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Replication of adenovirus type 2 DNA *in vitro*

(eukaryotic DNA replication/soluble nuclear extracts)

LEE M. KAPLAN, RONALD E. KLEINMAN, AND MARSHALL S. HORWITZ

Departments of Cell Biology, Microbiology and Immunology, and Pediatrics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

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ABSTRACT **A soluble replication system that synthesizes full-sized adenovirus DNA molecules has been developed. The extraction of infected HeLa nuclei solubilizes approximately 25% of the viral replicating DNA and leaves the HeLa chromatin associated with the insoluble nuclear material. Both the extracted viral DNA and the DNA product synthesized *in vitro* are intact and identical to the adenovirus DNA or its replicating intermediates produced in whole infected cells. The direction of elongation and the termini of replication of progeny DNA *in vitro* are essentially identical to those observed *in vivo*. Nuclear extract replication is dependent on Mg^{2+} and the four deoxyribonucleoside triphosphates and partially stimulated by ATP.**

Synthesis of viral DNA in infected cells has been studied extensively to explore mechanisms of prokaryotic and eukaryotic DNA replication. The use of soluble systems to allow manipulation of environmental conditions and the identification of genetic markers for synthetic functions have contributed significantly to our knowledge of the molecular mechanisms of DNA replication in prokaryotes (1–4). For eukaryotes, nuclei isolated from various types of uninfected and virus-infected cells have been used to study mechanisms and control of replication (5–9). Success with these systems, however, has been limited by the inability to manipulate conditions and to fractionate and characterize required components. Several subnuclear systems have been developed to overcome these difficulties (10–14). A nuclear membrane system for replication of adenovirus DNA has been reported that synthesizes short fragments of viral DNA (10); however, the relationship of such synthesis to replication is unknown. Soluble systems, derived from cells infected with simian virus 40, have been shown to complete replication of viral DNA molecules initiated *in vivo* (11, 12). These have provided a means to study the detailed structure of simian virus 40 replicating intermediates.

The structure and replication of adenovirus DNA presents several unusual features suitable for study in a subnuclear system. In addition, viral mutants in DNA synthesis are available and should prove useful for complementation studies *in vitro* (15–17). Adenovirus type 2 (Ad2) DNA is a 23×10^6 dalton, linear, double-stranded molecule (18) with an inverted terminal repetition (19, 20). It replicates in the nucleus of human cells, and host chromosomal replication is inhibited after infection (21). After the synthesis of early viral protein is completed, viral DNA replication no longer requires protein synthesis for the initiation of individual DNA molecules (22). There are proteins covalently linked to each of the 5′ ends of the viral DNA (23–25). These proteins can circularize the DNA, presumably by protein–protein interactions (23). However, it has not been established that adenovirus replicating DNA is circular. Each strand of adenovirus DNA replicates in the 5′ to 3′ direction from a separate initiation point near each end of the DNA molecule (26–30). There is considerable displacement of each parental strand by the elongation process such that replicating intermediates contain a sizable portion of single-stranded DNA (31).

We have isolated a soluble adenovirus DNA replication system extracted from infected HeLa cell nuclei in a manner analogous to the isolation of adenovirus transcriptional complexes (32, 33). The extract is free of chromosomal DNA and elongates Ad2 DNA to full size. This system, which can be obtained in large amounts, provides a means to characterize the template structure and the factors necessary for the replication of adenovirus DNA.

MATERIALS AND METHODS

Cells and Viruses. The sources of HeLa S3 cells and Ad2 have been described (34). Cells were grown in suspension culture and were infected at a multiplicity of 4000 virions per cell as described (35).

Radioactive Labeling of DNA. Uninfected HeLa cells were labeled for 22 hr prior to infection with 1 μCi of [^{14}C]thymidine per 100 ml of culture. Ad2-infected cells were labeled with either [^{3}H]thymidine or [^{14}C]thymidine as described for each experiment. Nuclear extracts were labeled with [^{3}H]dTTP as described in the legend to Table 2. ^{14}C-Labeled marker Ad2 DNA was isolated from purified virion as described (35).

Sedimentation Analysis of Extracted DNA. DNA from the nuclear extract was analyzed on 5–20% neutral or alkaline sucrose gradients without further purification (35). The neutral gradients, containing 1 M NaCl in 20 mM Tris·HCl (pH 7.3) and 10 mM EDTA, were formed over a 0.5-ml CsCl solution ($\rho = 1.8$) as a cushion. Samples for neutral gradients were diluted to 0.5 ml with 1 M NaCl and 10 mM EDTA. Duplicate samples for alkaline gradients were brought to 0.5 ml with 0.15 M NaCl and 10 mM EDTA. All gradients were run in the Beckman SW27.1 rotor at 24,000 rpm for 16 hr at 4°.

Quantitation of Protein and DNA. Protein in isolated nuclei and nuclear extracts was determined by the method of Lowry *et al.* (36). The concentration of DNA in these fractions was determined by measuring the absorbance at 260 nm after purification of the DNA (37). Labeled DNA was used to correct for losses during purification, and 22 absorbance units was equivalent to 1 mg of DNA.

Hybridization Analysis of DNA Products Formed *In Vitro*. The DNA products synthesized *in vitro* were purified from reaction mixtures and analyzed by DNA·DNA hybridization on nitrocellulose filters as described (38).

Abbreviations: Ad2 RI DNA, adenovirus type 2 DNA replicating intermediate comprising a double-stranded region and a single-stranded region of nonreplicated DNA and a double-stranded region of replicated DNA; BND-cellulose, benzoylated naphthoylated DEAE-cellulose; *Hpa* I, *Hemophilus parainfluenzae* restriction endonuclease I.

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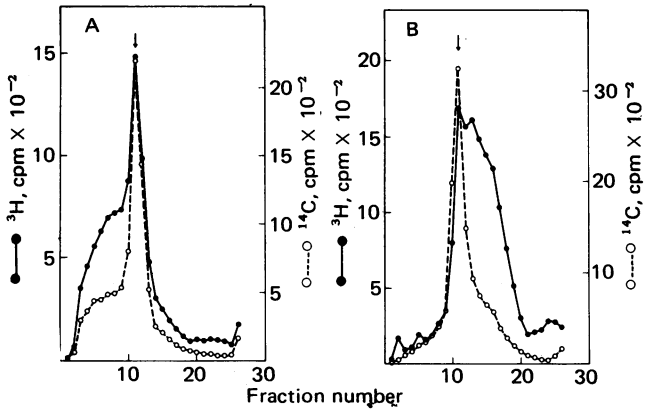

FIG. 1. Sedimentation analysis of Ad2 DNA present in nuclear extract. Infected cells (5×10^7) in 50 ml were labeled with 7 μCi of [^{14}C]thymidine at 37° for 30 min. The cells were then chased for 15 min with 20 μM unlabeled thymidine. After centrifugation of the cells to remove the thymidine, they were labeled in 5 ml with 0.5 mCi of [^{3}H]thymidine (18 Ci/mmol) for 10 min and a nuclear extract was prepared. Aliquots of the nuclear extract containing 1.48×10^4 cpm of ^{3}H and 1.44×10^4 cpm of ^{14}C were layered onto linear 5–20% neutral (*A*) and alkaline (*B*) sucrose gradients and centrifuged at 24,000 rpm in a Beckman SW27.1 rotor for 16 hr at 4°. (*A*) The arrow represents the position of 31S marker Ad2 [^{14}C]DNA (1×10^4 cpm) purified from virion and run in a parallel gradient. The recoveries of ^{3}H and ^{14}C were 99 and 95%, respectively. The CsCl cushion contained 2690 cpm of ^{3}H and 2510 cpm of ^{14}C. (*B*) The arrow represents the position of 34S marker Ad2 virion [^{14}C]DNA single strands (1×10^4 cpm). The recoveries of ^{3}H and ^{14}C were both 83%. The CsCl cushion contained 246 cpm of ^{3}H and 286 cpm of ^{14}C. The direction of sedimentation in these and all other gradients is from right to left.

BND-Cellulose Chromatography. Chromatography of purified deproteinized DNA on benzoylated naphthoylated DEAE-cellulose (BND-cellulose) was used to separate completed (double-stranded) DNA molecules from replicating intermediates, which contain single-stranded regions, as described (26).

Restriction Endonuclease. The digestion of purified DNA molecules by the restriction enzyme *Hpa* I (from *Hemophilus parainfluenzae*) the separation of the DNA fragments by electrophoresis on 1.6 × 30 cm 1.4% agarose gels, and the quantitation of radioactivity in the DNA fragments have been described (26).

Reagents. [^{3}H]Thymidine (18 and 60 Ci/mmol), [^{14}C]thymidine (53 mCi/mmol), and [^{3}H]dTTP (15 and 52 Ci/mmol) were purchased from Schwarz-Mann, Inc. All other nucleotides were purchased from either Schwarz-Mann or Sigma Chemical Co. Deoxyribonuclease I (ribonuclease free) and ribonuclease A were from Worthington Biochemicals. Ribonuclease T_1 and Pronase were from Calbiochem. BND-cellulose was purchased from Serva, and Nonidet P-40 was from Shell Oil Co.

RESULTS

Preparation of Nuclear Extracts. Nuclear extracts were prepared 16–18 hr after infection at the time of maximal viral DNA synthesis (35). Infected HeLa cells were pelleted and washed once with Earle's balanced salt solution at 4°. The pelleted cells were again washed with a volume of 10 mM Tris·HCl (pH 7.5), 10% sucrose, and 5 mM EDTA equal to the packed cell volume. Cells, resuspended in an equal volume of Tris/sucrose/EDTA, were lysed by adding Nonidet P-40 to a final concentration of 0.5%. Nuclei were freed of cytoplasm by sedimentation at 1000 × *g* for 5 min and washed once with an equal volume Tris/sucrose/EDTA. Except as noted in Table 3, nuclear extracts were prepared by resuspending pelleted nuclei in an equal volume of 10 mM Tris·HCl (pH 7.8)/4 mM 2-mercaptoethanol/0.3 M ammonium sulfate and incubating for 1.5–2 min at 37°. Although the nuclear morphology was markedly altered by this procedure, the nuclei did not completely lyse. After 3–5 min on ice and centrifugation at 7000 × *g* for 5 min in the Beckman Type 40 rotor, a clear supernatant (extract), free of structures visible by phase contrast microscopy, was removed from the nuclear pellet.

Table 1. Extraction of labeled DNA from nuclei

	Fraction		
	Nuclei		Nuclear extract, %
	cpm × 10^{-6}	%	
Ad2 DNA, 10-min ^{3}H pulse	3.38	100.0	26.2
Ad2 DNA, 30-min ^{14}C pulse + 25-min chase	0.62	100.0	22.6
Ad2 DNA, 600 min ^{14}C pulse	2.34	100.0	13.1
HeLa [^{14}C]DNA	1.72	100.0	2.1

Ad2 DNA pulsed for 10 min and Ad2 DNA pulsed for 30 min followed by a 25-min chase were labeled at 16–17 hr after infection as described in the legend of Fig. 1. At this time, greater than 85% of the incorporated label *in vivo* was found in viral DNA (35). For the isolation of labeled viral DNA after 600 min, 7×10^7 infected cells in 300 ml were labeled with 5 μCi of [^{14}C]thymidine starting at 9 hr after infection. Nuclear extracts were prepared after the radioactive labeling. HeLa cellular DNA was labeled with [^{14}C]thymidine prior to infection. Viral DNA was determined by the radioactivity sedimenting as Ad2 DNA in alkaline sucrose gradients. During the preparation of nuclei, fewer than 1.5% of the counts were recovered in the cytoplasm or nuclear wash fractions.

Characterization of DNA in Nuclear Extracts. Infected HeLa cells were pulsed with [^{14}C]thymidine and chased with unlabeled thymidine to label completed Ad2 DNA molecules. Viral replicating intermediates (RI) were labeled with short pulses of [^{3}H]thymidine. Nuclear extracts were prepared from the labeled cells and the DNA was sedimented in alkaline and neutral sucrose gradients. Most of the ^{14}C-labeled, pulsed and chased DNA found in nuclear extracts sedimented in neutral sucrose gradients as a sharp 31S peak, characteristic of full-sized, double-stranded Ad2 DNA (Fig. 1*A*). The remainder sedimented to the position of Ad2 RI DNA, in a broad, faster sedimenting shoulder to the 31S peak (Fig. 1A, fractions 2–9). On alkaline sucrose gradients, nearly all of the ^{14}C-labeled strands cosedimented as a sharp band with 34S Ad2 DNA single strands, indicating that few nicks or breaks were introduced during the extraction procedure (Fig. 1*B*). In cells pulsed with [^{3}H]thymidine for 10 min to label primarily the nascent strands of replicating DNA, at least 50% of the extracted DNA sedimented as RI in neutral sucrose (Fig. 1*A*). The labeled strands were heterogeneous in size in alkaline sucrose gradients, with the largest material sedimenting at 34 S (Fig. 1*B*). These data suggest that all DNA species in the replicating pool are present in the nuclear extract.

The amount of host and viral DNA extracted from the nuclei was determined using infected cells that were labeled for various intervals before and after infection. Approximately one-fourth of the replicating and recently completed Ad2 DNA molecules were extracted from the nuclei (Table 1). These species appear to be preferentially extracted, since only 13% of the nuclear viral DNA synthesized during a 10-hr period was found in the extract (Table 1). A small amount of HeLa cellular DNA (2%) labeled with [^{14}C]thymidine before infection was released from the infected cell nuclei, indicating a marked enrichment of viral DNA by the extraction procedure.

DNA Synthesis in Isolated Nuclei and Nuclear Extracts.

Table 2. DNA synthesis in isolated nuclei and nuclear extracts

Fraction analyzed	Protein content, μg/10 μl	DNA content, μg/10 μl	Incorporation of [^{3}H]dTMP, pmol/30 min per 10 μl
Ad2-infected HeLa			
Nuclei	144	31.7	4.6
Nuclear extract	68	4.0	3.4
Uninfected HeLa			
Nuclei	179	ND	2.1
Nuclear extract	92	ND	<0.1

Reaction mixtures (0.04 ml) containing 27 mM Tris·HCl (pH 7.5), 3 mM dithiothreitol, 6.25 mM $MgCl_2$, 0.25 mg of bovine serum albumin per ml, 2 mM ATP, 0.125 mM each of dATP, dCTP, and dGTP, 0.05 mM [^{3}H]dTTP (1640 cpm/pmol), and 10 μl of isolated nuclei or nuclear extracts were incubated at 30° for 30 min. The reaction was terminated by addition of 0.2 ml of 0.2 M NaOH and cooling to 4°. Two-tenths milliliter of 0.1 M sodium pyrophosphate, 50 μg of bovine gamma globulin, and 50 μg of yeast RNA were added and the mixture was precipitated with 2 ml of 20% trichloroacetic acid. After 15 min at 0°, the precipitate was collected on Whatman Type GF-C glass fiber filters, washed three times with 5% trichloroacetic acid, once with 95% ethanol, and dried. Radioactivity was determined in TT-21 liquid scintillation cocktail (Yorktown Research Corp.). Ten microliters of nuclear extract was derived from 3×10^6 infected nuclei. ND, not determined.

DNA synthesis catalyzed by isolated nuclei and nuclear extracts prepared 16–18 hr after infection was examined. Nuclear extracts were approximately 75% as active as isolated nuclei. No endogenous synthesis occurred in similarly prepared extracts from uninfected cells, despite significant incorporation in isolated nuclei (Table 2).

In vitro DNA synthesis by nuclear extracts from infected cells required Mg^{2+} and was stimulated approximately 40% by 2 mM ATP. Deoxyribonuclease I (100 μg/ml) added to the reaction mixture decreased the activity approximately 90% (Table 3). Dialysis of the nuclear extract decreased total activity approximately 50% (Table 3) without increasing the dependence on exogenous ATP (data not shown). Nuclear extracts prepared without the brief incubation at 37° were 75% as active as those prepared in the normal way.

Characterization of DNA Synthesized *In Vitro*. DNA·DNA filter hybridization analysis demonstrated that 95% of the DNA synthesized *in vitro* was Ad2 DNA. After incubation of infected nuclear extracts in reaction mixtures containing [^{3}H]dTTP, the labeled DNA was purified. Seventy-five percent (8360 cpm) of the DNA synthesized *in vitro* and 79% (4420 cpm) of the control viral DNA hybridized to 3 μg of Ad2 DNA bound to nitrocellulose filters. These data show that Ad2 sequences comprised 95% of the *in vitro* synthetic product.

Table 3. Requirements for Ad2 DNA synthesis by nuclear extracts

Additions	DNA synthesis, pmol*
Complete	3.4
−ATP	2.4
−dATP, dCTP, dGTP	0.4
−$MgCl_2$	0.1
−Bovine serum albumin	3.1
−Dithiothreitol	2.7
+Deoxyribonuclease I (100 μg/ml)	0.3
Complete; dialyzed extract†	1.5
Complete; extract prepared without 37° incubation	2.5

* Activity is expressed as pmol of [^{3}H]dTMP incorporated into acid-precipitable material in 30 min. Reaction conditions were as described in the legend of Table 2.

† Nuclear extract was dialyzed for 1 hr against 2000 volumes of 25 mM Tris·HCl (pH 7.4)/1.5 mM 2-mercaptoethanol/1 mM EDTA/and 10% glycerol at 4°.

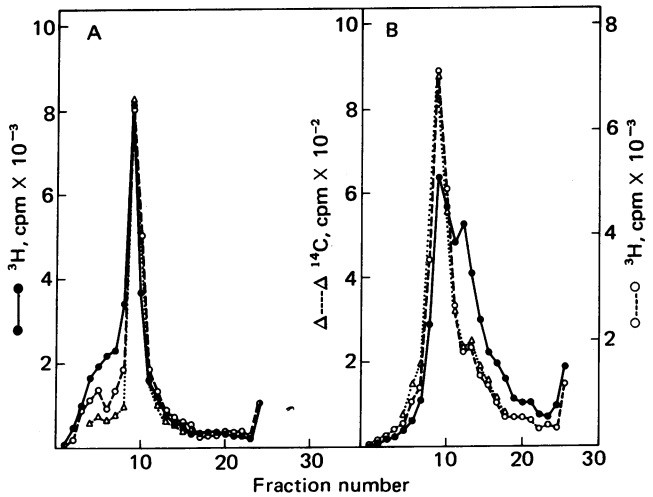

FIG. 2. Sedimentation analysis of Ad2 DNA labeled during *in vitro* synthesis. A nuclear extract (80 μl) containing 830 μg of protein was prepared from infected cells. The extract was incubated for 10 min in the *in vitro* reaction mixture (described in Table 2) containing 3.6 μM [^{3}H]dTTP (3.15×10^4 cpm/pmol) and was chased for 0 or 30 min with 250 μM unlabeled dTTP. The reaction was stopped by addition of 20 mM EDTA to aliquots at 4°. Equal aliquots of the reaction stopped after a 0-min chase (4.23×10^4 cpm) and a 30-min chase (4.49×10^4 cpm) were layered onto linear 5–20% neutral (*A*) and alkaline (*B*) sucrose gradients and centrifuged as in the legend of Fig. 1. (*A*) Ad2 [^{14}C]DNA (4×10^3 cpm) purified from virion (31 S) was included as a marker. The recoveries of ^{3}H from the gradients containing the 0-min and 30-min chase DNA were 88 and 81%, respectively. The CsCl cushions from the 0-min and 30-min chases contained 9830 and 6350 cpm, respectively. (*B*) Ad2 virion [^{14}C]DNA single strands (5×10^3 cpm) were included as a marker (34 S). The recoveries of ^{3}H from the gradients containing the 0-min and 30-min chase DNA were 93 and 92%, respectively. The CsCl cushions from the 0-min and 30-min chases contained 625 and 548 cpm, respectively. ●—●, 10-min ^{3}H pulse + 0-min chase; ○ - - ○, 10-min ^{3}H pulse + 30-min chase; Δ ··· Δ, Ad2 [^{14}C]DNA.

DNA synthesized *in vitro* was first associated with the heterogeneous sized strands of replicating intermediates and later with full-sized Ad2 DNA. After a 10-min incubation of nuclear extracts in reaction mixtures containing [^{3}H]dTTP, incorporated label sedimented in neutral sucrose gradients as a 31S peak with a broad, faster sedimenting shoulder in the region of RI DNA (Fig. 2*A*). Alkaline sucrose gradient analysis showed the label to be distributed in DNA strands of broad size range up to the 34S Ad2 marker DNA (Fig. 2*B*). A 30-min chase of this newly labeled DNA with an excess of unlabeled dTTP resulted in a decrease of label in the RI region of a neutral sucrose gradient (Fig. 2*A*). After this chase, nearly all of the label was associated with full-sized strands of Ad2 DNA, demonstrating that the nuclear extract was able to complete rounds of replication of at least a fraction of the DNA pool (Fig. 2*B*).

***In Vitro* Completion of DNA Chains Initiated in Whole Cells.** Nuclear extracts were prepared from infected cells pulsed briefly with [^{3}H]thymidine to label the Ad2 nascent strands. As described earlier, distribution of the extracted label on neutral sucrose gradients was in a 31S peak with a broad, faster sedimenting shoulder (Fig. 3*A*). Alkaline sucrose gradients showed a broad peak of heterogeneous sized DNA strands (Fig. 3*B*). Incubation of nuclear extracts in reaction mixtures containing all four unlabeled dNTPs resulted in a transfer of the prelabel to a sharp peak cosedimenting with Ad2 marker DNA

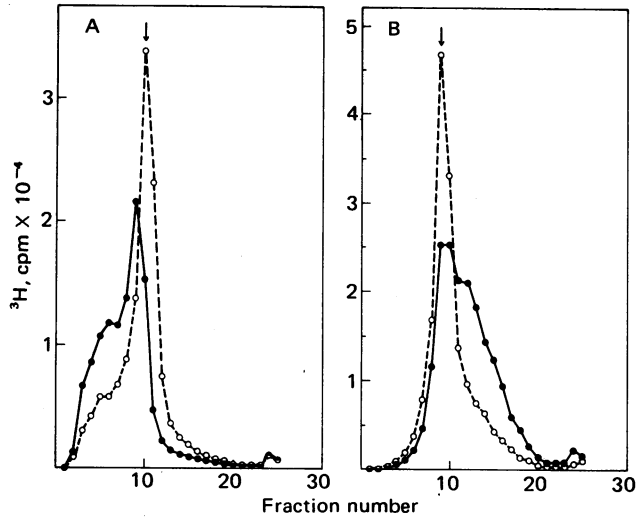

FIG. 3. Sedimentation analysis of Ad2 replicating intermediate DNA after elongation in nuclear extract. Infected cells (1.2×10^8 in 12 ml were labeled with 1 mCi of [^{3}H]thymidine (60 Ci/mmol) for 10 min and a nuclear extract was prepared. Aliquots (0.1 ml) of the extract were incubated for 60 min in the *in vitro* reaction mixture as described in the legend of Table 2, except that they contained 0.125 mM unlabeled dTTP. Reactions were stopped by addition of 20 mM EDTA at 4°. There was no increase in acid-precipitable radioactivity during the *in vitro* chase. Fifty microliters of the nuclear extract (1.79×10^5 cpm) and 0.5 ml of the 60-min reaction mixture (1.52×10^5 cpm) were layered onto linear 5–20% neutral (*A*) and alkaline (*B*) sucrose gradients and centrifuged as in the legend of Fig. 1. (*A*) The arrow represents 31S marker Ad2 [^{14}C]DNA (1×10^4 cpm) purified from virion. The CsCl cushions from the gradients with the nuclear extract and the 60-min *in vitro* chase contained 48,700 and 57,000 cpm, respectively. (*B*) The arrow represents 34S marker Ad2 virion [^{14}C]DNA single strands (1×10^4 cpm). The CsCl cushions from the gradients with the nuclear extract and the 60-min *in vitro* chase contained 2710 and 1860 cpm, respectively. The recovery of counts added to each gradient was 100% except for the neutral gradient with the nuclear extract, for which it was 93%. ●—●, Nuclear extract; ○ - - ○, nuclear extract + 60-min *in vitro* chase.

in both alkaline and neutral gradients. These data indicate that most of the DNA that has initiated replication *in vivo* can be elongated to completion in the *in vitro* system.

Direction of Chain Elongation *In Vitro*. The direction of chain elongation of Ad2 DNA *in vitro* was determined by methods previously described for intact infected cells (26). Ad2 DNA was partially labeled with [^{3}H]dTTP in the *in vitro* reaction mixture. After incubation for 20, 30, and 60 min, the DNA was purified and chromatographed on BND-cellulose to separate completed double-stranded DNA from replicating intermediates containing single strands. With short labeling times (20 or 30 min), the completed DNA is labeled predominantly near the terminus of replication. With a longer incubation (60 min), nearly all DNA molecules should be completed, including those labeled at or near the origin. Thus, completed molecules isolated after a long labeling period should show a more uniform distribution of label across the genome. As shown (Fig. 4), *Hpa* I digests of completed DNA indicate a preponderance of radioactivity at both molecular ends after labeling for 20 or 30 min and a more uniform distribution after 60 min. This pattern is entirely congruent with results obtained *in vivo*, indicating bidirectional replication (26).

Kinetics of Incorporation of [^{3}H]dTMP *In Vitro*. *In vitro*, dNMP incorporation at 30° was linear for 15 min, during which time DNA equivalent to 0.11% of the total amount of DNA present in the nuclear extract was synthesized. The reaction continued and was essentially complete by 120 min (Fig. 5).

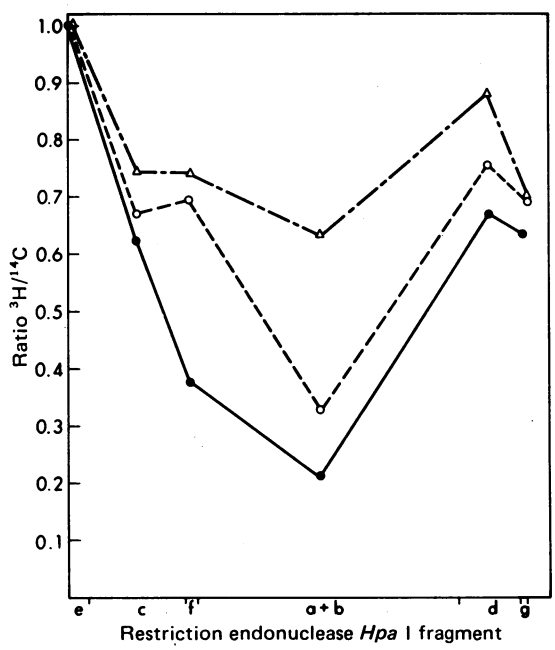

FIG. 4. Order of labeling of selected regions of Ad2 DNA synthesized in nuclear extracts. The nuclear extract was incubated in the *in vitro* reaction mixture described in the legend of Table 2 containing 3.6 μM [^{3}H]dTTP (3.15×10^4 cpm/pmol) for 20, 30, and 60 min and stopped by addition of 20 mM EDTA at 4°. The DNA was purified and chromatographed on BND-cellulose to isolate the completed double-stranded fraction. The double-stranded DNA was precipitated, resuspended in 0.3 ml of 0.01× SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate), and mixed with uniformly labeled Ad2 virion [^{14}C]DNA. The DNA mixture was digested with the *Hpa* I restriction endonuclease and the fragments were separated on 1.4% agarose gels. The ^{3}H and ^{14}C content in gel slices corresponding to each of the DNA fragments was measured. The ratio of [^{3}H]DNA (pulse labeled) to [^{14}C]DNA (uniformly labeled) is expressed as a function of position of each fragment on the genome. The ^{3}H/^{14}C ratio of the *e* fragment was arbitrarily set as 1, and the other values were normalized to it. The *a* fragment does not separate from the *b* fragment under these conditions of electrophoresis. ●—●, 20-min label; ○ - - ○, 30-min label; △ - - △, 60-min label.

Total synthesis at 120 min represented approximately 0.28% of the DNA present in the reaction.

The reaction kinetics provide a simple assay for the integrity of the *in vitro* synthetic product. During the course of these studies, we observed that infected cell extracts from several lines of HeLa and KB cells contained fragmented DNA and synthesized *in vitro* products that were small (data not shown). On two occasions, HeLa cells that had been used to make active adenovirus-infected nuclear extracts would no longer synthesize full-length Ad2 DNA *in vitro*. In all cases observed, synthesis of small products was correlated with reactions that were complete by 15 min (data not shown). This rapid test was used to determine the ability of extracts to catalyze DNA replication *in vitro*.

DISCUSSION

Replication of Ad2 DNA *in vitro* in nuclear extracts reflects closely the situation observed *in vivo*. By extracting nuclei, we have succeeded in separating virtually all contaminating cellular DNA while retaining viral DNA in good yield. Synthesis proceeds by continuous elongation of nascent strands to full size and occurs largely in intermediates that sediment faster than mature DNA on neutral sucrose gradients. Data defining the origin and direction of new synthesis are consistent with results obtained *in vivo* showing initiation near both ends of linear, double-stranded DNA molecules (26).

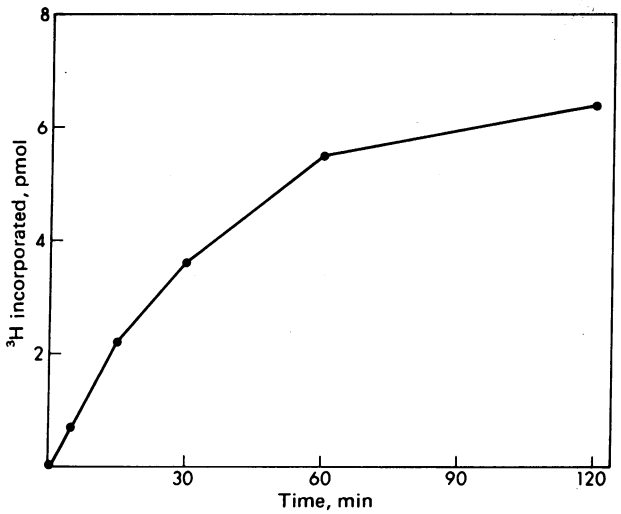

FIG. 5. Rate of incorporation of [^{3}H]dTMP into viral DNA. Nuclear extracts from infected cells (10 μl) containing 61 μg of protein and 4 μg of DNA were incubated in reaction mixtures containing 0.05 mM [^{3}H]dTTP (1640 cpm/pmol) as described in the legend to Table 2.

The *in vitro* reaction is slightly stimulated by ATP, proceeds in a linear fashion for 15 min, and is incomplete even after 30 or 60 min. We have exploited this last characteristic to identify cell cultures whose infected extracts synthesize only fragmented DNA.

The initial rate of nucleotide incorporation *in vitro* appears to be approximately one-tenth that observed *in vivo* (21); however, most of the nascent DNA strands initiated *in vivo* can be completed in the *in vitro* reaction. Since a significant fraction of replicating molecules were extracted from the nuclei, dNMP incorporation *in vitro* does not result from action on a minor species in the replicating pool.

The DNA synthetic activity in the infected nuclear extracts sediments as a diffuse complex larger than 100 S in neutral sucrose gradients in which NaCl was omitted. It is not known how this structure relates to adenovirus replication complexes isolated by other workers (10). One of the proteins present in this complex is the 5′ covalently linked peptide that we have found on replicating Ad2 DNA molecules (L. M. Kaplan and M. S. Horwitz, unpublished). A role has been proposed for this protein as an initiator of DNA synthesis (24); however, it is not yet known if the peptide is attached to the nascent or only the parental strands of the replicating DNA.

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