The EMBO Journal vol.5 no.8 pp.1939–1944, 1986

DNA helicase activity of SV40 large tumor antigen

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Communicated by H.Hoffmann-Berling

Large tumor antigen (T antigen) was extracted from SV40-infected African Green Monkey cells and purified to homogeneity by immunoaffinity chromatography. The purified T antigen preparations unwind DNA duplices of >120 bp in a reaction which is dependent on magnesium ions and ATP hydrolysis. Based on these and other properties of the reaction we classify this newly discovered enzymatic activity as a eukaryotic DNA helicase. The helicase and the known ATPase function of T antigen cosediment with the mono- or dimeric 4–6 S form of T antigen, but not with higher T antigen aggregates. The helicase activity seems to be an intrinsic function of SV40 T antigen. First, several different T antigen-specific monoclonal antibodies interfere with the DNA unwinding activity; monoclonals which are known to reduce the T antigen-specific ATPase most strongly inhibited the helicase reaction. Second, mutant T antigens with impaired ATPase function also showed a reduced DNA unwinding activity.

Key words: SV40/large tumor antigen/DNA helicase

Introduction

The M_r 94000 protein, coded for by the simian virus 40 (SV40) 'early' region, is commonly referred to as large tumor antigen (T antigen) because its gene seems to be both necessary and sufficient for transformation of primary and established cell lines. T antigen is also essential for the lytic SV40 infection cycle: it is required for the regulation of both viral and cellular genes, as well as for viral DNA replication (for reviews see Tooze, 1980; DePamphilis and Wassarman, 1982; Rigby and Lane, 1983).

T antigen binds specifically to several sites in the control region of the viral genome, can form complexes with the cellular p53 protein and possesses ATPase activity (reviewed in Rigby and Lane, 1983). The structural integrity of the binding site in the SV40 origin is an essential prerequisite for viral DNA replication (Shortle and Nathans, 1978; Myers and Tjian, 1980), and mutant T antigens which do not recognize these specific DNA sequences are therefore unable to initiate replication (Kalderon and Smith, 1984). The ATPase activity of T antigen is also necessary for DNA replication (Cole *et al.*, 1986; Huber *et al.*, 1985). However, the biochemical function of the ATPase remained obscure.

In replicating intermediates of SV40 minichromosomes a fraction of T antigen appears to be bound to origin distal sections of the viral genome (Stahl and Knippers, 1983), and evidence for a role of T antigen in replicative chain elongation has been presented (Stahl *et al.*, 1985).

T antigen binds non-specifically with higher affinity to single-stranded than to double-stranded DNA sequences (Spillman *et al.*, 1979), and certain single-stranded sequences such as polydeoxythymidylic acid stimulate the ATPase function of T antigen (Giacherio and Hager, 1979). This property of T antigen is reminiscent of a well-studied class of prokaryotic enzymes, DNA helicases, which are DNA-dependent ATPases and unwind DNA duplices at the expense of ATP (reviewed by Geider and Hoffmann-Berling, 1981). We now report that highly purified T antigen is associated with DNA helicase activity. This activity is strongly inhibited by several T antigen-specific monoclonal antibodies. Furthermore, mutant T antigens from SV40-transformed cells, known to be defective in ATPase function, also have reduced DNA helicase activities.

Our data suggest a functional relationship between the long established ATPase and the newly discovered DNA unwinding activity. Our experiments also explain earlier observations which have indicated a role for T antigen in the elongation phase of the viral DNA replication cycle.

Results

DNA helicase activity in immunopurified T antigen preparations

SV40 T antigen purified from extracts of lytically infected cells by immunoaffinity chromatography was free from contaminating proteins as judged by silver staining of polyacrylamide gels after electrophoresis in the presence of SDS (Figure 1A). Protein kinase, an enzymatic activity which copurifies with T antigen during conventional column chromatography (Tjian *et al.*, 1979; Baumann and Hand, 1979), was not detectable in our T antigen preparations. They were also free of DNA polymerase α, which has been reported to form complexes with T antigen (Balabanova *et al.*, 1981; Jones and Su, 1982).

Since previous work suggested a participation of T antigen in a DNA unwinding reaction during replicative chain elongation (Stahl *et al.*, 1985; M.Wiekowski, P.Dröge and H.Stahl, submitted) we searched for DNA helicase activity in immunopurified T antigen preparations.

Single stranded M13mp8 DNA, carrying an annealed ^{32}P-labeled 19-mer oligonucleotide was used as a substrate for DNA helicase (Figure 1C). Purified T antigen was reproducibly found to unwind the primer–M13 DNA duplex and released the 19-mer oligonucleotide. This reaction depended on the presence of magnesium salts and ATP (Figure 1B). A non-hydrolyzable ATP analogue, adenylyl-(β,γ-methylene)-diphosphonate (Boehringer-Mannheim), could not replace ATP in this reaction (Figure 1B) showing that ATP hydrolysis was required to unwind hydrogen-bonded DNA. This observation excludes the possibility that the 19-mer oligonucleotide may simply be displaced from its complementary strand due to the high-affinity binding of T antigen to single stranded DNA. Similar substrates have been used before to investigate DNA unwinding reactions and it has been shown that oligonucleotides remain annealed, even in the presence of high concentrations of cooperatively binding single strand-specific proteins (Bianchi *et al.*, 1985; Hübscher and Stalder, 1985).

With increasing amounts of T antigen an increasing fraction of DNA was unwound until 70–80% of all hydrogen-bonded 19-mer oligonucleotide was released from M13mp8 DNA (see

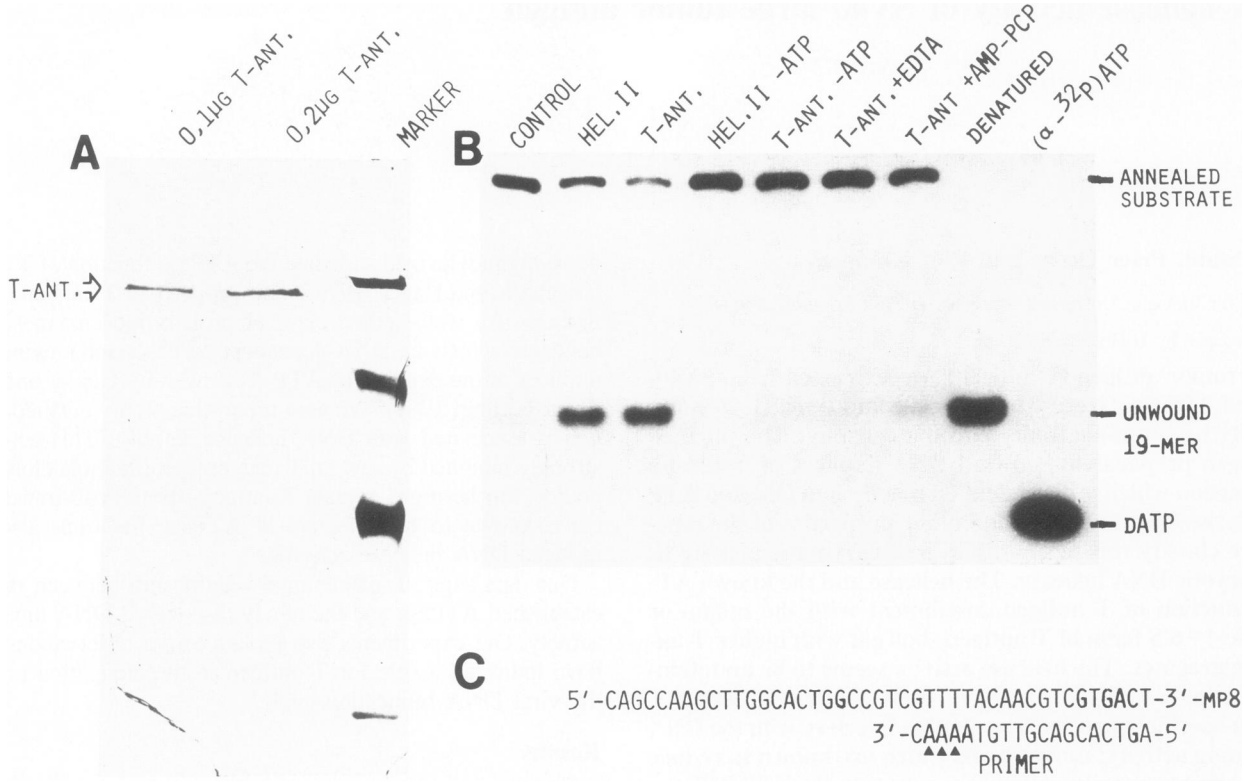

Fig. 1. An ATP-dependent DNA unwinding function of SV40 T antigen. **(A)** Purified T antigen. T antigen purified by immunoaffinity chromatography (see Materials and methods), was investigated by polyacrylamide gel electrophoresis in the presence of SDS (Laemmli, 1970). The gel was stained with silver salts according to Wray *et al.* (1981). The molecular mass markers are proteins of M_r 94000, M_r 67000, M_r 43000 and M_r 30000 (top to bottom). **(B)** DNA unwinding. M13mp8 DNA, carrying a hydrogen-bonded ^{32}P-labeled 19-mer oligonucleotide (see Materials and methods, and **panel C**), was used as the substrate for the DNA helicase assay. We show the autoradiogram of a gel. **Control**: standard reaction without enzyme. **Hel II**: 0.02 µg *E. coli* DNA helicase II, incubated under standard conditions or without ATP (−ATP). **T-ant.**: 0.2 µg SV40 T antigen, incubated under standard conditions or without ATP (−ATP) or with 2 mM adenylyl-(β,γ-methylene)-biphosphonate (+AMP-PCP) or without $MgCl_2$ in the presence of 4 mM EDTA (+EDTA). **Denatured**: heat denatured substrate. **dATP**: free (α-^{32}P)dATP. **(C)** Substrate. We show the nucleotide sequence of a section of the DNA which was used as substrate in the helicase reaction. A commercially available 15-mer oligonucleotide primer was hybridized to M13mp8 DNA and elongated at its 3'OH end using the Klenow fragment of bacterial DNA-Polymerase I in the presence of dCTP and [α-^{32}P]dATP (triangles).

below, Figure 6) in a time-dependent reaction (Figure 2).

In the experiment shown in Figure 1 and in the other experiments (see below), we used the well-characterized *Escherichia coli* DNA helicase II (Geider and Hoffmann-Berling, 1981) for comparison with T antigen-associated helicase. We found that both activities behaved similarly under our assay conditions (Figures 1 and 3).

To find out whether T antigen was able to unwind DNA duplices of > 19 bp we prepared a substrate using oligonucleotide-primed M13mp8 DNA and Klenow polymerase in the presence of all four deoxynucleotide triphosphates, including [α-^{32}P]dATP and didesoxyguanosine triphosphate (Sanger *et al.*, 1980). We thus obtained a spectrum of complementary DNA strands with up to 150 nucleotides as verified by DNA sequencing gels (not shown). We could demonstrate that the T antigen-associated DNA helicase was able to release hydrogen-bonded DNA strands of this length (Figure 3) in a reaction which was also dependent on the presence of magnesium salts and ATP hydrolysis (not shown).

Completely double-stranded DNA, when assayed under helicase reaction conditions, cannot be unwound by T antigen (data not shown). This was tested using the double-stranded 34-bp M13mp DNA fragment of Andersen *et al.* (1980) (obtained through Bethesda Research Laboratories and converted to complete double strandedness by a strand-filling reaction using Klenow's DNA polymerase I). We also tested a 109-bp fragment of the SV40 origin region (between the *Hin*dIII site at SV40 nucleotide 5171 and the *Nco*I site at nucleotide 36; see Tooze, 1980). T antigen binds specifically to this 109-bp origin fragment even in the presence of > 100-fold excess of competing non-specific DNA (not shown) but unwinding could not be detected. Thus, T antigen helicase, like its prokaryotic counterparts, needs free single-stranded DNA regions to perform helix unwinding and specific DNA binding seems to be neither sufficient nor required for this reaction.

In this context, we would like to point out that the helicase activity could be inhibited by the addition of increasing amounts of single stranded M13mp8 DNA (not shown), probably due to the fact that excess single strands compete effectively with the helicase substrate for T antigen binding. This may at least partially explain why relatively high T antigen concentrations were needed for optimal helicase activity since our DNA substrates contained long stretches of single-stranded DNA compared with the short double-stranded region (Figure 1C).

It is known that several subgroups of T antigen exist in lytically infected cells. These subgroups are defined by differential modifications of amino acid side chains including ADP-ribosylation (Goldman *et al.*, 1981) and phosphorylation (Scheidtmann *et al.*, 1982). Furthermore, a substantial fraction of T antigen is converted some time after its synthesis to tetramers or higher aggre-

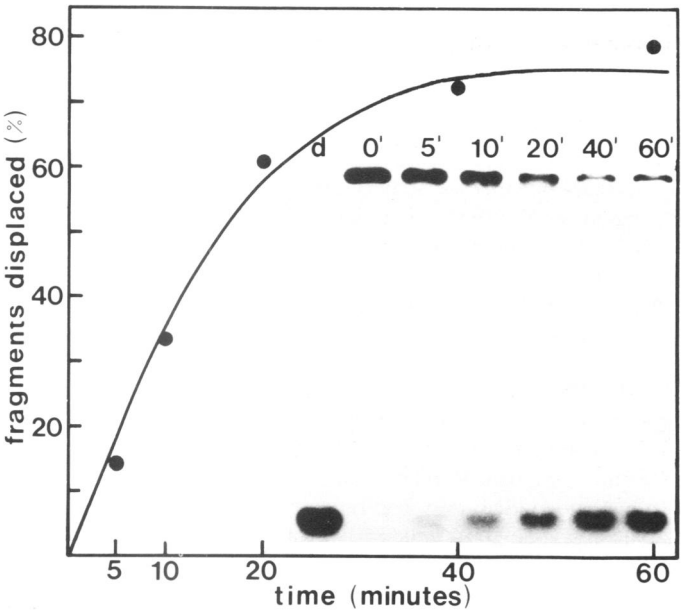

Fig. 2. Kinetics of the helicase function. The experiment was performed exactly as described in Figure 1B with 1.5 μg T antigen/0.4 ml reaction mixture. Samples of 0.05 ml were removed at the indicated times and subjected to gel electrophoresis and autoradiography (insert). The radioactivity of the released oligonucleotide was determined by cutting out the respective regions of the gel and scintillation counting. The results are expressed as percent of the radioactivity determined in the oligonucleotide released by heat denaturation (**lane d**, insert). Values obtained without incubation (<3%) were subtracted as background.

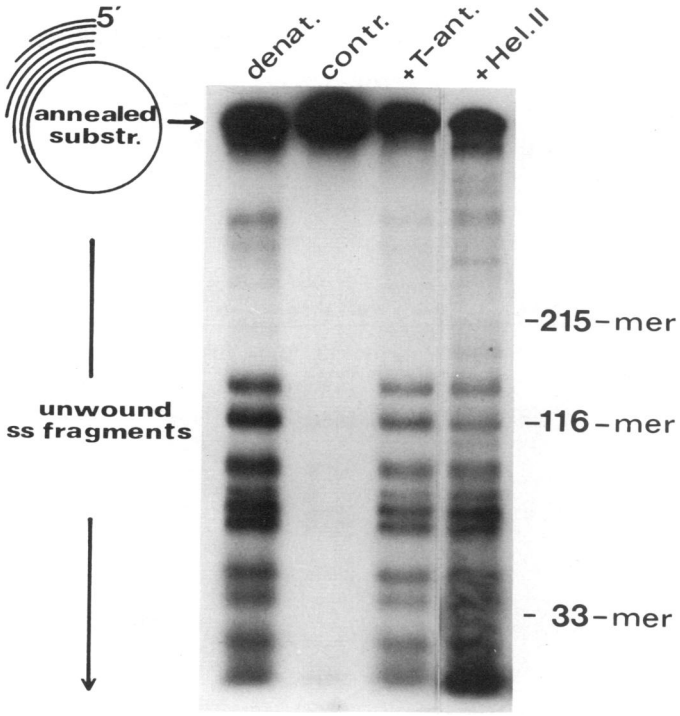

Fig. 3. Efficiency of the T antigen associated DNA unwinding activity. A schematic drawing of the substrate used in this experiment is shown at the left. Oligonucleotide-primed M13mp8 DNA was used in a sequencing reaction with 0.2 mM dideoxyguanosine triphosphate to produce ^{32}P-labeled complementary DNA strands of various length. Heat denaturation of the substrate yielded a spectrum of single stranded (SS) DNA fragments (**denat.**). As control (**contr.**), the substrate was incubated under standard conditions without T antigen. DNA unwinding reactions with T antigen (+ **T-ant**) and with *E. coli* DNA helicase II (+ **Hel.II**) were performed as described in Materials and methods. As markers (right) we used denatured restriction fragments of known lengths.

gated forms (Fanning *et al.*, 1981). Aggregation appears to affect at least one known function of T antigen, namely its specific binding to origin sequences (Burger and Fanning, 1983). To find out whether the state of aggregation has an influence on the DNA unwinding activity we fractionated a T antigen preparation (Figure 1A) by sucrose gradient centrifugation. We obtained at least two forms of T antigen; fast sedimenting, higher complexes and a 4–6S T antigen form, probably consisting of monomers and dimers (Figure 4A, insert). We have determined specific DNA binding, ATPase and DNA helicase activities in fractions of the gradient and found that these activities co-sedimented with the 4–6S T antigen form (Figure 4A and B; specific binding of T antigen to SV40 origin fragments is not shown; see Burger and Fanning, 1983). Neither DNA helicase nor ATPase activity could be detected in the gradient fractions containing higher T antigen aggregates (Figure 4).

DNA helicase activity is associated with T antigen

Protein extracts from uninfected, actively proliferating TC7 cells were chromatographed on the immunoaffinity columns, used for the preparation of T antigen (Figure 1). In this case no helicase activity could be recovered, suggesting that the DNA unwinding activity described above is closely associated with or intrinsic to T antigen. To further investigate this possibility we used a set of different T antigen specific monoclonal antibodies which bind to epitopes distributed throughout the protein. The collection includes the well characterized antibodies PAb 204 and 205 (Clark *et al.*, 1981, 1983) as well as PAb 1613 and PAb 1630 (Ball *et al.*, 1984; Stahl *et al.*, 1985) which recognize epitopes in central sections of T antigen. We also used two antibodies, PAb 101 and KT3, which react with the extreme C-terminal part (Deppert *et al.*, 1981; McArthur and Walter, 1984), and monoclonal PAb 108 which binds to the N-terminal section of T antigen (Gurney *et al.*, 1986). All antibodies reacted well with the immunopurified T antigen as demonstrated by immunoprecipitation experiments (data not shown).

Pretreatment of T antigen with these antibodies had different effects on the DNA unwinding functions (Figure 5). For a quantitative evaluation of the antibody effects the radioactivity of the released oligonucleotide was determined (Figure 2). We found that saturating amounts of monoclonals PAb 204 and 1613 inhibited the activity to 1–2% and that of monoclonal PAb 1630 to about 20% of the control. Monoclonals PAb 205 and PAb 108 inhibited about 50% of the helicase activity whereas the antibodies PAb 101 and KT3, reacting with the C-terminal part of T antigen, were clearly less effective (Figure 5, see also below, Table I).

None of the antibodies used inhibited the activity of *E. coli* helicase II (not shown). Their effects on the T antigen associated DNA unwinding function appear to be specific and we conclude therefore that DNA helicase is an intrinsic enzymatic activity of SV40 T antigen. To confirm this conclusion we present some experiments with mutant T antigens.

Impaired DNA helicase function of mutant T antigen

Many SV40-transformed cell lines express mutant T antigens which are unable to provide the functions required for SV40 DNA replication (Rigby and Lane, 1983). The mutant T antigen, expressed in SV40-transformed mKSA mouse cells, has greatly reduced ATPase actively (Huber *et al.*, 1985). We have purified

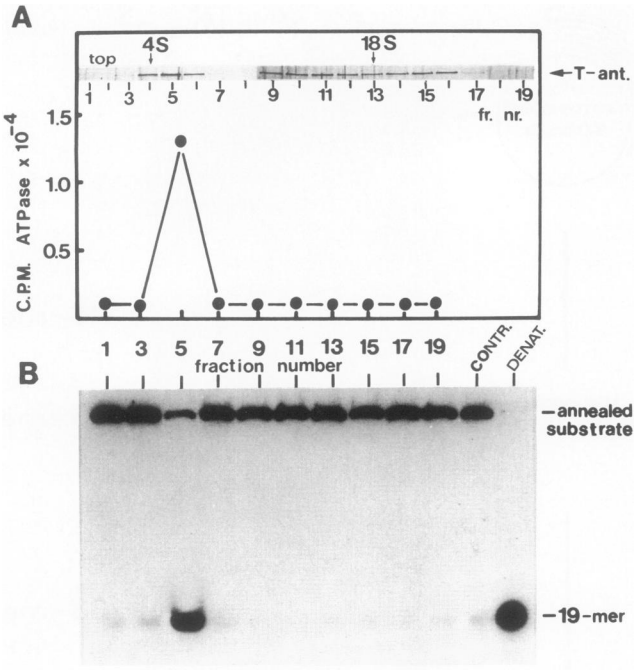

Fig. 4. Cosedimentation of T antigen associated DNA helicase and ATPase. About 15 μg of purified T antigen was centrifuged through a 20%–5% linear sucrose gradient, made up to 10 mM Tris HCl, pH 7.3, 1 mM dithioerythritol and 10% glycerol. Centrifugation was performed in the SW55 Beckman rotor at 39000 r.p.m. for 20 h and at 0°C. Twenty-two fractions were collected. **(A)** ATPase assays were performed using 0.04 ml aliquots of odd-numbered fractions under the conditions described by Clark *et al.* (1981). Insert: T antigen was determined in 0.04-ml aliquots of the gradient fractions by electrophoresis and silver staining as in Figure 1A. We show a section of the gel to indicate the position of the 4–6S T antigen form and of higher T antigen aggregates. Sedimentation markers, run in parallel centrifugation tubes, were bovine hemoglobin (4S) and SV40 form II DNA (18S). **(B)** DNA helicase activity was determined as described in Figure 1 in 0.04-ml aliquots of odd numbered gradient fractions. The control lanes show the DNA substrate before (**contr.**) and after heat denaturation (**denat.**).

the mKSA T antigen using the immunoaffinity procedure (Figure 1) and titrated its DNA unwinding activity in comparison with T antigen, prepared from lytically infected monkey TC7 cells. The mutant T antigen helicase was much less active than that of wild type T antigen (Figure 6). The residual activity of DNA helicase, about 17% of the wild type control, agrees well with the remaining ATPase activity of mKSA T antigen as reported by Huber *et al.* (1985). T antigen preparations from the SV40-transformed H65/90 hamster cell line also possess reduced ATPase (Huber *et al.*, 1985) and helicase activities (not shown). Both enzymatic activities are normal for the functional T antigen from SV40-transformed *cos*-1 cells (Gluzman, 1981).

Discussion

Previous attempts to detect DNA helicases in higher eukaryotes were mostly unsuccessful even though DNA-dependent ATPases are quite abundant in mammalian cells (Falaschi *et al.*, 1980). To our knowledge, only one report has been published describing a eukaryotic DNA helicase activity of one of the DNA-dependent ATPases from calf thymus tissues (Hübscher and Stalder, 1985).

We have shown that purified T antigen preparations contain an activity which enzymatically unwinds DNA duplices of 19 to more than 100 bp. The reaction is efficient, dissociating 70–80% of annealed DNA strand during an incubation time of 30–40 min. The T antigen-associated DNA unwinding function requires ATP and magnesium ions and needs free single-stranded DNA sequences. The activity is therefore comparable with DNA helicases from prokaryotic sources (Geider and Hoffmann-Berling, 1981).

We find it unlikely that a cellular DNA helicase copurifies during immunoaffinity chromatography because no DNA unwinding activity could be detected when a protein extract from uninfected, actively proliferating cells was subjected to the same purification procedure used for the preparation of T antigen (not shown). A possible explanation for the presence of helicase activity in our preparations is that a cellular helicase forms a tight complex with T antigen which remains stable during immunoaffinity chroma-

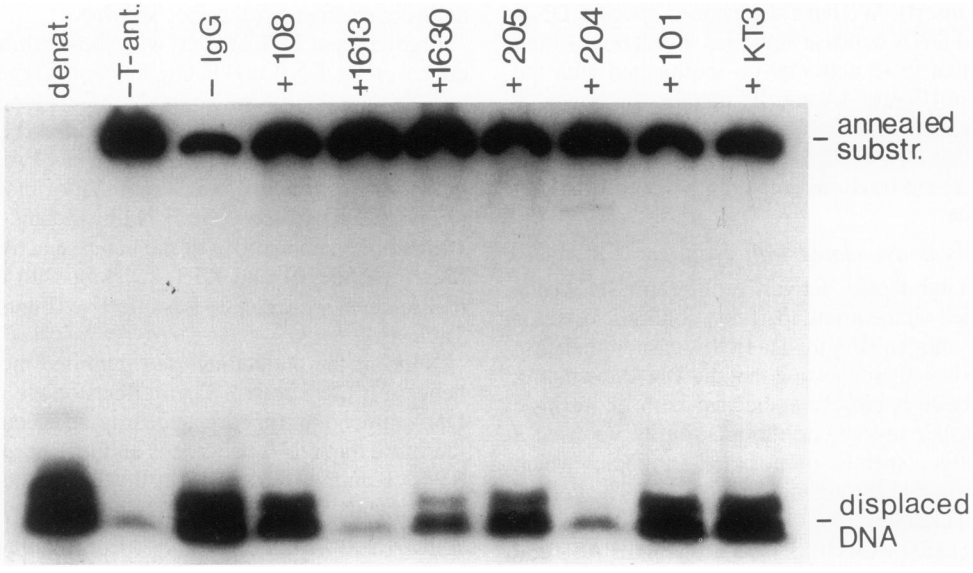

Fig. 5. Influence of T antigen specific monoclonal antibodies on the helicase activity. A ^{32}P-labeled M13mp8 oligomer substrate of maximally 29 nucleotides in length was synthesized in a reaction containing all nucleotide triphosphates except thymidine (see Figure 1C). To assay for antibody inhibition, 0.5 μg T antigen and 5 μg of highly purified monoclonal antibodies were mixed and kept on ice for 15 min. The activity of antibody-treated T antigen was then assayed under standard conditions. The numerical designations of the antibodies used (PAb numbers) are given on top of the corresponding lanes. The source of antibodies and some of their characteristics are given in the text.

Table I. Effects of specific monoclonal antibodies on the DNA unwinding and ATPase activity of purified T antigen

Antibody	Helicase (activity, % control)	ATPase
PAb 108	49	95
PAb 1613	1	32
PAb 1630	17	97
PAb 205	44	75
PAb 204	2	4
PAb 101	80	100
KT3	85	99
(no antibody	100	100)

The inhibition of the helicase activity was proven exactly as shown in Figure 5. The radioactivity of the displaced DNA fragments was quantitated as described under Figure 2. We give average values of three independent experiments with different T antigen preparations. Results obtained with T antigen in the absence of antibodies are taken as 100%. The assay of Clark *et al.* (1981) was used to determine the ATPase activity in untreated and in antibody-treated T antigen. The effects of the antibodies on T antigen ATPase agree well with those reported before (Clark *et al.*, 1981; Deppert *et al.*, 1981; Stahl *et al.*, 1985; Gurney *et al.*, 1986).

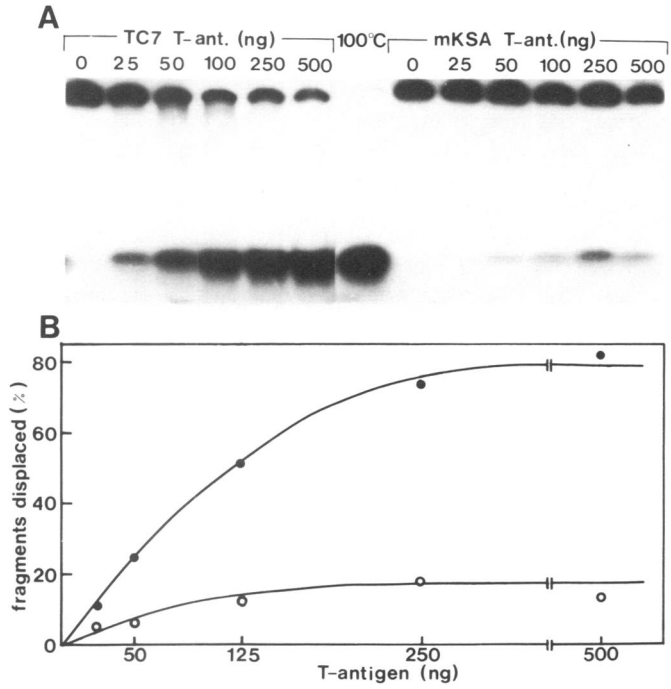

Fig. 6. Helicase activity of mKSA T antigen. T antigen was prepared from mKSA cells by immunoaffinity chromatography exactly as described for SV40-infected TC7 cells. Increasing amounts (ng) of wild type (TC7 T antigen) or mKSA T antigen were then tested for helicase activity. **(A)** Autoradiographical evaluation of the experiment. Amounts of T antigen tested are indicated at the top of each lane. **(B)** The displaced DNA was quantitated as described in Figure 2 and plotted as a function of the amount of T antigen used. Values obtained after incubation of substrate without T antigen were <3% of the input radioactivity and were subtracted as background.

tography. However, in this case we would not expect that several T antigen specific monoclonal antibodies, recognizing different epitopes throughout the protein, could influence the DNA unwinding reaction.

We note some correlation between antibody effects on T antigen helicase and on T antigen-associated ATPase, as previously reported. PAb 204 and PAb 1613 have been shown to strongly inhibit the ATPase activity whereas PAb 205 is less active (Clark *et al.*, 1981; Table I). Antibodies PAb 101 and KT3, reacting with the C terminus of T antigen, do not inhibit the ATPase and have small effects on the T antigen associated helicase (Figure 5, Table I). In contrast, monoclonal PAb 108, reacting with the N terminus, does not inhibit the ATPase activity (Gurney *et al.*, 1986) but blocked the T antigen associated helicase by >40%. This may be due to conformational reasons. The N-terminal section of T antigen plays an important role in the stabilization of the protein's tertiary structure (Clark *et al.*, 1983). The inhibitory effect of monoclonal PAb 1630 (Table I) is particularly interesting. This antibody strongly reduced *in vitro* replication of SV40 chromatin (Stahl *et al.*, 1985) even though its effects on ATPase are negligible. Thus, all antibodies which blocked the ATPase function of T antigen also inhibited the DNA helicase activity but helicase-inhibiting antibodies do not necessarily impair the ATPase function. The fact that different antibodies, binding to different epitopes on the protein, influence the helicase function strongly suggests that DNA unwinding is catalyzed by a function intrinsic to T antigen.

This conclusion is supported by our finding that mutant T antigens from transformed mKSA and Hb 65/90 cells possess reduced helicase activity while functional T antigen from transformed *cos*-1 cells is active. Thus, the most obvious interpretation of our data is that SV40 T antigen, in particular its 4–6S form, functions as a eukaryotic DNA helicase.

The calf thymus DNA helicase described by Hübscher and Stalder (1985) was isolated as a complex with DNA polymerase α suggesting a functional cooperation of both enzymes in replicative chain elongation. In this context, it is interesting to recall previous reports from two laboratories describing an association of SV40 T antigen and cellular DNA polymerase α (Balabanova *et al.*, 1981; Jones and Su, 1982) as if T antigen could replace the cellular helicase in viral DNA replication. Indeed, T antigen-specific antibodies inhibit ongoing SV40 DNA replication (Stahl *et al.*, 1985), and the same antibodies which affect the T antigen helicase probably interfere with a strand separation process in an *in vitro* replication system (Dröge *et al.*, 1985; M.Wiekowski, P.Dröge and H.Stahl, submitted).

It remains to be definitely shown whether the T antigen helicase operates during viral replication cycles. Analysis of *in vitro* SV40 replication systems revealed three distinct and essential T antigen functions at the initiation step, namely specific DNA binding, ATPase and a third undefined activity (Stillman *et al.*, 1985) which could quite possibly be related to the DNA unwinding reaction described in this report.

Materials and methods

Purification of T antigen

Twenty 14.5-cm monolayer plates of African Green Monkey kidney cell line TC7 were infected with wild type SV40. After 48 h a nuclear extract was prepared with 0.3 M NaCl at pH 9. The nuclear extract was centrifuged at 13 000 *g* for 45 min at 0°C. T antigen was isolated from the supernatant using 3 mg of highly purified monoclonal PAb 101 (Deppert *et al.*, 1981) cross-linked to 2 ml Protein A-Sepharose (Pharmacia). Nuclear extract and the antibody carrying sepharose were rolled for 10–12 h at 4°C. The material was then poured into a column and extensively washed with 1 M NaCl and 1% Nonidet-40 in 20 mM Tris–HCl, pH 7.6, 1 mM EDTA. The remaining proteins were finally eluted with 3.5 M $MgCl_2$ in Tris-buffer at pH 7.0 (Dixon and Nathans, 1985; Simanis and Lane, 1985) and dialyzed against 0.1 M NaCl; 20 mM Tris–HCl, pH 7.6; 1 mM EDTA; 1 mM dithioerythritol and 10% glycerol. Total yield was usually about 1 mg electrophoretically pure T antigen in 5 ml. T antigen preparations, kept at −20°C, remained enzymatically stable for ≥6 months.

T antigen from SV40-transformed cells was prepared by a related procedure.

Substrates

The 15-mer 'universal' primer (Bethesda Research Lab.) was annealed to 1 μg

M13mp8 DNA under the conditions used for DNA sequencing (Sanger *et al.*, 1980). The primer was then elongated using 5 U DNA polymerase I-Klenow fragment (Boehringer-Mannheim) in the presence of 0.1 mM each of dCTP and [α-^{32}P]dATP. After 20 min at 25°C, 1 mM dATP was added for 20 min. Unincorporated nucleotides were removed by gel filtration.

Substrates with longer complementary strands were produced using oligonucleotide-primed M13mp8 DNA in a sequencing reaction (Sanger *et al.*, 1980) with all four deoxynucleotide triphosphates (including ^{32}P-labeled dATP) and 0.2 mM dideoxyguanosine triphosphate.

DNA helicase assay

The standard reaction mixture was 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM dithioerythritol, 2 mM ATP, 0.1 mg/ml bovine serum albumin and 6–9 ng of labeled substrate. Unless stated otherwise, 0.2 μg purified T antigen were added to 0.05 ml of the reaction mixture and incubated for 30 min at 37°C. Control samples were incubated at 37°C without T antigen. The reactions were stopped by addition of 0.1 volume of 3.3% SDS and 0.5 M EDTA. Total reaction mixture were then electrophoresed on 8.0–12.5% polyacrylamide gels using a Tris–borate–EDTA buffer at pH 8.3.

In parallel experiments, 0.02 μg purified *E. coli* DNA helicase II (a gift of H.Hoffmann-Berling) were used under identical assay conditions.

Antibodies

The source of the antibodies is described in the text. The purification of antibodies from ascites fluids and hybridoma cell culture supernatants has been described in Stahl *et al.* (1985).

Acknowledgements

We are grateful to Maria Wiekowski who purified the antibodies used in this work. We thank H.Hoffmann-Berling for gifts of *E. coli* DNA helicases and for discussions. We also thank G.Brandner, W.Deppert, K.H.Hochkeppl and D.Lane for antibodies. This work was supported by Deutsche Forschungsgemeinschaft (SFB 156) and by a grant from Boehringer Ingelheim Fond to P.D.

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Received on 3 March 1986; revised on 26 May 1986