
Sequence independent duplex DNA opening reaction catalysed by SV40 large tumor antigen

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ABSTRACT

Simian virus 40 (SV40) large tumor antigen (T antigen) is mainly localized in the nucleus where it exhibits two biochemical properties: DNA binding and helicase activity. Both activities are necessary for viral DNA replication and may also enable T antigen to modulate cellular growth. Here we present biochemical and electron microscopic evidence that the helicase activity can start at internal sites of fully double-stranded DNA molecules not containing the SV40 origin of replication. Using T antigen specific monoclonal antibodies, this unwinding reaction can be biochemically divided in an initiation (duplex opening) and a propagation step. The duplex opening reaction (as well as the propagation step) does not depend on a specific DNA sequence or secondary structure. In addition, we have found that T antigen forms an ATP dependent nucleoprotein complex at double-stranded DNA, which may be an essential step for the sequence independent duplex DNA opening reaction.

INTRODUCTION

The simian virus 40 (SV40) large tumor antigen (T antigen) can be considered to be a virally encoded oncogene product (1, 2) whose function during cell transformation has yet to be explored.

T antigen is a DNA helicase (3), which has been shown to play an essential role during the initiation and elongation phase of viral DNA replication (4-9).

The DNA strand separation activity of T antigen was demonstrated in a standard DNA helicase assay, using partially double-stranded (ds) DNA with a single-stranded (ss) region at the 3'-end as substrate (3). T antigen preferentially binds to the ss/ds DNA junction of these substrates and processively unwinds the ds part with a 3' to 5' polarity (10, 11), implying that T antigen, like all known DNA helicases, needs a ss DNA region to initiate DNA unwinding (12-17).

However, during the initiation phase of viral DNA replication, T antigen must start at the SV40 origin, most probably within a fully ds DNA structure. Indeed, it was demonstrated in an *in vitro* SV40 DNA replication system, that T antigen catalyses an origin dependent strand opening reaction on SV40 DNA, followed by bidirectional unwinding of the DNA strands (18). The domain and spacer requirements of the core origin for DNA unwinding were reported to be identical with the origin requirements for SV40 DNA replication, suggesting that the duplex DNA opening activity of T antigen is highly sequence specific (19).

We were interested to find out whether T antigen is able to perform a duplex DNA opening reaction in a sequence independent manner. We have used duplex DNA fragments as substrates for the T antigen helicase and found characteristic biochemical properties demonstrating that the DNA strand separation reaction can start from internal sites independent of DNA sequence or secondary structure. The biochemical data were confirmed by electron micrographs of partially unwound DNA molecules.

MATERIALS AND METHODS

Reagents and enzymes

Nucleotides and analogs, restriction enzymes, DNaseI and *E. coli* DNA polymerase Klenow-fragment were purchased from Boehringer Mannheim, *E. coli* SSB from Pharmacia, phosphocreatine, creatine phosphokinase and papain (insoluble enzyme attached to beaded agarose) from Sigma, nitrocellulose filters (0.45 μ m) from Schleicher and Schuell.

DNA substrates

SV40 F I DNA was prepared from SV40-infected TC7 cells as previously described (20). The 46bp SV40 DNA fragment (SV40 nucleotides 1494-1539) was isolated after restriction of fragment G (obtained by HindIII and BamHI digestion of SV40 DNA, see Fig. 1) with Eco RI, the 53bp SV40 DNA fragment (SV40 nucleotides 4809-4861) after restriction of fragment A (Figure 1) with DdeI, the 65bp SV40 DNA fragment (SV40 nucleotides 3477-3541) after restriction of fragment E (Fig. 1) with Avall.

The construction of the two SV40 DNA containing plasmids, pSV C4 and pSV MO1, were described previously (21). The plasmid pSV C4 includes the 109bp SV40 HinfI-G fragment (SV40 nucleotides 4459-4568), pSV MO1 the 109 bp SV40 HindIII-NcoI fragment (SV40 nucleotides 5171-37). The latter fragment includes the two high affinity T antigen binding sites and the SV40 "core" origin. For unwinding and gel retardation assay the plasmids were linearized by Eco RI restriction.

The 143 bp DNA fragment (including the SV40 HinfI-G fragment) was recovered from the plasmid pSV C4 by Eco RI and HindIII restriction.

All restriction DNA fragments used in the unwinding assays originally had overhanging 5'-ends and were 3'-end labeled with [α -³²P] dNTPs by the *E. coli* DNA polymerase I Klenow-fragment. After labeling a chase was performed for 15 min in the presence of 100 μ M of each of the four unlabeled dNTPs to ensure that all recessed 3'-termini were completely filled (22).

T antigen and antibodies

SV40 T antigen and T antigen specific monoclonal antibodies were prepared as previously described (6).

For the preparation of Fab fragments, 1 mg of a highly purified antibody was incubated with 2 units of insoluble papain (attached to beaded agarose) for 12 h at room temperature in a buffer containing

20mM Tris-HCl (pH7.5), 20mM NaCl, 1mM dithiothreitol and 10mM Na-acetate (pH6.0) (23, 24). Fab fragments and Fc fragment were separated by chromatography in a Protein A-Sepharose CL-4B (Pharmacia) column (0.5cmx2cm). The Fab fragments in the flow through were free of any contaminating papain activity as tested by incubation with purified T antigen for two hours under standard unwinding reaction conditions (see below). No degradation of T antigen could be detected after NaDodSO₄-gelelectrophoresis (25) and silver staining (26).

Duplex DNA fragment unwinding assay

Unless otherwise indicated, unwinding reactions were carried out as follows: reaction mixtures (40μl) containing 35 pmol (as basepairs) [α -³²P] dATP labeled DNA fragments (1000-2000 cpm/pmol), 20mM Tris-HCl (pH7.5), 7mM MgCl₂, 1mM dithiothreitol, 2mM ATP, 50μg/ml creatine phosphokinase, 10mM phosphocreatine (diTris salt), 30μg/ml bovine serum albumine (BSA), 0.3μg of *E. coli* SSB and 150-500ng of SV40 T antigen were incubated for 90 min at 37°C. Reactions were stopped by the addition of 0.1 vol. 3% NaDodSO₄, 150mM EDTA, and the whole mixtures were electrophoresed on NaDodSO₄-polyacrylamide gels (12% for the short DNA fragments and 5% for linear plasmid pSV C4 as substrate) in 25mM Tris, 0.2M glycine, 0.1% NaDodSO₄. The duplex DNA fragments and the displaced strands were then visualized by autoradiography.

In experiments with T antigen specific monoclonal antibodies or with Fab fragments T antigen was preincubated with the respective antibody or the Fab fragment for 20 min on ice before unwinding reaction was carried out.

Electron microscopy

Glutaraldehyde-fixed samples were spread onto the hypophase (redistilled water) by the BAC method (27). The spreading solution contained 0.001% BAC and 16% formamide. Spread material was adsorbed to a positively charged carbonfilm (28) and contrasted in 80% ethanol containing 5mM uranylacetate followed by rotation shadowing with tungsten at an angle of 8°.

DNA gel retardation assay

To bind T antigen to DNA 1μg of T antigen was incubated with 180ng of DNA (linear forms of plasmid pSV C4 or pSV MO1) under the standard unwinding reaction conditions described above except that *E. coli* SSB was omitted. The binding reaction was carried out for 45 min at 37°C. Reactions were terminated by addition of glutaraldehyde to 0.1% and further incubated for 10 min at 37°C. After fixation, 0.1 vol. of gel loading buffer containing 20% Ficoll, 0.1M EDTA, 0.3% bromphenol blue was added and samples were electrophoresed in 1% agarose gels containing 90mM Tris, 90mM boric acid, 2.5mM EDTA for 12h at 4V/cm. DNA was visualized by UV illumination after ethidium bromide staining.

DNAseI protection assay

The SV40 DNA HindIII/BamHI restriction fragment E (see Fig. 1B) was nicktranslated as described

(29). Indicated amounts of T antigen were added to 5ng DNA (80 000cpm/ng) under helicase reaction conditions but without creatine phosphokinase and incubated at 37°C for 45 min. Samples were cooled to 0°C and 50 units of DNaseI and 0.1mM CaCl₂ were added to further incubate on ice for 8 min. 500µl of stop mix (10mM EDTA, 1mM DTT, 10µg/ml BSA, 10mM Hepes, pH7.3) were added and protein DNA complexes were bound to nitrocellulose filters. The filters were washed with 20mM sodium phosphate, 10mM EDTA, 1mM dithiothreitol, pH7.3 and bound DNA fragments were eluted from the filters by incubation with 50µl of 10mM Tris-borate (pH8.3), 0.2% NaDodSO₄, 10% glycerol, 1mM EDTA for 2 h. Eluted DNA fragments were analysed by electrophoresis (300V, 6h) on a 11% polyacrylamide gel (24cm in length) using a Tris-borate, EDTA buffer at pH8.3 followed by autoradiography.

RESULTS

Minimum length of duplex DNA fragments

We incubated duplex DNA fragments of increasing length (see Materials and Methods) with T antigen, ATP, and an ATP regenerating system, and tested for their ability to serve as T antigen DNA helicase substrates. As shown in Fig. 1A, a 46bp ds DNA fragment (as well as smaller ds DNA fragments, data not shown but see 11, 21) proved to be refractory to T antigen helicase even in the presence of the *Escherichia coli* single strand specific binding protein (SSB). However, duplex DNA fragments of 53bp and more were excellent substrates and could be efficiently unwound by T antigen, resulting in single strands with the same electrophoretic mobility as denatured substrate DNA (Fig. 1A).

In Fig. 1B we show the unwinding of a spectrum of ds DNA fragments isolated after Hind III and BamHI restriction of SV40 form I DNA. Some of these restriction fragments (fragment A, B, C, G) are known to contain conformationally distinct sites that deviate from common B-DNA form (30, 31) and may therefore be regarded as potential entry sites for the T antigen helicase. Nevertheless, quantitation of the reaction products (data not shown) revealed no preferred unwinding of any restriction fragment including the origin containing one (fragment B). We want to emphasize that we have chosen here and in the following unwinding experiments a T antigen/DNA ratio which is five- to tenfold higher than that used to determine the SV40 ori sequence specificity of duplex DNA fragment unwinding described before (10, 18, 21).

In contrast to partially ds DNA substrates used in previous experiments (11) unwinding of fully ds DNA fragments was stimulated by SSB (10) (Fig. 1B inset). However, as may be expected, the influence of SSB was less pronounced with the smaller DNA fragments as substrates than with the longer ones (compare Fig.1A lanes 2 and 4 and inset of Fig. 1B). We conclude that unwinding of duplex DNA fragments by T antigen requires a minimum length of the DNA substrate in contrast to partially ds DNA (11) and furthermore, that under the experimental conditions used here duplex DNA fragment

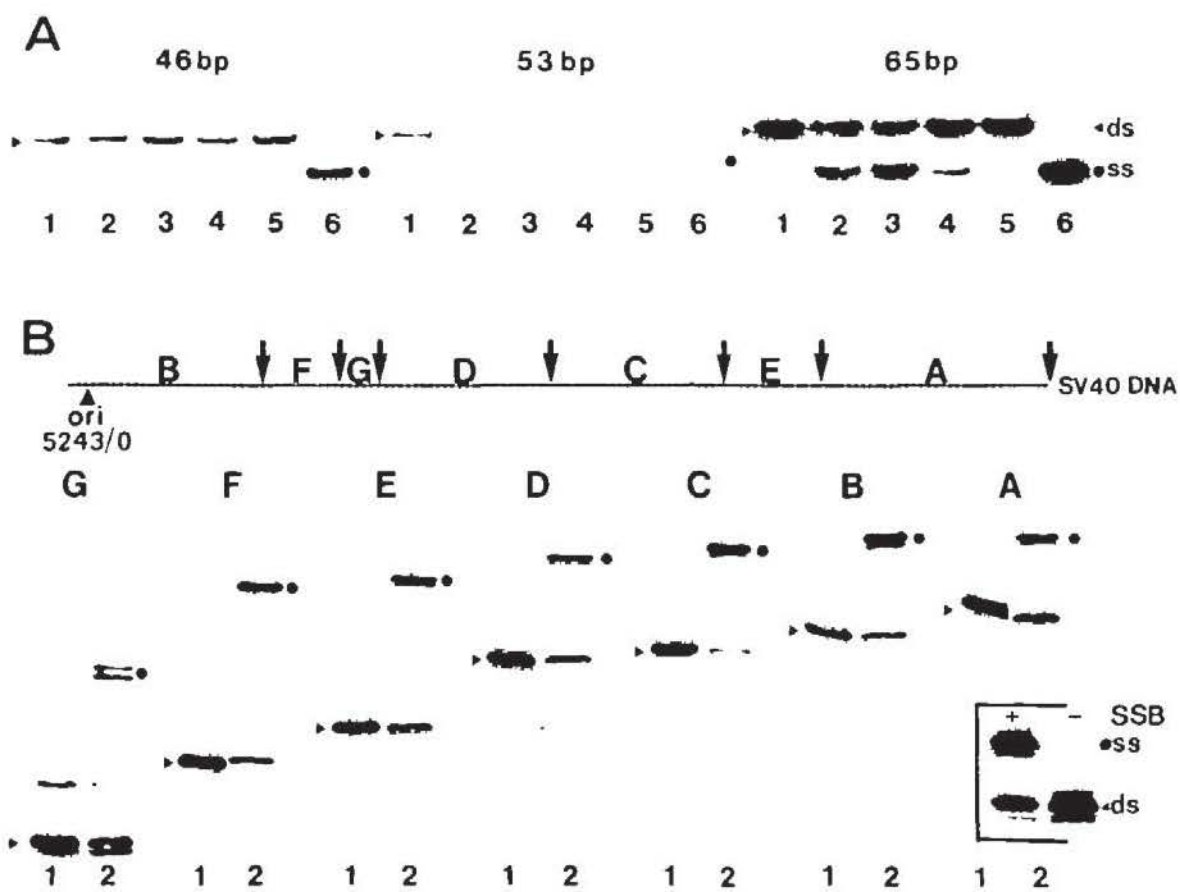


FIG.1 Unwinding of duplex DNA fragments.

Fully ds DNA fragments were incubated under standard helicase reaction conditions (see Materials and Methods). The samples were subjected to gel electrophoresis and autoradiography. The triangles and closed circles mark the position of the ds and ss forms, respectively, of the DNA fragments in the gel. **(A)** Minimal length of the DNA substrate. The length of the DNA fragments, in base pairs (bp), is indicated at the top. Lane 1, without T antigen; lane 2, reaction with 250ng of T antigen; lane 3, reaction with 500ng of T antigen; lane 4, reaction with 250ng of T antigen but without SSB; lane 5, substrate DNA; lane 6, heat denatured substrate DNA. **(B)** SV40 DNA restriction fragments as helicase substrates. At the top the HindIII, BamHI restriction map of SV40 DNA is shown. Arrows point to the restriction sites and the letters point to the position of the restriction fragments in the SV40 map. The size of the fragments is: A, 1169bp; B, 1119bp; C, 943bp; D, 825bp; E, 526bp; F, 447bp; G, 215bp. The origin of replication in fragment B at position 5243/0 (1) is indicated. The individual restriction fragments were isolated and converted to full double strandedness by the Klenow polymerase in the presence of all four deoxyribonucleotides and [α - 32 P] dATP and were used in the unwinding assays as indicated at the top of each panel. Lane 1, without T antigen; lane 2, reactions with 500ng of T antigen. The inset shows a direct comparison of DNA fragment A unwinding in the presence (+) or absence (-) of SSB. The position of the ss fragments were confirmed by electrophoresis of heat denatured substrate DNA. In comparison to Fig.1A, note that the ss forms of the larger DNA fragments used here run more slowly than their ds counterparts.

unwinding is apparently independent of DNA sequences and structures.

Monoclonal antibodies as probes for a T antigen catalysed duplex DNA opening reaction

Using partially ds DNA as a substrate we have shown before that some T antigen specific

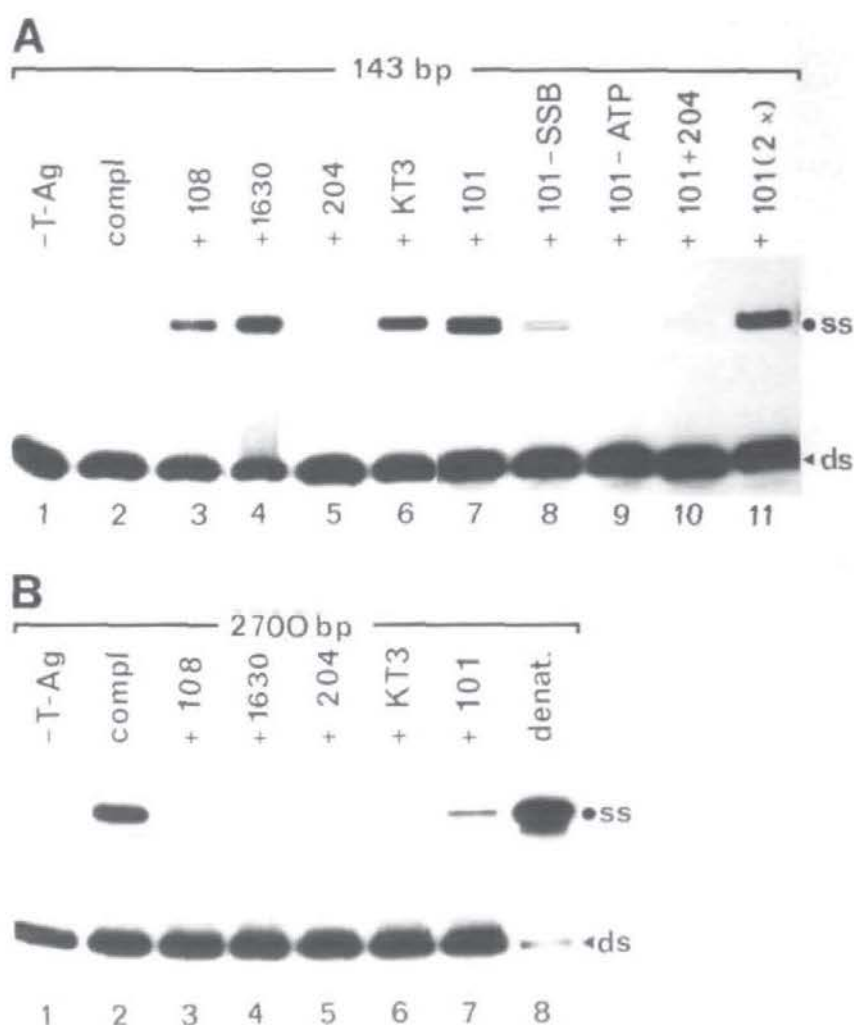


FIG.2 Duplex DNA fragment unwinding activity of T antigen immunocomplexes.

T antigen was preincubated with each one of the T antigen specific monoclonal antibodies, whose PAb numbers are indicated on top of the appropriate lanes (for source of antibodies and some of their characteristics see: 3, 8). (A) A 143bp DNA fragment (see Materials and Methods) was used as substrate. To show optimal stimulation, reactions were performed at relatively low T antigen concentrations. Lane 1, reaction without T antigen but in the presence of 0.75 μ g of PAb 101; lane 2, reaction with 0.15 μ g T antigen; lanes 3 to 10, 0.75 μ g of the indicated antibody was added; lane 8, SSB was omitted; lane 9, ATP was omitted; lane 10, PAb 204 (0.75 μ g) was included in the preincubation; lane 11, 1.5 μ g of Pab 101 was added. (B) Similar as (A) but a linear 2.7kbp DNA (pSV C4, see Material and Methods) was used as substrate in the presence of 0.5 μ g T antigen. Lane 8, heat denatured substrate DNA. The positions of the ds and ss forms of the DNA substrates are indicated.

monoclonal antibodies (PAb 101, KT3) exhibit weak inhibition of the T antigen helicase activity, whereas others have moderate (PAb 108, PAb 1630) or complete (PAb 204, PAb 1613) inhibitory effects (3,8). Testing the same antibodies in the T antigen unwinding reaction, with a 143 bp duplex DNA substrate, we found a dramatic stimulation of the helicase action by antibodies PAb 108, PAb 1630, KT3 and PAb 101 (Fig. 2A). The stimulation was about tenfold compared to control reactions and proved to be highly specific for unwinding of relatively short duplex DNA fragments (53 to about

500 bp in length; data not shown but see below). However, as expected, the T antigen ATPase inhibiting antibody PAb 204 completely inhibited the unwinding of the same DNA fragment, even in the presence of a stimulating (PAb 101) second antibody (Fig. 2A, lane 10). In addition, the finding that the unwinding reaction in the presence of stimulating antibodies is ATP and SSB dependent (Fig. 2A, lanes 8 and 9) demonstrates that the antibody effect is specific.

The opposite effects of the antibodies on T antigen dependent strand separation of duplex DNA fragments and of partially ds DNA substrates can be better understood when the process of T antigen unwinding of duplex DNA fragment is dissected into an initiation (or duplex opening) and a propagation step. The strand opening reaction is not necessary with partially ds DNA substrates whose ss/ds DNA junctions may mimic a replication fork and may therefore be directly accessible to T antigen. According to this model some antibodies may stimulate the strand opening reaction and simultaneously impede the propagation of T antigen unwinding. To test this hypothesis, we investigated the influence of the same antibodies on T antigen unwinding of large duplex DNA molecules, where the propagation step should be more prominent than with the 143bp fragment. As can be seen in Fig. 2B, T antigen efficiently unwound a 2.7 kbp duplex DNA molecule (up to 30% of input DNA were found to be in a single-stranded form after a reaction time of 90 min; lane 2, Fig. 2B) but strand separation was now inhibited by antibodies PAb 108, 1630, 101, KT3 to a similar extent as previously determined for partially ds DNA substrates (Fig. 2B, lanes 3 to 7). With a 1.1 kbp duplex DNA fragment we found neither a stimulation nor a inhibition of the strand separation demonstrating that the antibody effects on both steps (initiation and propagation) were in a equilibrium state with each other (data not shown).

Binding of a T antigen molecule (mono- to tetramer) to each arm of a monoclonal antibody could result in a proper orientation of two closely adjacent T antigen molecules, facilitating the duplex opening reaction as the first step in the unwinding process of duplex DNA fragments (see discussion). If this interpretation were correct, Fab fragments produced from PAb 108 or PAb 101 by digestion with papain should not possess the stimulating activities.

Indeed, highly purified Fab fragments produced from both antibodies (Fig. 3) impeded T antigen unwinding of the 143bp duplex DNA fragment to the same extent as found before for a partially ds DNA (8) and described above for long (2.7kbp) duplex DNA molecules. We postulate that the unwinding of duplex DNA fragments and of partially ds DNA by T antigen can be distinguished by a strand opening reaction that is necessary to start strand separation of duplex DNA fragments.

Analysis of the unwinding reaction by electron microscopy

To definitively show that T antigen is able to perform a strand opening reaction within duplex DNA structures we examined the unwinding of a linear duplex DNA molecule by electron microscopy. In these experiments the 2.7kbp DNA molecule used above was incubated with T antigen, ATP, an ATP regenerating system and SSB. To enrich partially unwound DNA molecules, the incubation time

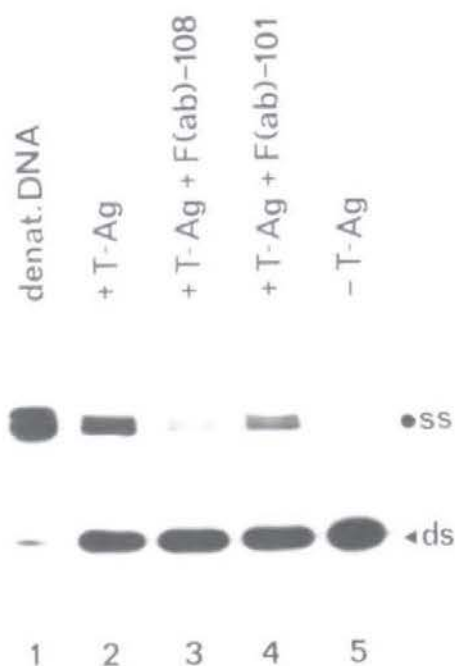


FIG.3 Unwinding of a short (143bp) duplex DNA fragment by T antigen-Fab complexes.

Fab fragments of antibodies PAb 108 and PAb 101 were prepared (see Materials and Methods) and preincubated with T antigen before standard helicase reactions were performed. Lane 1, heat denatured substrate DNA; lane 2, complete reaction with 0.5 μ g of T antigen; lane 3, reaction with 2.5 μ g of Fab prepared from PAb 108; lane 4, reaction with 2.5 μ g of Fab prepared from PAb 101; lane 5, without T antigen.

of the unwinding reaction was reduced to 20-30 min. Under these conditions only 5 to 10% of the input DNA was converted to ss (for efficiency of the unwinding of the 2.7kbp DNA during longer incubation times, see Fig. 2B). Unwinding was stopped by the addition of 0.22M NaCl (11), which also removes unspecifically bound proteins from DNA. The mixture was passed over a Sepharose CL-4B column and after fixation with glutaraldehyde (0.1%) the molecules were spread for electron microscopy. Partially unwound DNA molecules with ss regions of various lengths (representing 70% of reactive DNA molecules) as well as completely ss DNA molecules were observed (Fig. 4). The unwound single strands were coated with SSB and thus appeared thickened relative to duplex DNA (note that SSB-coated strands are condensed in length, about 2.7 times relative to ds DNA (32)). About 20% of the partially unwound DNA molecules showed ss bubbles (Fig. 4A to D) which were located within various internal sections of the DNA indicating a sequence unspecific start of the unwinding reaction. In Fig. 4E and F Y-formed DNA structures are shown. These structures, at least in part, would be the result of expanding unwinding bubbles that have reached one end of the DNA molecule. In addition, the T antigen duplex opening reaction may be facilitated near the ends of duplex DNA fragments. For a detailed analysis, longer DNA molecules such as phage T7 DNA are required. However, the electron micrographs presented in Fig. 4A to D show typical bubbled DNA structures after a T antigen helicase reaction that were never observed in controls performed without T antigen or in the absence of ATP (data not shown). This clearly demonstrates that T antigen is able to perform a duplex opening reaction in the absence of the SV40 ori sequence.

Previous studies have shown that T antigen is salt stably associated with replicating SV40 chromatin (33) at the replication fork (6). In our *in vitro* assays, T antigen of this kind may account for the large knobs that are often seen in the unwound bubbles at the ss/ds junctions (arrows Fig. 4).

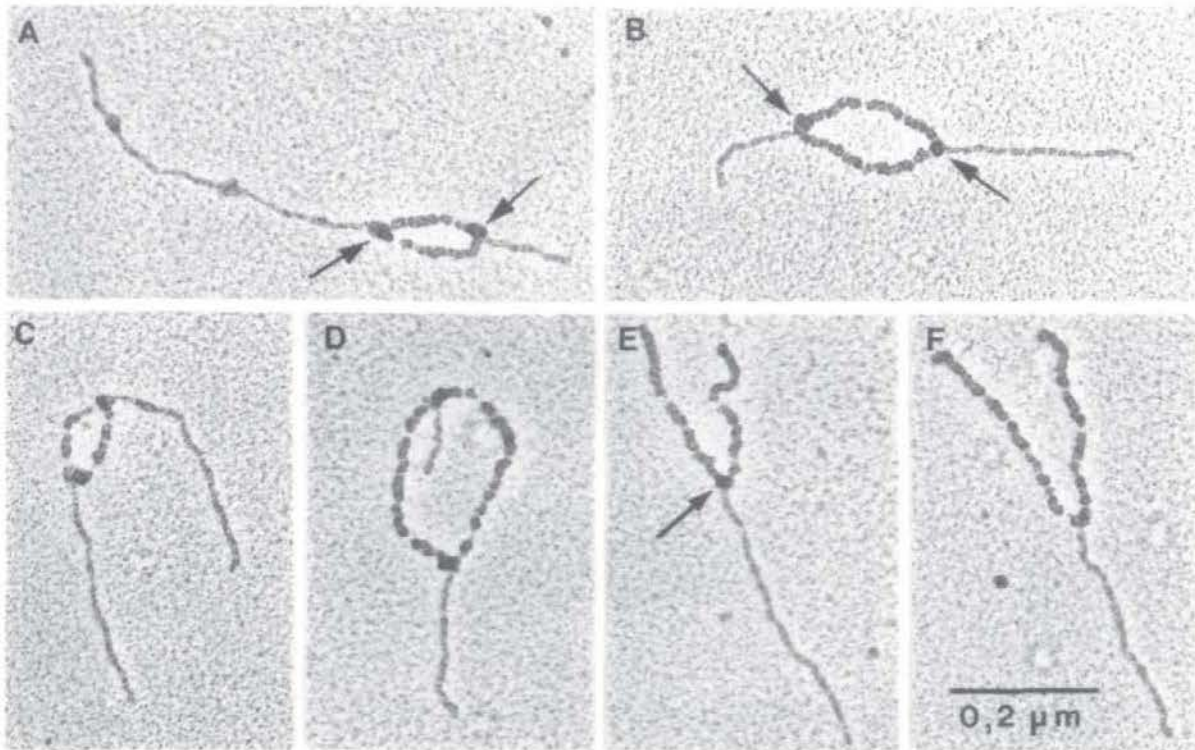


FIG.4 Electron micrographs of partially unwound duplex DNA.

The 2.7kbp DNA (pSV C4) was used as substrate. Unwinding was performed for 20 to 30 min and stopped by the addition of 0.22 M NaCl. Samples were passed through Sepharose CL-4B, subsequently fixed with 0.1% of glutaraldehyde, and used directly for electron microscopy. In the experiment shown a total of 2000 DNA molecules were analysed.

Although the knobs were mostly seen on both unwinding forks of a bubble, it remains uncertain whether the unwinding mediated by T antigen starts uni- or bidirectional.

T antigen interaction with duplex DNA in the presence of ATP

Previous reports on the role of T antigen during the initiation of SV40 DNA replication suggest that the opening of the origin sequences is probably preceded by a sequence specific binding of T antigen resulting in an ATP dependent formation of a specialized nucleoprotein structure (34,35). Since we expected a similar mechanism for the sequence independent duplex opening reaction, the influence of ATP on T antigen binding to ds DNA was examined. First, the binding of T antigen to ds DNA was analysed by the nucleoprotein induced, gel mobility shift assay (36). T antigen was bound to linearized forms of plasmids pSV C4 and pSV MO1, differing only in the presence or absence of a 109bp fragment containing the SV40 origin region, including T antigen binding sites I and II. Both DNA molecules exhibited a reduced mobility in an agarose gel after binding of T antigen in the absence of ATP and after fixation with glutaraldehyde (Fig. 5A). Addition of ATP resulted in an increase of the number of DNA molecules involved in nucleoprotein complex formation and in a greater reduction of their gel electrophoretic mobility in both cases (ori^+ and ori^- DNA) (Fig. 5A, lanes 4). ATP hydrolysis

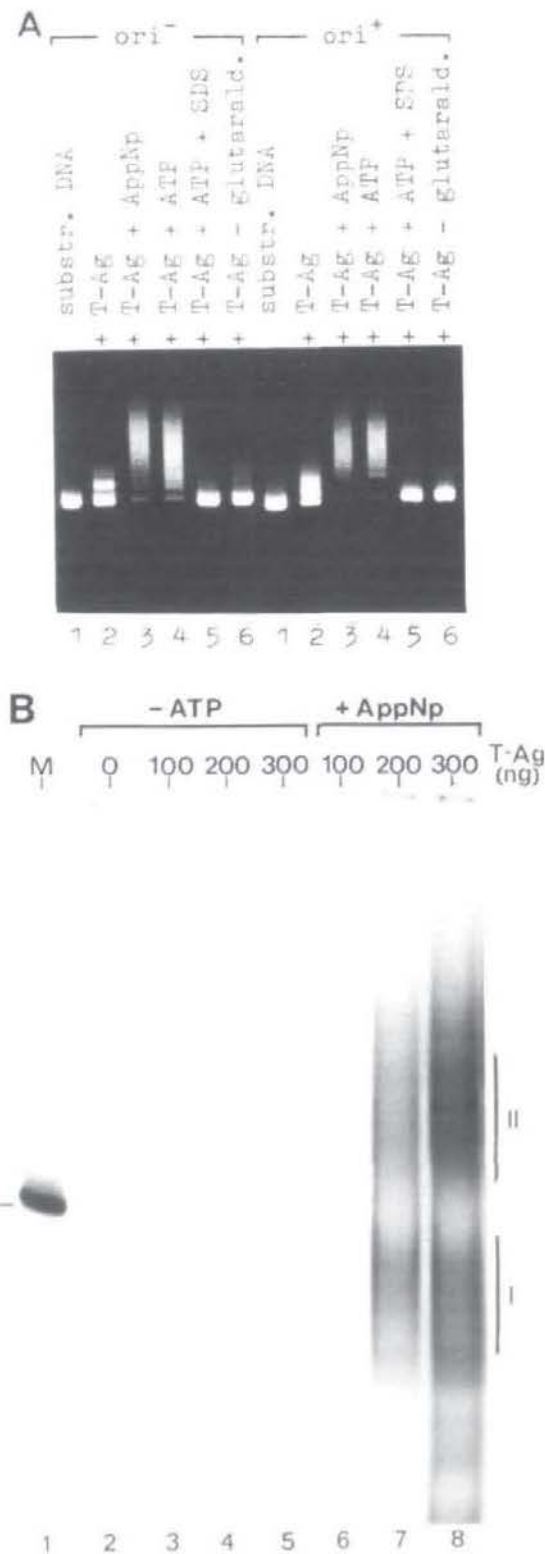


FIG.5 T antigen binding to duplex DNA in the presence of ATP or AppNp.

(A) Agarose gel electrophoresis of nucleoprotein complexes. T antigen was incubated with linear forms of plasmids pSV C4 (ori⁻) or pSV MO1 (ori⁺), as described in Material and methods. Lane 1, substrate DNA; lane 2, without ATP; lane 3, AppNp (4mM) was added; lane 4, ATP (4mM) was added; lane 5, the sample was incubated with 0.5% NaDodSO₄ before electrophoresis; lane 6,

glutaraldehyde treatment was omitted. (B) DNaseI fragment protection of T antigen bound to duplex DNA. Increasing amounts of T antigen (indicated at the top) were bound to 5ng of nick-translated DNA (SV40 fragment E, Fig. 1) in the absence of ATP or in the presence of AppNp (4mM). Analysis of protected fragments was performed similar as described (35, see Materials and Methods). The marker 65bp-fragment (lane M) was obtained by restriction of the fragment E with Avall.

is not required for enhancement of complex formation, since the nonhydrolysable ATP analog AppNp efficiently substituted for ATP in the reaction (lanes 3). When glutaraldehyde fixation was omitted the mobility shift was not detected, indicating a short half-life of the formed nucleoprotein complexes (lanes 6). Comparison of lanes 2 and 4 shows that the origin-containing substrate produces a more ordered band structure with T antigen probably reflecting sequence specific binding. Nevertheless, the overall ATP stimulated DNA binding of T antigen is obviously origin independent.

To examine whether ATP also alters the conformation of the DNA protein complex, we used a DNase protection assay (35). T antigen was incubated at 37° C with a nicktranslated ds SV40 DNA fragment not containing the SV40 ori (fragment E, Fig. 1) under helicase reaction conditions. The complexes were digested with a large excess of DNaseI and isolated by binding to nitrocellulose filters. The DNA fragments retained on the filters were analysed by gel electrophoresis followed by autoradiography (Fig. 5B). In the absence of ATP, T antigen bound only poorly to DNA, mainly protecting fragments of about 60 bp or smaller (lanes 3 to 5, class I). In the presence of the nonhydrolysable ATP analog AppNp or ATP (data not shown) the binding efficiency increased about tenfold as revealed by counting the filter bound radioactivity (data not shown, but compare lanes 4 and 5 with lanes 7 and 8). Gel electrophoretic analysis detected continuous DNA stretches ranging from about 70 to 150 bp (as revealed by co-electrophoresis of DNA marker fragments; classII in Fig. 5B). Very small amounts of larger fragments were seen in the absence of ATP, and then only at higher T antigen concentrations (lane 5). The sudden increase in binding efficiency obtained at T antigen concentrations above 100ng (compare lane 6 and 7) may indicate a cooperative binding mechanism of T antigen to ds DNA in the presence of ATP. The information obtained from DNaseI protection assay is consistent with the result of the gel retardation assay and demonstrates that T antigen binding to ds DNA is both qualitatively and quantitatively changed by ATP or a nonhydrolysable ATP analog.

DISCUSSION

It was suggested that the T antigen catalysed duplex opening reaction is a highly DNA sequence specific process preceded by an ATP dependent binding of T antigen to its recognition sites in the SV40 origin (7, 8, 19, 34, 35). We show here that this reaction is not limited to the SV40 origin but instead T antigen is able to start its helicase activity within duplex DNA structures of apparently any sequence. However, further studies have to show whether the unwinding of substrates without the SV40 origin sequence occurs in a completely random manner or initiates preferentially at sites

with some similarity to replication origins. This reaction is not dependent on any superhelicity but is efficiently performed on linear DNA. Our results may explain earlier reports that SV40 replication can start from nonorigin regions though with low efficiency (37).

We have shown before that T antigen binds directly to ss/ds junctions of partially ds DNA and processively unwinds the ds region with a 3' to 5' polarity. Accordingly T antigen helicase can efficiently unwind very small DNA fragments consisting of only a 5 bp ss tail at the 3'-end of a 20bp duplex. In contrast, similar small but fully ds DNA fragments were not unwound (Fig. 1A; 11, 21). We demonstrate now that a minimum length of about 60bp is necessary for unwinding of duplex DNA fragments by T antigen indicating that several T antigen molecules may be essential for this reaction. The reaction is dependent on SSB in contrast to partially ds DNA substrates which are efficiently unwound in the absence of a single strand specific binding protein (10, 11). Unwinding of both types of DNA substrates can therefore be well distinguished biochemically, most probably demonstrating two different initiation mechanisms. The sequence independent duplex opening reaction could also be performed on circular, covalently closed DNA molecules but only in the presence of a topoisomerase (data not shown).

For efficient duplex DNA fragment unwinding, the T antigen/DNA ratio must be raised about tenfold compared to ori⁺ ds DNA fragments (M. Scheffner, unpublished data). This may be explained by both the high affinity binding of T antigen to the SV40 origin (38-40) and by the AT-rich DNA sequence in the SV40 core origin of replication which may also facilitate the unwinding of ori⁺ DNA as has been reported for DNA unwinding of yeast replication origins (41).

The sequence specific binding of T antigen to its recognition sites in the SV40 origin of replication may result in orientation of T antigen molecules that is optimal for initiation of the duplex opening reaction (19). A similar orientation allowing intermolecular interaction may be achieved in some T antigen immunocomplexes, since several monoclonal antibodies (PAb 108, PAb 1630, KT3, PAb 101) dramatically stimulate the unwinding of a 143bp duplex DNA fragment without the SV40 origin sequence. The proposed role of the antibodies in orienting T antigen molecules is supported by our finding that the Fab fragments of these antibodies do not stimulate the unwinding reaction. Moreover, the same set of monoclonal antibodies which stimulate the unwinding reaction of a 143bp fragment impede the unwinding of a 2.7kbp ds DNA as well as of partially ds helicase substrates (as shown before, 3, 8). This apparent paradox can be resolved by formally dissecting the T antigen unwinding reaction of duplex DNA fragments into two distinct steps. The first step involves most probably the proper interaction of T antigen molecules bound to duplex DNA (of any nucleotide sequence) resulting in localized DNA unwinding. In our *in vitro* assays this reaction seems to be preferentially performed by some T antigen antibody complexes. The second step includes the movement of T antigen in the 3'-5' direction of the strand to which it is bound and the separation of the DNA strands by the helicase activity. This reaction seems to be hindered by a panel of monoclonal antibodies though to different

extents (see Fig. 2B; 3, 8). It is important that neither step, localized unwinding or propagation, requires the presence of the SV40 origin sequence in the DNA substrate.

T antigen unwinding of duplex DNA structures in the absence of the SV40 origin was confirmed by electron microscopic analysis. The electron micrographs (Fig. 4A to D) demonstrate unambiguously that T antigen is able to start its helicase reaction at internal sites of duplex DNA molecules to produce ss bubbles similar in shape to replication eyes. The open end structures observed may be the result of expanding unwinding bubbles that have reached one end of the DNA molecules. In addition, the biochemical differences described above for unwinding of partially and fully ds DNA molecules exclude the possibility that melting at the ends of the DNA fragments provides ss regions which are capable of binding T antigen, to start the helicase reaction in a similar way as observed at ss/ds DNA junctions, just as has been proposed by others (10). Our data, therefore, extend previous work (9) where unwinding of covalently closed circular plasmids not containing the SV40 origin sequence has been described. However, the results obtained in this earlier study did not allow to discriminate between unwinding with respect to i) wrapping of the DNA around T antigen molecules ii) altering the number of base-pairs for each turn of the DNA helix and iii) duplex DNA opening.

The origin specific DNA binding of T antigen is known to be stimulated and qualitatively altered by ATP (34, 35). It has been proposed that this effect is origin specific and a prerequisite for the SV40 ori dependent DNA unwinding. We have shown here (Fig. 5) that the ATP effect on DNA binding is not restricted to the T antigen interaction with the SV40 origin. Gel mobility shift and DNaseI protection experiments demonstrate that T antigen binding to duplex DNA in general is strongly enhanced and qualitatively altered by ATP or its nonhydrolysable analogs. Thus we suggest that T antigen can perform the duplex opening reaction upon the ATP dependent formation of specialized nucleoprotein complexes most probably in a sequence independent manner. Several T antigen molecules (mono- to tetramers) must participate in this process as indicated by the stimulating effect of some antibodies on unwinding of small duplex DNA fragments, their minimal essential length (about 60bp) as substrates and by the ATP effect on T antigen binding to ds DNA.

Finally we note that the SV40 origin independent duplex DNA opening activity described here should enable T antigen to unwind any accessible region of chromosomal DNA *in vivo*. The resulting ss DNA structures must be considered as potential sites for the start of DNA synthesis, transcription or recombination. An uncontrolled duplex DNA opening activity may therefore be of biological relevance for T antigen producing cells and could possibly influence these processes.

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