Unwinding of Duplex DNA from the SV40 Origin of Replication by T Antigen

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The T antigen specified by SV40 virus is the only viral-encoded protein required for replication of SV40 DNA. T antigen has two activities that appear to be essential for viral DNA replication: specific binding to duplex DNA at the origin of replication and helicase activity that unwinds the two DNA strands. As judged by electron microscopy, DNA unwinding is initiated at the origin of replication and proceeds bidirectionally. Either linear or circular DNA molecules containing the origin of replication are effective substrates; with closed circular DNA, a topoisomerase capable of removing positive superhelical turns is required for an efficient reaction. Presence of an origin sequence on duplex DNA and a single-strand DNA-binding protein appear to be the only requirements for T antigen to catalyze unwinding. This reaction mediated by T antigen defines a likely pathway to precise initiation of DNA replication: (i) the sequence-specific binding activity locates the origin sequence, (ii) the duplex DNA is unwound at this site, and (iii) the DNA polymerase and primase begin DNA replication. A similar pathway has been inferred for the localized initiation of DNA replication by bacteriophage λ and by *Escherichia coli* in which a sequence-specific binding protein locates the origin and directs the DnaB helicase to this site. Observations with the SV40 system indicate that localized initiation of duplex DNA replication may be similar for prokaryotes and eukaryotes.

THE INITIATION OF GENOME DUPLICATION typically requires the precise selection and controlled utilization of specific sites on duplex DNA (*1, 2*). In spite of substantial progress in understanding this process in prokaryotic replication systems (*2, 3*), the recognition mechanisms for eukaryotic origins have been elusive (*3*). The SV40 virus has been a widely studied model system for eukaryotic DNA replication because of a defined origin sequence (*ori*SV40) and the identification of the T antigen protein as the only viral-encoded protein required for SV40 replication (*4, 5*).

Analysis of SV40 replication has progressed largely because of development of an in vitro system that depends on *ori*SV40 (*6*) and techniques for producing large amounts of T antigen (*7, 8*). Many properties of the in vitro replication reaction have been determined and correlated with the in vivo process, including the requirement for T antigen and the specific origin sequence (*6, 9–17*). Replication in vitro proceeds bidirectionally from the origin (*9, 10, 12*), mimicking the in vivo reaction (*18, 19*).

The role of T antigen in SV40 DNA replication appears to involve at least two distinct properties of the protein: (i) specific binding to duplex DNA in the origin region (*20–23*) and (ii) unwinding of duplex DNA containing *ori*SV40 (*24*) by an intrinsic helicase activity of T antigen (*25*). In addition, T antigen may guide the DNA polymerase α–primase complex to the origin region; polymerase α is immunoprecipitated with T antigen (*26*), and the replication reactions of SV40 and polyoma work only with the replicative enzyme from a permissive cell type (*15, 16*).

The sequence of events inferred for the initiation of SV40 DNA replication is similar to the initiation of replication at the origin region of *Escherichia coli* (*ori*C) (*27–29*) and bacteriophage λ (*ori*λ) (*30–31*). For these prokaryotic systems, a sequence-specific DNA-binding protein localizes the origin by formation of a multiprotein nucleoprotein structure and initiates a series of protein addition reactions culminating in localized unwinding of the duplex DNA by the DnaB helicase (*2, 27–31*). For *E. coli* and phage λ, the sequence-specific binding proteins and the unwinding proteins are different. For SV40, the T antigen appears to be used for both reactions. We now report visualization by electron microscopy of a bidirectional unwinding reaction mediated by T antigen that is specific for the *ori*SV40 sequence.

The origin-containing plasmid DNA, pSVLD, was chosen as the substrate because its large size (9979 bp) allowed the observation of molecules with extensive regions of unwound DNA. The DNA was incubated with SV40 T antigen in the presence of adenosine triphosphate (ATP), an ATP regenerating system, HeLa topoisomerase I, and *E. coli* single-strand binding protein (Ssb). The *E. coli* Ssb was chosen to coat the unwound single strands because its structure has been characterized by electron microscopy (*1, 31*). The nucleoprotein complexes were fixed by cross-linking with glutaraldehyde and spread for electron microscopy (Fig. 1). The two unwound single strands were coated with Ssb and appeared thickened relative to the intact duplex DNA. The Ssb-coated strands were condensed in length about 2.7 times relative to duplex DNA (*1*). Circular DNA molecules unwound into the theta structure associated with replication of closed circular DNA are shown in Fig. 1, A and B (*33*). The unwound circular DNA was cut once by the restriction enzyme Aat II to localize the unwinding with respect to *ori*SV40 (Fig. 1, C to E) and cleaved twice by Eco RI, liberating linear SV40 viral DNA (Fig. 1, F to H). The reaction required T antigen, ATP, and a single-strand binding protein (Table 1). Similar properties have been found for the unwinding reaction when assayed by gel electrophoresis (*24*). As described below, the reaction also required *ori*SV40 and, in the case of a closed circular DNA substrate, a topoisomerase.

We analyzed DNA molecules cleaved with restriction endonuclease Aat II, which cuts pSVLD once and places the SV40 origin 35% from one end of the linearized DNA, to determine whether the unwinding mediated by T antigen was unidirectional or bidirectional (Fig. 1, C to E). In a second experiment, unwound molecules were cut with Eco RI, which allows visualization of unwinding on the SV40 DNA subfragment of the pSVLD plasmid (Fig. 1, F to H); on these smaller DNA molecules, the positions of less extensively unwound regions could be mapped more precisely.

The distances from the DNA ends to the unwound region were measured and plotted for DNA molecules cleaved with Aat II (Fig. 2A) or Eco RI (Fig. 2B). The unwound regions span the replication origin, suggesting that the unwinding reaction is

Table 1. Requirements for DNA unwinding mediated by T antigen. Unwinding reactions were carried out as described in the legend to Fig. 1, except that reaction components were omitted as indicated. A molecule was scored as unwound if it carried an Ssb-coated region.

T antigen	ATP	Ssb	Total molecules examined (No.)	Unwound (%)
+	+	+	300	24
−	+	+	300	<0.3
+	−	+	300	<0.3
+	+	−	300	<0.3

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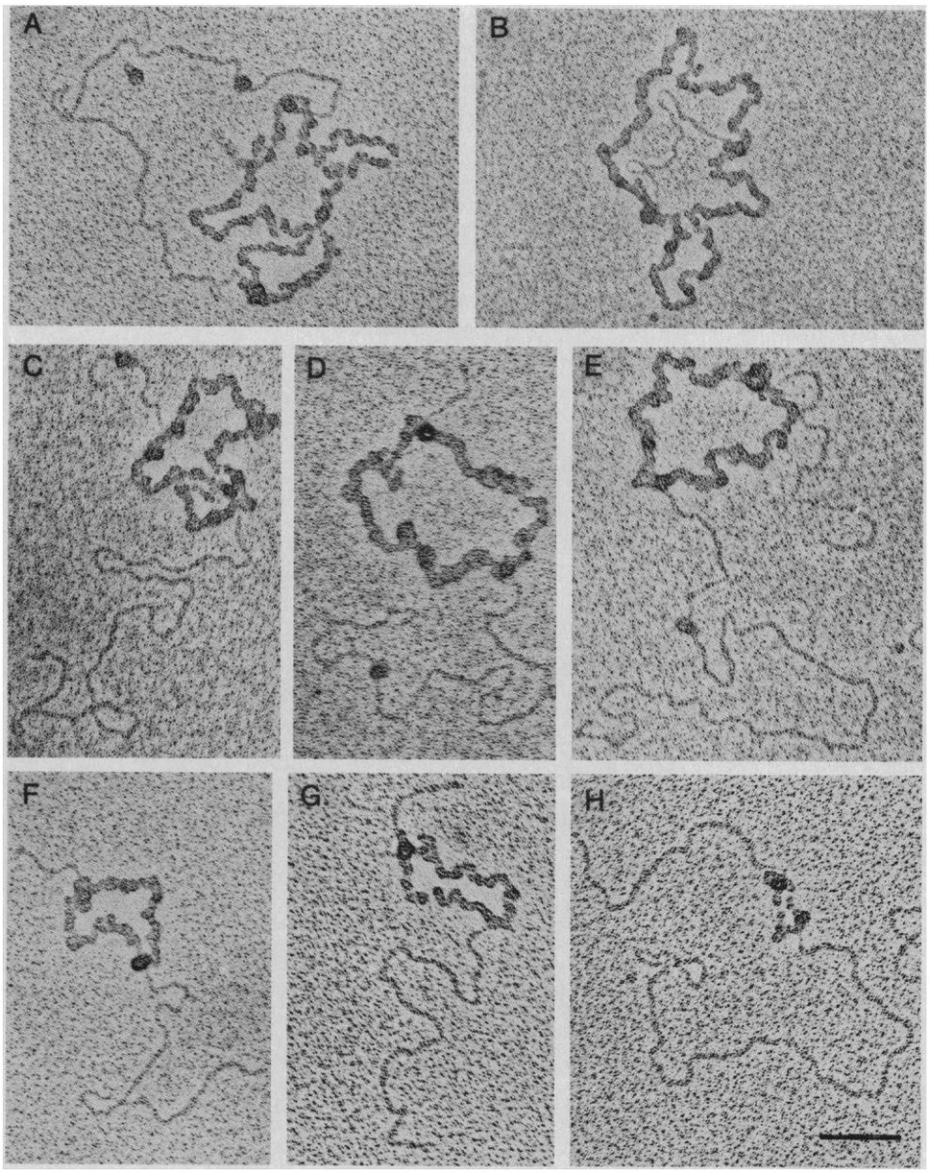

Fig. 1. Electron micrographs of DNA molecules after unwinding with T antigen. (**A** and **B**) Partially unwound circular plasmid DNA molecules. (**C, D,** and **E**) Partially unwound molecules linearized by restriction endonuclease Aat II. (**F, G,** and **H**) Partially unwound molecules cleaved with restriction endonuclease Eco RI to generate a linear, 5243-bp SV40 viral DNA. Knobs that appear in a region of duplex DNA, as in (A), may either be topoisomerase I or nonspecifically bound T antigen. The substrate was relaxed circular DNA of plasmid pSVLD (*24*). Unwinding reactions (30 μl) contained 40 m*M* creatine phosphate (disodium salt, *p*H 7.7), 7 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 4 m*M* ATP, creatine kinase (33 μg/ml), 0.36 μg of relaxed pSVLD DNA (9979 bp), 10 units of HeLa topoisomerase I, 0.6 μg of *E. coli* Ssb, and 0.7 μg of T antigen. The reaction mixtures were incubated for 2 hours at 37°C, terminated by the addition of glutaraldehyde to 0.1%, and incubated again for 15 minutes at 37°C. The mixtures were then passed over a Sepharose 4B column (4 by 0.5 cm) previously equilibrated with buffer (40 m*M* Hepes-KOH, *p*H 7.6, 11 m*M* magnesium acetate). The peak fraction was diluted 1 to 4 in column buffer to a final DNA concentration of about 1 μg/ml, and electron microscopy was carried out by the polylysine technique of Williams (*32*). Grids were rotary shadowed with tungsten. Samples to be cut with restriction enzyme after fixation in glutaraldehyde were passed through a Sepharose 4B column that had been equilibrated with the appropriate restriction buffer. The peak fraction was treated with restriction enzyme, the products were again passed over a Sepharose 4B column, and the nucleoprotein complexes were examined by electron microscopy as described above without further dilution. The lengths of projected molecules were measured on a Numonics 2400 digitizing board. All other techniques and materials were as described (*24, 31*). (Bar = 0.1 μm).

nearly always bidirectional and proceeds at about the same rate in each direction. The center of each unwound region was calculated and plotted (Fig. 3) and shown to distribute symmetrically around the position of the replication origin for DNA molecules cleaved with either Aat II (Fig. 3A) or Eco RI (Fig. 3B) (a few exceptions can be seen, presumably examples of asynchronous bidirectional unwinding).

Topoisomerase was expected to be required with closed circular DNA only to remove positive turns and not as an intrinsic component of the unwinding reaction. This was tested with a linear DNA substrate. After cleavage by Sal I, the plasmid pSVLD DNA was incubated with T antigen, ATP, and *E. coli* Ssb under the standard reaction conditions in the absence of a topoisomerase. Electron microscopy of the DNA revealed extensive unwinding, showing that linear DNA is active as a substrate. Three classes of product molecules were observed (Fig. 4): (i) DNA with unwound regions localized within the interior of the molecule (unwinding bubbles) (Fig. 4, A and D, right), (ii) DNA with an unwound region

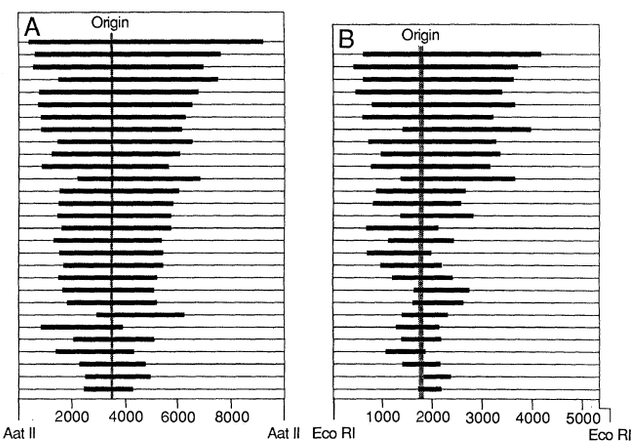

Fig. 2. Position of unwound regions in pSVLD DNA with respect to the SV40 replication origin. After the unwinding reaction, the nucleoprotein complexes were cleaved with either (**A**) Aat II, which linearized the 9979-bp plasmid, or (**B**) Eco RI, which cut the plasmid twice and liberated a 5243-bp fragment that contained the SV40 origin (the SV40 genome). Molecules were photographed, and the lengths of the two duplex DNA arms were measured from the projected micrographs. The shorter DNA arm was taken to be the one closest to the replication origin; this arm is placed to the left in the figure, and the molecules are arranged in decreasing order of the size of the unwound region. Thin lines represent duplex DNA arms; solid bars represent the unwound DNA region coated by Ssb. The position of the SV40 replication origin is shown. The 2972-bp plasmid pSV01ΔEP (*12*) was included as an internal length standard; the standard deviation of the length measurements was 4.5%.

Fig. 3. Bidirectional DNA unwinding by T antigen. The center of the unwound region in each molecule shown in Fig. 2 was calculated. The positions of the centers were plotted relative to distance along the origin-containing DNA molecule for (**A**) the Aat II–generated linear DNA, and (**B**) the Eco RI–generated fragment. A diagram of the fragment with the replication origin is shown below each graph.

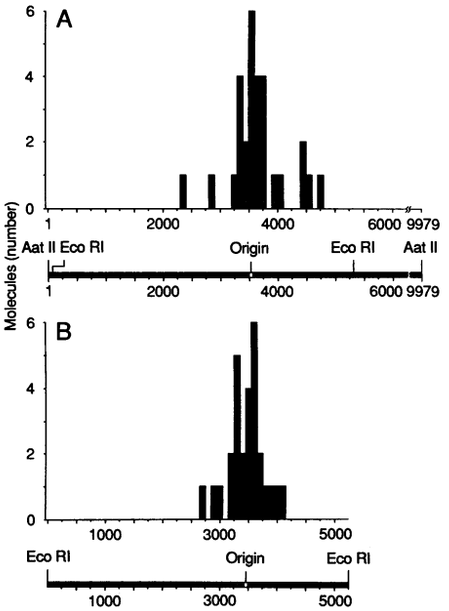

Fig. 4. Electron micrographs of unwound structures produced on linear pSVLD DNA. The DNA substrate was linear pSVLD plasmid DNA produced by digestion with Sal I. The unwinding reaction was carried out and the products were analyzed as described in the legend to Fig. 1. The Ssb-coated structures were either confined to the interior of the linear molecule (molecules with bubbles) (**A** and **D**), extended to one end of the molecule (**B** and **C**), or covered the entire molecule (fully unwound molecules) (D).

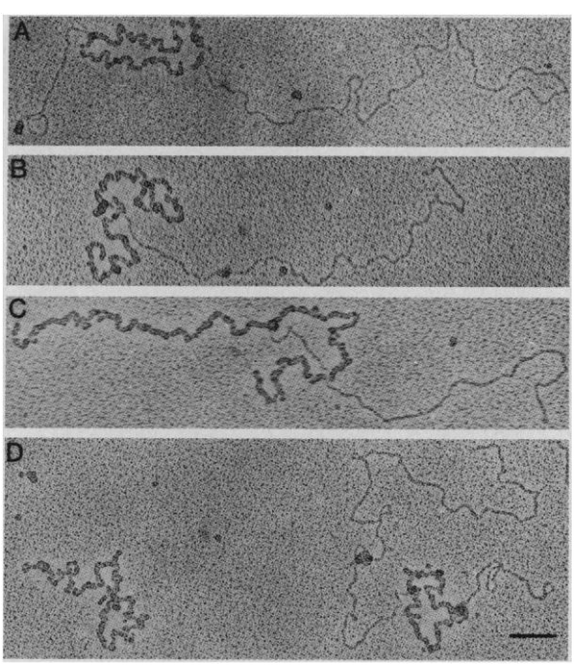

Table 2. Origin specificity and topological requirements of the DNA unwinding reaction. Unwinding reactions were carried out with either circular or linear DNA, as indicated. The *ori*SV40 plasmids were pSVLD and pSVLD 6-1 (*24*); the *ori*λ plasmid was pRLM4 (*31*). Molecules carrying an Ssb-coated region were classified as indicated in Fig. 4.

DNA substrate	Distribution of unwound molecules				
	With bubbles (No.)	Unwound to an end (No.)	Fully unwound (No.)	Total examined (No.)	Unwound (%)
*ori*SV40 circle	73	13*	64	400	37
*ori*SV40 6-1 circle	2	3*	2	400	2
*ori*λ circle	0	0	0	300	<0.3
*ori*SV40 linear	25	65	92	400	45
*ori*SV40 6-1 linear	1	19	14	400	8
*ori*λ linear	0	0	0	300	<0.3

*Partially unwound circular molecules in which the DNA has broken in one or more places during the reaction were arbitrarily placed under the heading of "unwound to an end."

extending out to one end of the molecule (Y forms) (Fig. 4, B and C), and (iii) fully unwound linear DNA (Fig. 4D, left). As for the circular molecules, the location of the unwinding bubbles was consistent with bidirectional replication from *ori*SV40.

In other experiments, the origin specificity of the reaction was verified. To ensure that unwinding of the linear DNA did not originate from the ends generated by the restriction enzyme, because the helicase activity of T antigen can act on duplex DNA bordered by a single-strand region (*24, 25*), we used as substrates the DNA molecules pSVLD, pSVLD 6-1 (*24*), and pRLM4 (*30, 31*). Plasmids pSVLD and pSVLD 6-1 are identical, except that pSVLD 6-1 carries a 6-bp deletion in *ori*SV40 that renders it defective for replication both in vitro (*24*) and in vivo (*34*); pRLM4 is a 6138-bp plasmid DNA containing the replication origin of *ori*λ. For circular DNA substrates, *ori*λ DNA gave no unwinding at all, and the percent of unwound *ori*SV40 6-1 was reduced by a factor of 18 compared to *ori*SV40 (Table 2). For the linear DNA molecules generated by cleavage with Aat II, *ori*λ DNA was also completely unreactive, and unwound *ori*SV40 6-1 was reduced to one-sixth that of *ori*SV40. Similar results were obtained with linear DNA produced by cleavage with Sal I. Thus, origin specificity was clearly demonstrated with both circular and linear substrates. The mutant SV40 origin permits more unwinding with linear than with circular DNA; apparently the residual binding capacity of T antigen is more effective with the linear substrate. Since circular and linear DNA with *ori*SV40 are equally efficient substrates for T antigen (Fig. 4 and Table 2), no topological constraint is necessary to initiate unwinding, and the only role that topoisomerase plays in the process is to remove topological strain that builds up during unwinding of circular molecules.

Our observations are consistent with a critical role for T antigen in providing for bidirectional DNA replication in vivo and in vitro (*9, 10, 12, 18, 19*). The T antigen is presumably located at the unwinding fork and might account for the large knob that is often seen in the unwound DNA molecules at the junction between the single-stranded and duplex DNA regions (Fig. 1, A, B, and F to H). Previous studies have shown that T antigen was preferentially associated with replicating rather than nonreplicating SV40 chromatin in vivo (*35–37*) and that a monoclonal antibody specific for T antigen inhibited the elongation in vitro of SV40 DNA chains initiated in vivo (*37*). It appears that T antigen is both a site-specific DNA-binding protein and a DNA-unwinding protein.

Furthermore, it does not require any superhelical strain to initiate or propagate the unwinding reaction. The 10-kbp linear DNA can be unwound to completion, which suggests that T antigen requires no auxiliary factors except a single-strand binding protein. This inference is consistent with the finding that linear or circular DNA with *ori*SV40 can be replicated effectively by a purified replication system containing T antigen, HeLa topoisomerase I, a single-strand DNA-binding protein from HeLa cells, and DNA polymerase α–primase (*38*).

Although T antigen can carry out the DNA unwinding reaction without auxiliary factors, a lengthy (2-hour) reaction in vitro is necessary to accumulate the large number of unwound molecules that we have used for our quantitative studies. The slow step is probably the initiation of unwinding because the propagation is highly symmetric, and most of the observed molecules for the linear substrate are fully unwound (Table 2). The rate-limiting step may be the conversion of T antigen from its initial interaction as a localized DNA-binding protein to its general helicase activity. The efficient replication of SV40 in vivo requires sequences outside of the minimal origin that are responsible for control of transcription of viral genes (21-bp and 72-bp enhancer elements) (*39, 40*). Proteins bound to these sequences might facilitate the transition of T antigen from its binding to unwinding mode by an interaction similar to that required to activate transcription (*2, 40*).

Two extensively studied prokaryotic replication systems, *E. coli* and phage λ, exhibit similarities to the SV40 system. The origin region is recognized by sequence-specific proteins, *E. coli* DnaA and λ O, that undergo multiple DNA-protein and protein-protein interactions to generate a specialized nucleoprotein structure (*2, 27, 30*). These structures guide a series of protein addition reactions culminating in localized unwinding of the origin region by the DnaB helicase (*2, 28, 29, 31*). For *E. coli*, the DnaB-DnaC protein complex is required for association with DnaA at *ori*C. Phage λ acquires the host DnaB protein through a tight interaction with λ P protein in an O-P-DnaB structure. In turn, this structure must be partially disassembled by the DnaJ and DnaK heat shock proteins to allow DnaB to function as a helicase. These prepriming reactions define the initiation of DNA replication by DNA primase and DNA polymerase III holoenzyme (*28, 29, 41*). In contrast, for SV40, T antigen provides a highly simplified initiation system; T antigen has both of the two critical activities required for localized initiation of DNA replication: multiple site-specific DNA binding and helicase. Although the detailed mechanisms differ and the examples are limited, it appears that site-specific DNA-binding and unwinding reactions may be a widely used mechanism for localized and controlled initiation of DNA replication with duplex DNA.

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