Proc. Natl. Acad. Sci. USA
Vol. 76, No. 2, pp. 655–659, February 1979
Biochemistry

Adenovirus DNA replication *in vitro*

(eukaryotic DNA replication)

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Communicated by John W. Littlefield, November 9, 1978

ABSTRACT **A soluble extract from the nuclei of HeLa cells infected with adenovirus 5 (Ad5) carries out the semiconservative replication of exogenously added Ad5 DNA *in vitro*. Maximal DNA synthesis is observed when DNA–protein complex, isolated from Ad5 virions, is added as template. DNA–protein complex from virions of the closely related virus, adenovirus 2, is also active. In contrast, very little *in vitro* DNA synthesis is observed when deproteinized Ad5 DNA or DNA from a heterologous source (bacteriophage T7) is added as template. The product of the *in vitro* reaction consists of long Ad5 DNA strands that are hydrogen-bonded, but not covalently linked, to the input DNA template. During the course of the *in vitro* reaction, branched molecules with structural features identical to *in vivo* replication intermediates are formed. These findings support the conclusion that replication in the *in vitro* system closely resembles adenovirus DNA replication *in vivo*. The system provides an assay that should be useful for the purification and subsequent characterization of viral and cellular proteins involved in DNA replication.**

Much of the recent dramatic progress in understanding prokaryotic DNA replication is due to the development of soluble *in vitro* systems that carry out the replication of exogenously added DNA templates of defined structure (1–4). These systems have made it possible to study in detail the biochemical mechanisms involved in the initiation and elongation of DNA chains and to purify and characterize the proteins required for these processes. It is clear that the development of analogous *in vitro* systems would be extremely valuable for dissecting the mechanisms of eukaryotic DNA replication.

The human adenoviruses have several advantages as model systems for studying eukaryotic DNA replication. The adenovirus genome is easily isolated in intact form from purified virions, and its structure and genetic organization are well defined (5, 6). In addition, a good deal is known about the general mechanism of adenovirus DNA replication *in vivo* (7). This makes it possible to assess the biological relevance of any synthetic reactions observed *in vitro*. The adenovirus genome is a linear, duplex DNA molecule with a molecular weight of $20–25 \times 10^6$ (8). It possesses two novel structural features of possible significance for DNA replication. First, the two ends of the genome have identical nucleotide sequences (refs. 9–11; J. R. Arrand and R. J. Roberts, personal communication), and second, the 5′ ends of both DNA strands are covalently linked to a polypeptide of molecular weight 55,000 (12–16). *In vivo* studies (reviewed in ref. 7) have shown that initiation of adenovirus DNA replication takes place at or near either end of the viral genome. Daughter strand synthesis then proceeds in the 5′ to 3′ direction with concomitant displacement of the parental strand with the same polarity. Synthesis of the complementary strand is initiated at or near the 3′ end of the displaced parental strand and also proceeds in the 5′ to 3′ direction. Thus, the overall pattern of adenovirus DNA replication appears rather simple. At each growing point only one of the two parental strands is replicated, and, at least in principle, there is no requirement for a discontinuous mechanism of DNA synthesis. This simplicity makes the adenovirus system especially attractive for biochemical studies.

In this paper we describe a soluble enzyme system from adenovirus-infected cells that is capable of replicating exogenously added adenovirus DNA molecules. We have shown by several criteria that the DNA synthesis that occurs in this *in vitro* system closely resembles adenovirus DNA replication *in vivo*.

METHODS

Preparation of Nuclei from Adenovirus-Infected Cells. HeLa cells were maintained at 37°C in suspension culture in Eagle's minimal essential medium supplemented with horse serum (5%). One liter of cells at a density of $4–5 \times 10^5$ cells/ml was infected with adenovirus 5 (Ad5) at a multiplicity of 10 plaque-forming units/cell. At 2 hr after infection, 10 ml of 1.0 M hydroxyurea was added. At 21 hr after infection, the cells were collected by centrifugation at $3000 \times g$ for 5 min and washed once with 20 ml of cold hypotonic buffer (20 mM Hepes, pH 7.5/5 mM KCl/0.5 mM $MgCl_2$/0.5 mM dithiothreitol) containing 0.2 M sucrose (17). The cells were resuspended in 5 ml of cold hypotonic buffer without sucrose, allowed to swell for 10 min on ice, and then lysed by 10 strokes of a tight-fitting Dounce homogenizer. The resulting lysate was centrifuged at $2000 \times g$ for 5 min. The nuclear pellet was resuspended in 2.5 ml of 50 mM Hepes, pH 7.5/10% sucrose and frozen in 1.0-ml aliquots in liquid nitrogen. The supernatant was clarified by centrifugation at $15,000 \times g$ for 20 min, and the resulting cytoplasmic extract was frozen in liquid nitrogen. Both fractions were stored at −13°C.

Preparation of Nuclear Extract. An aliquot (1.0 ml) of frozen nuclei was allowed to thaw on ice. After addition of 20 μl of 5 M NaCl, the suspension was incubated on ice for 1 hr. During this procedure, the nuclei maintained their typical morphology (as determined by phase contrast microscopy) and the suspension remained nonviscous. The nuclei were then centrifuged at $15,000 \times g$ for 20 min at 4°C. The clear supernatant was removed with a pasteur pipette and stored on ice. The protein concentration of the nuclear extract from both infected and uninfected cells was 3.5–4.0 mg/ml as determined by the method of Lowry *et al.* (18). The *in vitro* DNA synthesis activity of this extract was stable at 0°C for at least 1 week.

Conditions for *In Vitro* DNA Synthesis. The standard reaction mixture for *in vitro* DNA synthesis contained (in 0.1 ml) 50 mM Hepes (pH 7.5); 5 mM $MgCl_2$; 0.5 mM dithiothreitol; 50 μM [α-^{32}P]dTTP [specific activity: 0.25–1 mCi/mmol ($0.925–3.7 \times 10^7$ becquerels/mmol) (New England Nuclear)]; 50 μM each dCTP, dATP, and dGTP; 2 mM ATP (Schwarz/Mann); 150 ng of Ad5 DNA–protein complex; and 25 μl of nuclear extract. Incorporation of nucleotides into DNA was

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Abbreviations: Ad5, adenovirus 5; Ad2, adenovirus 2.

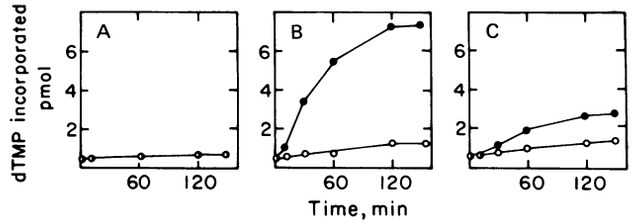

FIG. 1. Kinetics of Ad5 DNA synthesis *in vitro*. The kinetics of DNA synthesis were measured by using the standard reaction conditions described in *Methods*. The reaction mixtures contained nuclear extract from either Ad5-infected (●) or uninfected (○) cells, and either no added DNA (*A*), 150 ng of Ad5 DNA–protein complex (*B*), or 150 ng of deproteinized Ad5 DNA (*C*).

monitored as acid-precipitable radioactivity. To isolate the product of the reaction after incubation, EDTA was added to a concentration of 25 mM and the reaction mixture was incubated with either Pronase (Calbiochem) at 1.0 mg/ml in the presence of 0.6% sodium dodecyl sulfate or proteinase K (EM Biochemicals, Elmsford, NY) at 0.1 mg/ml at 37°C for 2 hr. The resulting solution was extracted three times with an equal volume of neutralized phenol and dialyzed, first against 10 mM Tris·HCl, pH 8.0/1.0 M NaCl/1 mM EDTA, and then against 10 mM Tris·HCl, pH 8.0/1 mM EDTA. For electron microscopy, the deproteinized reaction mixture was incubated with pancreatic RNase (Calbiochem) at 2 μg/ml at 37°C for 2 min.

Preparation of DNA Templates. Ad5 and Ad2 virions were purified as described (19). DNA–protein complex was prepared from virions by sedimentation through sucrose gradients containing 4 M guanidinium Cl as described by Sharp *et al.* (13). Fractions containing DNA–protein complex were pooled and dialyzed extensively against 10 mM Tris·HCl, pH 8.0/1 mM EDTA. Deproteinized Ad5 DNA was prepared by incubating the Ad5 DNA–protein complex in 0.6% sodium dodecyl sulfate with Pronase (at 1 mg/ml) at 37°C for 2 hr. The resulting solution was extracted twice with neutralized phenol and dialyzed against 10 mM Tris·HCl, pH 8.0/1 mM EDTA.

RESULTS

DNA Synthesis on Exogenous Templates by a Soluble Nuclear Extract from Adenovirus-Infected Cells. Nuclear extract was prepared from Ad5-infected HeLa cells at 21 hr after infection. In order to avoid a high background of *in vitro* synthesis on partially replicated endogenous viral DNA templates (20–22), we added hydroxyurea to the culture shortly after infection. Hydroxyurea treatment blocks DNA replication *in vivo*, but allows the synthesis of proteins required for viral DNA replication (23). The Ad5-infected-cell extract and an extract from uninfected HeLa cells prepared by the same procedure were tested for their ability to support the *in vitro* replication of several natural DNA templates (Fig. 1, Table 1). Neither extract was active in DNA synthesis in the absence of added DNA (Fig. 1*A*, Table 1). The uninfected-cell extract was also inactive in the presence of all of the exogenous DNA templates tested (Fig. 1 *B* and *C*). In contrast, the infected-cell extract catalyzed extensive DNA synthesis when incubated with Ad5 DNA–protein complex isolated from purified virions (Fig. 1*B*). Significantly less DNA synthesis in infected-cell extracts was observed when deproteinized Ad5 DNA or DNA from a heterologous source (bacteriophage T7) was employed as template (Fig. 1*C*, Table 1). With DNA–protein complex as template, DNA synthesis was linear for about 1 hr and ceased after about 2 hr, at which time the extent of incorporation was approximately 20–25 pmol of total nucleotide per standard reaction mixture. This corresponds to the replication of approximately 6% of the input DNA. Since the reaction mixture contained extract from about 5×10^6 cells, the observed incorporation was equivalent to the synthesis of about 60 molecules of adenovirus DNA per cell.

Table 1. Requirements for Ad5 DNA synthesis *in vitro*

Conditions	dTMP incorporation, pmol
Complete	7.8
+ cytoplasmic extract	8.0
− DNA–protein complex	0.4
+ deproteinized Ad5 DNA (0.15 μg)*	2.2
+ T7 DNA (0.15 μg)*	2.0
− dATP, dGTP, dCTP	0.9
− ATP	3.0
+ CTP, GTP, UTP (0.75 mM each)	6.1

DNA synthesis activity of the nuclear extract of Ad5-infected cells was measured in the standard assay described in *Methods*. Incubation was for 2 hr at 37°C. Cytoplasmic extract was added in an amount equal to one-half the cell equivalent of nuclear extract.
* Reactions containing other DNAs did not contain Ad5 DNA–protein complex.

Other requirements for *in vitro* DNA synthesis by the Ad5-infected-cell extract are shown in Table 1. The reaction required the four deoxyribonucleoside triphosphates. It was stimulated 2- to 3-fold by ATP but was not stimulated further by the addition of the other ribonucleoside triphosphates. Synthesis was not significantly enhanced by the addition of the cytoplasmic extract from infected cells.

The radioactive product of the *in vitro* reaction was analyzed by cleavage with restriction endonuclease *Bam*HI (Fig. 2). The cleavage pattern observed was that expected for Ad5 DNA. To demonstrate that the template for *in vitro* synthesis is the exogenously added DNA, we also analyzed the product synthesized by Ad5-infected-cell extract in response to DNA–protein complex prepared from adenovirus 2 (Ad2) virions. The extensive homology between the genomes of Ad2 and Ad5 (28) suggested that the two DNAs should be interchangeable as templates for the *in vitro* reaction, and such was in fact the case. The rate of DNA synthesis in Ad5-infected-cell extract was virtually identical with either Ad5 or Ad2 DNA–protein complex as substrate (data not shown). When the radioactive product synthesized in response to Ad2 DNA–protein complex was analyzed by digestion with the *Bam*HI restriction endonuclease, the observed cleavage pattern was that expected for Ad2 DNA (Fig. 2). This result established that the exogenously added DNA serves as template *in vitro* and rules out the possibility that a significant fraction of the incorporation is the result of stimulation of synthesis on an endogenous template by a factor(s) present in the DNA–protein complex preparations.

Size Distribution of Ad5 DNA Synthesized *In Vitro*. The Ad5 [^{32}P]DNA synthesized during a 2-hr *in vitro* reaction with Ad5 [^{3}H]DNA as template was analyzed by sedimentation through neutral and alkaline sucrose gradients (Fig. 3). In the neutral gradient, most of the ^{32}P-labeled product cosedimented with the intact Ad5 [^{3}H]DNA substrate. In the alkaline gradient, about 40–60% of both the ^{32}P-labeled product and the ^{3}H-labeled template sedimented as full-length Ad5 strands, with the rest of the radioactivity in a broad distribution of shorter strands. None of the radioactivity sedimented more rapidly than full-length strands. Thus, the newly synthesized DNA resides in unit-length duplex molecules composed predominantly of long strands, many of which are full length. The data also show

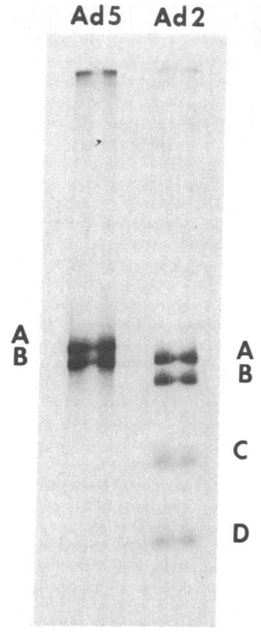

FIG. 2. Restriction enzyme analysis of DNA synthesized *in vitro*. A standard *in vitro* reaction mixture containing [α-^{32}P]dTTP and either Ad5 or Ad2 DNA–protein complex as template was incubated at 37°C for 2 hr. The reaction mixtures were then deproteinized and incubated with 2 units of the *Bam*HI restriction endonuclease under described conditions (24). The digests were fractionated by electrophoresis on a 1.4% agarose slab gel as described (25). The gel was then dried and autoradiographed. The heading at the top of each lane denotes the source of the DNA–protein complex added as template to the *in vitro* reaction. The positions of the radioactive bands were identical to those of the *Bam*HI restriction fragments of the input DNAs as determined by ethidium bromide staining. The reported sizes in kilobases (kb) of the fragments produced by cleavage of Ad5 and Ad2 DNA by *Bam*HI are as follows (26, 27): Ad5-A, 21 kb; Ad5-B, 14.7 kb; Ad2-A, 14.7 kb; Ad2-B, 10 kb; Ad2-C, 6.5 kb; Ad2-D, 4.7 kb.

that extensive degradation of the input DNA does not occur during the *in vitro* reaction.

Evidence for Semiconservative Replication. To rule out the possibility that the *in vitro* product consists of short DNA chains that are covalently linked to the template DNA, as would be expected for a repair reaction, Ad5 DNA was synthesized in a reaction mixture containing 5-bromodeoxyuridine triphosphate in place of dTTP and analyzed by equilibrium centrifugation in CsCl gradients (Fig. 4). Between 60 and 70% of the *in vitro* product banded in a narrow zone at the density of hybrid DNA. The remainder of the product banded in a broad peak at densities slightly greater than the unsubstituted marker DNA. When the DNA was denatured prior to centrifugation, 60–70% of the product banded at the density expected for Ad5 DNA strands fully substituted with bromouracil, and the rest was heterogeneously distributed at lower densities. Thus a major fraction of the Ad5 DNA synthesized *in vitro* consists of long DNA strands that are hydrogen-bonded, but not covalently linked, to the input DNA template. These data are consistent with the hypothesis that the extract can initiate the synthesis of DNA strands *de novo*. However, we cannot rule out the possibility that synthesis is initiated on very short primers present in the input DNA.

Structure of Ad5 DNA Molecules Replicating *In Vitro*. A standard reaction mixture containing Ad5 DNA–protein complex and infected-cell extract was deproteinized after incubation for 10 min at 37°C and examined by electron microscopy. Linear, duplex DNA molecules containing one or two single-stranded branches (Fig. 5) were observed at a frequency of about 5% of the input Ad5 DNA molecules. Length measurements revealed that the structural features of these molecules were the same as those of authentic replicating intermediates isolated from infected cells (19). In particular, the total length of the duplex DNA in each molecule was equal to that of the mature Ad5 genome, and the length of the single-stranded branch was equal to one of the duplex arms defined by the branch point. The lengths of the single-stranded branches were variable, ranging from 5 to 40% of the Ad5 genome (single-stranded branches up to 90% of unit length were observed in reaction mixtures incubated for periods greater than 10 min). Branched molecules with these characteristics were not present in reaction mixtures deproteinized at zero time nor in reaction mixtures incubated in the absence of added Ad5 DNA–protein complex. They were similarly absent from reaction mixtures in which Ad5 DNA–protein complex was incubated with extract from uninfected cells under otherwise identical conditions.

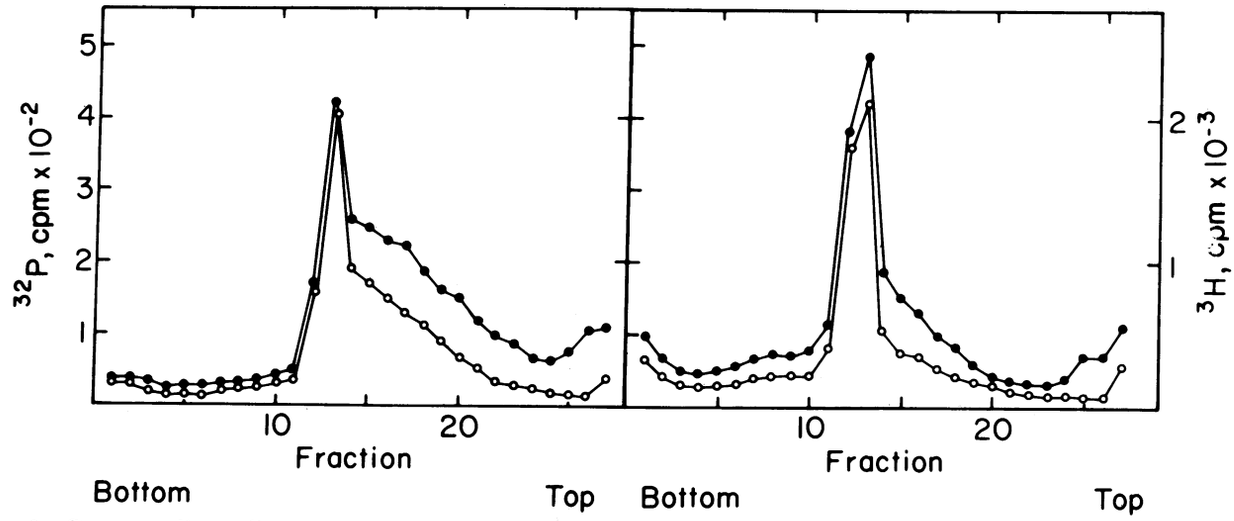

FIG. 3. Sucrose gradient sedimentation of DNA synthesized *in vitro*. DNA was synthesized in a standard *in vitro* reaction mixture containing Ad5 [^{3}H]DNA–protein complex and [α-^{32}P]dTTP. After incubation at 37°C for 2 hr, the reaction mixture was deproteinized by using proteinase K. Aliquots were then layered onto either an alkaline (*Left*) or a neutral (*Right*) sucrose gradient (5–20%) and sedimented at 50,000 rpm for 1.5 hr at 20°C in an SW 50.1 rotor. Gradients contained 1 mM EDTA and either 10 mM Tris·HCl, pH 8/1 M NaCl (neutral) or 0.2 M NaOH/0.8 M NaCl (alkaline). ●, Product [^{32}P]DNA; ○, input [^{3}H]DNA.

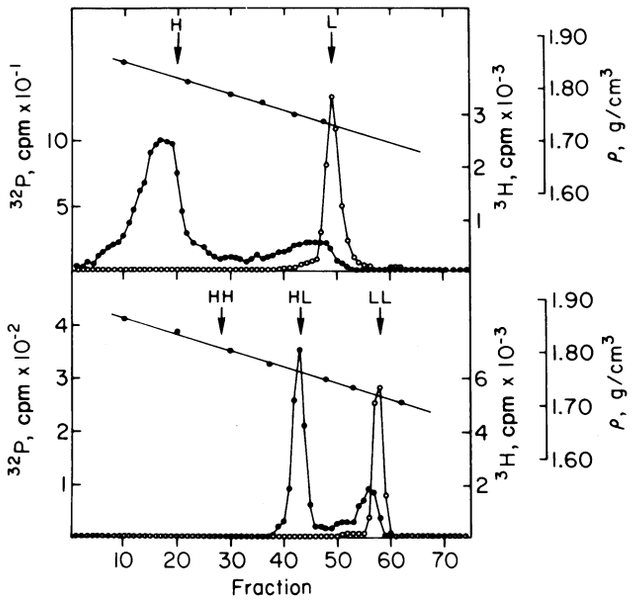

FIG. 4. Isopycnic centrifugation of DNA synthesized *in vitro* in the presence 5-bromodeoxyuridine triphosphate. A standard *in vitro* reaction mixture containing Ad5 DNA–protein complex, 50 μM [α-^{32}P]dCTP, and 100 μM 5-Br-dUTP (in place of dTTP) was incubated at 37°C for 2 hr. After deproteinization, Ad2 [^{3}H]DNA was added as a density marker, and aliquots of the mixture were analyzed by isopycnic centrifugation in neutral CsCl gradients (ρ = 1.76 g/cm^3) with (*Upper*) and without (*Lower*) prior denaturation. Denaturation was accomplished by addition of NaOH to 0.1 M and subsequent neutralization with Tris·HCl, pH 7.2. Sedimentation was at 35,000 rpm for 70 hr at 20°C in an SW 50.1 rotor. The density of selected fractions relative to the Ad2 [^{3}H]DNA marker was determined by refractive index measurements. The arrows indicate the expected positions for fully substituted (HH), hybrid (HL), and unsubstituted (LL) Ad5 duplexes, and for fully substituted (H) and unsubstituted (L) Ad5 single strands (29). To ensure that the substituted DNA had not been extensively degraded during isolation, the ^{32}P-labeled product was also analyzed by alkaline sucrose gradient sedimentation under the conditions described in the legend to Fig. 3. The average length of the product DNA strands was about one-half that of full length Ad5. ●, Product [^{32}P]DNA; ○, marker [^{3}H]DNA.

A detailed description of the electron microscopic data will be presented elsewhere.

DISCUSSION

In this communication we have described a soluble enzyme system from the nuclei of adenovirus-infected cells that carries out semiconservative replication of exogenously added Ad5 DNA *in vitro*. The ability of this system to replicate an exogenous template is in contrast to all previously studied subcellular DNA replication systems from eukaryotic cells with the exception of a recently described system derived from the cytoplasm of *Xenopus laevis* eggs (31). Several laboratories have described DNA synthesis on endogenous adenovirus DNA templates in isolated nuclei (32, 33) or subnuclear "replication complexes" (20–22).

Several characteristics of the *in vitro* DNA replication system described here attest to its biological relevance. The system exhibits a specific requirement for an extract derived from adenovirus-infected cells. Extracts obtained from uninfected cells by the same procedure did not promote the replication of any of the double-stranded DNA templates tested, including adenovirus DNA–protein complex. These findings strongly suggest that the DNA replication observed in the system requires the participation of one or more virus-coded proteins or virus-induced cellular proteins or both. The system also exhibits

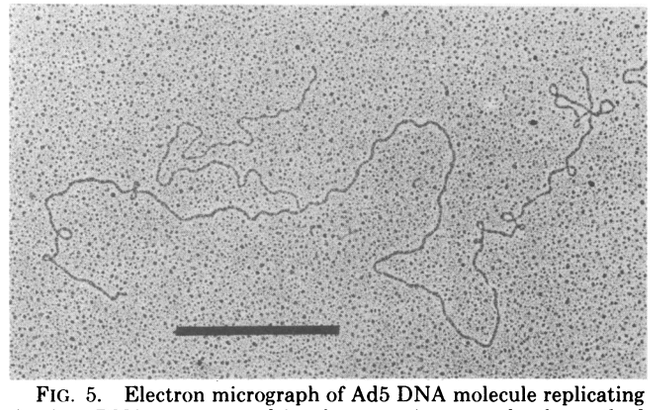

FIG. 5. Electron micrograph of Ad5 DNA molecule replicating *in vitro*. DNA was mounted for electron microscopy by the method of Davis *et al.* (30). Bar = 1 μm.

a striking preference for a particular template, namely, a DNA–protein complex isolated from purified adenovirus virions. Deproteinized adenovirus DNA or DNA from a heterologous source is significantly less effective in supporting DNA synthesis *in vitro*. These observations suggest that a protein(s) present in preparations of DNA–protein complex plays an important role in the replication process (see below). The product of the *in vitro* reaction consists of long adenovirus DNA strands that are hydrogen-bonded to input DNA strands. At least some of the newly synthesized strands approximate full length. The bulk of the product is not covalently linked to the template DNA, indicating that the DNA synthesized by the extract is not the product of a repair reaction; however, the resolution of our density shift experiments is not sufficient to rule out the possibility that a very short segment of the template DNA serves as a primer for DNA synthesis. Electron microscopic observations revealed that branched molecules with structural features identical to *in vivo* replication intermediates (19) are produced during the course of the *in vitro* reaction. Thus, it seems likely that adenovirus DNA replication in the *in vitro* system proceeds by a displacement mechanism as it does *in vivo*. In addition, the structure of the branched molecules strongly suggests that DNA replication in the *in vitro* system starts at or near the termini of the template DNA as is also the case *in vivo* (7). We have recently obtained independent confirmation of this suggestion by examining the distribution of radioactivity in the *in vitro* reaction product as a function of time of incubation. Our data clearly show that early in the course of the *in vitro* reaction, the terminal restriction fragments of the product are preferentially labeled (unpublished results). In summary, all of our findings support the conclusion that DNA replication in the *in vitro* system closely resembles adenovirus DNA replication *in vivo*.

It seems likely that *de novo* initiation of DNA chains takes place in the *in vitro* system although this has not been directly demonstrated. In particular, we cannot at present rule out the possibility that the input DNA includes molecules that had initiated DNA synthesis *in vivo* shortly before encapsidation and that such molecules serve as substrates for chain elongation *in vitro*. The observed preference of the system for DNA–protein complex may have important implications for the initiation mechanism. Preparations of complex consist principally of duplex adenovirus DNA molecules whose 5′ termini are covalently linked to a protein of 55,000 daltons (12–16). It is possible that the terminal protein may serve to facilitate initiation of DNA replication, perhaps via interaction with proteins present in the infected-cell extract. In this context it is of interest to note that the specific infectivity of adenovirus DNA–protein complex is considerably greater than that of deproteinized adenovirus DNA (13).

Only a fraction of the input Ad5 DNA molecules appears to participate in extensive DNA synthesis *in vitro*. The maximal extent of incorporation of precursors that we have observed to date corresponds to 5–10% of the input DNA, yet the average size of the DNA chains synthesized *in vitro* is considerably greater than 5–10% of unit length. There are several possible explanations for the limited utilization of the input DNA. It is possible, as mentioned above, that preinitiated molecules are the exclusive substrates for *in vitro* synthesis. Alternatively, the harsh conditions used to obtain DNA–protein complex may promote the inactivation of an essential replication protein(s). Finally, some required component of the extract may be limiting.

The system described in this communication should prove valuable for probing the biochemical mechanisms of eukaryotic DNA replication. The requirement of the system for exogenous DNA offers an opportunity to define the specific structural features of the template that are important in replication. In addition, the development of this system makes possible a rational approach to the purification and functional characterization of viral and cellular proteins involved in adenovirus DNA replication.

We thank Michael Stern for expert technical assistance and Drs. Gary Ketner, Sharon Price, and Daniel Nathans for helpful discussions. This work was supported by U.S. Public Health Service Grant CA13478 from The National Cancer Institute.

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