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Simian virus 40 DNA replication *in vitro*: Study of events preceding elongation of chains

(simian virus 40-encoded large tumor antigen/simian virus 40 origin/RNase effects/ammonium sulfate fractionation)

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ABSTRACT **We have evidence for the formation of a stable preelongation complex during the replication of simian virus 40 (SV40) origin containing DNA (ori$^+$ DNA) *in vitro*. Preincubation of ori$^+$ DNA with HeLa cytosolic extracts and SV40-encoded large tumor antigen (T antigen) in the absence of deoxynucleoside triphosphates eliminates a lag that normally precedes replication. This effect requires ATP and is inhibited by RNase A; subsequent elongation is inhibited by aphidicolin but not by RNase A. A T antigen and SV40 origin-dependent complex can be isolated by gel-filtration chromatography of preincubation reaction mixtures. In both cases, the products formed by replication after complex formation resemble those formed during *in vitro* replication reactions described previously. HeLa cytosolic extract was separated into two ammonium sulfate fractions: a 0–40% fraction (AS 40) that shows low levels of DNA synthesis and a 40–65% fraction (AS 65) that is inactive by itself but stimulates synthesis when added to the AS 40 fraction. DNA synthesis by these combined fractions has the same requirements as crude extract, occurs in two stages as described above, and is sensitive to RNase A. Pretreatment of both fractions with micrococcal nuclease eliminated replication activity, whereas the combination of a pretreated fraction (either AS 40 or 65) with an untreated fraction was active. A heat-inactivated (55°C, 5 min) AS 65 fraction restored replication activity to the combination of micrococcal nuclease-treated AS 40 and AS 65 fractions.**

In prokaryotes, the *in vitro* study of DNA replication has been aided by the use of viral model systems (1, 2). Experiments with simian virus 40 (SV40) *in vivo* or in permeabilized infected cells have provided numerous insights into the mechanism of semidiscontinuous DNA replication in higher eukaryotes, since the only viral-encoded protein needed for replication is SV40-encoded large tumor antigen (T antigen) (3). The recent development of *in vitro* DNA replication systems for SV40 (4–7) should allow detailed investigation of the enzymatic processes involved in host DNA replication. This system has been used recently to examine the role of DNA polymerase α-primase in SV40 DNA replication (8, 9) and progress has been made in isolating multiple fractions required for replication activity (9, 10). One feature of prokaryotic replication that must be true of eukaryotic systems is that DNA replication occurs in separable, ordered steps, each with its own enzymatic requirements (11–13): prepriming, priming, elongation, and termination. The study of these isolated steps has helped define the roles of the large number of proteins involved in the overall replication process.

In this paper we describe conditions under which replication of SV40 ori$^+$ DNA [DNA containing the SV40 origin of replication (pSVO1ΔEP)] can be divided into two stages: (*i*) a preelongation step that occurs in the absence of dNTPs and (*ii*) elongation. We present evidence for the formation of an isolable intermediate during the preelongation stage of replication that requires ori$^+$ DNA, T antigen, and cellular factors. We have also examined the previously reported sensitivity of the *in vitro* replication reaction to bovine pancreatic ribonuclease A (RNase A) (7) and find that the preelongation step, but not the elongation step, is sensitive to this enzyme. In addition, we describe the fractionation of crude extract by ammonium sulfate precipitation and show that pretreatment of fractions with micrococcal nuclease results in a loss of activity that can be restored by adding untreated fractions.

MATERIALS AND METHODS

Preparation of Extracts and Ammonium Sulfate Fractions. T antigen, SV40 ori$^+$ DNA, and cytosolic extracts of HeLa cells were prepared as described (7). To prepare ammonium sulfate fractions, saturated ammonium sulfate solution (4°C, pH 8.0) was added to the cytosolic extract to 40% saturation (0.667 ml/ml of extract) and the precipitate was removed by centrifugation at 10,000 × *g* for 30 min. The supernatant was then adjusted to 65% saturation (0.714 ml/ml of fraction) and centrifuged as above. The precipitates were dissolved in buffer A (20 mM Tris·HCl, pH 8.0/0.1 mM EDTA/1 mM dithiothreitol/10% glycerol; 1/10 to 1/5 the starting volume of the extract) and dialyzed against two changes of buffer A containing 50 mM NaCl and 25% glycerol. These fractions, designated AS 40 and AS 65, respectively, were stored at −20°C, where they were stable for at least 1 month.

Replication Assays and Preincubation Conditions. Replication assays (30 μl) contained 30 mM Tris·HCl (pH 8.5), 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 4 mM ATP, 200 μM CTP, GTP, and UTP, 100 μM dATP, dCTP, and dGTP, 40 mM creatine phosphate (CrP), 20 μg of creatine phosphokinase (CrPK) per ml, 20 μM [*methyl*-^{3}H]dTTP (500 cpm/pmol), 0.5–1.0 μg of T antigen, and 180 ng of ori$^+$ DNA. Indicated amounts of fractions were added and the reaction mixtures were incubated as described in the figure legends.

Preincubation assays were performed as follows: 600 ng of ori$^+$ DNA, 1.6 μg of T antigen, and 600 μg of HeLa extract protein were incubated at 37°C as indicated in replication reaction mix (50 μl; see above) lacking dNTPs. dATP, dCTP, dGTP (100 μM each), and [^{3}H]dTTP (20 μM, 500 cpm/pmol) were then added and the incubation was continued at 25°C for the designated times. Reactions were terminated and acid-

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Abbreviations: SV40, simian virus 40; T antigen, SV40-encoded large tumor antigen; ori$^+$ DNA, DNA containing the SV40 origin of replication (pSVO1ΔEP); RNase A, bovine pancreatic ribonuclease A; CrP, creatine phosphate; CrPK, creatine phosphokinase; RF I, superhelical circular duplex DNA; RF II, relaxed circular duplex DNA containing at least one single-strand break; bp, base pair(s).

insoluble radioactivity was determined as usual (14). In some cases, 20 μM [α-^{32}P]dCTP (5000 cpm/pmol) was used in place of [^{3}H]dTTP.

Complex Isolation by Gel-Filtration Chromatography. ori$^+$ DNA (1.2 μg), 3 μg of T antigen, and 1.2 mg of HeLa extract were incubated in 100-μl reaction mixtures under preincubation conditions (see above) for 30 min at 37°C. After chilling on ice for 5 min, the total reaction mixture was chromatographed at 4°C on a 0.7 × 18.5 cm Bio-Gel A-15m column (Bio-Rad) in buffer containing 50 mM Tris·HCl (pH 8.0), 1 mM dithiothreitol, 1 mM $MgCl_2$, 0.2 mM ATP, 100 μg of bovine serum albumin per ml, and 10% glycerol at a flow rate of 5 ml/hr, and 200-μl fractions were collected. Aliquots (70 μl) of each fraction were assayed for replication activity in 100-μl reaction mixtures (see above) in the absence of added DNA and T antigen.

Micrococcal Nuclease Treatment. Aliquots (50 μl) of the ammonium sulfate fractions described above were adjusted to 1 mM $CaCl_2$, 30 units of micrococcal nuclease (P-L Biochemicals) was added, and the reaction mixtures were incubated at 30°C for 60 min. Digestion was terminated by the addition of 2 mM EGTA.

RESULTS

Time Course of SV40 DNA Replication with and Without Preincubation. When replication reaction mixtures were incubated at 25°C there was a lag of 20–30 min before nucleotide incorporation began that was abolished by preincubation at 37°C in the absence of dNTPs (Fig. 1*A*). Preincubation for <10 min was ineffective at stimulating subsequent incorporation, consistent with the 10-min lag in replication at 37°C described previously (7). Longer preincubations, up to 30 min, increased nucleotide incorporation, but preincubation for >30 min had little further effect (Fig. 1*B*). The incorporation during a 10-min second incubation was the same at 37°C and 25°C (data not shown). Similar observations have been made with the φX174 and *oriC* replication systems *in vitro* and are consistent with the formation of a preelongation complex.

A 1:10 dilution of preincubation reaction mixtures with replication buffer before the second-stage incubation did not affect incorporation. If the diluted preincubation reaction mixtures were incubated at 37°C in the absence of dNTPs before the second step, the stimulatory effect of preincubation declined with a half-life of 18 min (unpublished observations).

Products Formed During Second Incubation. ^{32}P-labeled products formed during the second-step incubation were similar to those formed during normal replication reactions (7) (Fig. 2*A*), with the exception that little or no superhelical circular duplex DNA (RF I DNA) was observed. When products labeled for short times from the start of the reaction were digested with *Dde* I, label first appeared in origin-proximal fragments (Fig. 2*B* and *Inset*) and, with time, in fragments distal to the origin, the fragment 180° opposite to the origin (165 bp) being labeled last. This suggests that replication initiated at the origin and proceeded bidirectionally to the expected termination point. Another possibility that cannot be ruled out by these results is that replication proceeded unidirectionally in either direction on different molecules with equal frequency. The time required to label the 165- and 410-bp fragments suggests that the elongation rate is 100–200 nucleotides per min, which compares favorably with the *in vivo* rate of SV40 replication (16, 17).

Requirements for the Preincubation Effect. As described above, the amount of incorporation during the first 10 min of the second-step incubation was dependent upon the length of the preincubation. As shown in Table 1, this effect absolutely required T antigen and was dependent upon HeLa extract and ATP. ori$^+$ DNA was also required during the preincubation; if ori$^+$ DNA was omitted or replaced by ori$^-$ DNA, incorporation was reduced by a factor of >10. Addition of aphidicolin, at concentrations that selectively inhibit DNA polymerase α, to the second reaction completely inhibited nucleotide incorporation. In addition, the synthesis normally observed in the absence of T antigen (1–2 pmol) was markedly reduced by preincubation (0.2–0.3 pmol). When RNase A was included in the preincubation reaction mixture, subsequent replication was inhibited. However, virtually no effect was seen when RNase A was added to the second reaction (Table 1).

Gel-Filtration Chromatography of Preincubation Products. To determine if an isolable intermediate were formed in the SV40 system, ori$^+$ DNA was preincubated with HeLa extract and T antigen followed by gel-filtration chromatography on Bio-Gel A-15m (exclusion limit, 1.5 × 10^7 Da). Replication assays of the fractions revealed a peak of activity coinciding with the DNA, well-separated from the bulk (>90%) of the eluted protein in the void volume fractions (Fig. 3*A*), suggesting that a complex had formed during preincubation. No replication activity was seen if ori$^+$ DNA was replaced with ori$^-$ DNA during the preincubation, even if ori$^+$ DNA was added after gel filtration (Fig. 3*A*) or if the preincubation step was omitted (Fig. 3*B*). T antigen was required during the preincubation; addition of T antigen to the second incubation did not result in nucleotide incorporation (Fig. 3*C*).

Incorporation by the peak fractions was the same whether the replication reaction mixture was incubated at 37°C or 25°C (Fig. 3*B*), similar to the preincubation experiments (see above). A comparison of the replication activity at 25°C in the peak fractions (23 pmol, Fig. 3*B*, fractions 8 and 9) with the amount of replication observed in the preincubation experi-

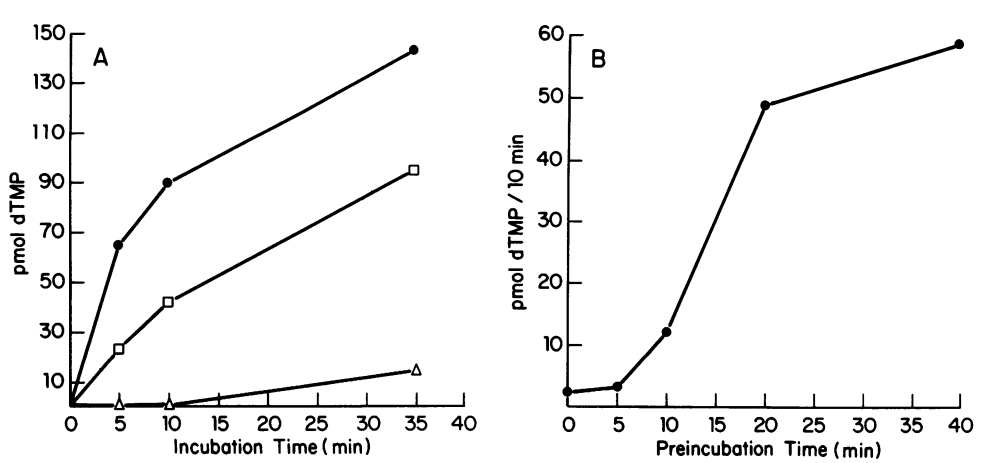

FIG. 1. (*A*) Time course of replication of ori$^+$ DNA at 25°C with or without preincubation at 37°C. Samples were preincubated for 0 min (△), 10 min (□), or 20 min (●). dNTPs and [^{3}H]dTTP were added, incubation was continued at 25°C, and acid-insoluble radioactivity was determined at the times indicated. (*B*) Nucleotide incorporation at 25°C as a function of preincubation time at 37°C. Samples were preincubated as described above at 37°C for the times indicated on the abscissa, dNTPs and [^{3}H]dTTP were added, and the acid-insoluble incorporation after 10 min at 25°C was determined.

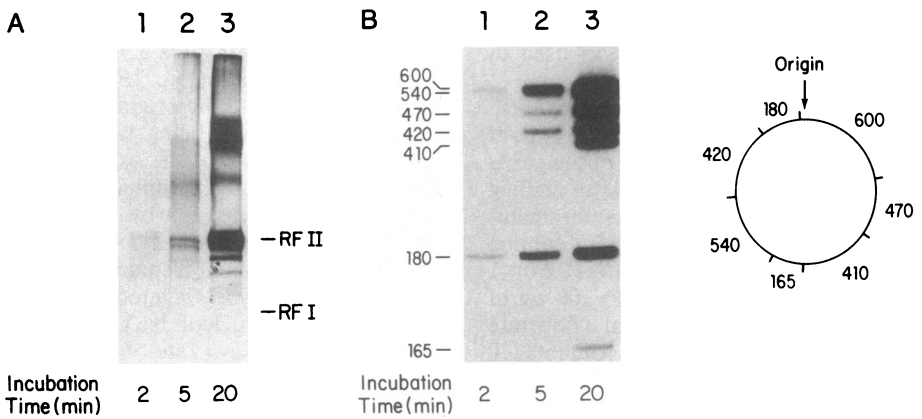

FIG. 2. (*A*) Agarose gel electrophoresis of products formed during second incubation. Reactions containing [α-^{32}P]dCTP were terminated at the times indicated. Equal volumes of resuspended ethanol precipitates were loaded onto a 15-cm 1.5% agarose gel in Tris, pH 8.5/acetate/EDTA buffer (15) and electrophoresed at 90 V for 3 hr. The gel was dried and the bands were visualized by autoradiography with Kodak XAR-5 film. RF I and RF II (relaxed circular duplex DNA containing at least one single-strand break) refer to positions of ethidium bromide-stained pSVO1ΔEP24 (ori$^+$ DNA) markers. (*B*) *Dde* I digest of products during time course of second incubation. Reactions labeled as above were terminated at the times indicated and equal volumes of the phenol-extracted, ethanol-precipitated products were digested with *Dde* I. Samples were loaded directly onto a 15-cm 5% polyacrylamide gel (20:1, acrylamide/bisacrylamide) in Tris/borate/EDTA buffer (15) and electrophoresed at 100 V for 90 min. Numbers at left indicate sizes of *Dde* I restriction fragments in base pairs (bp). (*Inset*) Location of *Dde* I fragments on pSVO1ΔEP24.

ments (Fig. 1 and Table 1) indicated that the recovery of replication activity was efficient.

The products formed by the gel-filtration column fractions resembled those formed during normal replication reactions (7) or in the preincubation experiments (Fig. 2*A*) (unpublished data). As with the preincubation experiments, little or no monomer RF I DNA was observed and the bulk of the products consisted of high molecular weight material.

Preparation of Ammonium Sulfate Fractions and Effect of Micrococcal Nuclease Treatment. We have prepared two ammonium sulfate fractions from the crude extract, AS 40 and AS 65. The AS 40 fraction had low replication activity that was stimulated >5-fold by AS 65, which was inactive by itself (Table 2). The requirements for, and products synthesized by, these fractions were similar to crude extract (Table 2 and ref. 10). A background of T antigen-independent incorporation catalyzed by the AS 40 fraction was inhibited by the AS 65 fraction. The replication catalyzed by these fractions was inhibited by aphidicolin and was sensitive to an amount of RNase A (5 μg/ml) much lower than that required to inhibit replication by crude extract (20–50 μg/ml) (7). As with crude extracts, a preelongation stage was observed in replication with these fractions that was selectively inhibited by RNase A (Table 1).

Table 1. Requirements for preincubation effect

Component omitted (−) or added (+)		dTMP incorporated, pmol/10 min
First incubation	Second Incubation	
Complete	Complete	37.2
− preincubation		1.0
− ATP	+ ATP	6.7
− ATP, CrP, CrPK	+ ATP, CrP, CrPK	6.0
− extract	+ extract	2.5
− T antigen	+ T antigen	<1
− DNA	+ ori$^+$ DNA	2.0
+ ori$^-$ DNA	—	<1
—	+ 0.1 mM aphidicolin	<1
+ 50 μg of RNase A per ml	—	3.6
—	+ 50 μg of RNase A per ml	33.3
+ AS 40, + AS 65	—	25.3
− AS 40, − AS 65	+ AS 40, + AS 65	2.1
+ 5 μg of RNase A per ml, + AS 40, + AS 65	—	<1
—	+ 5 μg of RNase A per ml, + AS 40, + AS 65	26.0

Replication assays (50 μl) were carried out. Following the second incubation at 25°C for 10 min, acid-insoluble [^{3}H]dTMP was determined where indicated, and 60–100 μg of AS 40 and 60–100 μg of AS 65 were added to each reaction mixture in place of crude extract. Incubations were carried out as described above.

To further study the nature of nuclease-sensitive factors in the *in vitro* replication reaction, we have used micrococcal nuclease to pretreat AS fractions. As shown in Table 3, when both AS fractions were micrococcal nuclease digested, replication activity was lost, whereas mock-treated fractions (EGTA added prior to micrococcal nuclease) were still active. However, combination of an untreated fraction with a micrococcal nuclease-treated fraction restored full replication activity, suggesting that the nuclease-sensitive component(s) could be supplied by either fraction. The low replication activity present in the AS 40 fraction in the absence of the AS 65 fraction was abolished by micrococcal nuclease treatment.

The AS 65 fraction was also sensitive to heat, and incubation at 55°C for as little as 2 min resulted in complete loss of stimulatory activity. Combination of the heat-treated and nuclease-treated AS 65 fractions, however, again allowed replication with a nuclease-treated AS 40, suggesting that heat and micrococcal nuclease treatment inactivate different factors.

RNase A Inhibits Multiple Rounds of Replication. One corollary of the observation that RNase A inhibits preelongation but not elongation is that addition of RNase A to preincubated reaction mixtures will inhibit multiple rounds of replication, since reinitiation would be blocked. To test this, reaction mixtures were preincubated as above and then incubated at 37°C for extended periods of time with dNTPs in the presence or absence of RNase A, and the products were analyzed by digestion with *Mbo* I. Since *Mbo* I will cleave at the sequence GAT C only if the adenosine residues on both strands are unmethylated, and since the substrate plasmid was maintained in a *dam*$^+$ strain of *Escherichia coli* (HB101), *Mbo* I digestion products should be seen only after

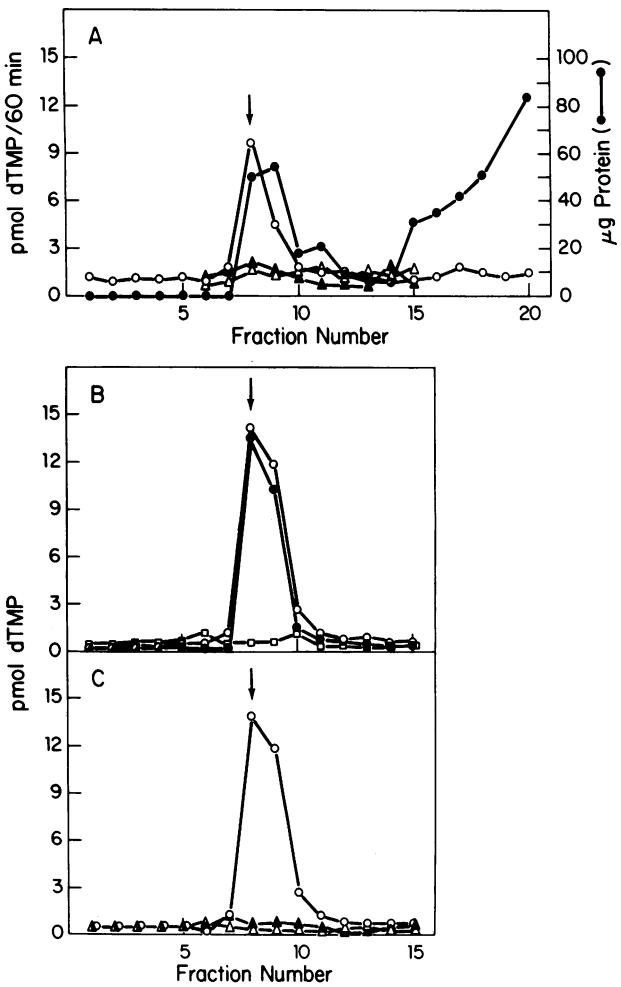

FIG. 3. Bio-Gel A-15m chromatography of preelongation complexes. (*A*) DNA requirement for complex formation. Preincubations were carried out in the presence of SV40 ori$^+$ (○) or ori$^-$ (△, ▲) DNA. Aliquots of column fractions were assayed for replication activity at 37°C either directly (○, △) or after the addition of 480 ng of ori$^+$ DNA (▲). Total μg of protein per fraction was determined by using the Bio-Rad protein assay reagent under the manufacturer's recommended conditions. (*B*) Effect of preincubation time on complex formation. Samples were preincubated for 0 min (□) or 30 min (○, ●), chromatographed, and assayed for replication activity at either 37°C for 60 min (○, □) or 25°C for 20 min (●). (*C*) T antigen requirement for complex formation. Samples were preincubated in the presence (○) or absence (△, ▲) of T antigen. Fractions were assayed for replication activity at 37°C for 60 min in the absence (△) or presence (▲) of 1.6 μg of T antigen. Arrow indicates the void volume fraction (elution position of plasmid DNA).

more than one round of replication. Using this assay, we previously showed that under normal conditions, crude extracts are capable of multiple rounds of replication (7). In the presence of RNase A, the products remained resistant to *Mbo* I, even after 2 hr in the replication reaction mixture (Fig. 4, lanes 1 and 5), whereas in its absence, the expected *Mbo* I-sensitive material was produced (lanes 3 and 7). This inhibitory effect of RNase A was not observed in the preincubation experiments (Table 1) since reinitiation could not have occurred during the brief second incubation at 25°C.

DISCUSSION

We have presented evidence that SV40 DNA replication *in vitro* proceeds through the formation of a preelongation intermediate, which occurs in the absence of dNTPs, followed by elongation, which requires dNTPs and DNA polymerase α, and that these two stages can be separated. Studies on φX174 (11, 12) and *oriC* (13) DNA replication *in vitro* with purified proteins have demonstrated that a lag preceding replication could be eliminated by preincubating specific proteins with DNA in the absence of dNTPs. It was shown that this lag was due to the formation of a preinitiation complex that could be isolated by gel-filtration chromatography and was competent to carry out efficient replication upon addition of dNTPs and elongation proteins.

We have found that preincubation of SV40 ori$^+$ DNA, T antigen, and HeLa extract at 37°C in the absence of dNTPs markedly stimulated synthesis at a lower temperature upon addition of dNTPs. This stimulation was also dependent on ATP and the length of the preincubation period. The products formed during replication of preincubated samples were similar to those formed during normal replications (7), except that little or no RF I DNA was observed.

The lag before DNA synthesis could reflect the time required to form a preelongation complex, analogous to those described for φX174 and *oriC* (see above), or to synthesize the first primer. Since the preincubations were done in the absence of dNTPs and since replication activity could be recovered in gel-filtration void volume fractions of reaction mixtures preincubated in the presence of aphidicolin (data not shown), it is unlikely that deoxynucleotide incorporation by DNA polymerase α during the first stage is necessary for

Table 2. Requirements for replication by ammonium sulfate fractions

Component added (+) or omitted (−)	dTMP incorporated, pmol/60 min
Complete	32.7
− AS 65	6.0
− AS 65, − T antigen	3.0
− AS 40	<1
− ATP, CrP, CrPK	6.1
− T antigen	<1
− DNA	<1
− ori$^+$ DNA, + ori$^-$ DNA	<1
− CTP, GTP, GTP	31.6
− dATP, dCTP, dGTP	1
+ 0.1 mM aphidicolin	<1
+ 5 μg of RNase A per ml	<1

Replication reaction mixtures (30 μl) contained 60–100 μg of AS 40 and 60–100 μg of AS 65 in the standard replication reaction mixture. Reaction mixtures were incubated at 37°C for 60 min and acid-insoluble ^{3}H was determined.

Table 3. Effect of micrococcal nuclease and heat on ammonium sulfate fractions

Fraction added	dTMP incorporated, pmol/60 min
Untreated AS 40 + untreated AS 65	31.9
MN-treated AS 40	
− AS 65	<1
+ untreated AS 65	27.2
+ MN-treated AS 65	1.7
+ mock MN-treated AS 65	25.1
+ heated (55°C, 5 min) AS 65	3.5
+ MN-treated AS 65 + heated AS 65	18.4
MN-treated AS 65	
− AS 40	<1
+ untreated AS 40	23.6

Replication assays (25 μl) contained 60–100 μg of AS 40, 60–100 μg of AS 65, 180 ng of ori$^+$ DNA, and 0.6 μg of T antigen plus the standard replication assay components. Following incubation at 37°C for 60 min, acid-insoluble radioactivity was determined. MN, micrococcal nuclease.

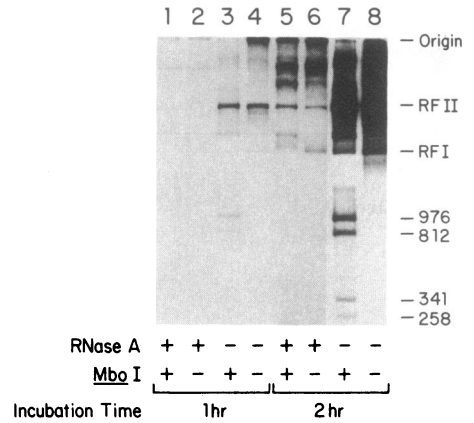

FIG. 4. *Mbo* I digestion of products formed in the presence and absence of RNase A. ori$^+$ DNA, T antigen, and HeLa extract were preincubated as before, dNTPs and [α-^{32}P]dCTP were added, and incubation was continued at 37°C in the presence (lanes 1, 2, 5, and 6) or absence (lanes 3, 4, 7, and 8) of 50 μg of RNase A per ml. Products were divided in two equal aliquots; one aliquot was digested with *Mbo* I (lanes 1, 3, 5, and 7) and one was not (lanes 2, 4, 6, and 8), and samples were electrophoresed on a 1.5% agarose gel (see Fig. 2). Numbers at right indicate sizes of *Mbo* I restriction fragments in bp (see ref. 7 for positions of fragments).

the preincubation effect. Due to the lack of an assay for initiation (i.e., primer synthesis), it was not possible to determine whether primer synthesis was required for the preincubation effect.

The gel-filtration experiments provide further evidence that the replication can be divided into two stages and that an intermediate generated during the first stage can be isolated and subsequently elongated. The observation that the excluded fractions efficiently carried out elongation upon addition of dNTPs suggests that most, if not all, of the proteins required for elongation have become associated with the DNA. It is possible that many of these proteins coeluted with the DNA due to T antigen- or origin-independent interactions and it should be noted that DNA polymerase α was observed in void volume fractions of ori$^+$ and ori$^-$ DNA-containing reactions (unpublished observations). Thus, the excluded fractions probably contain nonspecific complexes in addition to those dependent on preincubation in the presence of T antigen and ori$^+$ DNA.

The time required to complete one round of replication of the plasmid (5–20 min) is similar to *in vivo* observations (18, 19) and slower than the rate of mammalian chromosomal replication (16). The reason for this discrepancy is not clear, although others have suggested that it reflects a slower replication of origin-distal fragments (17, 20).

The inhibitory effect of RNase A during preincubation but not during elongation suggests that a nuclease-sensitive component is required prior to elongation. This was substantiated by the inhibition of multiple rounds of replication by RNase A (Fig. 4). The elongation reaction catalyzed by HeLa extracts on a poly(dT)-tailed linear duplex structure (8) was also not inhibited by RNase A (unpublished observation).

We have extended the observations from the RNase A inhibition experiments by pretreating two ammonium sulfate fractions required for replication with micrococcal nuclease and demonstrating a resultant loss of replication activity. This suggests that a preexisting nucleic acid is required for replication and provides an assay for the purification of such a component. The fact that a heat-inactivated AS 65 fraction can complement micrococcal nuclease-treated fractions suggests that this component is heat stable and that at least one other, heat-labile component exists in the AS 65 fraction. Whether the nuclease-sensitive component plays a direct role in the catalysis of DNA replication or an indirect role, such as binding nucleases or other potential inhibitors of replication, is not clear at present. It is interesting to note that at least one cellular RNA, the 7S-K nuclear RNA, has homology to a portion of the SV40 control region (21), although the homologous sequences can be deleted from origin-containing plasmids with no apparent loss of replication activity *in vitro* (22). Regulation of replication initiation by an origin-complementary polyribonucleotide has been shown to occur in the case of the ColE1 replicon (23). Answers to questions regarding the nature and mechanism of action of the nuclease-sensitive component(s) in SV40 DNA replication *in vitro* await further fractionation of the system.

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