JOURNAL OF VIROLOGY, Feb. 1987, p. 411–418
0022-538X/87/020411-08$02.00/0

Vol. 61, No. 2

Monoclonal Antibodies as Probes for a Function of Large T Antigen during the Elongation Process of Simian Virus 40 DNA Replication

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Received 3 March 1986/Accepted 23 October 1986

Various monoclonal antibodies specific for simian virus 40 large tumor antigen (T antigen) inhibit the elongation process of viral DNA replication in an in vitro system. The results provide strong evidence for a function intrinsic to T antigen during ongoing replicative-chain elongation. The antibody inhibition studies were further used to establish a correlation between the known biochemical activities of T antigen and its function during the elongation phase. The data demonstrate that, in addition to DNA binding and ATPase, a third function of T antigen is required for replicative chain elongation. This function is most probably related to the recently described DNA helicase activity of T antigen. This conclusion is based on the following results: (i) aphidicolin treatment of actively replicating simian virus 40 minichromosomes causes a partial uncoupling of parental DNA strand separation and DNA synthesis; the strand separation reaction is blocked by the same monoclonal antibodies which strongly inhibit the elongation process. (ii) DNA helicase activity of isolated T antigen is equally well inhibited by the same set of monoclonal antibodies that affect minichromosome replication in vitro.

The structure of the simian virus 40 (SV40) minichromosome is similar to that of cellular chromatin and therefore serves as a good model for study of the replication of cellular chromatin. The viral genome contains a single origin of DNA replication from which DNA synthesis proceeds bidirectionally until the two forks meet (reviewed in references 13 and 26).

The viral A genes encode a multifunctional protein, the large tumor antigen (T antigen), which is required for the regulation of transcription of viral genes, for viral DNA replication and for cell transformation (for review, see reference 29). The biochemical functions of T antigen in SV40 DNA replication are not well understood.

It has been shown that T antigen possesses at least two biochemical activities essential for viral DNA replication, namely DNA binding, specific for several sites near the origin of DNA replication (9, 11, 32, 45–47), and an ATPase activity (7, 10, 17).

Specific DNA binding to the replication origin is most likely required for the initiation of each round of viral DNA replication in vivo (25, 33) and in vitro (22, 40).

During chain elongation, T antigen appears to be preferentially bound to early replicating minichromosomes (42) and dissociates from viral chromosomes with the completion of a replication cycle (31). The nature of T-antigen interaction with replicating viral DNA structures probably differs from binding to origin DNA sequences as observed in in vitro studies. This is concluded from the following observations. (i) Most T antigen remains associated with replicative intermediate DNA under conditions which destabilize in vitro-formed specific T antigen-DNA complexes (27, 37, 39). (ii) The structure of T antigen bound to SV40 chromatin changes as replicating molecules are converted to mature minichromosomes (43). (iii) A large fraction of tightly bound T antigen is not located at the origin sites but most probably in the vicinity of replication forks (38). The presence of T antigen at the replication forks is consistent with reports describing complexes between T antigen and DNA polymerase α, the enzyme which serves as the deoxyribonucleotide polymerizing function during SV40 DNA replication (3, 20, 48).

Mutant T antigens with impaired ATPase activity are unable to sustain viral DNA replication (10, 40). According to recent results the ATPase activity is intimately associated with another in vitro function of T antigen, namely, the ability to unwind DNA duplexes in a manner analogous to bacterial DNA helicases (37a). We ask whether evidence can be obtained showing that the DNA unwinding activity of T antigen might be involved in the elongation process during the replication cycle.

We used an experimental system consisting of a nuclear extract from SV40-infected cells containing SV40 chromatin as a template and a complex mixture of replication factors, supplied as cytosol from proliferating TC7 cells (13, 41). The system allows the completion of in vivo-initiated rounds of viral DNA replication. The time course of DNA synthesis and density shift experiments clearly showed that initiation of new replication rounds did not occur in this system (12). The inhibition of SV40 DNA synthesis by a T antigen-specific monoclonal antibody in this elongation system was therefore interpreted to indicate a participitation of T antigen in the elongation process of SV40 DNA replication (38).

In this communication we describe experiments with a set of T antigen-specific monoclonal antibodies binding to various determinants destributed over the entire polypeptide chain. The antibodies were analyzed for their ability to inhibit specific DNA binding in vitro, the T antigen–associated ATPase and DNA helicase activities, and, most importantly, SV40 replication in the in vitro elongation system.

Using an experimental system which allows an uncoupling of fork movement from replicative DNA synthesis (16), we obtained evidence that T antigen is involved in the process of parental strand separation during SV40 DNA replication.

The elongation function is most probably related to the helicase activity of T antigen. We conclude this because the antibody inhibition data obtained from our studies with the in vitro replication system strongly correlate with those

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obtained by measuring the ATPase and helicase activity of immunopurified T antigen.

MATERIALS AND METHODS

Chemicals. Nucleoside triphosphates and deoxynucleoside triphosphates were obtained from Boehringer GmbH. The monoclonal antibody purification system was from BioRad, [^{3}H]thymidine, [^{32}P]phosphate, [γ-^{32}P]ATP, and [α-^{32}P]dATP were purchased from Amersham Corp. Anti-mouse immunoglobulin G (IgG)–agarose was from Sigma Chemical Co., and poly(dT) (10S) was from P-L Biochemicals. Aphidicolin was a gift from Imperial Chemical Industries.

Preparation of antibodies. Tumor serum and mouse control serum were prepared as described previously (38). All monoclonal antibodies were prepared from mouse hybridoma cell lines except PAb 204 and 205, which were isolated from ascites fluids. The antibodies were purified after an 60% ammonium sulfate precipitation by the monoclonal antibody purification system. If necessary for higher purification in a second step an anti-mouse IgG–agarose column was used.

After isolation, the immunoglobulins were dialyzed against 50 mM potassium acetate–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.8, and stored in working samples at −20°C. Each preparation was tested for DNAase activity with SV40 F1 DNA and single-stranded M13mp8 DNA as described before (38). The antibodies were free of any contaminating phosphatase activity as tested by the incubation with ^{32}P-labeled, purified T antigen for several hours. No reduction of T antigen labeling or release of radioactive isotope could be detected.

T-antigen assays. The preparation of crude cell extracts from SV40-infected cells and conditions for immunoprecipitation of T antigen as well as sodium dodecyl sulfate-gel electrophoresis have been described before (38, 39). T antigen was purified by immunoaffinity chromatography (34) with the monoclonal antibody PAb 108 coupled to protein A–Sepharose Cl-4B (Pharmacia Fine Chemicals). For an ATPase assay (6), 0.2 μg of purified T antigen was preincubated with indicated amounts of antibodies for 10 min on ice in a buffer containing 25 mM HEPES (pH 7.8), 5 mM $MgCl_2$, 0.1 M NaCl, 1 mM dithiothreitol, 0.01% albumin, and 10% glycerol. The reaction was started by the addition of 20 μM [γ-^{32}P]ATP (250 cpm/pmol). After incubation for 20 min at 37°C the unreacted ATP was precipitated by acid-washed charcoal (20% in 0.25 M HCl–0.25 M H_3PO_4), and after centrifugation the free ^{32}P in the supernatant was counted in Aquasol (Dupont).

A target bound DNA-binding assay for T antigen was performed by using minor modifications of the methods described elsewhere (19a). SV40 DNA was *Hin*dIII restricted, and the fragments were labeled at the 3′ end with Klenow polymerase in the presence of [α-^{32}P]dATP. Binding conditions were as described by McKay (23).

The DNA helicase assay was performed as described elsewhere (37a) and contained in a standard reaction mixture 20 mM Tris hydrochloride (pH 7.5), 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 2 mM ATP, 0.1 mg of bovine serum albumin per ml, 6 to 9 ng of labeled substrate, and 0.5 μg of purified T antigen.

In vitro chromatin replication. SV40 chromatin was prelabeled with [^{3}H]thymidine in vivo for 1 h and prepared in hypotonic buffer from TC7 cells 40 h after infection with SV40 as previously described (41). To the nuclear extract the individual monoclonal antibodies were added in the amounts given below. The samples were incubated on ice for 30 min before the reaction was started by the addition of cytosol prepared from serum of stimulated, uninfected TC7 cells and nucleotides with [α-^{32}P]dATP as a tracer. Conditions for in vitro DNA replication were exactly as described before (28, 38). Incubation was at 30°C for 40 min. The incorporation of ^{32}P into DNA was determined by precipitation of the samples with 10% trichloracetic acid on ice (28).

For pulse experiments replicating minichromosomes were labeled with [^{32}P]dATP for 3 min, followed by a chase with a 600-fold excess of unlabeled dATP for 37 min at 30°C. The incubation was stopped by the addition of 0.2 volume of 1% sodium dodecyl sulfate–50 mM EDTA and analyzed by neutral sucrose gradient centrifugation (38). Neutral and alkaline agarose gel electrophoresis was performed exactly as described previously (16, 38).

RESULTS

Characterization on monoclonal antibodies. For our experiments, we have chosen the following 12 well-characterized T antigen-specific monoclonal antibodies: PAb 1604, 1613, 1614, 1617, 1619, 1623, and 1630 (4); PAb 101 (13); PAb 108 (18); PAb 204 and 205 (6, 8); and KT3 (22). The antibodies were highly purified and free of DNAase, ATPase, and phosphatase activities (see Materials and Methods).

All antibodies are reported to have a high affinity for SV40 T antigen. We have confirmed this for our particular preparations; all monoclonal antibodies, used in saturating amounts, were at least as effective as immunoglobulins prepared from polyclonal mouse tumor serum in immunoprecipitating ^{32}P-labeled T antigen from crude extracts of SV40-infected TC7 cells (Fig. 1A).

In the present study the monoclonal antibodies were used as tools to probe for a proposed function of T antigen during the elongation phase of viral DNA replication. Since mutant studies had shown that specific DNA binding and the ATPase activity of T antigen are necessary for viral DNA replication (40), we have investigated whether the monoclonal antibodies had effects on T antigen-specific DNA binding and ATPase activity, respectively.

To test for specific DNA binding, we used a modification of the target-bound binding assay of Hinzpeter et al. (in press). T antigen was first immunoprecipitated from a crude extract by using the monoclonal PAb 108, which binds at the very amino terminal end of the T-antigen polypeptide chain and does not interfere with DNA binding (19a). The individual monoclonal antibodies were added to the T antigen-loaded immunoprecipitates. DNA binding was then assayed by the addition of ^{32}P end-labeled *Hin*dIII SV40 DNA restriction fragments as described previously (38). The antibodies did not interfere with specific DNA binding of T antigen to the *Hin*dIII C fragment (Fig. 1B) containing the SV40 origin (47). There was one exception: antibody PAb 1604, which drastically reduced specific DNA binding of T antigen. Quantitative analysis by densitometry revealed that the inhibition was in the range of 80 to 90% when compared with the control (PAb 108). Monoclonal antibody PAb 1604 was also able to dissociate preformed T antigen-DNA complexes (data not shown).

The influence of the monoclonal antibodies on unspecific DNA binding was also tested by using a filter binding assay and purified T antigen (5, 15). Monoclonal PAb 1604, but none of the other antibodies, caused a decrease of T antigen-DNA interaction (data not shown).

Finally, we tested whether the binding of antibodies interferes with the T antigen intrinsic ATPase activity.

Immunopurified T antigen was preincubated with increasing amounts of each monoclonal antibody. ATPase activity was then tested as described previously (7). The maximal inhibition rates were determined by comparing saturating antibody concentrations with a control without antibodies (Fig. 1C). As reported before (6), we found that PAb 204 inhibited the T antigen-associated ATPase activity almost completely (to about 95%), whereas PAb 205 only inhibited it to about 25%. We also found a remarkable inhibition of ATPase activity by monoclonal PAb 1613, which reduced the activity to the same extent as polyclonal T antigen-specific antibodies.

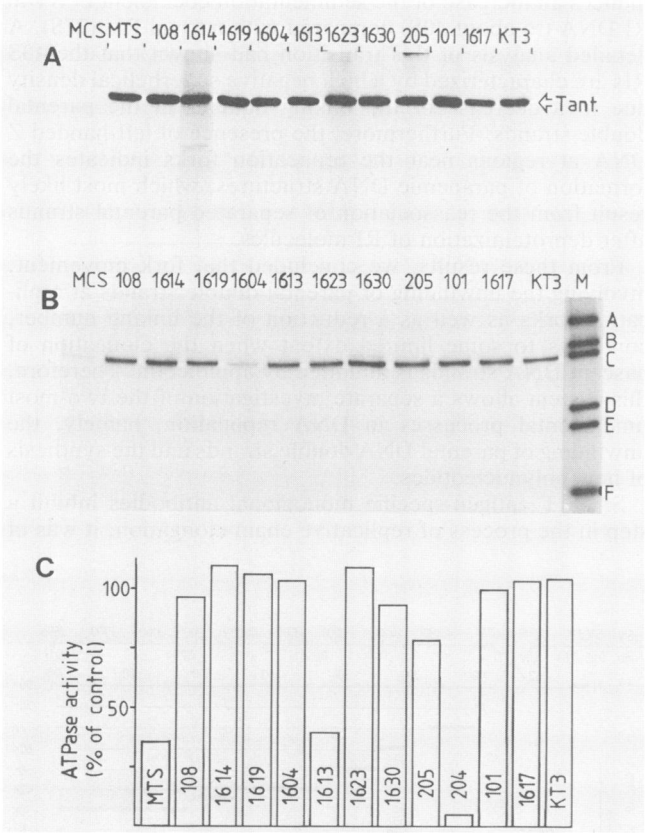

FIG. 1. Characterization of T antigen-specific monoclonal antibodies. (A) Specificity and affinity. TC7 monkey cells were labeled 37 h after infection with SV40 for 3 h with [^{32}P]phosphate. For each assay a sample of the crude extract representing 0.2 to 0.4 μg of T antigen was immunoprecipitated with 5 μg of IgGs and analyzed by sodium dodecyl sulfate-gel electrophoresis. The numerical designations of the monoclonal antibodies are indicated at the top; MCS, mouse control serum; MTS, mouse tumor serum. We show the autoradiogram of a polyacrylamide gel after 15 h of exposure. (B) Effects on the formation of specific T antigen-DNA complexes. T antigen (0.2 μg per probe) was immunoprecipitated from a crude extract of SV40-infected cells with antibody PAb 108 and *Staphylococcus aureus*. Immunoglobulins, purified from mouse control serum, were used as a control. To each sample 2 μg of a given monoclonal antibody was added as indicated. Specific DNA-binding activity of T antigen in the immunocomplex was subsequently tested with ^{32}P-labeled, *Hin*dIII-restricted SV40 DNA. We show the autoradiogram of the electrophoretically separated DNA fragments (M, 1/10 of the input DNA as a marker). The *Hin*dIII C fragment contains the SV40 origin (47). (C) ATPase activity. T antigen (0.2 μg), homogeneously purified by immunoaffinity chromatography (37a), was incubated with 0.5 μg of the individual antibody. ATPase activity was tested in the presence of 20 ng of poly(dT). The activity of a charge without antibodies was taken as 100%.

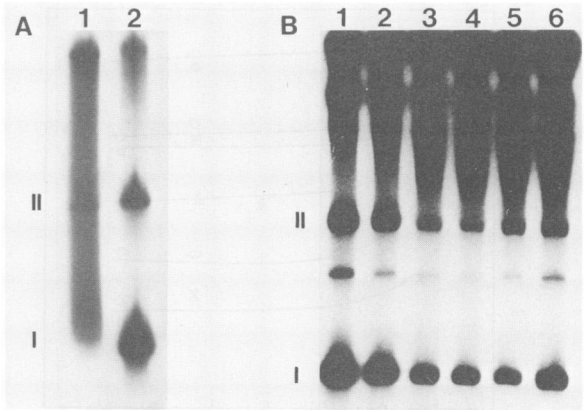

FIG. 2. Analysis of in vitro replicated DNA by neutral gel electrophoresis. SV40 chromatin, prelabeled for 12 to 16 h with [^{3}H]thymidine in vivo, was pulse-labeled for 3 min in the presence of [α-^{32}P]dATP. The samples were then incubated for 15 min on ice in the presence of T antigen-specific monoclonal antibodies in saturating amounts (Fig. 3), followed by a chase with an excess of unlabeled dATP for 37 min at 30°C. After deproteinization and neutral sucrose gradient centrifugation the DNA was recovered by ethanol precipitation and applied to the gel slots. (A) Pulsed (track 1) and chased (track 2) DNA without antibodies. (B) Chased DNA without antibodies (track 1) or in the presence of PAb 108 (track 2), PAb 1604 (track 3), PAb 1613 (track 4), PAb 1630 (track 5), or PAb 205 (track 6). The positions of form I DNA (I) and form II DNA (II) are indicated.

Some noninhibiting antibodies, like PAb 1614 or PAb 1623, stimulated even the ATPase activity to a small but significant extent (Fig. 1C).

Inhibition of in vitro SV40 DNA replication. Replicating SV40 minichromosomes extracted from nuclei of infected cells were used as substrates for in vitro DNA replication. In the presence of cytosol from proliferating uninfected cells, in vivo-initiated DNA replication cycles are completed in vitro without apparent repair synthesis. The initiation of new replication rounds has never been detected in this in vitro system (12, 41). We used this system to test whether the monoclonal antibodies (Fig. 1) show an effect on SV40 DNA replication as has been previously reported for PAb 1630 and 101 (38). SV40 chromatin was prelabeled in vivo with [^{3}H]thymidine for 60 min. The ^{3}H label appeared almost exclusively in mature SV40 DNA and served as a marker during further analysis of the replication products.

During in vitro SV40 DNA replication, polymerization of deoxyribonucleotides proceeded at an almost linear rate for about 30 min (data not shown) (38). When in vitro pulse-labeling with [α-^{32}P]dATP was followed by a chase with an excess of unlabeled dATP, most replicative intermediates (RIs) were converted to mature form I and form II DNA (Fig. 2A). A small fraction of the chase products appeared in an electrophoretically slowly migrating form (Fig. 2A, track 2), most probably representing late Cairns structures and intermediates in the process of seggregation (36).

The effects of some inhibiting antibodies on SV40 DNA replication are shown in Fig. 2B. When added at the beginning of the chase period the antibodies reduced the conversion of RIs to mature SV40 DNA forms. The extend of inhibition varied from antibody to antibody. However, even the most effective antibodies (PAb 1630 and 1613) did not completely suppress DNA replication (Fig. 2B, tracks 4 and 5; see below for a quantitative evaluation). The pulse-labeled

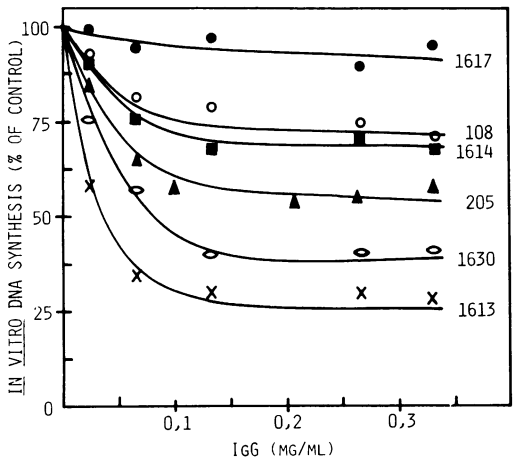

FIG. 3. Effect of T antigen-specific antibodies on SV40 chromatin replication in vitro. Samples of SV40 chromatin labeled long term with ^{3}H were pretreated with the indicated amounts of antibodies per milliliter of assay mixture. In vitro DNA replication was started by the addition of cytosol und nucleotides, including [α-^{32}P]dATP (38). In vitro replication was for 40 min. The results were recorded as the amount of ^{32}P incorporated relative to the amount of ^{3}H label in template DNA and are expressed as the percentage of the rate of DNA synthesis in the absence of antibodies.

RIs continued DNA replication to some limited extent, producing RI DNA with reduced electrophoretic mobility and a small amount of mature SV40 DNA. Analysis by alkaline agarose gel electrophoresis revealed an incomplete maturation of nascent DNA strands and limited ligation of smaller DNA fragments exactly as described before with polyclonal mouse tumor serum and PAb 1630 (38).

For a more quantitative evaluation we tested the inhibition of in vitro DNA synthesis at increasing antibody concentrations (Fig. 3).

The monoclonal antibodies PAb 1617 and 101 (as well as KT3, data not shown) had almost no effect on in vitro DNA replication even at very high antibody concentrations. Other antibodies significantly reduced the incorporation of deoxyribonucleotides, although to different extents. The strongest inhibition (to about 75%) was achieved with monoclonal antibody PAb 1613 (Fig. 2) or PAb 204 (data not shown, but see below). We point out that higher inhibition rates of DNA replication were not induced when a combination of inhibiting antibodies was used, in accordance with results obtained before with T antigen-specific polyclonal antibodies (38).

In Fig. 4, the effects of all antibodies on SV40 DNA replication are plotted relative to their binding domains on the T antigen polypeptide chain. As mentioned before, the highest reduction in replicative DNA synthesis was observed when SV40 chromatin was treated with the monoclonal antibody PAb 204 or 1613. Even though these antibodies are reported to bind to different domains they strongly inhibit the ATPase activity (Fig. 1C) (6). PAb 205, a weak inhibitor of ATPase, had moderate effects on in vitro replicative chain elongation (about 40%), whereas PAb 1604, which blocks specific and unspecific DNA binding of T antigen, strongly inhibited these processes.

Generally, antibodies which recognize epitopes in more centrally located regions of T antigen were stronger inhibitors than those recognizing epitopes at the termini of the polypeptide chain. This is also true for PAb 1630 and 1623, which have no effects on the T antigen-associated ATPase and DNA-binding activity (these monoclonal antibodies recognize closely adjacent, but not identical epitopes, [4]).

Finally, antibodies reacting with the N-terminal section of T antigen had relatively small but significant effects on SV40 DNA replication, whereas antibodies with binding specifities close to the C terminus (PAb 101 and 1617 and KT3) were completely inactive.

Effect of T antigen-specific monoclonal antibodies on aphidicolin-induced supercoiling in SV40 RIs. Aphidicolin, a specific inhibitor of DNA polymerase α, causes a prompt and almost complete block of SV40 DNA replication (14, 38). In a recent report (16), we could show that this inhibition induces an increase of the sedimentation coefficient of SV40 RI DNA (to about 40S) compared with normal RIs (27S). A detailed analysis of this transition had shown that the 40S RIs are characterized by a high negative superhelical density due to a decrease of the linking number in the parental double strands. Furthermore, the presence of left-handed Z DNA at regions near the replication forks indicates the formation of paranemic DNA structures, which most likely result from the reassociation of separated parental strands after deproteinization of RI molecules.

From these results, we concluded that fork movement, involving the unwinding of parental double strands at replication forks as well as a reduction of the linking number, continues to some limited extent when the elongation of nascent DNA strands is inhibited by aphidicolin. Therefore, this system allows a separate investigation of the two most fundamental processes in DNA replication, namely, the unwinding of parental DNA double strands and the synthesis of new polynucleotides.

Since T antigen-specific monoclonal antibodies inhibit a step in the process of replicative chain elongation, it was of

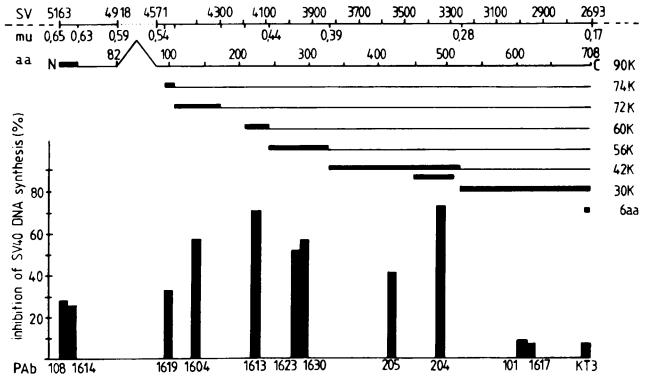

FIG. 4. Inhibition of SV40 DNA replication by T antigen-specific antibodies in relation to the position of their binding sites. The inhibition of SV40 DNA replication at saturating antibody concentrations was determined as described in the legend to Fig. 3. The values are the means of three independent experiments. The domains of most antibody-binding sites had been mapped by using various truncated forms of T antigen coded for by adenovirus-SV40 hybrid viruses (4). The relative molecular masses of these proteins are given in kilodaltons (K). The binding region of the monoclonal PAb 204 was further limited by immunoprecipitation of mutant T antigen (24). The monoclonal antibody KT3 had been raised against a hexapeptide representing the last six amino acids at the carboxy terminus of T antigen (22). The SV40 nucleotide numbers (SV) and map units (mu) on the A gene as well as the amino acid (aa) positions on the T-antigen polypeptide chain are given as described previously (34). N and C represent the amino and carboxy termini of T antigen, respectively.

interest to determine whether these antibodies had any effect on the aphidicolin-induced supercoiling in RI DNA.

SV40 minichromosomes extracted from nuclei of infected cells were incubated under in vitro replication conditions and pulse-labeled for 3 min with [α-^{32}P]dATP. Samples were then treated with different T antigen-specific monoclonal antibodies for 15 min at 0°C and further incubated for 40 min at 30°C in the presence of a 600-fold excess of unlabeled dATP and aphidicolin. The pulse-labeled and -chased DNA samples were deproteinized and analyzed by zone velocity sedimentation (Fig. 5).

The ^{32}P pulse-labeled DNA sedimented as a broad peak with 27S (Fig. 5A), the sedimentation coefficient of normal SV40 RI DNA (30). After the chase, the ^{32}P-labeled RIs sedimented either as torsionally stressed RI DNA with 40S or as normal RI molecules with 23S to 27S, depending on the antibody present in the reaction mixture (Fig. 5, Table 1). Monoclonal antibody PAb 101, which does not inhibit chain elongation (Fig. 3 and 4), also had no effect on the formation of 40S RI DNA, whereas PAb 1630 and 1613 strongly inhibited the 27S-to-40S transition (Fig. 5C and D). The effects of all tested monoclonal antibodies in this experimental system are summarized in Table 1.

The results of the sedimentation experiments were confirmed by analysis of the chase products by gel electrophoresis in the presence of chloroquine. This DNA-binding drug unwinds DNA double helices and consequently relaxes negative superhelical turns in torsionally stressed RI molecules. At low chloroquine concentrations the 40S RI DNA clearly showed an increased electrophoretic mobility compared with normal RI DNA, indicating that the 40S RIs exhibit a higher degree of negative superhelical turns (data not shown) (16).

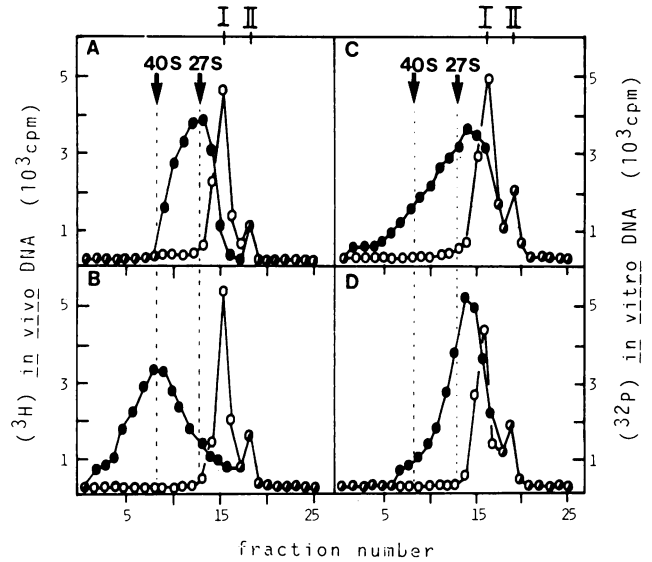

FIG. 5. Effects of T antigen-specific antibodies on the formation of 40S RI DNA. SV40 chromatin prelabeled for 12 to 16 h with [^{3}H]thymidine in vivo (○) was used for in vitro chromatin replication in the presence of [α-^{32}P]dATP (●) performed exactly as described (16). (A) Pulse label for 3 min at 30°C in the presence of [α-^{32}P]dATP. (B) Pulse label for 3 min, followed by a 15-min incubation with PAb 101 (0.3 mg/ml) at 0°C. The sample was then chased for 40 min at 30°C with an excess of unlabeled dATP in the presence of aphidicolin (20 μg/ml). (C) Same as (B), except that PAb 1630 (0.3 mg/ml) was used instead of PAb 101. (D) Same as (B), except that PAb 1613 (0.3 mg/ml) was used instead of PAB 101. After the pulse and chase, the DNA was deproteinized by sodium dodecyl sulfate treatment and centrifuged through neutral sucrose gradients. The positions of superhelical SV40 form I DNA (I) and of relaxed form II DNA (II) in the gradients are indicated.

TABLE 1. Inhibition of T-antigen activities by monoclonal antibodies

Antibody	Inhibition of activity[a]				
	DNA binding	ATPase activity	In vitro DNA replication	27S-to-40S transition	Helicase activity
PAb 1614	−	−	±	−	ND
PAb 108	−	−	±	−	+
PAb 1619	−	−	±	−	+
PAb 1604	++	−	+	+	++
PAb 1613	−	++	++	++	++
PAb 1623	−	−	+	+	+
PAb 1630	−	−	+	+	+
PAb 205	−	±	+	−	+
PAb 204	−	++	++	ND	++
PAb 1617	−	−	−	−	±
PAb 101	−	−	−	−	−
KT3	−	−	−	−	−

[a] −, No or weak inhibition, less than 15%; ±, moderate inhibition, less than 35%; +, inhibition, less than 65%; ++, inhibition, above 65%; ND, not determined.

Inhibition of T antigen-associated DNA helicase activity. We have recently shown that immunopurified T-antigen preparations contain an activity which enzymatically unwinds DNA duplexes in a reaction reminiscent of bacterial DNA helicases (37a). Since the results described above are consistent with a role of T antigen in the strand separation process during fork movement, we analyzed whether the monoclonal antibodies have an effect on the helicase activity of immunopurified T antigen.

As a substrate, we used oligonucleotide-primed M13mp8 DNA which was elongated by Klenow polymerase in the presence of all four deoxynucleotide triphosphates (including [α-^{32}P]dATP) and dideoxyguanosine triphosphate. We thus obtained a spectrum of complementary DNA strands with up to 200 nucleotides (37a). This DNA template was incubated under appropriate conditions with purified T antigen. We observed the release of DNA fragments of up to at least 150 nucleotides (Fig. 6). The reaction was absolutely dependent on the hydrolysis of ATP (data not shown), and the displaced oligonucleotides appeared to be identical to those obtained after incubation with *Escherichia coli* DNA helicase II or after heat denaturation at 100°C (Fig. 6).

Antibodies PAb 1613 and 204 almost completely inactivated the T antigen-unwinding activity, whereas others had negligible (PAb 101 and 1617 and KT3) or moderate (PAb 108 and 205) effects. The inhibition of the helicase activity by the monoclonal antibodies was quantitated as described elsewhere (37a). The results are summarized in Table 1, where they are compared with the antibody effects on the other T-antigen activities tested.

We note that the helicase-inactivating antibodies also inhibited strongly the ATPase activity, the 27S-to-40S transition, and the in vitro DNA replication.

DISCUSSION

We have used T antigen-specific monoclonal antibodies as probes to test for a function of SV40 large T antigen during the elongation phase of viral DNA replication. We first

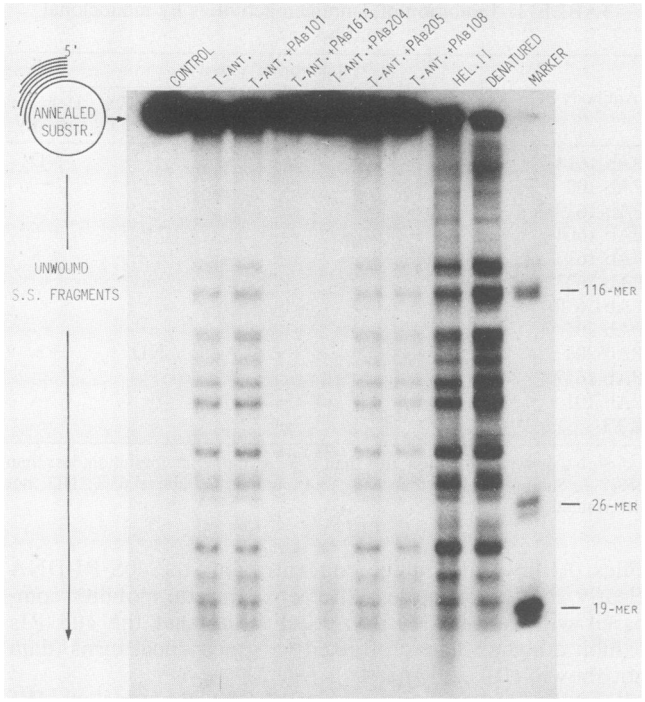

FIG. 6. Influence of T antigen-specific monoclonal antibodies on the helicase activity. A schematic drawing of the helicase substrate used in this experiment is shown at the left. Oligonucleotide-primed M13mp8 DNA was used in a sequencing reaction with 0.2 mM dideoxyguanosine triphosphate to produce ^{32}P-labeled complementary DNA strands of various length. Heat denaturation of the substrate yielded a spectrum of single-stranded (S.S.) DNA fragments (denatured). To assay for antibody inhibition, 0.5 μg of T antigen was incubated either in the presence or absence of 5 μg of highly purified monoclonal antibodies 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 the top of the corresponding lanes. As a control (contr.), the substrate was incubated under standard conditions without T antigen. DNA unwinding reaction with 0.1 μg of *E. coli* DNA helicase II (Hel. II) was performed exactly as described for T antigen (T-Ant.). As markers (right), we used denatured restriction fragments of known length.

analyzed the purified immunoglobulins for their effects on DNA-binding and ATPase activity of T antigen. It is important to note that differences caused by variable binding affinities can be excluded, since all antibodies are able to precipitate essentially all soluble T antigen from crude extracts of SV40-infected cells (Fig. 1A).

One monoclonal antibody, PAb 1604, reduced T antigen-specific and unspecific DNA-binding activity (Fig. 1B). This antibody interacts with a domain of T antigen that has previously been shown to be involved in DNA binding (7).

Two monoclonal antibodies strongly inhibited the T antigen-associated ATPase activity: PAb 204 most likely binds to amino acid residues located within the active site for ATP hydrolysis at 0.24 to 0.20 map units (24); however, PAb 1613 binds to an epitope outside the postulated active center (Fig. 4). It appears likely that binding of this antibody changes the tertiary structure of T antigen.

Some antibodies inhibited the elongation of SV40 DNA replication in our in vitro system, which in contrast to more recently developed replication systems (1, 30, 40) is not able to initiate new replication cycles (41). Our results therefore provide additional evidence that T antigen participates in the elongation process during SV40 minichromosome replication (38). The weakly inhibiting or noninhibiting antibodies serve as an appropriate control. In particular, antigenic binding sites for PAb 1617 and 101 and KT3 at the carboxy-terminal half of T antigen are apparently not essential for the function during replicative chain elongation. Antibodies PAb 108 and 1614, which bind to the N-terminal part, showed low but significant inhibition of DNA synthesis, probably by interfering with the conformation of the T-antigen polypeptide chain (Fig. 4). We note that others have proposed an involvement of the amino-terminal part of T antigen in the ATPase reaction (7).

Inhibition of DNA synthesis by aphidicolin induces supercoiling in SV40 replicative intermediate DNA in vitro and in vivo. It was shown that this induction is most likely due to the separation of parental DNA strands, which continued to some limited extent when DNA synthesis is blocked by aphidicolin (16). Since the same antibodies which most strongly inhibit in vitro DNA replication also prevent supercoiling, this implicates T antigen with the active separation of parental DNA strands during the elongation phase of SV40 DNA replication. Furthermore, the comparison of the antibody inhibition data (Table 1) shows that several biochemical activities of T antigen are necessary for this function.

First, T antigen must be bound to the replicating DNA molecules. This binding occurs in the vicinity of replication forks as shown before (38) and is therefore probably not sequence specific. The T antigen-DNA interaction is disrupted by monoclonal PAb 1604 (Fig. 1B), which could be the reason why this antibody inhibits the elongation of nascent DNA strands.

Second, T antigen must most probably retain its ATPase activity to perform its proposed function during the elongation phase. This conclusion can be drawn because the ATPase-inhibiting antibodies, PAB 1613 and 204, were found to be the strongest inhibitors of SV40 DNA synthesis (Fig. 4). Our results are in agreement with previous studies with SV40 *ts*A mutants (*ts*A16, *ts*A209, and *ts*A58) which are temperature sensitive for the initiation of viral DNA replication but not for ongoing chain elongation (44). The ATPase activity of T antigen from these mutants was shown to be as stable at the nonpermissive temperature in vitro as that of wild-type T antigen (8).

Two monoclonal antibodies, PAb 1630 and 1623, interfered neither with T antigen DNA-binding activity nor with its ATPase activity, but nevertheless efficiently reduced strand separation and the elongation process during SV40 DNA replication (Table 1). These results show that a third biochemical activity of T antigen might be necessary for the proposed participation of T antigen in the elongation process.

Highly purified T antigen is able to unwind DNA double helices when tested with appropriate substrates containing single-stranded DNA regions. This DNA helicase activity of T antigen depends on the presence of magnesium ions and ATP hydrolysis (37a) and most interestingly is equally well inhibited by PAb 1630 and 1623 (Table 1). Furthermore, all antibodies which efficiently reduced the ATPase activity of T antigen as well as in vitro DNA replication also blocked DNA helicase activity (Table 1).

Thus, we conclude that T antigen, beside its still unknown reaction at the origin, performs a DNA-unwinding reaction during SV40 chain elongation much like the bacterial DnaB protein, a DNA helicase, which is involved in the initiation

as well as in the elongation process of bacterial DNA replication (2).

ACKNOWLEDGMENTS

We are grateful to R. Knippers for his helpful discussions and critical reading of the manuscript. We thank T. Kapitza for his excellent technical assistance and I. Lühr for typing. We also thank W. Deppert for his kind gift of hybridoma cells producing PAb 101 and 108 and KT3, G. Brandner and K. H. Hochkeppel for an ample supply of hybridoma supernatants used to prepare PAb 1604, 1613, 1614, 1617, 1619, 1623, and 1630, and D. Lane for ascites fluids containing PAb 204 and 205.

This research was supported by the Deutsche Forschungsgemeinschaft through SFB 156 and a grant from Boehringer Ingelheim Fond to P.D.

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