences from *Hin*dIII/*Sal* I in pBR322. The Ori Δ6 circle is derived from the 506-bp *Ssp* I/*Sal* I fragment from pSV-ori Δ6. pSV-ori Δ6 contains the 206-bp *Hin*dIII/*Sph* I fragment of the SV40 origin, with a 6-bp deletion at the *Bgl* I site, inserted into the *Hin*dIII/*Sph* I sites of pML I. The Ori-A/T circle is

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Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen.

derived from a 607-bp *Sca* I/Nco I fragment from the plasmid pOR.1134. This plasmid contains the *Hin*dIII/*Nco* I region of the SV40 genome (nucleotides 5171–338), with an internal deletion of nucleotides 13–314, cloned into the *Hin*dIII/*Nco* I sites of pKP55 (23). Note that the predominant topoisomer formed upon recircularization depends precisely on the length of the DNA fragment. All preparations were checked to demonstrate that >90% of the isolated circles were covalently closed by heating to 100°C for 3 min, running on acrylamide gels as described above, and determining the fraction of templates that were converted to single-stranded circles and linears.

Reactions. Topology shift reactions contained 0.5 ng of circular DNA (1000–2000 cpm), 200 ng of immunoaffinity-purified SV40 T antigen, 5 units of calf thymus topoisomerase I (BRL), 2 mM ATP, 8 mM $MgCl_2$, 40 mM Hepes-KOH (pH 7.4), 50 mM NaCl, 0.5 mM dithiothreitol, and 100 ng of 3T3 cell DNA as carrier in a final vol of 25 μl. Reactions were carried out for 1 hr at 37°C, stopped by adding 1/10th vol of 1% SDS, 0.5 M EDTA, incubated with 20 μg of proteinase K at 37°C for 15 min, and extracted with phenol/chloroform. Reaction products were separated by electrophoresis through a 3.5% polyacrylamide gel (acrylamide/bis-acrylamide, 10:1) at 7 V/cm for 12 hr. Gels were vacuum dried and the reaction products were visualized by autoradiography. When tested, we used 1.5 μg of *E. coli* SSB, 40 mM phosphocreatine, and 1 μg of creatine phosphokinase.

Dissociation Rates. ^{32}P-end-labeled linear origin DNA fragment (0.5 ng) was incubated with 100 ng of SV40 T antigen at 37°C for 10 min in a buffer containing 8 mM $MgCl_2$, 40 mM Hepes-KOH (pH 7.4), 50 mM NaCl, 0.5 mM dithiothreitol, and 150 ng of 3T3 cell DNA as carrier. The extent of binding was measured by vacuum filtration through Millipore 0.45-μm HA nitrocellulose filters, followed by washing with 10 ml of 20 mM Hepes-KOH (pH 7.4), 50 mM NaCl, 1.5 mM $MgCl_2$. Under these conditions, control DNA fragments containing no T-antigen binding sites were not retained on the filter, while 50–100%, depending on the ATP concentration, of the DNA fragments containing T-antigen binding sites were retained. Dissociation rates were determined by adding 3 μg of the plasmid pSV-ori, after the initial binding, and following the extent of filter binding at the indicated times. The rate constant (k) for dissociation is the slope of the curve of ln(percent bound) versus time. Dissociation rates were determined without ATP, with 2 mM ATP, and with 2 mM adenosine 5′-*O*-(3-thiotriphosphate).

RESULTS

CTo study the interactions between SV40 T antigen and the replication origin, T antigen was bound to a 571-bp, covalently closed relaxed DNA circle containing the complete SV40 replication origin in a reaction mixture containing calf thymus topoisomerase I. In a control reaction, treatment of this molecule with topoisomerase I alone yielded an equilibrium distribution of products consisting primarily of the relaxed DNA form, plus ≈20% of the +1 topoisomer and a very small amount of the −1 topoisomer (Fig. 1*A*, lane 3). This equilibrium distribution is determined by the integral number of helical turns in the circle (24, 25). Addition of T antigen and ATP to this reaction mixture yielded three specific products, the −1, −2, and −5 topoisomers (lane 4). Since T antigen itself has no topoisomerase activity, T-antigen binding to the origin in the absence of exogenous topoisomerase I produced no topologic change in the template DNA (lane 2).

The linking difference for each reaction product was determined in the following way. First, the reaction products were compared to a set of markers generated by treatment of either the purified relaxed or +1 topoisomer with DNA gyrase. The topoisomer standards obtained by treatment of

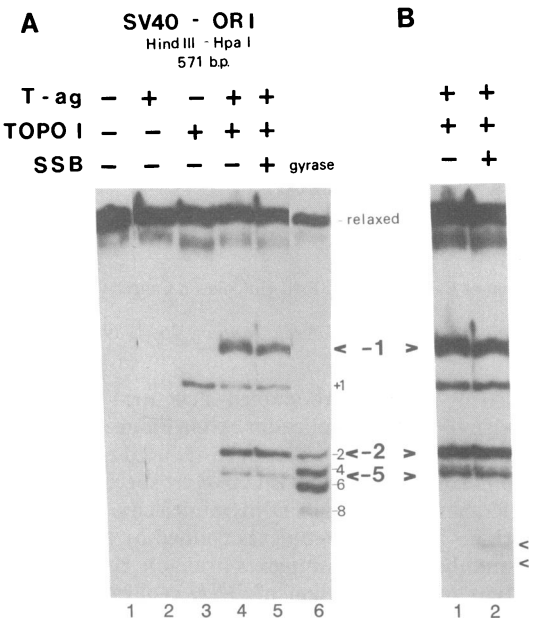

FIG. 1. T antigen changes the conformation of origin DNA. A ^{32}P-labeled 571-bp relaxed double-stranded DNA circle containing the *Hin*dIII/*Hpa* I region of the SV40 genome was prepared. This circle contained the complete SV40 replication origin. The circle was incubated in the presence or absence of SV40 T antigen, calf thymus topoisomerase I, and *E. coli* single-stranded DNA binding protein for 60 min at 37°C. The DNA reaction products were purified, separated by electrophoresis through a 3.5% polyacrylamide gel, and visualized by autoradiography. The relaxed input DNA template is indicated as are the three T-antigen-dependent reaction products: the −1, −2, and −5 topoisomers. Lane 5 contains a set of markers generated by treatment of the relaxed DNA circle with *Micrococcus luteus* DNA gyrase and the linking differences of the supercoiled products are indicated. (*B*) A long exposure of two lanes from *A* to demonstrate the two *E. coli* SSB-dependent products (<).

the relaxed circle with gyrase are shown (recall that gyrase changes linking number in steps of 2) (Fig. 1*A*). Second, we purified each of the T-antigen-induced reaction products and monitored the intermediates obtained by gradual relaxation with limiting amounts of either eukaryotic or prokaryotic topoisomerase I (data not shown). The intermediates obtained by relaxation with either topoisomerase were those expected for a circle with the assigned linking difference. Since prokaryotic topoisomerase I will not relax positively supercoiled DNA (26), the reaction products were unambiguously shown to be negatively supercoiled.

SV40 T antigen contains a helicase activity that, in the presence of a single-stranded DNA binding protein, can extensively unwind the template DNA. Inclusion of *E. coli* SSB in the reaction did not stimulate the formation of either the −1, −2, or −5 topoisomers (Fig. 1*A*, lane 5), demonstrating that these products result from a localized origin–T-antigen interaction. *E. coli* SSB did, however, promote the formation of two new reaction products (Fig. 1*B*, lane 2). These are probably single-stranded DNA circles (plus and minus strands) produced by complete unwinding of nicked template molecules since these forms comigrate precisely with single-stranded circles obtained either by heat denaturation or *E. coli* exonuclease III treatment of nicked double-stranded circles.

We observed that ATP was required for the T-antigen-dependent change in origin circle topology, and that an ATP regenerating system did not increase the yield of product (Fig. 2*A*). We found that 2 mM ATP was saturating for this reaction, and that 2 mM dATP, TTP, dCTP, or UTP could partially or completely substitute for ATP. These nucleotides (and their relative efficacy) are exactly the same as those that

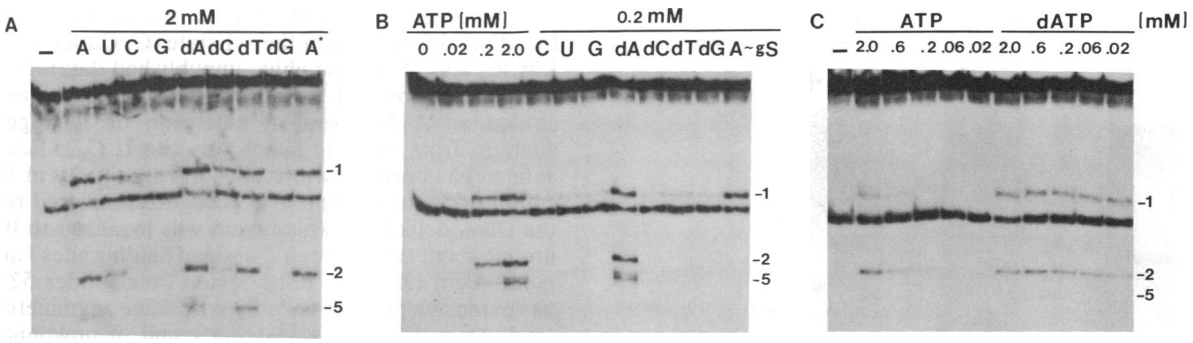

FIG. 2. Nucleotide cofactor requirements for T-antigen-dependent changes in DNA conformation. The 571-bp origin DNA circle was incubated with T antigen, topoisomerase I, and the indicated concentration of deoxy- or ribonucleoside triphosphate at 37°C for 60 min. Reaction products were visualized as in Fig. 1. Lanes – contained no added nucleoside triphosphate. (*A*) Lane A* contained ATP plus creatine phosphokinase and phosphocreatine. (*B*) Lane A~gS contained the nonhydrolyzable ATP analog adenosine 5′-[γ-thio]triphosphate.

have been shown to function as cofactors for the T-antigen helicase (27). This suggests that the T-antigen helicase plays an important role in the change in origin DNA conformation.

With an ATP level of 200 μM, the extent of the reaction was ≈50% of that obtained with 2 mM ATP (Fig. 2*B*). When various other nucleotides were tested at this concentration, only dATP was able to function as a cofactor (Fig. 2*B*). A direct comparison of the ability of ATP and dATP to support the T-antigen–origin interactions demonstrated that 10 to 30 times lower levels of dATP were able to support maximal reaction rates (Fig. 2*C*). Thus, T-antigen-dependent changes in origin structure had a strong preference for dATP as a cofactor.

We also found that the nonhydrolyzable ATP analogs adenosine 5′-[γ-thio]triphosphate (Fig. 2*B*) and AMP-PNP (data not shown) would support the formation of the −1 topoisomer but not the subsequent interactions that result in the −2 and −5 reaction products. T antigen will bind to the replication origin in the absence of ATP. However, we found that the stability of the T-antigen–origin DNA interaction (as determined by measurement of off-rate) was increased 2- to 3-fold by ATP and 4- to 5-fold by adenosine 5′-*O*-(3-thiotriphosphate) (Fig. 3*A*). Therefore, after binding to the replication origin, T antigen, in a reaction that requires ATP binding but not ATP hydrolysis, forms a stable protein–DNA complex in which the origin DNA is relatively underwound.

The T-antigen-induced changes in circle topology were specific for DNA molecules containing the replication origin. A 622-bp circle derived from a *Hin*dIII/*Sal* I fragment of pBR322 showed no T-antigen-induced reaction products (Fig. 4*A*).

Binding of T antigen to the origin was necessary, but not sufficient, to induce changes in origin conformation; DNA sequences important for origin function were also required. A 6-bp deletion within T-antigen binding site II completely eliminates origin function (28). A 541-bp circle containing this mutation, but with intact T-antigen binding sites I and III, showed no T-antigen-dependent topology shift (Fig. 4*B*). Site I binds T antigen at least as strongly as site II (29–31).

The 20-bp A+T-rich region adjacent to T-antigen binding site II is part of the minimal core origin of replication. This sequence does not affect the affinity of T antigen for the replication origin (Fig. 3*B*). However, deletion of this region abolishes origin function (22). Binding of T antigen to a 629-bp circle containing a deletion of the A+T-rich region yielded only the −1 topoisomer (Fig. 4*C*). As observed for the wild-type origin, formation of the −1 topoisomer required ATP but not ATP hydrolysis (data not shown). These observations suggested that the initial T-antigen–origin interactions could occur with this mutant, but that subsequent functional interactions (as defined by the −2 and −5 products) required the A+T-rich region.

The circle containing the A+T region deletion also did not contain T-antigen binding site III. Control reactions with a circle deleted for site III but retaining the A+T region demonstrated that deletion of site III did not result in a qualitative change in the reaction products. However, deletion of either T-antigen site I or III did have quantitative effects on the yield of specific topoisomers, indicating that these auxiliary binding sites outside the minimal origin play important roles in origin function (C. Gutierrez, Z. Guo, J.M.R., and M. DePamphlis, unpublished data).

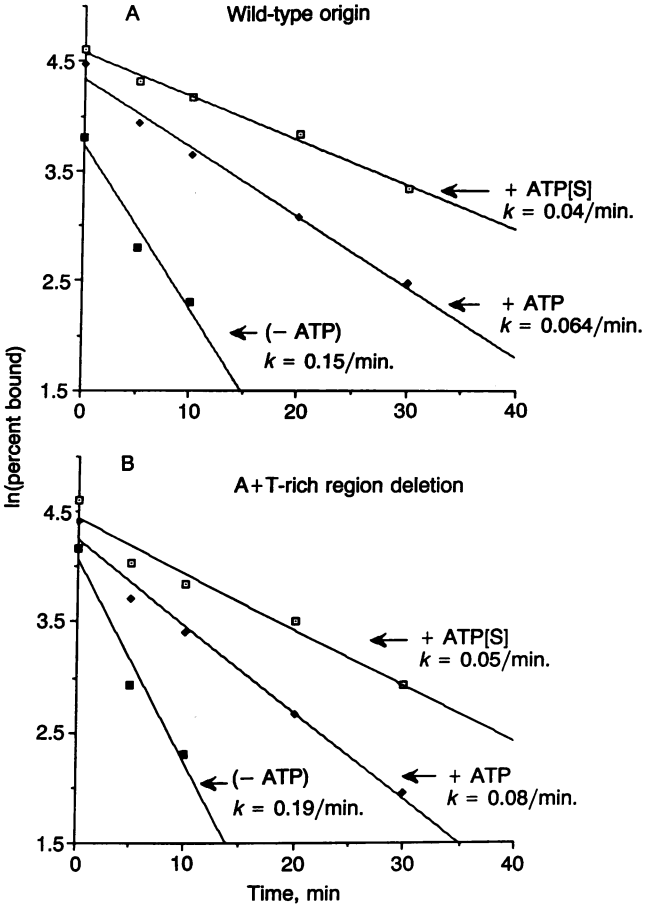

FIG. 3. ATP affects the affinity of T antigen for the replication origin. Filter binding was used to measure the rate of dissociation of T antigen from linear DNA fragments containing either the complete SV40 replication origin (*A*), or an origin containing a deletion of the A+T-rich region (*B*). Measurements were carried out in the absence of ATP or in the presence of 2 mM ATP or 2 mM adenosine 5′-[γ-thio]triphosphate (ATP[S]).

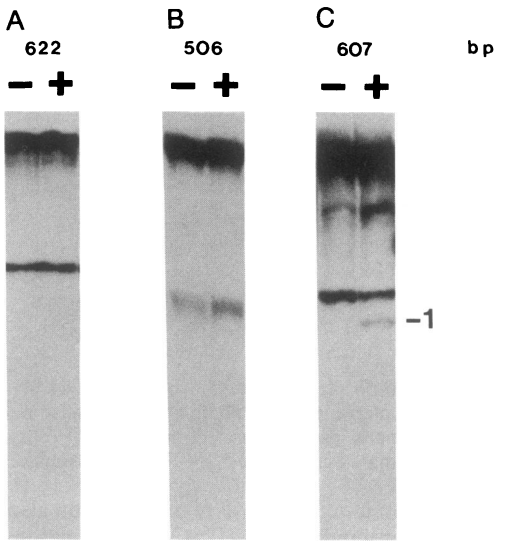

FIG. 4. Specific DNA sequences are required for T-antigen-dependent changes in DNA conformation. DNA circles containing either pBR322 DNA (*A*), SV40 origin DNA with a 6-bp deletion at the *Bgl* I site within T-antigen binding site II (*B*), or SV40 origin DNA with a deletion of the A+T-rich region of the replication origin (*C*) were each mixed with ATP and topoisomerase I, with (lanes +) and without (lanes −) SV40 T antigen, and the reaction products were visualized as described above. The sizes of the DNA circles are indicated.

DISCUSSION

We describe three specific changes in the conformation of the SV40 replication origin upon binding of the initiator protein SV40 T antigen. These three complexes, defined on the basis of their DNA linking number difference, represent a minimal number of T-antigen/origin configurations, since this assay can distinguish only among complexes that differ by a linking difference of at least one. Our data are consistent entirely with the simple interpretation that the linking difference of each origin conformation was a consequence of a change in superhelicity. However, we cannot exclude the possibility that the reaction products also included topologic forms that behaved, in the assays described above, similarly to the behavior predicted for simple superhelical topoisomers. It is unlikely that the −1 and −2 states arose from thermal fluctuations in topology within a single complex, since at least two conditions (i.e., absence of ATP hydrolysis and deletion of the A+T-rich region) suppress formation of only the −2 topoisomer. We conclude that each topoisomer probably represents (at least) one unique T-antigen–origin complex.

Our data suggest that a specific sequence of events occurs during the initiation of DNA replication at the SV40 origin; these events can be defined by the magnitude of the change in origin DNA structure in specific origin–T-antigen complexes. During formation of the initial origin–T-antigen complex, the DNA underwent a conformational change with a net −1 difference in linking number. Formation of the initial complex can occur only through an interaction between T antigen and T-antigen binding site II. The A+T-rich region, which is also part of the minimal core replication origin, was not required.

What is the nature of the conformational change in the −1 complex? Electron microscopy shows that T antigen causes no apparent change in DNA length upon binding to the origin (32). This implies that the origin DNA does not wrap about a T-antigen core, but coiling around just one or a few T-antigen monomers, rather than a large multimeric complex, might not have been detected. Alternatively, interactions between T-antigen molecules bound to sites I and II might unwind the intervening DNA helix. We have shown that site I facilitated formation of the −1 state (C. Gutierrez, Z. Guo, J.M.R., and M. DePamphlis, unpublished data). Also, electron microscopy of T-antigen–origin complexes has demonstrated an ATP-dependent extension of T-antigen–origin contacts from site I to site I plus site II (32). Recently, T antigen has been shown to alter the sensitivity of the replication origin to reagents that recognize unpaired regions of the DNA helix (33). This region was localized to the palindromic sequence between T-antigen binding sites I and II. As pointed out (33), this 10-bp region (nucleotides 5210–5220) has extensive polypurine/polypyrimidine asymmetry, which tends to promote helix instability and, if unwound, would generate a topologic change of the magnitude we observe in the −1 complex.

ATP, but not ATP hydrolysis, was necessary to form the initial complex. In the presence of nonhydrolyzable ATP analogs, T antigen bound to the replication origin and formed a stable complex with a half time for dissociation of ≈20 min. In the absence of ATP, the T-antigen–origin complex was unstable with a half time for dissociation of 4 min. Apparently, ATP hydrolysis destabilized the origin–T-antigen interaction since the half time for dissociation in the presence of ATP was ≈8 min. Perhaps weakening of the initial T-antigen–origin interaction by ATP hydrolysis is necessary for T antigen to modify its contacts with the origin DNA and engage in the subsequent initiation steps defined by the −2 and −5 complexes. In this lower affinity configuration, interactions between T antigen bound to sites II and III may be necessary to stabilize the initiation complex.

In addition to the initial −1 complex, we identified two other origin–T-antigen complexes with linking differences of −2 and −5. We suspect, but cannot prove, that the −1 complex was a precursor to these increasingly underwound states for the following reasons. Nonhydrolyzable ATP analogs caused the accumulation of the −1 topoisomer relative to ATP controls [compare the amounts of the −1 form obtained with 0.2 mM ATP and 0.2 mM adenosine 5′-*O*-(3-thiotriphosphate); Fig. 2*B*]. Consistent with this conclusion, some origin DNA deletions that prevented formation of the −2 and −5 products also caused an accumulation of the −1 complex. Also, in reaction mixtures containing excess T antigen and the wild-type origin, the abundance of the −1 complex reached steady state within 3 min, the −2 complex in ≈30 min, and the −5 complex took >30 min to reach steady-state levels.

Formation of the −2 and −5 complexes required ATP hydrolysis, and required the A+T-rich region flanking T-antigen binding site II. We considered the possibility that the −5 state actually reflected an unusual topologic structure—for example, a knot. However, since the purified −5 DNA can be relaxed efficiently to the −1 state with topoisomerase I, formation of this complex could not have involved any topologic alteration reversible only by double-stranded DNA breaks. One interpretation is that the −5 complex resulted from a discrete interaction between T-antigen molecules bound on either side of the A+T-rich region. We have observed that T-antigen binding site III significantly enhances formation of the −5 complex (C. Gutierrez, Z. Guo, J.M.R., and M. DePamphlis, unpublished data). One prediction of this model is that the spacing between binding sites II and III might affect the degree and/or efficiency of unwinding. We could not determine whether the transition to the −2 and −5 states preserved the conformational change in the −1 complex, or whether reversion of the −1 state was used to drive subsequent structural changes.

It has been shown that in the presence of ATP, T antigen will induce a deformation of the A+T-rich origin region such that it becomes sensitive to $KMnO_4$, which recognizes distortions of the DNA helix, and not to reagents that recognize

unpaired bases (33). It was suggested, therefore, that the A+T-rich region underwent a conformational change more consistent with bending or untwisting (i.e., increased number of bases per helical turn) than with complete unwinding. The magnitudes of the topologic changes we observed impose some constraints on these models. For example, these types of helical deformation could explain the origin of the −2 complex. However, a region of untwisted DNA would have to extend significantly beyond the boundaries of the domain sensitive to $KMnO_4$ to generate a linking difference of −5 (or even −3, if the −2 state was independent and preserved). Also, DNA coiling, rather than bending, would need to be invoked to explain the −5 state. It therefore seems more likely that the −5 complex does indeed contain unwound bases. These might arise within the A+T-rich region of DNA following its initial deformation in the −2 complex. The absence of complexes with linking differences between −2 and −5 is not understood, but note that a cooperative transition from a deformed helix (the −2 complex) to unwound DNA (the −5 complex) could explain the absence of intermediate configurations that might have appeared as −3 and −4 topoisomers.

There are striking parallels between the events we observed at the SV40 replication origin and the sequence of events that occurs during initiation at the *E. coli* replication origin (34). The initial DNA–protein complex at the *E. coli* origin contains the dnaA protein bound to specific origin DNA sequences (3). Formation of this complex requires an ATP-bound form of dnaA, but not ATP hydrolysis (35). In the initial complex, the origin DNA wraps once about a multimeric complex of the dnaA protein (3). The initial −1 complex at the SV40 origin contained a DNA conformational change of similar magnitude, although at the SV40 origin the change may be due to DNA unwinding rather than wrapping about a protein core. Nevertheless, the energy stored in this conformation may be important, in both cases, for driving further steps in the initiation process. The next identified step at the *E. coli* origin is DnaA-mediated ATP-dependent unwinding of an A+T-rich region flanking the DnaA binding sites to form an open complex (36). Formation of the OriC open complex may be analogous to the ATP-dependent deformation of the A+T-rich region in the SV40 origin to form the −2 complex. The OriC open complex provides the DnaB helicase access to the template leading to more extensive unwinding of the flanking origin sequences to form the prepriming complex (36, 37). Similarly, at the SV40 origin we observed the formation of a more extensively unwound T-antigen helicase-dependent −5 complex. We do not know yet whether the −5 complex is the template for the priming reaction and the start of DNA synthesis, or whether more extensive template unwinding beyond this stage is necessary. The basic similarities between the initiator protein–origin interactions in SV40 and *E. coli* suggest significant evolutionary conservation in the mechanisms that initiate DNA replication.

We have been interested in the control of DNA replication during the eukaryotic cell cycle and have demonstrated that a cellular factor necessary for unwinding origin DNA is induced at the G_1 to S transition (21). Induction of this unwinding activity is sufficient to account for the difference in the ability of G_1 as compared to S-phase extracts to replicate SV40 DNA in a cell-free system. We expect that the assay described here will be useful in determining the role that this S-phase-specific cellular unwinding activity plays in the initiation of DNA replication.

Measurement of the change in circular DNA linking number has been helpful in studying other DNA–protein interactions, particularly the DNA unwinding induced by RNA polymerase during formation of transcription initiation complexes. In these experiments, linking differences were detected as changes in the distribution of topoisomers of larger DNA circles (5, 38). Our use of small circular templates offered significant advantages and possibly some disadvantages. It is possible that small circles might impose energetic barriers to topologic change that would not be present in larger templates and thereby inhibit the formation of certain DNA–protein complexes. However, the use of small DNA circles greatly increases both the precision and resolution of this assay. This assay is useful particularly in determining the magnitude of changes in DNA conformation and in resolving individual states in a complex pool of sequential or independent DNA–protein configurations. The series of events occurring at other replication origins may be clarified by this approach.

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1. Dodson, M., Roberts, J., McMacken, R. & Echols, H. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4678–4683.
2. Echols, H. (1986) *Science* **233,** 1050–1056.
3. Fuller, R., Funnell, B. & Kornberg, A. (1984) *Cell* **38,** 889–900.
4. Saucier, J. & Wang, J. (1972) *Nature (London) New Biol.* **239,** 167–170.
5. Wang, J., Jacobsen, H. & Saucier, J. (1977) *Nucleic Acids Res.* **5,** 1225–1241.
6. Hochschild, A. & Ptashne, M. (1986) *Cell* **44,** 681–687.
7. Irani, M., Orosz, L. & Adhya, S. (1983) *Cell* **32,** 783–788.
8. Dunn, T., Hahn, S., Ogden, S. & Schlief, R. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 5017–5020.
9. Hahn, S., Hendrickson, W. & Schlief, R. (1986) *J. Mol. Biol.* **188,** 355–367.
10. Takahashi, K., Matthes, H., Wildeman, A., Zenke, M. & Chambon, P. (1985) *Nature (London)* **319,** 121–126.
11. Wang, J. & Giaever, G. (1988) *Science* **240,** 300–304.
12. Wasserman, S. & Cozzarelli, N. (1986) *Science* **232,** 951–960.
13. Rigby, P. & Lane, D. (1983) *Adv. Virol. Oncol.* **3,** 31.
14. Li, J. & Kelly, T. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 6973–6977.
15. Stillman, B. & Gluzman, Y. (1985) *Mol. Cell. Biol.* **5,** 2051–2060.
16. Stahl, H., Droge, P. & Knippers, R. (1986) *EMBO J.* **5,** 1939–1944.
17. Dodson, M., Dean, F., Bullock, P., Echols, H. & Hurwitz, J. (1987) *Science* **238,** 964–967.
18. Dean, F., Bullock, P., Murakami, Y., Wobbe, R., Weissbach, L. & Hurwitz, J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 16–20.
19. Wold, M., Li, J. & Kelly, T. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 3643–3647.
20. Dean, F., Borowiec, J., Ishimi, Y., Deb, S., Tegtmeyer, P. & Hurwitz, J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8267–8271.
21. Roberts, J. & D'Urso, G. (1988) *Science* **241,** 1486–1489.
22. Tegtmeyer, P., Deb, S., DeLucia, A., Deb, S., Tsui, S., Parsons, R., Partin, K., Baur, C., Dean, F. & Hurwitz, J. (1988) in *Cancer Cells,* eds. Kelly, T. & Stillman, B. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), Vol. 6, pp. 123–132.
23. Li, J., Peden, K., Dixon, R. & Kelly, T. (1986) *Mol. Cell. Biol.* **6,** 1117–1128.
24. Horowitz, D. & Wang, J. (1984) *J. Mol. Biol.* **173,** 75–91.
25. Shore, D. & Baldwin, R. (1983) *J. Mol. Biol.* **170,** 983–1007.
26. Wang, J. (1971) *J. Mol. Biol.* **155,** 523–533.
27. Goetz, G., Dean, F., Hurwitz, J. & Matson, S. (1988) *J. Biol. Chem.* **263,** 383–392.
28. Gluzman, Y., Sambrook, J. & Frisque, R. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 3898–3902.
29. Deb, S. & Tegtmeyer, P. (1987) *J. Virol.* **61,** 3649–3654.
30. DePamphlis, M. & Bradley, M. (1986) in *The Papovaviridae,* ed. Salzman, N. P. (Plenum, New York), Vol. 1, pp. 99–214.
31. Borowiec, J. & Hurwitz, J. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 64–68.
32. Dean, F., Dodson, M., Echols, H. & Hurwitz, J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8981–8985.
33. Borowiec, J. & Hurwitz, J. (1988) *EMBO J.* **7,** 3149–3158.
34. Kornberg, A. (1988) *J. Biol. Chem.* **263,** 1–4.
35. Sekimizu, K., Bramhill, D. & Kornberg, A. (1987) *Cell* **50,** 259–265.
36. Bramhill, D. & Kornberg, A. (1988) *Cell* **52,** 743–755.
37. Funnell, B., Baker, T. & Kornberg, A. (1987) *J. Biol. Chem.* **262,** 10327–10334.
38. Amouyal, M. & Buc, H. (1987) *J. Mol. Biol.* **195,** 795–808.