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Multiple rounds of adenovirus DNA synthesis *in vitro*

(eukaryotic DNA replication/soluble nuclear extracts/soluble cytoplasmic extracts/DNA–protein interaction)

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ABSTRACT **Adenovirus (Ad) type 2 DNA synthesis can be initiated in the presence of a soluble extract of uninfected HeLa cell nuclei, a 25–60% saturated ammonium sulfate fraction of infected cytoplasm and viral DNA covalently linked to a 5′-terminal protein (Ad DNA-prot). As the purification, from either the nuclei or cytoplasm, of factors active in DNA replication proceeded, various nonreplicative reactions which incorporate labeled deoxynucleotides were uncovered. In order to distinguish replicative from repair reactions, an assay was developed in which the Ad DNA-prot was digested with *Xba* I, all of the fragments so produced were used (without separation) in a replication reaction, and the products were assayed by electrophoresis on neutral agarose gels. In replicative reactions, most of the radioactivity was incorporated into the terminal fragments, with the internal fragments remaining unlabeled. Infected cytoplasm contains a "discrimination" function in addition to specific factors for Ad DNA replication. The discrimination factors inhibit the nonspecific nucleotide incorporation by uninfected HeLa nuclear extracts on Ad DNA-prot. The specific replicative incorporation into the terminal Ad DNA-prot fragments has also allowed an independent assay for reinitiation of progeny molecules synthesized *in vitro*. After the first round of replication, the 5′ strand of the progeny duplex from each end is labeled. These same labeled strands will be displaced during the second round of replication and appear in new bands which have been shown to be the single-strand equivalents of the terminal fragments. Thus, at least two rounds of Ad DNA synthesis can initiate at each terminus *in vitro*. The appearance of displaced single strands requires DNA replication because the addition of dideoxycytidine triphosphate after the first round of synthesis prevents the displacement reaction. Both the progeny single- and double-stranded DNA appear to be linked to protein.**

Several *in vitro* systems that use infected cell extracts for the synthesis of adenovirus (Ad) DNA have been reported (1–4). These cell fractions, which preserve many of the unusual features of Ad DNA synthesis demonstrated *in vivo*, replicate only one strand in the 5′-to-3′ direction at replication forks and simultaneously displace the parental strand of the same polarity (5). We have recently found that an ammonium sulfate fraction of infected HeLa cell cytoplasm and a low-salt extract of uninfected HeLa nuclei synthesize full-sized DNA on an exogenously added Ad template which maintained the 5′-terminal, covalently linked protein (Ad DNA-prot). Purification of the active components from both the nuclei and cytoplasm has shown that, as the cell extracts are separated into various fractions, some of the fractions become active in abortive synthesis on the exogenous DNA template. These aberrant patterns of incorporation of labeled deoxynucleoside triphosphates result in the production of acid-insoluble radioactivity that does not reflect actual DNA synthesis.

In order to differentiate rapidly between replication and gap-filling or other repair-type reactions, we have developed an assay that relies on the synthesis of Ad DNA on restriction endonuclease-cleaved, defined fragments of Ad DNA-prot. By adding a mixture of the two terminal fragments containing the 5′-linked protein and the internal fragments devoid of this protein, the specific replicative pattern can readily be distinguished from the repair-type reaction: Replicative synthesis needs the terminal protein and is therefore confined to the end fragments, whereas the nonspecific reaction indiscriminantly adds radioactivity to all fragments as a function of their molecular weight.

During the development of this assay, bands of radioactivity that did not correspond to any of the marker, restriction endonuclease fragments were produced. Investigation of these extra discrete bands revealed that they were the single-strand equivalents of the two terminal fragments and most likely represent the strand displaced during replication. Because the displaced single strand had already been labeled *in vitro* during the first round of replication, the appearance of this label in the single strand indicated reinitiation at the origin and at least two rounds of replication *in vitro*.

MATERIALS AND METHODS

Preparation of Components of the DNA Synthesis Reaction. Ad DNA-prot was purified from Ad2 that had been banded twice in CsCl gradients (6). The Ad DNA-prot from guanidine-lysed virion was sedimented on sucrose gradients containing 4 M guanidine hydrochloride as described (7). The Ad DNA-prot (0.15 μg) was digested with 7 units of *Xba* I, 3 units of *Bam*HI, or 2 units of *Eco*RI in 20 μl of 20 mM Hepes, pH 7.4/10 mM NaCl/7 mM $MgCl_2$ for 60 min at 37°C. Ad2 cytoplasm was prepared, at 21 hr after infection, from 3.5 liters of infected cells (4000 virions per cell) to which 3 mM hydroxyurea had been added at 2 hr after infection (2). The nuclei were removed by centrifugation after Dounce homogenization, and the cytoplasm was fractionated by ammonium sulfate precipitation. The cytoplasmic proteins precipitated by 25% saturated ammonium sulfate (0.144 g added to 1 ml of solution) were removed by centrifugation at 15,000 rpm in the type 40 Beckman Spinco rotor for 20 min. The supernatant solution was adjusted to 60% saturation with ammonium sulfate (0.230 g added to 1 ml of solution) and the collected precipitate was suspended in 3.5 ml of 0.025 M Tris, pH 7.5/1.5 mM 2-mercaptoethanol/1 mM EDTA/50 mM NaCl/10% (vol/vol) glycerol and dialyzed twice against 200 vol of the same buffer. The uninfected nuclear extracts were made from logarithmically growing HeLa cultures as described for the infected cells but no hydroxyurea was added (8).

Reaction Conditions for *in Vitro* Ad DNA Synthesis. After a 1-hr digestion with restriction endonuclease, the cleaved DNA was added to a reaction mixture (100 μl) which contained 25 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.05 mM

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Abbreviations: Ad, adenovirus; Ad DNA-prot, Ad DNA covalently linked to a 5′-terminal protein on each strand; ddCTP, dideoxycytidine triphosphate; BND-cellulose, benzoylated naphthoylated DEAE-cellulose.

dATP, 0.05 mM dGTP, 0.05 mM dCTP, 3.75 mM ATP, 1.5 μM [α-^{32}P]dTTP, 20 μl of nuclear extract (protein concentration, 2.5 mg/ml), and 2.5 μl of Ad2 cytoplasm (25–60% ammonium sulfate precipitate; protein concentration, 20 mg/ml). The reaction was continued for 60 min at 37°C and then was terminated with 0.2% NaDodSO$_4$. Pronase (10 μg) was added, the mixture was incubated for 20 min at 37°C to remove the covalently linked terminal protein, and the DNA was precipitated for 16 hr with 2.5 vol of ethanol in the presence of 0.5 M NaCl. The DNA was dissolved in 50 μl of 40 mM Tris/1 mM EDTA/5 mM sodium acetate, pH 7.8 and electrophoresed on 1.4% agarose slab gels containing the same buffer, until the bromphenol blue dye reached the lower edge of the gel. The gels were stained with ethidium bromide (0.5 μg/ml), photographed, dried, and autoradiographed (usually for 18 hr). The acid-precipitable radioactivity in DNA was quantitated in aliquots of the terminated reaction mixtures.

Chromatography of Ad DNA-prot on Benzoylated Naphthoylated DEAE (*BND*)-Cellulose. Samples from DNA synthesis reactions were processed for BND-cellulose chromatography by stopping the reaction with 10 mM EDTA/90 mM sodium pyrophosphate. After dialysis against three changes of 500 vol of 10 mM Tris, pH 7.4/1 mM EDTA, the sample was divided into two portions. One portion was digested with Pronase (40 μg/ml) for 30 min at 37°C; both were adjusted to 0.3 M NaCl and loaded onto BND-cellulose columns as described (9, 10). Deproteinized double-stranded DNA was eluted with 1 M NaCl; the single-stranded DNA was eluted by the addition of 2% caffeine in the same buffer. Single- and double-stranded DNA covalently linked to protein remained bound until eluted with 1% NaDodSO$_4$/8 M urea. All samples containing radioactivity were precipitated with ethanol, dissolved in 50 μl of 40 mM Tris/1 mM EDTA/5 mM sodium acetate, pH 7.8/0.2% NaDodSO$_4$ and electrophoresed on 1.4% agarose gels with the tray and gel buffer containing 0.2% NaDodSO$_4$.

Materials. *Xba* I and *Bam*HI were purchased from BBL, S1 nuclease was from Calbiochem, and *Eco*RI was purified in this laboratory by Karen Scharff by described methods (11). The [α-^{32}P]dTTP was obtained from Schwarz/Mann, the agarose was from Sargent-Welch, and the ACS II scintillation fluid was from Amersham.

RESULTS

DNA Synthesis on *Xba* I Fragments of Ad2. Ad DNA-prot was digested for 1 hr with *Xba* I and the resulting fragments (without any further purification) were added to a DNA synthesis reaction containing extracts of both Ad-infected cytoplasm and uninfected HeLa nuclei. After 1 hr of incubation, the viral DNA fragments were deproteinized, precipitated with 2.5 vol of ethanol, and electrophoresed on an agarose gel. The *in vitro* DNA synthesis reaction labeled primarily terminal DNA fragments C and E (Fig. 1). When the terminal protein was removed from the DNA before the synthesis reaction, there was only a small amount of radioactive incorporation distributed simply according to the size of the fragments and therefore appearing primarily in the larger fragments (A and B). When synthesis was attempted on the Ad DNA-prot with the uninfected nuclear extract alone, there was considerable nonspecific incorporation in a distribution that smeared through most of the molecular weight ranges of the gel with some accentuation in the regions of the *Xba* I bands. Synthesis with Ad DNA-prot and infected cytoplasm alone gave barely detectable products, but the radioactivity concentrated in the terminal fragments. There was no detectable degradation of the *Xba* I internal fragments A and B during the synthesis reaction, as evidenced by the ethidium bromide staining of the viral DNA in the reaction mixture.

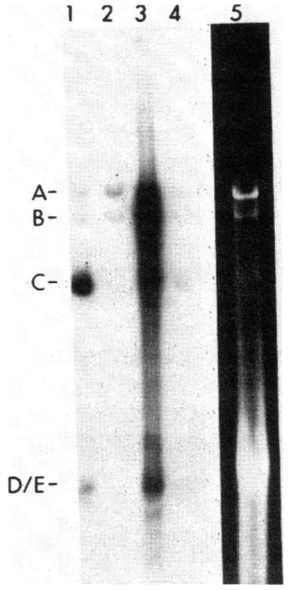


FIG. 1. DNA synthesis on *Xba* I fragments of Ad2. Ad DNA-prot or deproteinized DNA was digested with *Xba* I and the cleaved DNA fragments were added to a synthesis reaction mixture containing various combinations of Ad2 cytoplasmic extract and uninfected HeLa nuclear extract. The radioactive DNA products of the synthesis reaction are shown following electrophoresis and autoradiography. Lanes: 1, Ad DNA-prot (0.15 μg DNA), Ad2 cytoplasm, and uninfected HeLa nuclear extract; 2, identical to lane 1 except that Ad2 DNA (deproteinized) replaced the Ad DNA-prot; 3, Ad DNA-prot with uninfected nuclear extract alone; 4, Ad DNA-prot with the Ad2 cytoplasm alone; 5, ethidium bromide stain of the synthesis reaction in lane 1. The letters designate the location of the five *Xba* I fragments. Fragments D and E did not separate. Fragments C and E represent the right and left termini of Ad2 DNA, respectively (see Fig. 2). The diffuse ethidium bromide stain that appeared near the D/E band (lane 5) was RNA in the cell extract and not the product of *Xba* I digestion of Ad2 DNA-prot; this band was not present when the sample was treated with RNase A (100 μg/ml, 30 min, 37°C) before electrophoresis.

Ad DNA Synthesis on Fragments Generated by Three Restriction Endonucleases. The terminal fragments of *Bam*HI and *Eco*RI digestions were more easily separated than were those of *Xba* I digestion [the internal D fragment (4%) was difficult to separate from the left-hand terminal E fragment (3.85%)]. Synthesis in the presence of terminal protein was on the *Xba* I C and E fragments, the *Eco*RI A and C fragments, or the *Bam*HI A and B fragments (Fig. 2). All of these fragments of Ad2 DNA are located at the termini. In contrast, deproteinized DNA incorporated radioactivity poorly and with a distribution that conformed to the molecular weights of the fragments. If the incorporation of [α-^{32}P]TTP which began at the terminus represented only a short stretch of end labeling rather than synthesis for the entire extent of the *Bam*HI A and B fragments, subsequent cleavage with *Xba* I enzyme would have yielded label only in *Xba* I fragments C and E, a terminal subset of *Bam*HI fragments A and B, respectively. However, the results in Fig. 2, lane 4, suggest that the entire fragments A and B were labeled with [α-^{32}P]TTP because, after the second cleavage, there were large internal subfragments of *Bam*HI fragments A and B that contained radioactivity.

Multiple Rounds of Synthesis on Ad2 DNA Are Initiated *in Vitro*. During the replication reaction on Ad DNA terminal fragments, one parental strand should be displaced by the progeny-growing chain. In the first round of synthesis *in vitro*, the dis-

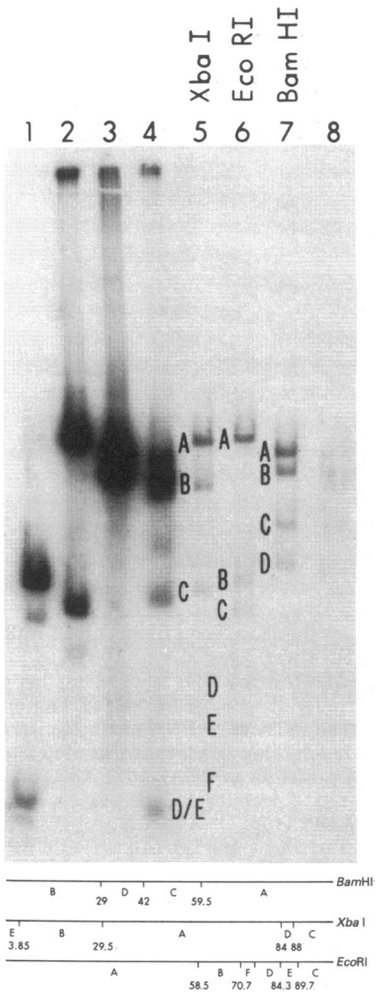


FIG. 2. Comparison of Ad DNA synthesis on *Xba* I, *Eco*RI, and *Bam*HI fragments. Ad DNA-prot (0.15 μg; lanes 1–4) or Ad DNA (0.15 μg; lanes 5–8) were digested for 1 hr with *Xba* I (lanes 1 and 5), *Eco*RI (lanes 2 and 6), or *Bam*HI (lanes 3 and 7). The digestion products were used in a synthesis reaction which contained Ad2-infected cytoplasm and uninfected HeLa nuclear extract. Lanes 4 and 8 are *Bam*HI digests identical to lanes 3 and 7, respectively, except that, after the 1-hr synthesis reaction, the products were digested with *Xba* I for an additional 1 hr. Lanes 5, 6, and 7 are labeled to show the mobility of the fragments generated by each of the restriction endonucleases. Size and location of the various restriction endonuclease products are shown diagramatically (12).

placed single strand would not be detected by autoradiography because it was only minimally labeled with [^{14}C]thymidine (150 cpm added per reaction). However, if the terminal fragments underwent another round of replication, the second strand displaced would have been labeled with [^{32}P]dTMP during the first round of replication. Accordingly, bands of radioactivity that might represent displaced single strands were sought and their kinetics of labeling during the *in vitro* DNA synthesis reaction were measured. The general strategy was to label the *Xba* I fragments during a 20-min DNA synthesis reaction *in vitro* and to continue the reaction but stop the radioactive incorporation with large excesses of unlabeled dTTP. If a labeled single-stranded DNA were displaced during the subsequent synthesis of Ad DNA *in vitro*, it should appear with a short delay. The resultant single-stranded intermediate should be stable in alkali but digestable with S1 nuclease. The results of such an experiment are shown in Fig. 3.

After a 20-min synthesis reaction, *Xba* I fragments C and E were prominently labeled (Fig. 3). Thirty minutes later, radioactivity appeared in two other bands whose electrophoretic mobility was faster than that of the C and E bands. Radioactivity, chased from the C and E bands, accumulated in the new bands (C′ and E′) over the next 3 hr. The acid-precipitable radioactivity remained constant during the chase period. The sample at 3 hr was divided and one part was denatured with alkali before processing for electrophoresis. At increased pH, virtually all the radioactivity was converted from C and E to C′ and E′ bands. When samples were treated with S1 nuclease (a single-strand-specific nuclease) before electrophoresis, the C′ and E′ bands completely disappeared. Thus, the new bands had the characteristics expected of single strands displaced from the duplex Ad DNA restriction endonuclease fragments.

Densitometric tracings showed that the kinetics of conversion of radioactivity to C′ and E′ were identical when corrections were made for the length of the fragments, indicating identical rates of initiation at both ends of the Ad DNA-prot (data not shown). The appearance of label in the single strands of the terminal restriction endonuclease fragments indicates that strands synthesized during initial rounds of DNA replication *in vitro* are further replicated. The products presumably are displaced labeled single strands replicated in the type I synthesis reaction (13).

Displacement of Single Strands Requires DNA Replication. The appearance of the terminal fragment single strands could have resulted from displacement replication or, alternatively, could have resulted from a nonreplicative unwinding reaction of the terminal fragments. To distinguish between these possibilities, various inhibitors of DNA synthesis were added to the replication reaction. After 30 min of normal *in vitro* DNA synthesis on the *Xba* I fragments, ddCTP was added to the reaction mixture and the fate of the DNA synthesized in the preceding interval was followed for an additional 150 min. The presence of ddCTP sufficient to inhibit DNA synthesis prevented the appearance of C′ and E′ single-stranded bands (Fig. 4). However, it did not prevent further nonspecific low-level

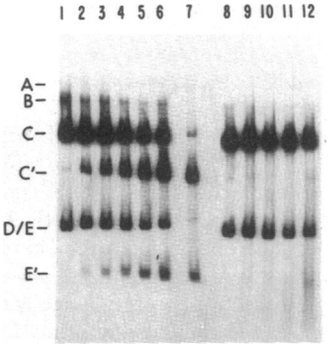


FIG. 3. Single-stranded DNA is displaced from *Xba* I fragments C and E. Ad DNA-prot (0.15 μg) was digested with *Xba* I and the subsequent synthesis reaction with [α-^{32}P]dTTP (1.5 μM) was allowed to proceed for 20 min. Then, unlabeled dTTP (100 μM) was added to each reaction and the incubation was continued for 0 min (lanes 1 and 8), 30 min (lanes 2 and 9), 60 min (lanes 3 and 10), 90 min (lanes 4 and 11), 120 min (lanes 5 and 12), and 180 min (lanes 6 and 7). The reaction was stopped by the addition of EDTA and cooling to 0°C. In lanes 8–12 the samples were digested with S1 nuclease (0.1 unit/ml) in 30 mM sodium acetate, pH 4.5/100 mM NaCl/0.3 mM $ZnSO_4$ for 15 min at 37°C. The sample in lane 7 was processed by adding 0.2 M NaOH and holding at 0°C for 10 min before neutralization with HCl. All samples were subsequently deproteinized and processed for electrophoresis and autoradiography. The quantity of sample in lane 7 was 50% of that shown in all other lanes.

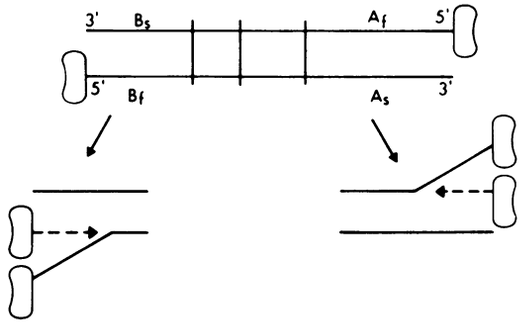
labeling of the A and B internal fragments. Aphidicolin, another inhibitor of DNA synthesis, likewise inhibited the synthesis of single-stranded progeny DNA molecules (data not shown).

Determination of DNA Strand Synthesized *in Vitro*. In order to determine which of the strands at each end of the DNA molecule was synthesized by the *in vitro* synthesis reaction, the replication mixture containing [α-^{32}P]dTTP was incubated for 60 min in the presence of the ^{14}C-labeled fragments of Ad DNA-prot previously digested with the *Bam*HI. The *Bam*HI fragments were separated on 0.7% neutral agarose gels and quantitated by measurement of Cerenkov radioactivity. This confirmed that most (91%) of the ^{32}P radioactivity was in the A and B terminal double-stranded fragments. The gel fragments containing the A and B bands were soaked in 0.2 M NaOH to denature the DNA without eluting it from the gel. After removal of the alkali from the gel fragments, they were repolymerized into 0.7% neutral agarose gels and the single strands were separated by electrophoresis (13). The results are shown in Table 1. The ^{32}P radioactivity was found predominantly in one strand at each terminus and that strand represented the 5′ end. These results are consistent with the known models for Ad DNA replication *in vivo*.

Ad2 DNA Synthesized *in Vitro* is Linked to Protein. Another prediction of the model of replication is that the terminal fragments synthesized *in vitro* would contain the 5′-terminal protein on the progeny duplex molecules as well as on the displaced single strand. The presence of the covalently linked terminal protein was measured by chromatography on BND-cellulose columns which can distinguish deproteinized double-stranded DNA from single-stranded DNA and both of these species from any DNA covalently linked to protein. When the synthesis reaction on *Xba* I fragments was stopped by NaDodSO$_4$ followed by Pronase digestion, the usual pattern of end labeling in the C and E double-stranded fragments and C′ and E′ single-stranded fragments was observed after 180 min of synthesis (Fig. 5). The double-stranded fragments C and E of deproteinized DNA were readily separated from the single-stranded C′ and E′ products on BND-cellulose. There was no further radioactivity eluted by 1% NaDodSO$_4$/8 M urea during the chromatography of deproteinized DNA (data not shown). When the products of the synthesis reaction were not deproteinized prior to BND-cellulose chromatography, no C, E, C′, or E′ bands were eluted either by 1 M NaCl or 1 M NaCl/2% caffeine. These four bands were successfully eluted by 1% NaDodSO$_4$/

Table 1. Determination of DNA strand synthesized *in vitro*

*Bam*HI Fragment	Strand	Label, cpm ^{14}C	^{32}P
A	Slow	145	72
A	Fast	188	524
B	Slow	136	74
B	Fast	179	739



A *Bam*HI digestion and DNA synthesis reaction identical to those in Fig. 2, lane 3, were performed with Ad DNA-prot. The deproteinized products were electrophoresed (35 × 0.6 cm; 0.7% agarose cylindrical gel) at 80 V for 16 hr. The fragments were located by ethidium bromide staining for 15 min in the dark, and the A and B bands were cut out with minimal exposure to the UV light source. Denaturation of the DNA within the gel slice and subsequent electrophoresis were performed as described (14) except that the gel slices were assayed in ACS II scintillation fluid (10 ml). ^{14}C represents radioactivity in the input DNA-prot; ^{32}P represents incorporation during the *in vitro* synthesis reaction. The A fragment fast-moving strand represents the right-hand 5′ terminus and the B fragment fast-moving strand represents the left 5′ terminus (15). The model of replication for the terminal restriction fragments of Ad DNA-prot is shown. The symbol at the 5′ ends of the DNA represents the covalently linked protein (5).

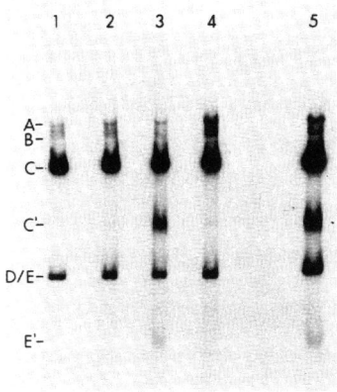


FIG. 4. ddCTP inhibits conversion of double-stranded DNA to single-stranded. *Xba* I fragments were prepared from Ad DNA-prot and the synthesis reaction with [α-^{32}P]dTTP was allowed to proceed normally for 30 min. At that time the reaction mixture was divided into five aliquots. Lanes: 1, promptly stopped with NaDodSO$_4$ (lanes 2–5 were further incubated for 150 min); 2, 50 μM ddCTP plus 100 μM dTTP during the chase; 3, 100 μM dTTP but no inhibitor; 4, 50 μM ddCTP; 5, no further additions for the 150-min chase period. All reactions were stopped with NaDodSO$_4$ and deproteinized before electrophoresis on 1.4% agarose gels.

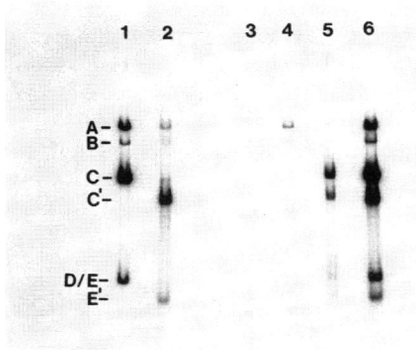


FIG. 5. Ad 2 synthesized *in vitro* is linked to protein. Five aliquots of Ad DNA-prot (0.15 μg in each) were digested with *Xba* I for 1 hr. The DNA synthesis reaction was begun with the addition of uninfected HeLa nuclear extract, Ad2-infected cytoplasm, and the necessary nucleotides. After 180 min of synthesis, one reaction was terminated by the addition of NaDodSO$_4$ and Pronase. The four others were stopped by EDTA and pyrophosphate, pooled, and processed for BND-cellulose column chromatography. Lanes: 1 and 2, electrophoresis of deproteinized DNAs eluted by 1 M NaCl and 1 M NaCl/2% caffeine, respectively; 3–5, DNAs recovered from the chromatography of DNA-protein samples eluted by 1 M NaCl (lane 3), 1 M NaCl/2% caffeine (lane 4), or 1% NaDodSO$_4$/8 M urea (lane 5); 6, intact sample treated with NaDodSO$_4$ and Pronase without the BND-cellulose chromatography separation step.

8 M urea. The small amount of nonspecific labeling in the A and B fragments of deproteinized DNA which eluted as both double- and single-stranded DNA did not appear in any fraction eluting with protein. Thus, it seems that both the double-stranded and single-stranded products of the *in vitro* DNA synthesis reaction are bound to protein.

DISCUSSION

DNA can be efficiently synthesized *in vitro* in extracts of uninfected HeLa nuclei supplemented with a 25–60% saturated ammonium sulfate fraction of infected cytoplasm, provided that the exogenously supplied Ad2 DNA has the terminal protein covalently linked to its 5′ end. The reaction on deproteinized Ad2 DNA, which probably still has several amino acids blocking the 5′ terminus, was minimal as was the replication of the internal restriction endonuclease DNA fractions. The reaction was dependent on ATP because the specific synthesis on the end fragments was decreased by 89% in the absence of exogenous ATP. DNA-prot and infected cytoplasm with the appropriate reaction mixture produced small amounts of Ad DNA which originated at the termini. However, the nuclear extract was necessary to amplify this reaction by a factor of 8- to 13-fold. Nuclear extracts from various sources will complement the Ad replication system (unpublished data). Both G_1 and S phase nuclei from HeLa cells, as well as random nuclei from Chinese hamster ovary cells, were able to substitute for the randomly growing HeLa nuclei used in these experiments (16). Purified preparations of DNA polymerase β isolated from HeLa cells could replace 70–80% of the nuclear requirement (16).

The infected cytoplasmic extract not only furnished the factors that specifically result in synthesis starting at both ends of the viral DNA molecule but also had a factor(s) which repressed the nonspecific dTMP incorporation in other segments of the DNA. This factor, which we refer to as a "discrimination" function, is also present in similar extracts from uninfected HeLa cytoplasms.

The new radioactive bands (C′ and E′) appearing during the chase of radioactivity from the positions of the C and E restriction endonuclease bands have been shown to be single-stranded DNA both by selective susceptibility of these bands to S1 nuclease and by conversion of the double-stranded DNA from the C and E bands to C′ and E′ upon the addition of alkali. Approximately 50% of the radioactivity was chased with identical kinetics from the C and E into the C′ and E′ bands, respectively, in 3 hr. However, there was a 2.5-fold greater increase of absolute radioactivity in the C′ compared to E′ because the C fragment (12%) is approximately 3-fold longer than the E fragment (3.85%). Of the radioactivity that ultimately was found in the C′ and E′ bands, 45% appeared between the second and third hours of incubation; however, some radioactivity was discernible in these bands by 30 min after the chase began. The second round of replication, which resulted in the displacement of labeled single strand, therefore began asynchronously more than several hours after the first round of replication.

The appearance of single-stranded DNA could also have been the result of an unwinding process uncoupled from the replication reaction. This explanation was eliminated, however, because inhibition of DNA replication completely prevented the appearance of the single strand.

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