JOURNAL OF VIROLOGY, Apr. 1975, p. 744-758

Vol. 15, No. 4
Printed in U.S.A.

Adenovirus Type 2 DNA Replication

I. Evidence for Discontinuous DNA Synthesis

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Received for publication 24 October 1974

Isolated nuclei from adenovirus type 2-infected HeLa cells catalyze the incorporation of all four deoxyribonucleoside triphosphates into viral DNA. The observed DNA synthesis occurs via a transient formation of DNA fragments with a sedimentation coefficient of 10S. The fragments are precursors to unit-length viral DNA, they are self-complementary to an extent of at least 70%, and they are distributed along most of the viral chromosome. In addition, accumulation of 10S DNA fragments is observed either in intact, virus-infected HeLa cells under conditions where viral DNA synthesis is inhibited by hydroxyurea or in isolated nuclei from virus-infected HeLa cells at low concentrations of deoxyribonucleotides. Under these suboptimal conditions for DNA synthesis in isolated nuclei, ribonucleoside triphosphates determine the size distribution of DNA intermediates. The evidence presented suggests that a ribonucleoside-dependent initiation step as well as two DNA polymerase catalyzed reactions are involved in the discontinuous replication of adenovirus type 2 DNA.

Okazaki et al. (24) were the first to demonstrate that chain growth during DNA replication in *Escherichia coli* occurs discontinuously, i.e., via the transient formation of DNA fragments (Okazaki fragments) which are subsequently joined into nascent DNA strands. In the meantime similar observations have been made in a number of other procaryotic (16, 17) as well as eucaryotic systems (8, 9, 10, 13, 19). The present studies were undertaken to investigate whether the concept of discontinuous DNA synthesis can be extended to the replication of adenovirus DNA.

Adenovirus type 2 (Ad2) or type 5 (Ad5) virions contain nonpermuted linear double-stranded DNA genomes of a molecular weight of 23×10^6 (11). Replicative intermediates have been identified in both systems which are characterized by (i) the presence of replicating daughter strands which, although they vary in size, never exceed genome length (7, 15, 33); (ii) an increased sedimentation rate in neutral sucrose gradients (7, 15, 29, 33); and (iii) an increased buoyant density of 6 to 10 mg/ml in neutral cesium chloride as compared to mature viral DNA (15, 26, 29, 33, 36). The increased buoyant density of replicative intermediate DNA is sensitive to a treatment with the single-stranded, DNA-specific endonuclease from *Neurospora crassa* (26, 29). This has been interpreted to indicate the presence of extensive single-stranded regions in replicating Ad2 DNA.

Based on these results as well as on electron microscopy observations, Sussenbach et al. (32, 35) have proposed a model for the replication of Ad5 DNA. According to this model, Ad5 DNA replication starts at the molecular right end by displacement synthesis on the light strand, whereas synthesis on the displaced, complementary strand begins only with a considerable delay. This model of displacement synthesis does not necessarily require the formation of Okazaki-type fragments to replicate DNA in the 5′ to 3′ direction as replication of the two complementary daughter strands occurs highly asynchronously (34).

Nevertheless, short pieces of virus-specific DNA have been observed in Ad2-infected HeLa cells (15) as well as in Ad5-infected KB cells (7). In addition, Sussenbach et al. (33, 36) described the presence of fragmented DNA in isolated nuclei from Ad5-infected KB cells which had been incubated for 30 min under optimal conditions for DNA synthesis. These DNA fragments, however, have never been identified and characterized as Okazaki pieces since they did not seem to accumulate preferentially during short pulses. In contrast, 10 to 12S DNA fragments have recently been identified as intermediates during replication of CELO virus, an avian adenovirus (1).

The evidence for discontinuous DNA synthesis of Ad2 DNA, discussed in the present study, was obtained in part during studies on viral

DNA synthesis in isolated nuclei obtained from Ad2-infected HeLa cells. Under suitable conditions (33, 36), such nuclei catalyze the incorporation of deoxyribonucleoside triphosphates into virus-specific 10S DNA fragments which are precursors to unit-length viral DNA. The properties of these fragments will be described.

Evidence for discontinuous DNA synthesis could be obtained in Ad2-infected HeLa cells which were starved for deoxyribonucleotides by treatment with hydroxyurea. This drug, a potent inhibitor of polyoma (20, 21) and SV40 (18) DNA synthesis, rapidly inhibited Ad2 DNA synthesis. Analysis of newly synthesized viral DNA from inhibited cells indicated that it consisted mainly of 10S virus-specific DNA fragments.

The combined evidence from both the in vitro and the in vivo systems indicates that discontinuous DNA synthesis does occur during replication of the linear, double-stranded DNA genome of Ad2.

MATERIALS AND METHODS

Cells. Human HeLa cells (obtained from J. Williams, Glasgow) were grown in suspension cultures supplemented with 15% calf serum (Flow Laboratories) or in monolayers in Dulbecco modified Eagle medium in the presence of 10% calf serum. Cultures were tested bi-monthly for mycoplasma contamination (4).

Virus and viral DNA. Ad2 (from W. Rowe, N.I.H., Bethesda, Md.) was plaque purified on HeLa cells and propagated in suspension cultures (4×10^5 cells/ml) of HeLa cells at low multiplicity of infection (0.1 PFU/cell). For the preparation of virus stocks, cells were harvested 40 h after infection by low-speed centrifugation at $1,000 \times g$ for 10 min at 4 C, resuspended at 5×10^7 cells/ml in 20 mM Tris-chloride (pH 8.0) and sonicated at 4 C for 3×30 s, with 1-min intervals, in a Branson sonifier at 65 mA. Extracts were cleared by extraction with Freon 113 and either kept at −80 C in suitable aliquots or purified through three cycles of equilibrium centrifugation in cesium-chloride density gradients (5, 6). Purified virus was found to retain its infectivity for several months when stored in buoyant CsCl at 4 C in concentrations of not more than 20 optical density units at 280 nm per ml. Plaques assays were performed on HeLa cells as described previously (37). Labeled virus stocks were obtained by adding [^{14}C]formate (50 mCi/mmol; 1 μCi/ml) or [^{3}H]thymidine (26 Ci/mmol; 1-3 μCi/ml) to infected cultures 8 to 10 h after infection. Isolation and purification were carried out subsequently as described above. Ad2 DNA was prepared from purified stocks of unlabeled or labeled virus as described by Doerfler et al. (5, 6).

Chemicals. [^{3}H]dTTP (17 Ci/mmol) was obtained from Schwarz-Mann, Orangeburg, N.Y., and [^{3}H]dATP (14.5 Ci/mmol), [^{3}H]thymidine (27 Ci/mmol), [^{14}C]formate (50 mCi/mmol), [2-^{14}C]thymidine (50 mCi/mmol), and [^{14}C]dATP (450 mCi/mmol) were obtained from Amersham Buchler GmbH, Braunschweig, Germany, 5'-Bromo-deoxyuridinetriphosphate (dBrUTP), unlabeled deoxyribonucleoside triphosphates, phosphoenolpyruvate, and pyruvate kinase were purchased from Boehringer, Mannheim, Germany. Proteinase K (12) was kindly provided by H. Lang, Merck, Darmstadt; CsCl, 2,2'-p-phenylene-bis-(5-bisoxazole), and 2,5-diphenyloxazole were obtained from Merck, Darmstadt; ethylene - glycol - bis(β-amino-ethylether)-*N,N'*-tetraacetic acid and agarose were obtained from Sigma; HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethane sulfonic acid), acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*,tetramethylenediamine, and Genetron 113 were from Serva, Heidelberg. Soluene 100 is a product of the Packard Instrument Corp., Breda, The Netherlands. Dithiothreitol was purchased from Carl Roth, Karlsruhe.

Buffers. The compositions of hypotonic HEPES and isotonic HEPES have been described (38, 39). Tris is a tris-buffered saline (40). The TIS scintillation mixture is composed of 900 ml of toluene, containing 5 g of 2,5-diphenyloxazole and 0.3 g of 2,2'-p-phenylene-bis-(5-phenyloxazole), 50 ml of 80% isopropanole in water, and 50 ml of toluene 100 (all reagents analytical grade).

Isolation of nuclei. The standard procedure, performed at 4 C, will be described for a 500-ml suspension culture (3 to 5×10^5 cells/ml) infected with a virus multiplicity of 50 PFU/cell. At 20 h after infection, the culture was mixed with 1 volume of a slurry of frozen Tris and centrifuged at $1,000 \times g$ for 10 min. The pellet was washed and recentrifuged three times with 30 ml of Tris and finally suspended in 15 ml of hypotonic hepes. After 10 min, the swollen cells were broken with six strokes of a tight fitting Dounce homogenizer (Braun, Melsungen), and the homogenate was immediately diluted into 1 volume of 0.6 M sucrose and centrifuged for 15 min at $1,000 \times g$. The nuclei pellet was resuspended in 20 volumes of isotonic HEPES and recentrifuged, and the pellet was resuspended in 2 volumes of isotonic HEPES. The resulting suspension, at a concentration of 4×10^7 to 7×10^7 nuclei/ml, was used in the in vitro experiments. As judged from phase contrast microscopy, it contained less than one intact cell in 1,000 nuclei.

Standard conditions for in vitro DNA synthesis. Nuclei were incubated at 25 C under conditions similar to those described previously (38, 39). A final reaction mixture contained 60 mM NaCl, 4 mM $MgCl_2$, 2 mM ethylene-glycol-bis-(β-amino-ethylether)-*N,N'*-tetraacetic acid, 2 mM ATP, 0.06 mM each of dATP, dCTP, and dGTP, 0.02 mM [^{3}H]dTTP (3,000 to 7,000 counts/min per pmol), 5 mM phosphoenol-pyruvate, pyruvate kinase (20 μg/ml), 0.06 mM each of GTP, UTP, and CTP, 180 mM sucrose, 40 mM HEPES (pH 8.0), 0.4 mM dithiothreitol, and 0.4 mM $CaCl_2$. Reactions were stopped through the addition of 5 volumes of 50 mM HEPES, 5 mM EDTA, and 0.22 M sucrose. Lysis was performed either in 1 volume of 0.5 M NaOH and 10 mM EDTA on top of alkaline sucrose gradients or, if total DNA had to be isolated, through incubation of the nuclear suspension at 37 C for 60 min in the presence of 0.5% sodium dodecyl sulfate (SDS), 5 mM EDTA, and 100 μg of

proteinase K per ml. After three phenol and two chloroform-isoamylalcohol (24:1) extractions, followed by dialysis against three changes of 500 volumes of 10 mM Tris-hydrochloride and 5 mM EDTA (pH 8.0), the extracted nuclear DNA was used for CsCl equilibrium centrifugation or DNA-DNA hybridization experiments. A selective extraction of viral DNA under conditions described by Hirt (14) for the isolation of polyoma DNA (molecular weight 3×10^6) could only be achieved with a yield of 30 to 50%.

To determine total incorporation, 5-μl aliquots of an incubated nuclei suspension were dissolved in 100 μl of 0.3 M NaOH and kept for 1 h at 80 C. The incubation mixture was then diluted with 1 ml of 10% trichloroacetic acid-0.1 M sodium pyrophosphate, and the acid-insoluble radioactivity was determined as described (39). Specific activities were obtained from 5-min incubations and are defined as picomoles of [^{3}H]dTTP or [^{3}H]dATP incorporated/minute per milligram of total DNA. The method of Burton (3) was used to determine DNA concentrations in aliquots of the nuclear suspensions. Specific activities of 30 to 40 pmoles/min per mg of DNA were obtained regularly for incorporation into total nuclear DNA.

Fragmentation of total nuclear DNA by the EcoRI restriction endonuclease. The EcoRI restriction endonuclease was prepared according to methods developed by Yoshimori (Ph.D. thesis, Univ. of California, San Francisco, 1973). The material eluting from DEAE-cellulose between 0.38 and 0.48 M NaCl was used throughout this study (4). This preparation cleaved Ad2 DNA into six specific fragments (27) and was free of any single-stranded, DNA-specific nuclease activity when assayed with single-stranded Ad2 DNA. After incubation under standard conditions for DNA synthesis, nuclei were diluted with 9 volumes of 20 mM HEPES (pH 8.0), 1 mM dTTP, 1 mM EDTA, centrifuged at $1,000 \times g$ for 10 min, and resuspended in 300 μl of 10 mM $MgCl_2$, 1 mM dTTP, 50 mM HEPES (pH 7.4), 0.22 M sucrose, and 25 μl of endonuclease R·RI. After incubation at 37 C for 1 h, the nuclei were lysed in the presence of 0.5% SDS, 5 mM EDTA, and 100 μg of proteinase K per ml at 37 C for 30 min. The resulting DNA preparation was extracted with phenol and chloroform-isoamylalcohol as described above and dialyzed extensively against $^1/_{10}$ concentrated electrophoresis buffer (see below).

Analytical gel electrophoresis on mixed polyacrylamide-agarose gels. Conditions for gel electrophoresis of fragmented DNA have been described recently (4). Fragments were isolated from gels after electrophoresis by incubation of 0.8-mm slices in 0.5 ml of 0.1% SDS for 18 h at 37 C. Under these conditions, between 75 to 90% of the applied acid-insoluble radioactivity could be recovered.

Lysis of nuclei and zonal centrifugation in alkaline or neutral sucrose gradients. The method developed and described by Burger and Doerfler (2) for the lysis of intact cells on alkaline sucrose gradients was adopted with minor modifications.

After incubation under standard conditions for DNA synthesis, nuclei were suspended in 50 mM HEPES (pH 8.0), 10 mM EDTA, and 0.22 M sucrose at a concentration of 2×10^6 nuclei/ml. Aliquots, containing between 20,000 to 500,000 nuclei or 0.3 to 7 μg of DNA, were layered on top of alkaline sucrose gradients.

Sucrose gradients in Spinco SW41 tubes were composed of a 1-ml cushion of 70% sucrose in 0.7 M NaCl, 0.3 M NaOH, and 5 mM EDTA, 11 ml of a 5 to 20% gradient in 0.7 M NaCl, 0.3 M NaOH, and 10 mM EDTA, and 0.2 ml of a lysis layer containing 0.5 M NaOH and 10 mM EDTA. Lysis was allowed to proceed for 12 to 18 h on top of the gradients in a was added to the gradients just prior to the nuclei. Gradients were centrifuged at 4 C in a SW41 rotor for 5 h at 40,000 rpm. After centrifugation, fractions were collected from the bottom of the tubes, acid precipitated with 1 ml of 10% trichloroacetic acid-0.1 M sodium pyrophosphate and prepared for scintillation counting as described above.

For neutral gradients, the SW41 tubes contained a 1-ml cushion of 70% sucrose in 1 M NaCl, 10 mM EDTA, and 10 mM Tris-hydrochloride (pH 8.0) beneath 11 ml of a linear 5 to 20% sucrose gradient in 1 M NaCl, 5 mM EDTA, 10 mM Tris-hydrochloride (pH 8.0), and 0.5% sarcosyl. Lysis of nuclei containing up to 3 μg of DNA (ca. 200,000 nuclei) was allowed to proceed for 12 to 18 h on top of the gradients in a 0.2-ml layer of 1% sarcosyl. Centrifugation was performed for 4 h and 40,000 rpm at 4 C. Fractions were collected and analyzed for radioactivity as described above.

Equilibrium centrifugation in neutral cesium chloride or cesium sulfate gradients. Total intranuclear DNA was extracted from a 50-μl incubation mixture as described. After extensive dialysis against several changes of 0.25 M NaCl, 0.01 M Tris-hydrochloride (pH 8.0), and 0.005 M EDTA, cesium chloride was added to the DNA solution to a final density of 1.715 g/cm^{-3}. Samples of 4 ml were overlaid with paraffin oil and centrifuged in a Beckman 50 Ti tube at 20 C and 35,000 rpm for 60 h. About 60 fractions were collected from the bottom of the tubes and the density gradient profile was determined as previously described (22). Radioactivity was determined as mentioned above.

Composition of neutral and alkaline cesium sulfate gradients as well as centrifugation conditions have been described previously (22).

DNA-DNA hybridization. DNA-DNA hybridization experiments were performed essentially as described by Horwitz (15). Ad2 DNA (0.5, 1, and 2 μg) or HeLa cell DNA (10 μg) were immobilized on Sartorius nitrocellulose filters (25-mm diameter). Samples from alkaline sucrose gradients were neutralized, diluted to 300 μl with water, sonicated, heat denatured, and diluted with 18 × SSC (0.15 M NaCl plus 0.15 M sodium citrate) and 10% SDS to final concentrations of 3 × SSC and 0.1% SDS. Final volumes were 0.6 ml. Total nuclear DNA was sheared through sonication, heat denatured, and diluted with SSC and SDS as described.

Reassociation of small DNA fragments. Nuclei were incubated for 1 min in the presence of dBrUTP instead of dTTP and [^{14}C]dATP instead of dATP. After lysis on top of alkaline sucrose gradients and subsequent sedimentation, as described, fractions

from six Beckman SW41 gradients containing the 10S DNA fragments were pooled and centrifuged to equilibrium in alkaline cesium sulfate (22). Centrifugation was performed at 20 C in an SW56 rotor at 29,000 rounds/min for 96 h. Fractions from various regions of the density gradient were pooled (see Fig. 6), dialyzed against two changes of 200 volumes of 10 mM NaCl, 10 mM Tris-hydrochloride, and 5 mM EDTA (pH 8.0), and heat denatured. Reassociation experiments were performed at 68 C in a total volume of 1.6 ml in 1 M NaCl, 10 mM Tris-hydrochloride, 5 mM EDTA (pH 8.0), supplemented with 1 μg of sheared salmon sperm DNA. The reannealing reaction was followed by determination of the increase in acid-insoluble radioactivity after incubation of aliquots of the reassociation mixture with the single-stranded DNA-specific nuclease S_1 from *Aspergillus oryzae* (23). The enzyme was prepared as described (4). Aliquots of 50 μl were withdrawn at various times, diluted to 1 ml to give a final concentration of 5 mM $ZnSO_4$, 0.3 M NaCl, 0.025 M sodium acetate (pH 4.5), and 10 μg of calf thymus DNA. After addition of 10 μl of S_1 nuclease, solutions were incubated at 45 C for 2 h and acid-insoluble radioactivity was determined.

Control experiments included both the reassociation of labeled Ad2 DNA (0.30 μg) and addition of unlabeled Ad2 DNA (2 and 10 μg) to reassociating ^{3}H-labeled 10S fragments.

RESULTS

Parameters for optimal DNA synthesis in isolated nuclei. The ability of isolated nuclei to incorporate [^{3}H]dTTP into acid-insoluble material was studied both as a function of time after infection and of virus multiplicity. Nuclei were prepared at various times after infection from HeLa cells, infected at a multiplicity of 50 PFU/cell, and incubated under standard conditions for DNA synthesis. As shown in Fig. 1, maximal values for [^{3}H]dTTP incorporation could be observed between 16 to 22 h postinfection. Specific activities within this time interval varied between 26 to 29 as compared to values of 1 to 3 for mock-infected cells.

In a second series of experiments, specific activities of isolated nuclei were studied as a function of virus multiplicities. At various multiplicities between 50 and 400 PFU/cell, the observed specific activities remained almost unchanged although the peak activity shifted from 16 to 22 h at 50 to 220 PFU/cell to 12 to 18 h at 400 PFU/cell (Fig. 1). A maximal value of only 18 was observed at a multiplicity of 5 PFU/cell at 30 h after infection (not shown). It can be concluded from these data that DNA synthesis in isolated nuclei from Ad2-infected HeLa cells occurs optimally between 16 to 22 h after infection at a multiplicity of 20 to 100 PFU/cell.

The kinetics of [^{3}H]dTTP incorporation was studied at 25 and 37 C in 20-h nuclei from cells infected at a multiplicity of 50 PFU/cell. As demonstrated in Fig. 2, the rate of DNA synthe-

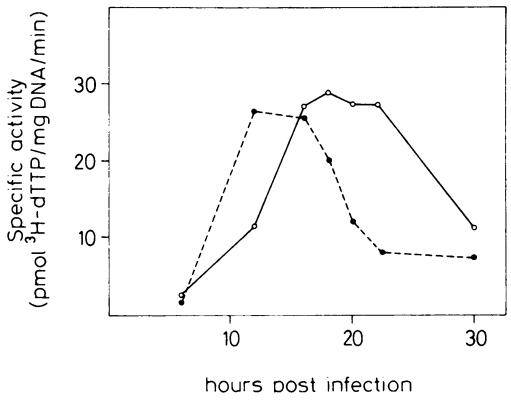

FIG. 1. *Specific activities for [^{3}H]dTTP incorporation of isolated HeLa cell nuclei as a function of time after infection and virus multiplicity. Nuclei were isolated at various times after infection with 50 or 400 PFU/cell of Ad2. After incubation at 25 C under optimal conditions for DNA synthesis, specific activities were determined from 5-min incubation periods as described. Symbols: ○, 50 PFU/cell; ●, 400 PFU/cell.*

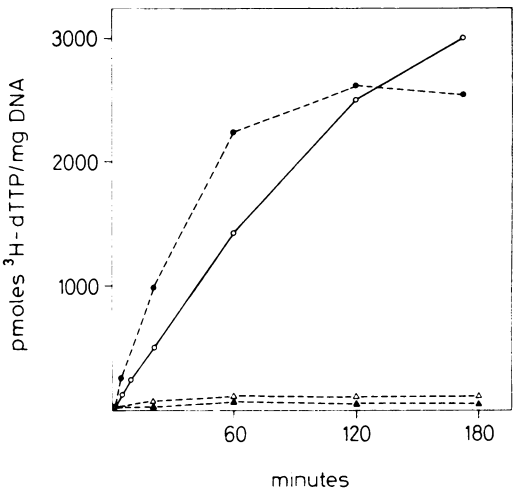

FIG. 2. *Kinetics of [^{3}H]dTTP incorporation into total DNA in isolated nuclei as a function of temperature and dNTP concentration. Nuclei from infected cells, isolated 20 h after infection, were incubated either at 25 and 37 C under optimal conditions for DNA synthesis or at 25 C in the absence of exogenous dATP, dCTP, and dGTP or without dATP only. Total nuclear DNA was extracted and determined as described. Symbols: ●, incorporation at 37 C; ○, incorporation at 25 C; △, incorporation minus dATP; ▲, incorporation minus three extra dNTP.*

sis at 25 C is linear for almost 60 min, and for at least 30 min at 37 C. A plateau value seems to be reached at 37 C above 60 to 90 min of incubation, whereas synthesis at 25 C is still proceeding, albeit at reduced rates. This observed decrease in activity can be due only to a limited extent to physical inactivation of the nuclei, as nuclei, preincubated at 25 C for 60 min in the absence of dNTP, could resume DNA synthesis at only a slightly lower rate than normal after addition of the substrates (specific activity of 22 versus 31). In addition, the data in Fig. 2 show the strong dependence of the reaction on the presence of deoxyribonucleoside triphosphates. In the absence of three triphosphates except dTTP, the initial rate of incorporation decreases to 1 to 2% of the normal values. If only one triphosphate is omitted, slightly higher specific activities are observed which, however, never surpass the 5% level of the full incorporation rate under optimal conditions. The nature of the products, synthesized under suboptimal conditions, will be described below.

Analysis of newly synthesized DNA in cesium chloride density gradients. Newly synthesized DNA from isolated nuclei was analyzed through CsCl equilibrium density centrifugation and, subsequently, DNA-DNA hybridization to determine relative amounts of viral and cellular DNA synthesis. Under neutral conditions, cellular DNA bands at 1.699 g/cm^{-3}, whereas mature viral DNA and replicating viral DNA have been shown to band at 1.715 and 1.723 g/cm^{-3}, respectively (7, 26, 33). When total nuclear DNA from isolated nuclei, incubated under standard conditions for 45 min, was subjected to equilibrium centrifugation in CsCl, the pattern depicted in Fig. 3 was obtained. Over 90% of the incorporated radioactivity banded at a density characteristic for viral DNA, whereas 5 to 10% can be identified as cellular DNA. Newly synthesized DNA from control nuclei of mock-infected cells showed the expected broad banding pattern of cellular DNA (Fig. 3).

The main band of the radioactivity incorporated during 45 min into isolated nuclei shows a density shift of 2 mg/ml over mature viral DNA, indicating that the extent of single-stranded regions in these molecules is considerably smaller than in replicative intermediates, isolated after 30- to 60-s pulses from intact, infected cells (26, 29). However, this is consistent with data which will be presented below which demonstrates that after 60 min of incubation in vitro at least 80% of the incorporated radioactivity appears in unit-length progeny strands.

Total DNA from a 45-min incubation was further characterized through DNA-DNA hybridization experiments. The results, which are summarized in Table 1, indicate that the newly synthesized DNA is virus specific. This further supports the conclusion previously reached from the density gradient experiments that late

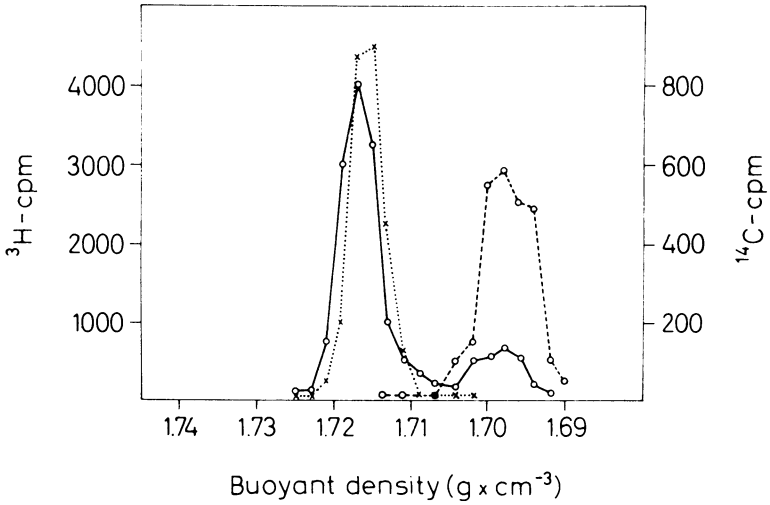

FIG. 3. *Equilibrium centrifugation of nuclear DNA in neutral cesium chloride Total DNA from a 45-min incubation of isolated nuclei was extracted and subjected to equilibrium centrifugation in neutral cesium chloride together with ^{14}C-labeled Ad2 marker DNA as described. In a separate experiment, ^{3}H-labeled HeLa cell DNA from nuclei of mock-infected cells was centrifuged to equilibrium under identical conditions with the same ^{14}C-labeled Ad2 marker DNA. Results from both gradients are superimposed relative to the ^{14}C-labeled Ad2 marker position. Symbols:* O——O, *^{3}H-labeled DNA from isolated nuclei;* ×, *^{14}C-labeled Ad2 marker DNA;* O----O, *^{3}H-labeled HeLa marker DNA.*

TABLE 1. *DNA-DNA hybridization of newly-synthesized DNA from isolated nuclei to DNA filters*

Labeled DNA	Counts/ min applied	DNA on filter	% Hybridized
Nuclei (20 h)	1,800	None	1.4
	1,800	Ad2 (2 μg)	73
	1,800	HeLa (10 μg)	4.1
Mock	1,400	None	1.1
	1,400	Ad2 (2 μg)	0.9
	1,400	HeLa (10 μg)	6.8

after infection most of the DNA synthesis in vitro occurs in viral DNA. These data are in good agreement with those obtained by Burger and Doerfler (2) and van der Eb (7) from [^{3}H]thymidine pulse experiments in Ad2-infected KB or HeLa cells, respectively. In both systems, DNA synthesis late in infections is restricted almost entirely to viral DNA replication. DNA synthesis in isolated nuclei thus reflects conditions observed in infected cells.

Analysis of newly synthesized DNA in alkaline sucrose gradients. The size distribution of newly synthesized DNA from isolated nuclei was studied in alkaline sucrose gradients. To minimize shearing, nuclei were lysed on top of preformed alkaline sucrose gradients for 18 h at 4 C. The sedimentation pattern of the acid-insoluble radioactivity, synthesized in 60 min, indicates that (i) more than 80% of the total radioactivity sediment at the position of marker Ad2 DNA; (ii) that approximately 5% of the incorporated radioactivity can be found at the bottom of the tube together with the bulk of the unlabeled host cell DNA, and (iii) that a small amount of trailing occurs to lower sedimentation coefficients (Fig. 4a). In a 2-h pulse, this proportion would still amount to about 10% of the total incorporated radioactivity.

The marker used in these experiments originated from a pulse with [^{14}C]thymidine, performed at 14 to 18 h postinfection, prior to the isolation of the nuclei. Its sedimentation pattern is indistinguishable from that of purified viral DNA as determined in a separate experiment.

A quite different pattern is observed when a similar sedimentation analysis was performed on newly synthesized viral DNA pulse labeled for only short periods at 25 C. As shown in Fig. 4a, about 70% of the total radioactivity from a 30-s pulse sediments in a discrete peak with a sedimentation coefficient of 10*S*. In this gradient, the smallest of the six specific fragments generated by digestion of ^{14}C-labeled viral DNA with the restriction endonuclease EcoRI was added as a marker. Its position in the gradient indicates that the observed *S* value for the newly synthesized viral DNA fragments corresponds to a molecular weight of 550,000 (27). The remaining radioactivity incorporated within a 30-s pulse period is distributed throughout the gradient.

With increasing pulse length, proportionally less radioactivity is found to sediment at 10*S* until, as discussed above, at least 80% of the total radioactivity cosediments with unit-length Ad2 DNA marker.

Aliquots of various fractions from an alkaline gradient of newly synthesized DNA from a 1-min pulse were subjected to DNA-DNA hybridization. The results, presented as black bars in Fig. 4b, indicate that the observed DNA fragments are indeed virus specific. A small but nevertheless significant amount of hybridization is observed in material sedimenting faster than unit-length viral marker. Although this material has not been studied in detail, it might represent the fast sedimenting, integrated viral DNA observed by Burger and Doerfler (2) in Ad2-infected KB cells.

A product-precursor relationship between the 10*S* fragments and marker viral DNA could be demonstrated in pulse-chase experiments. Nuclei were first incubated for 1 min at 25 C in the presence of labeled dTTP. After dilution with a 50-fold excess of cold dTTP, the incubation was continued for 30 min. Alkaline sucrose gradient analysis (Fig. 4b) of the pulse-labeled and the pulse-chased samples indicates that the observed fragments are indeed precursors to unit-length Ad2 DNA.

From the data presented it is concluded that Ad2 DNA synthesis occurs discontinuously through the transient formation of DNA fragments, 550,000 daltons long, which are subsequently ligated to form intact Ad2 DNA.

10S DNA fragments occur at all stages of replication. The question whether the observed DNA fragments are formed along either the whole length of the replicating Ad2 genome or only at specific replication sites was investigated in the following experiment.

Isolated nuclei were incubated for 30 s under standard conditions for DNA synthesis when about 70% of the total incorporated radioactivity can be found in 10*S* fragments as described above (Fig. 4a). The reaction was stopped and the nuclei were incubated subsequently in the presence of the EcoRI restriction endonuclease. During the digestion period, the nuclei contained ^{14}C-labeled marker DNA from a 4-h prelabel period with [^{14}C]thymidine prior

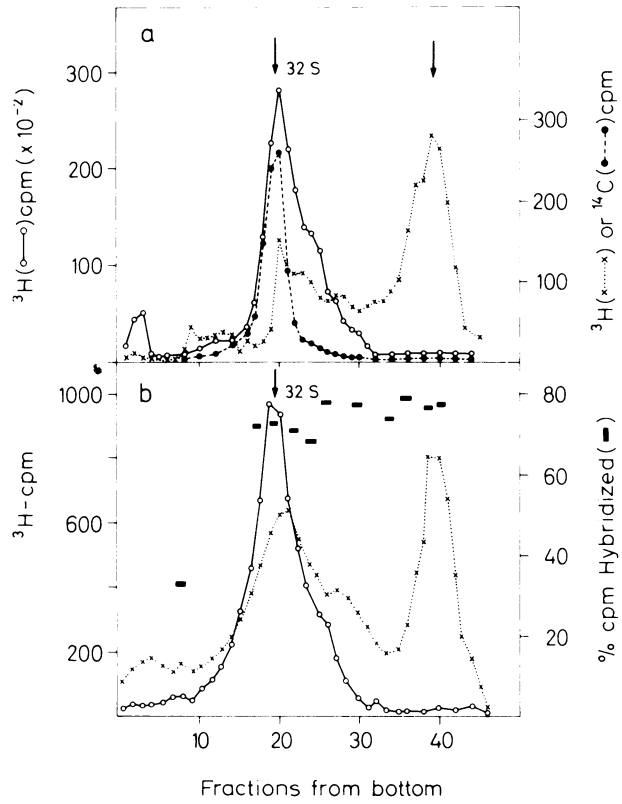

FIG. 4. *Analysis of newly synthesized DNA from isolated nuclei on alkaline sucrose gradients. Nuclei were isolated 20 h after infection from Ad2-infected HeLa cells. Prior to the isolation of the nuclei, from 16 to 20 h after infection, [^{14}C]thymidine had been added to label viral DNA. (a) Nuclei were incubated under optimal conditions from DNA synthesis for either 30 s or 60 min, lysed on top of alkaline gradients, and centrifuged as described. The sedimentation patterns from both incubation periods are superimposed and aligned with respect to the position of the ^{14}C-labeled Ad2 marker, present in both nuclei preparations. The unmarked arrow marks the peak banding position of the isolated endonuclease R·RI fragment F, obtained from ^{14}C-labeled marker DNA. The banding pattern of the fragment was determined in a separate centrifugation experiment. Symbols: ×, 30-s incubation; O, 60-min incubation; ●, ^{14}C-labeled Ad2 DNA. (b) Two nuclei incubation mixtures were incubated for 60 s in the presence of [^{3}H]dTTP. Nuclei from one experiment were lysed on top of an alkaline gradient, whereas the remaining mixture was incubated for additional 30 min at 25 C in the presence of a 50-fold excess of unlabeled dTTP prior to lysis on a preformed alkaline sucrose gradient. Both gradients are superimposed and aligned via the ^{14}C-labeled Ad2 DNA marker, the position of which is indicated by an arrow. In a separate experiment, nuclei from the same 60-s incubation were lysed and centrifuged on a preparative SW41 alkaline gradient. Fractions were isolated and analyzed by DNA-DNA hybridization as described. Black bars indicate the extent of hybridization against Ad2 containing nitrocellulose filters. Symbols: ×, 60-s incubation; O, 60-s incubation plus a 30-min chase.*

to isolation of the nuclei.

Total DNA was extracted as described and subjected to gel electrophoresis on composite polyacrylamide-agarose gels. The distributions of the two isotopes from the in vivo prelabel period (^{14}C) and the in vitro incorporation (^{3}H) are given in Fig. 5.

^{14}C radioactivity appears in eight peaks. Six of these peaks with 90% of the total radioactivity, correspond to the six fragments A through F, characteristic of an EcoRI digest of Ad2 DNA (27). In addition, the proportion of radioactivity in each of the six fragments corresponds to values expected from their known molecular weight (27). The digest therefore appears to have been complete.

The two additional peaks adjacent to fragment B, marked C′ and B′, consistently appear after digests of viral DNA within isolated nuclei labeled either in vitro or in vivo. Fragment C′, after elution from gel slices, was found to hybridize to an extent of 69% to filter-bound

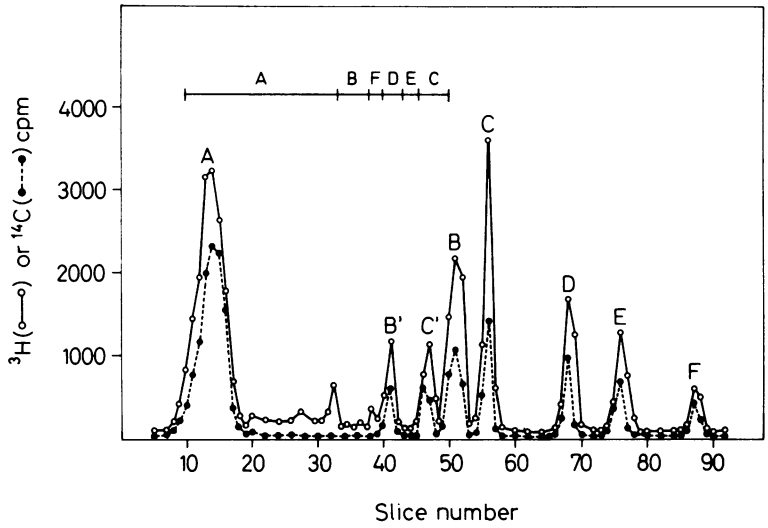

FIG. 5. *Endonuclease R·RI fragmentation pattern of viral DNA from isolated nuclei. Nuclei from infected cells were incubated at 25 C for 30 s, washed, and incubated for an additional hour in the presence of endonuclease R·RI. Total DNA was extracted and subjected to gel electrophoresis. The anode is towards the right. Peaks are designated A to F, B' and C' as described in the text. Locations of the fragments A to F on the linear Ad2 genome are indicated schematically in the upper left hand corner. Symbols:* O, *^3H-labeled DNA from a 30-s pulse in isolated nuclei;* ●, *^{14}C-labeled marker Ad2 DNA from a prelabel period as described in the legend to Fig. 4.*

Ad2 DNA. Since this result is similar to those obtained upon hybridization of isolated virus-specific EcoRI fragments B and C, fragment C' thus is of viral origin. After a similar analysis, fragment B' showed only background hybridization to filter-bound Ad2 DNA, whereas 4.7% hybridized to HeLa DNA, indicating that fragment B' represents cell specific DNA.

The distribution of radioactivity from incorporated [^{3}H]dTTP is quite similar; six peaks can be identified as the characteristic EcoRI fragments, comprising 85% of the total radioactivity recovered. Two additional peaks appear at positions identical to those of the ^{14}C-labeled DNA marker peaks B' and C'. The cell DNA peak B' corresponds to 4 to 7% of the total radioactivity. In addition, a background level, representing another 5 to 10% of the incorporated radioactivity, is distributed unspecifically between fragments A and B.

Ratios of radioactivity incorporated during 30 s from [^{3}H]dTTP versus ^{14}C-labeled racioactivity in marker DNA were determined as A = 1.05, B = 1.61, C = 1.85, D = 1.50, E = 1.54, and F = 1.00. It is not clear whether the reduction of relative incorporation into fragments A and F as compared to fragments B, C, D, and E is significant, and, thus suggesting that replication of Ad2 DNA occurs with variable rates along the viral chromosome.

The following conclusions can be drawn from these data: (i) 85 to 90% of the total radioactivity, incorporated either in intact cells or in isolated nuclei, is found in virus-specific EcoRI fragments, whereas 5 to 15% is found in cell-specific DNA. This is in agreement with our data from DNA-DNA hybridization and neutral cesium chloride density gradients. (ii) 10*S* DNA fragments are generated along almost the whole length of the Ad2 DNA molecule. Discontinuous DNA synthesis is thus not restricted to particular segments of the virus genome. (iii) At the time of isolation, isolated nuclei appear to contain replicative intermediates of Ad2 DNA at all stages of replication since, within the limits discussed above, the distribution of radioactivity in newly synthesized DNA is relatively uniform along the entire viral chromosome.

Self-annealing of 10S DNA fragments. Self-annealing experiments were performed with isolated 10*S* DNA fragments to establish whether they originate from one or both parental strands of the replicating molecules. These experiments could, however, be subject to misinterpretation if the labeled fragments were contaminated with unlabeled, fragmented viral DNA. Although, as mentioned above, we did not observe any breakdown of long-term labeled viral DNA in isolated nuclei, it cannot be

excluded that small amounts of viral DNA could remain from early events of the infection cycle. Therefore, short fragments were recovered from alkaline sucrose gradients of newly synthesized viral DNA extracted from isolated nuclei, incubated for 60 s in the presence of dBrUTP and [^{3}H]dATP. When the isolated fragments were subjected to equilibrium centrifugation in alkaline cesium sulfate, more than 80% of the radioactivity could be found at the expected heavy density position (Fig. 6). This indicates that the DNA fragments are indeed initiated de novo during synthesis in isolated nuclei. Fractions from the density gradient were combined in two pools, A and B, as shown in Fig. 6. After heat denaturation, the DNA was incubated at 68 C in 1 M NaCl for various amounts of time under conditions allowing reassociation.

The amount of double-stranded DNA was estimated from its resistance to the action of the single-stranded, DNA-specific S_1 nuclease from *Aspergillus oryzae*.

Results of the reannealing studies together with those of a number of controls are summarized in Table 2. Controls include experiments in which (i) DNA fragments were reannealed to an extent of more than 80% in the presence of unlabeled, sonicated Ad2 DNA and in which (ii) sonicated and heat-denatured ^{3}H-labeled Ad2 DNA self-annealed to an extent of 86%.

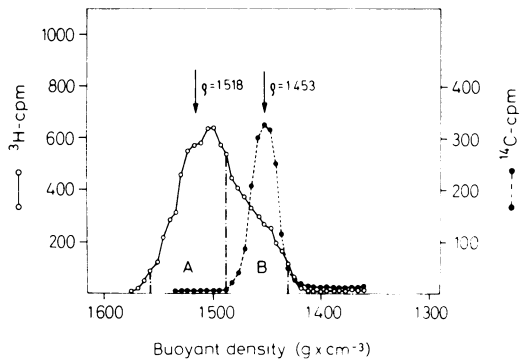

FIG. 6. *Purification of 10S viral DNA fragments. Nuclei were incubated for 60 s in the presence of dBrUTP and [^{3}H]dATP. After lysis and alkaline sucrose gradient centrifugation, fractions containing 10S DNA from six Beckman SW41 gradients were pooled and subjected to alkaline cesium sulfate equilibrium centrifugation. Arrows indicate the banding positions of light and heavy Ad2 DNA. Pools A and B were collected from gradient fractions as indicated. Marker ^{14}C-labeled DNA was present in the nuclei from a prelabel period with [^{14}C]thymidine prior to the isolation of the nuclei. Symbols: O, dBrUTP and ^{3}H-labeled viral DNA; ●, ^{14}C-labeled light Ad2 DNA.*

TABLE 2. *Self-annealing of isolated 10 to 12S fragments*

Determinants	Hybrid fraction (%)[a]			
	0 h[b]	10 h	18 h	48 h
Pool A	1.8	36	51	68
Pool B	1.1	49	69	88
Pool A + 0.30 μg of Ad2 DNA	1.2	54	59	81
Ad2 DNA	1.3	51	74	86

[a] Percentages represent an average of two experiments.
[b] Time of hybridization.

Under optimal conditions, the amount of self-annealing of the DNA fragments obtained from density-labeled, newly synthesized DNA was shown to reach 64 to 72%. Addition of unlabeled, denatured, and fragmented Ad2 DNA raised these values to over 80%. This result would indicate that the transiently formed DNA fragments do originate on both complementary parental strands. The limited extent, however, of the self-annealing reaction could possibly be explained by the following factors. A first, although unlikely, possibility is a selective loss of fragments from a particular region of the genome. A second possibility which cannot be excluded is that the concentration of DNA in the reannealing mixture was too low to permit a complete reaction in the 48-h period of the experiment. Finally, it appears possible that certain areas of the Ad2 genome are not replicated discontinuously. The last explanation does not appear to be totally unlikely as it has never been possible to develop conditions in which more than 70% of the total radioactivity was present in small fragments (Fig. 4a). In addition, the lower than expected incorporation of [^{3}H]dTTP during a 30-s pulse into the EcoRI fragment A and F is certainly consistent with this possibility.

Fragmented DNA is generated in isolated nuclei in the absence of exogenously added deoxyribonucleotides. When isolated nuclei are incubated in the absence of exogenously added dGTP, dCTP, and dATP, but in the presence of 20 μM [^{3}H]dTTP and at otherwise optimal conditions, the amount of incorporation after 45 min at 25 C was reduced 50-fold as compared to a standard experiment (Fig. 2). The residual DNA synthesis is due to deoxyribonucleoside triphosphates (dNTP) remaining in the nuclei after two washes with isotonic buffer. Actual pool sizes, however, have not been determined.

Nuclei incubated under these limiting conditions were lysed on top of alkaline sucrose gradients. Newly synthesized DNA was subsequently analyzed by zonal centrifugation. The sedimentation pattern, as shown in Fig. 7, demonstrates that a large proportion of the radioactivity is incorporated into a heterogenous population of DNA molecules, sedimenting between 16 and 26S. These molecules are precursors to unit-length viral DNA since radioactivity incorporated under limited dNTP supply could be chased into mature viral DNA through restoration of optimal conditions by addition of 20 μM of each of the ommited dNTP as well as a 30-fold excess of unlabeled dTTP (not shown).

Under suboptimal conditions, the sedimentation pattern is influenced by the presence of ribonucleoside triphosphates (rNTP) in the incubation mixture. In the presence of 60 μM rNTP, a majority of the newly synthesized DNA now sediments at lower sedimentation rates with a discrete peak around 12S (Fig. 7). Again, all the radioactivity can be chased into unit-length viral DNA upon addition of the missing dNTP as described above. An increase of the rNTP concentration from 60 to 300 μM has an even more pronounced effect on the size of the DNA intermediates which now sediment uniformly between 9 and 11S. The implication of these results on existing models for viral DNA replication will be discussed below.

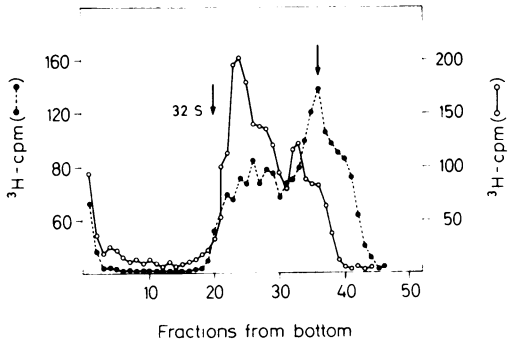

FIG. 7. *Alkaline sucrose gradient centrifugation of DNA from isolated nuclei synthesized under conditions of deoxyribonucleotide starvation. Nuclei from infected cells were incubated with [^{3}H]dTTP as the only exogenous dNTP source in the absence or presence of 60 μM rNTP. After a 45-min incubation period, nuclei were lysed and analyzed by alkaline sucrose gradient centrifugation as described. Arrows indicate the position of internal ^{14}C-labeled marker Ad2 DNA (32S) or the peak position of fragmented DNA synthesized in the presence of rNTP corresponding to a sedimentation coefficient of 12S. Symbols:* ○, *DNA synthesis in the absence of rNTP;* ●, *sedimentation pattern in the presence of rNTP.*

10S DNA fragments are generated in vivo in the presence of hydroxyurea. Accumulation of small, virus-specific DNA fragments has also been observed during a study of viral DNA synthesis in infected cells in the presence of hydroxyurea (HU). HU is a potent inhibitor of mammalian cell DNA synthesis acting through inactivation of the B2 subunit of the intracellular ribonucleotide reductase, thereby reducing available pools of dNTP required for DNA synthesis (30).

The effect of the drug on Ad2 DNA replication in HeLa cells was studied 20 h postinfection when cells were treated with 0.4, 2, and 10 mM HU. After a series of successive 10-min pulse experiments with [^{3}H]thymidine and subsequent determination of the incorporated acid-insoluble radioactivity, it can be concluded that the drug shuts off viral DNA synthesis to 12, 5, and 2%, respectively, of control values within 1 to 2 min after its addition. These residual levels of DNA synthesis are maintained at all three HU concentrations for at least 1 h after addition of the drug.

To characterize the DNA synthesized under these restricted conditions, cells exposed to 10 mM HU for 20 min and then pulse labeled for 15 min with [^{3}H]thymidine were lysed on top of alkaline sucrose gradients as described above. The sedimentation pattern as shown in Fig. 8 (upper panel), indicates that more than 80% of the incorporated radioactivity sediments at S values of approximately 10S. The remaining radioactivity was consistently found to sediment slightly slower than ^{14}C-labeled marker DNA which was either added prior to centrifugation or was present in the cells from a 16- to 20-h prelabel period. The presence of unfragmented marker DNA in these gradients, labeled in vivo as indicated, demonstrates that the presence of 2 μM unlabeled thymidine, the slow-sedimenting DNA fragments could be chased within 15 min into full length viral DNA molecules (Fig. 8, lower panel). This particular experiment, in contrast to the one described in Fig. 8 (upper panel) was performed in the presence of only 2 mM HU. At this concentration the sedimentation pattern is considerably more heterogenous with already 30 to 40% of the radioactivity sedimenting around 34S.

The viral nature of the 10S fragments was, in addition to the chase, ascertained by DNA-DNA hybridization. After isolation from a preparative alkaline sucrose gradient (Spinco SW27 rotor), radioactivity from the 10 to 12S

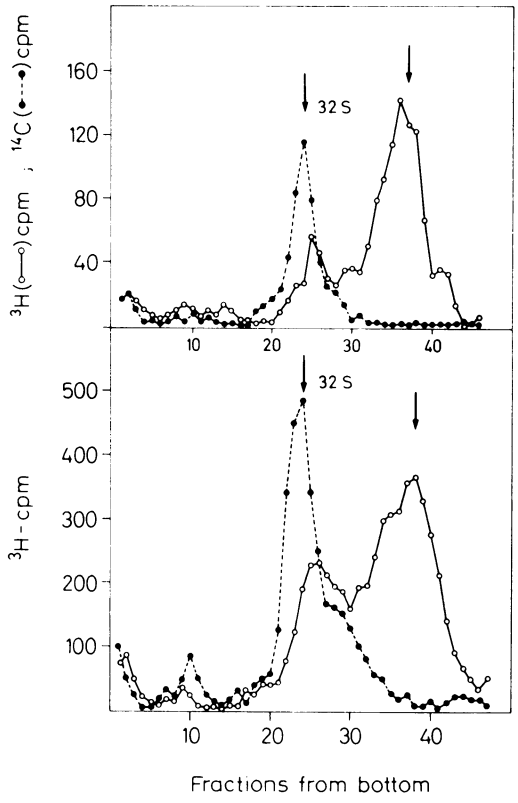

FIG. 8. *Analysis by alkaline sucrose gradient centrifugation of viral DNA labeled in Ad2-infected HeLa cells in the presence of HU. (Upper panel) Sedimentation pattern of DNA labeled 20 min after the addition of HU to infected cells during a 15-min pulse with [^{3}H]thymidine. Conditions of lysis of nuclei and centrifugation have been described. Arrows indicate the positions of either ^{14}C-labeled marker DNA (32S), present in the nuclei from a prelabel period as described in the legend to Fig. 4, or of the isolated endonuclease R·RI fragment F, determined in a separate experiment. Symbols: O, ^{3}H-labeled Ad2 DNA synthesized in the presence of HU; ●, ^{14}C-labeled Ad2 marker DNA. (Lower panel) HU-inhibited Ad2-infected HeLa cells were pulsed as described above but chased for additional 30 min with unlabeled thymidine in the absence of HU. Arrow positions have been described above. The sedimentation pattern of the ^{14}C-labeled marker DNA is omitted. Symbols: O, ^{3}H-labeled DNA synthesized in the presence of 2 mM HU; ●, ^{3}H-labeled DNA after a chase in the absence of HU.*

region of the gradient was shown to hybridize to an extent of 78 to 80% to immobilized Ad2 DNA on nitrocellulose filters.

In self-annealing experiments similar to those described for the fragments prepared from isolated nuclei, self-annealing levels of around 70% could be observed. These experiments are open to the criticism that the fragments might have been contaminated with fragmented viral DNA since, in the absence of a density label, they could not be purified away from contaminating DNA.

Nevertheless, the data are consistent with those obtained during DNA synthesis in isolated nuclei after short pulses or at suboptimal dNTP concentrations, and indicate a transient formation of 10*S* DNA fragments during Ad2 DNA replication.

10S viral DNA fragments contain RNA. Experimental evidence, discussed in the previous paragraph, established an influence of rNTP on the synthesis of DNA intermediates under suboptimal conditions in isolated nuclei. These results raise the question if, in addition to this regulatory phenomenon, rNTP serve as primers in the initiation of fragment DNA synthesis. In analogy to the situation in polyoma DNA synthesis, short fragments would thus be expected to contain a short sequence of ribonucleotides at their 5′ end.

Isolated nuclei were therefore incubated for 5 min under optimal conditions for DNA synthesis in the presence of [^{3}H]UTP, [^{3}H]CTP, and [^{14}C]dATP. The nuclei were lysed on top of neutral sucrose gradients in the presence of 0.5% sarcosyl and centrifuged as described above. Fractions containing the 10*S* DNA fragments were subjected to equilibrium centrifugation in neutral cesium sulfate. As shown in Fig. 9a, a majority of the radioactivity, incorporated from RNA precursors, was found to band at a density of 1.685 g/cm^{-3}, characteristic for RNA (21). However, a shoulder of this material reached into the DNA position, characterized by the radioactivity introduced from [^{14}C]dATP. When the material in this region of the gradient was collected as indicated by the black bar in Fig. 9a and recentrifuged in neutral cesium sulfate, a prominent peak appeared at the marker DNA position (Fig. 9b). A similar pattern was obtained when, before recentrifugation, the heat-denatured gradient fractions were subjected to chromatography on poly(U) sepharose (4). This step is expected to remove any poly (A)-containing viral transcripts noncovalently linked to the DNA fragments.

As a control experiment, DNA fragments obtained as indicated in Fig. 9a were kept for 1 h at 80 C in 0.3 M NaOH. Under these conditions over 90% of the material became acid soluble. Upon subsequent centrifugation, only radioactivity incorporated from [^{14}C]dATP could be found in the expected DNA position

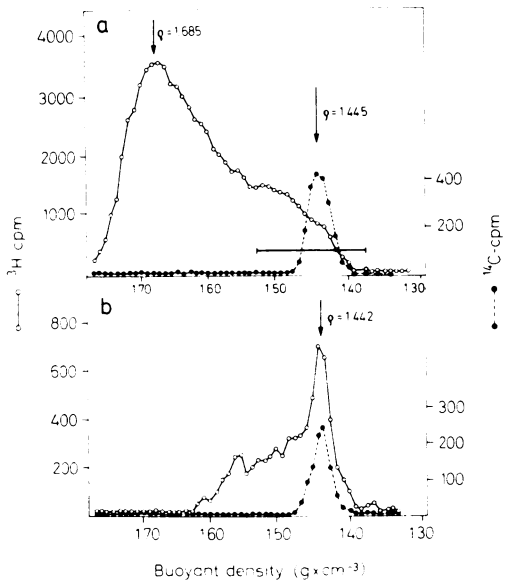

FIG. 9. *Equilibrium centrifugation in neutral cesium sulfate of viral DNA synthesized in isolated nuclei from [³H]UTP, [³H]CTP, and [¹⁴C]dATP. After a 5-min incubation period in the presence of [³H]UTP (60 μM; 52 Ci/mmol), [³H]CTP (60 μM; 22 Ci/mmol), and [¹⁴C]dATP (40 μM; 450 mCi/mmol), nuclei were lysed on top of neutral sucrose gradients containing 0.5% sarcosyl. Viral DNA-containing fractions were pooled, phenol extracted, denatured by heating in 10 mM Tris-hydrochloride and 5 mM EDTA (pH 8.0) at 100 C for 5 min, and subjected to equilibrium centrifugation. (a) Banding pattern of radioactivity incorporated from [³H]UTP, [³H]CTP (○), and [¹⁴C]dATP (●). Arrows indicated peak positions of RNA (ρ = 1.685) and viral DNA (ρ = 1.445). (b) Fractions covered by the black bar in (a) were pooled, heat denatured as described above, and recentrifuged in neutral cesium sulfate.*

whereas radioactivity from [³H]UTP and [³H]CTP had disappeared.

In a separate centrifugation experiment (not shown), ³²P-labeled Ad2 DNA and radioactivity incorporated from [¹⁴C]dATP banded together at a density of 1.442 g/cm^{-3}. This indicates that the amount of RNA in the Ad2 DNA fragments must be comparatively small. If one assumes a linear relationship between RNA content and banding position in the cesium sulfate gradient, the RNA content must have been less than 1% of the total weight of the fragment. At a fragment molecular weight of approximately 500,000, this represents less than 20 bases. A definite proof of the presence and localization of RNA stretches in Ad2 DNA fragments will only be obtained from analysis of the RNA-DNA linkage by ³²P transfers from deoxy- to ribonucleotide monophosphates, as performed by Pigiet et al. (28) in the polyoma system. Present data however already point to a strong association of ribonucleotides to DNA fragments generated during Ad2 DNA synthesis.

DISCUSSION

Cell-free systems consisting of isolated nuclei from mammalian cells have recently been developed as potent tools for the study of eucaryotic DNA replication. Under the appropriate conditions these subcellular systems catalyze the polymerization of dNTP with a fidelity which reflects conditions in the intact cell (13, 19). DNA synthesis in isolated nuclei, however, is not restricted to chromosomal DNA. Thus, it has been possible to demonstrate incorporation of DNA precursors into virus-specific DNA in nuclei, isolated from polyoma- (38, 39) or Ad5-infected (36) cells. In the polyoma system, the observed in vitro DNA synthesis occurs discontinuously (21) and represents an elongation of daughter strands already initiated in vivo (22).

The present study describes characteristics of viral DNA synthesis in isolated nuclei from Ad2-infected HeLa cells. The initial rate of [³H]dTTP incorporation is at least one order of magnitude higher than that observed in nuclei from uninfected, synchronized *S*-phase HeLa cells (13). The reaction, however, proceeds linearly only for 30 min to 1 h at 25 C and approaches a final plateau value after 2 h of incubation at 37 C. This is only partially due to a physical inactivation of the nuclei but reflects mainly, as will be reported in a subsequent publication, a partial failure of the system to initiate new rounds of viral DNA replication.

In alkaline sucrose gradients, significant differences were observed between the sedimentation patterns of viral DNA, synthesized in short and long pulse periods. Although newly synthesized DNA from a 45-min incubation period cosedimented with viral marker DNA, a large proportion of the radioactivity incorporated during a 30-s pulse sedimented with an *S* value of 10*S*. These fragments, which are intermediates in viral DNA synthesis in isolated nuclei, could arise in one of three ways: (i) fragmentation could result from random endonucleolytic degradation of viral DNA during preparation and incubation of isolated nuclei. This explanation is highly unlikely since viral DNA, labeled prior to the isolation of the nuclei, remains intact in the isolated nuclei. (ii) DNA fragments might result from initiation events at the origin(s) of replication only or (iii) from initiation along all or large parts of the Ad2 genome in the

unsynchronized population of replicating molecules. These latter two possibilities could be distinguished by an analysis of the distribution of radioactivity in double-stranded DNA fragments, generated by digestion of labeled DNA from a 30-s pulse with the endonuclease R·RI from *E. coli*. Under these conditions, when approximately 70% of the radioactivity is present in 10*S* fragments, radioactivity was distributed among all the expected digestion products in amounts proportional to their molecular weight. The second possibility is thus excluded. Even though incorporation into the larger Eco RI fragment A is slightly lower than expected, discontinuous DNA synthesis seems to occur along large parts of the Ad2 genome and, as we have documented, from both strands.

Accumulation of short, virus-specific DNA fragments is not restricted to short pulses in isolated nuclei but could also be observed during inhibition of viral DNA synthesis by HU. This drug, a potent inhibitor of polyoma and SV40 DNA synthesis (18, 20, 21), rapidly decreases the rate of Ad2 DNA synthesis to levels of 2 to 10% of normal when added late in infection. The observed residual DNA synthesis, which remains constant for at least 1 h after drug addition, occurs almost exclusively into 10*S* DNA fragments. These fragments do not represent an aberrant form of Ad2 DNA since they can be chased into unit-length viral DNA after withdrawal of the drug.

The question remains as to the origin of these fragments. In polyoma and SV40-infected cells, where similar observations have been made, it has been suggested that HU causes a selective inhibition of a gap-filling step during discontinuous DNA synthesis which was proposed to proceed in at least five reaction steps (18, 20, 28): (i) initiation of DNA synthesis by an RNA primer; (ii) synthesis of the DNA moiety of the fragment; (iii) specific digestion of the RNA moiety of the fragment; (iv) repair DNA synthesis of the resulting gap; and (v) a polynucleotide ligase catalyzed closure of remaining nicks. Since HU drastically reduces the intracellular dATP and dGTP pools in tissue cultured cells (30), accumulation of DNA fragments could be explained if two DNA polymerases with different K_m values for dNTP were involved in steps (ii) and (iv) of the proposed reaction sequence. In the presence of HU, the polymerase responsible for step (ii) with a comparatively low K_m for dNTP would still be able to synthesize the fragments, albeit at lower rate. The other polymerase, normally involved in step (iv), would be unable to perform the assigned gap-filling step. Although it is known (31) that human cells in tissue culture do contain at least two DNA polymerases with the proposed properties, it is not known at present whether they are involved in viral DNA synthesis in Ad2-infected cells.

If the proposed reaction sequence was correct, one could make two predictions: (i) isolated DNA fragments should contain a covalently linked RNA primer. This has, at present, only been documented by cesium sulfate equilibrium centrifugation (Fig. 9), and the exact position of the RNA moiety is not known. (ii) A depletion of dNTP levels during Ad2 DNA synthesis in isolated nuclei should not only result in a reduction of the overall rate of DNA synthesis but also in an accumulation of fragmented DNA. This prediction, as demonstrated in Fig. 2 and 7, has indeed been verified, although the sedimentation pattern of the fragmented DNA in alkaline sucrose is quite heterogenous.

The observed size distribution of viral DNA, synthesized at low dNTP concentrations, can be influenced by addition of rNTP which do not affect the rate of [^{3}H]dTTP incorporation. In the presence of 60 μM rNTP, a much larger proportion of the radioactivity incorporated from [^{3}H]dTTP now sediments more uniformly but with lower sedimentation rates, ranging from 10 to 18*S*. This size variation can be explained if the model of a reaction sequence for the synthesis of Ad2 DNA is extended to include kinetic considerations. It is thus proposed that the size of Okazaki-type fragments is determined by the relative rates of initiation (step i) and chain elongation (steps ii to v). If, according to this assumption, the rate of initiation (step i) was rate-limiting, Okazaki-type fragments would be comparatively larger than in a situation where the rate of propagation was relatively slow as compared to the rate of initiation. Olivera and Bonhoeffer have arrived at similar conclusions based on studies of *E. coli* DNA replication (25).

As these different situations can be simulated in isolated nuclei under suboptimal conditions for DNA synthesis through variation of the rNTP concentration alone, I would like to suggest that the rate of initiation of Okazaki-type fragment synthesis is determined by the intracellular rNTP concentration. The competition between initiation and propagation rate could thus be regulated simply by the relative intracellular dNTP and rNTP concentrations. It is obvious that the balance between the two rates could be different in different virus-cell systems. The observed differences between the size of

DNA intermediates in chromosomal (9, 10), polyoma (21), SV40 (8), and Ad2 DNA replication would then find a reasonable explanation. In addition, the failure to demonstrate an accumulation of a discrete size class of viral DNA fragments after [^{3}H]thymidine pulses of Ad2-infected cells (7, 15) could likewise be explained by a change, in these particular virus-cell systems, in the nature of one or more rate-limiting step in the reaction complex responsible for fragment synthesis. Finally, it should be pointed out that although we have established a role for rNTP and dNTP in the synthesis of Okazaki fragments, the molecular relationships and interactions between the low-molecular-weight precursors, the participating enzymes, and the replicating DNA remain unknown.

ACKNOWLEDGMENTS

This research was supported by the Deutsche Forschungsgemeinschaft (SFB 74).

I am grateful to C. Einhäuser and R. Schilling for their excellent technical assistance. I thank Els Marczinski-Verheugt and Marianne Stupp for the preparation of culture media and H. Zimmermann for the construction of laboratory equipment. Endonuclease R·RI was a gift from B. Weiser-Burger which I gratefully acknowledge. Finally, I am indebted to W. Doerfler for his continuous support and interest.

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