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Intermediates in the Synthesis of Type 2 Adenovirus Deoxyribonucleic Acid

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Intermediates in the synthesis of adenovirus type 2 deoxyribonucleic acid (DNA) were studied in HeLa cells. Pieces of DNA smaller than the viral genome were demonstrated after labeling with ^{3}H-thymidine for 10 to 240 sec. Intermediates as small as the Okazaki fragments (8 to 10*S*) do not predominate at any of the above times. No detectable addition of nucleotides to parental genome could be shown, nor was there any breakdown of recently synthesized viral DNA. The DNA intermediates were of viral origin for they hybridized to viral DNA and were made at a stage of the cell cycle (G_2) when host DNA is not synthesized.

Many models have been proposed for the replication of the deoxyribonucleic acid (DNA) of bacteria and their infecting viruses. Okazaki has described a model in which DNA grows by the polymerization of deoxyribonucleotides into strands approximately 1,000 nucleotides in length. These segments are joined by the enzyme, ligase (19). This model predicts the initial labeling of small DNA pieces and their elongation by segments to make the whole genome. Many of the predictions of this model have been confirmed. Alternate formulations which include the covalent addition of nucleotides to the ends of parental DNA have been proposed (8). Circular intermediates in the replication of *Escherichia coli* (3) and bidirectional growth during the replication of lambda (22) have been shown.

In studies of mammalian cells and viruses similar observations have been made. Small DNA pieces have been shown during vaccinia virus replication (16), but their nature has not been further investigated. Replicating circles have been shown in cells infected with the circular polyoma virus (12) and simian virus 40 (SV40) (15). Bidirectional growth of the Chinese hamster chromosome was demonstrated by autoradiography (14). The present work examines the replication of adenovirus type 2 DNA (Ad2 DNA) in HeLa cells.

Adenovirus DNA is a linear duplex with a molecular weight of 23×10^6 to 24.4×10^6 daltons (11). By demonstrating unique areas rich in adenine plus thymine bases in electron micrographs of partially denatured Ad2 DNA, Doerfler and Kleinschmidt proved that the genome is not circularly permuted (5).

The shutoff of host DNA synthesis is dependent on input virus multiplicity. However, even in infections with 100 plaque-forming units per cell, some host DNA is synthesized hours after the onset of viral DNA replication (9).

The adenoviruses are of particular interest because of their oncogenic potential for newborn hamsters. Three serotypes (12, 18, and 31) are highly oncogenic. Type 2, used in these investigations, is nononcogenic but does cause transformation of rat embryo cells in tissue culture (7).

This report demonstrates adenovirus DNA intermediates smaller than the completed genome. The size of the DNA was determined on alkaline sucrose gradients on which DNA is separated on the basis of its single-stranded size. Ad2 DNA as small as the Okazaki fragments does not predominate after short labeling times. No detectable addition of nucleotides to parental genome could be shown. The intermediates are not breakdown products because prelabeled DNA is stable under identical conditions. Likewise the intermediates have been shown to represent viral rather than host DNA in that (i) they are made during the phase of the cell cycle (G_2) when host DNA synthesis is eliminated; (ii) they elongate to the size of whole viral DNA; and (iii) they are identical to viral DNA in DNA-DNA hybridization reactions.

MATERIALS AND METHODS

Cells and virus. The origins of the stocks of adenovirus type 2, KB, and HeLa S3 cells have been described previously (17). Cells were maintained in suspension culture with Eagle's medium (6) supplemented with 5% fetal calf serum. Virus was purified as described previously (17) with the exception that virions from the first preformed CsCl gradient were

diluted to a density of 1.17 g/ml with 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 8.1) and recentrifuged for 16 hr on an 8-ml preformed CsCl gradient (1.2 to 1.4 g/ml) at 35,000 rev/min in the SW 41 rotor of a Spinco ultracentrifuge. Virions used for subsequent infection of cells were stored in 0.1% bovine serum albumin, 0.85% NaCl and 0.01 M Tris-hydrochloride (*p*H 8.1) at a concentration of 5×10^{11} to 10^{12} particles per ml (17). Virions, which were labeled with ^{14}C-thymidine and used for sedimentation markers in alkaline sucrose gradients, were stored in CsCl (1.34 g/ml) in 0.01 M Tris-hydrochloride buffer, *p*H 8.1.

Infection of cells. After being washed twice with serum-free Eagle's medium, the cells were concentrated to 10^7 ml in Puck's saline (18) and infected by incubation with 1,000 to 5,000 particles per cell for 15 min at room temperature. The infected cells were diluted to a concentration of 1.5×10^5 to 2.0×10^5 ml in media which had been prewarmed to 37 C, and the infection was allowed to continue in suspension culture in the presence of 5% serum.

Band sedimentation of DNA on alkaline sucrose gradients. Gradients were preformed in cellulose nitrate tubes (0.5 by 4 in.) for centrifugation either in the SW 25.3 or SW 27 rotor of a Spinco ultracentrifuge. Over 0.5 ml of CsCl in water ($\rho = 1.8$), a linear 15-ml gradient was formed by mixing 5 and 20% sucrose prepared in 1.0 M NaCl, 0.19 N NaOH, and 0.01 M disodium ethylenediaminetetraacetate (EDTA). A 0.5-ml amount of the NaOH-NaCl-EDTA solution without sucrose but containing 0.5% sodium deoxycholate was placed over the preformed gradient. Cells in 0.5 ml of 0.15 M NaCl were placed intact on the gradients. Purified virions, containing ^{14}C-thymidine-labeled DNA as a sedimentation marker, were mixed with the infected HeLa cells. Centrifugation was performed at 4 C for 6.5 to 16 hr at 18,000 to 24,000 rev/min. Sedimentation was independent of the DNA concentrations used in these studies. Gradients were fractionated by pumping equal portions (approximately 0.6 ml) through a probe placed 1.5 cm above the bottom of the tube. The "pellet" refers to approximately 2 ml of solution remaining at the bottom of the gradient after fractionation. Viral DNA can be separated from intact HeLa cell DNA due to large differences in size (13). With the sedimentation conditions employed in the following experiments, nonreplicating HeLa cell DNA was pelleted into the CsCl cushion and virus DNA appeared in the gradient.

Synchronized cells. HeLa cells were synchronized by arrest with 2 mM thymidine during the S phase of the cell cycle (20). Thymidine was added to logarithmically growing HeLa cells. Twelve hours later, the cells were centrifuged at $200 \times g$ for 5 min and suspended in fresh prewarmed Eagle's spinner medium. Eight hours later, 2 mM thymidine was again added, and after 12 hr, cells were infected in the usual manner. Uninfected controls were similarly treated and mock-infected with the albumin-NaCl-Tris-hydrochloride diluent used to store infectious virus.

DNA-DNA hybridization. DNA was purified by methods previously described (21). HeLa cell DNA was purified from nuclei which were prepared by Nonidet-P 40 (NP-40) lysis of cells (1). Adenovirus DNA was isolated from virions which were purified by two centrifugations in CsCl. The viral DNA was purified without the ribonuclease digestion step. DNA obtained from alkaline sucrose gradients was neutralized by dialysis against 0.01 × SSC (SSC is 0.015 M sodium citrate plus 0.15 M sodium chloride) and also purified without the ribonuclease step. Similar hybridization results were obtained when fractions of alkaline gradients were dialyzed against 0.01 × SSC and used directly.

Denatured DNA was immobilized on nitrocellulose membranes (Millipore HA) as modified from the procedure of Green (10). DNA was denatured by boiling in 0.01 × SSC for 15 min and rapidly cooled to 0 C. DNA was diluted in 4 × SSC to produce, in 10 ml of solution, the designated amount of DNA for each filter. After 4 hr of drying in air at room temperature, the filters were dried in vacuo at 80 C for an additional 4 hr. The DNA to be hybridized was dissolved in 0.01 × SSC and sonicated at 0 C for 10 min in a Branson sonic oscillator (model S75) set at 2. The amount of DNA required for hybridization with each filter was denatured by heating at 100 C for 15 min and diluted in 2× SSC and 0.1% sodium dodecyl sulfate. The hybridization reaction was performed at 60 C for 20 hr. Thereafter, the filters were serially rinsed in two 300-ml beakers of 0.003 M Tris-hydrochloride buffer (*p*H 9.4) and each side was washed 10 times under suction with 10 ml of the same buffer. Either 3.0 μg of viral DNA or 10 μg of HeLa cell DNA was immobilized on each filter.

Radioactive labeling of DNA. DNA was labeled with either ^{14}C- or ^{3}H-thymidine by exposing cells at concentrations of 2×10^5 to 20×10^5 cells/ml to various amounts of radioactivity. Purified virus labeled with ^{14}C-thymidine was prepared by incubating 1 liter of adenovirus-infected KB cells (2×10^5 cells/ml) with 50 μCi of ^{14}C-thymidine from 8 to 36 hr after infection. Virus was purified in the manner described above.

^{3}H-thymidine (10 to 23 Ci/mmole) and ^{14}C-thymidine (57 mCi/mmole) were purchased from Schwarz BioResearch Inc., CsCl (optical grade) from Harshaw Chemical Co., deoxyribonuclease from Worthington, and colchicine from Nutritional Biochemicals Corp. "Spinner salts" refers to a solution of Eagle's "minimum essential medium" (6) lacking amino acids, vitamins, and serum.

Determination of acid-precipitable radioactivity was done by methods previously reported (13) except that scintillation counting was done with a Triton-toluene-2,5-diphenyloxazole mixture with appropriate corrections for channel overflow. Samples containing only ^{14}C radioactivity were counted in a Nuclear Chicago model 8700 gas flow counter.

RESULTS

Rate of synthesis of viral DNA. Under the conditions of infection used in these studies (Materials and Methods), newly synthesized viral DNA detected by DNA-DNA hybridization appeared in HeLa cells at 8 to 9 hr after virus ab-

sorption. The rate of viral DNA synthesis was measured by pulse-labeling infected cells with thymidine and separating viral from host DNA on alkaline sucrose gradients. The rate was maximum at 16 to 18 hr after infection (Fig. 1A, B).

Viral DNA intermediates. To look for intermediates in the synthesis of viral DNA, HeLa cells infected for 17 hr were pulse-labeled for short times with ^{3}H-thymidine. Figure 2 shows that the majority of radioactivity, even after a 6-min pulse, remained in pieces of DNA smaller than the ^{14}C-viral genome added to the gradient.

Stability of viral DNA in the infected cell. In order to eliminate the possibility that small pieces of DNA found after short labeling periods were breakdown products of larger DNA, the stability of viral DNA was measured. At 17 hr after infection, HeLa cells were labeled with ^{3}H-thymidine for 50 min, washed free of radioactivity, and incubated an additional 60 min. ^{14}C-thymidine was added, and the size of newly formed ^{14}C-DNA intermediates was assessed after 5- and 10-min labeling times. Figure 3 shows that the ^{3}H prelabel appearing in viral DNA was not degraded during a period when ^{14}C radioactivity was appearing in small intermediates. The only other ^{3}H acid-precipitable radioactivity was in the pellet and was presumed to be reduced levels of host cell DNA (Table 1).

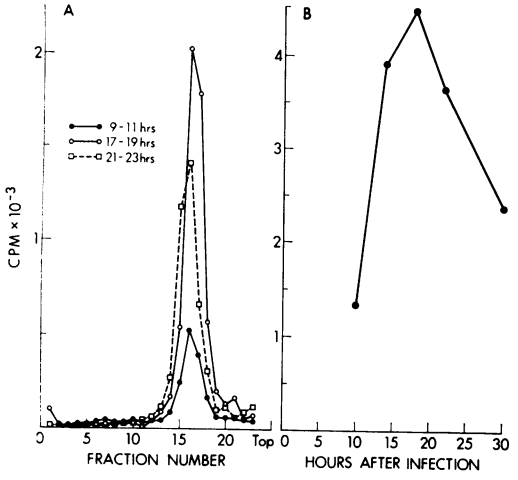

FIG. 1. *Rate of synthesis of adenovirus type 2 DNA in HeLa cells. At several times in the infection cycle, 100-ml amounts of HeLa cells (1.5 × 10⁵ cells/ml) infected with adenovirus type 2 were labeled with 5 μCi of ^{14}C-thymidine for 2 hr. The cells were washed free of radioactivity and suspended in prewarmed medium, and the infection was continued. At 35 hr after infection, cells from each time point were prepared and placed intact onto alkaline sucrose gradients for centrifugation at 24,000 rev/min for 6.25 hr. Viral DNA sediments to fraction 16 and host into the pellet (13). Gradients from some of the pulses are shown in panel A. Counts per minute in pellet: 3,165 (●——●), 1,821 (○——○), 1,684 (□- - -□). The amount of viral DNA was calculated from the radioactivity in the virus region of the gradients which were run in duplicate. Panel B shows the amount of viral DNA at the midpoint of each pulse period.*

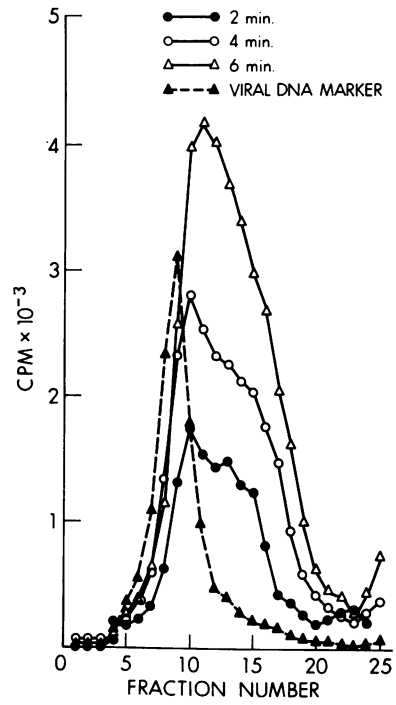

FIG. 2. *Viral DNA intermediates. At 17 hr after adenovirus infection, 60 ml of HeLa cells was resuspended in 6 ml of the same medium at a cell concentration of 2 × 10⁶ cells/ml; 250 μCi of ^{3}H-thymidine was added; and 2-ml samples of cells were removed at 2-min intervals and placed into ice-cold spinner salts to stop radioactive incorporation. After centrifugation, each of the three samples was resuspended in 1.0 ml of NaCl. Purified adenovirus type 2, labeled with ^{14}C-thymidine, was added to half of each sample. All samples were centrifuged on alkaline sucrose gradients for 15 hr at 24,000 rpm. The six gradients were fractionated, and acid-insoluble radioactivity was determined in the 2-, 4-, and 6-min samples containing ^{14}C virus marker. From the other three gradients, fractions 15, 16, and 17 were pooled, dialyzed against 0.01 × SSC, and used in hybridization studies (Table 2). Portions of cells were found to linearly incorporate radioactivity into acid-precipitable counts. Counts per minute in pellet: 2 min, 1,466; 4 min, 1,437; 6 min, 3,115; viral DNA marker, 329.*

HeLa cell DNA intermediates. HeLa cell DNA synthesis was much reduced by 16 hr after infection but not completely shut off even at high input multiplicities of adenovirus. Under the condi-

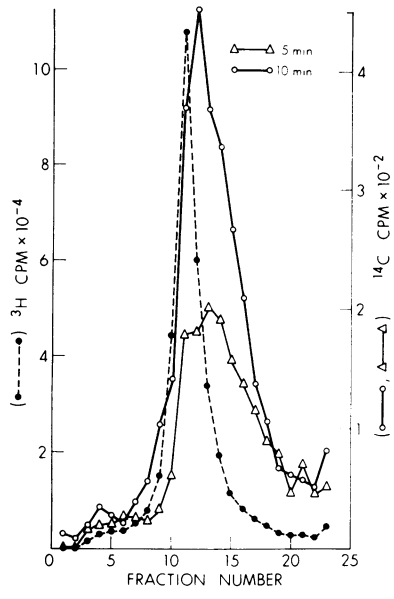

FIG. 3. *Stability of viral DNA in the infected HeLa cell. At 17 hr after infection, HeLa cells were concentrated to 1.5 × 10^6 cells/ml and labeled with 125 μCi of ^{3}H-thymidine for 50 min. The radioactivity was removed by washing the cells with 200 ml of cold medium. After 60 min of incubation in fresh medium, the cells were again washed and pulse-labeled at a density of 4 × 10^6 cells/ml; 8 μCi of ^{14}C-thymidine per ml was added, and samples were removed into ice-cold spinner salts at 5 and 10 min. The cells were placed onto alkaline sucrose gradients and centrifuged at 20,000 rev/min for 18 hr. Counts per minute in pellets: ^{3}H, 72,000; ^{14}C (5 min), 473; ^{14}C (10 min), 2,105.*

tions of infections employed in these experiments, 10 to 15% of the total DNA synthesized was HeLa cell DNA. This explains the radioactivity seen in the pellets of the sucrose gradients (Fig. 1, 2, and 3).

Uninfected HeLa cells were pulse-labeled with ^{3}H-thymidine to examine the intermediates of HeLa DNA appearing after short synthesis times. Figure 4 shows that some of the HeLa cell DNA intermediates labeled for 4 min cosedimented on alkaline sucrose gradient with whole virus DNA; thereafter the modal distribution of HeLa DNA pieces was larger than virus DNA. Another difference between the infected and uninfected cells was the accumulation of most of the radioactivity in the pellets of gradients in which uninfected cells were sedimented.

To eliminate the possibility that the synthesis of host DNA was responsible for the small DNA pieces in the infected cell, several methods were employed to prove the viral origin of the slowly

TABLE 1. *Hybridization of deoxyribonucleic acid (DNA) from pellets of alkaline gradients with adenovirus DNA*[a]

Length of pulse (min)	Counts hybridized to 3 μg of adenovirus DNA[b]	Total acid-precipitable radioactivity applied to filter
2	38	894
4	51	710
6	69	1,732

[a] ^{3}H-thymidine-labeled DNA extracted from the pellets of the alkaline sucrose gradients described in Fig. 2 was hybridized to viral DNA. The hybridization was performed as described in Materials and Methods. Comparative hybridizations using purified ^{14}C-labeled viral or ^{3}H-labeled HeLa cell DNA are shown in Table 2.

[b] Nonspecific counts attached to blank filters were subtracted.

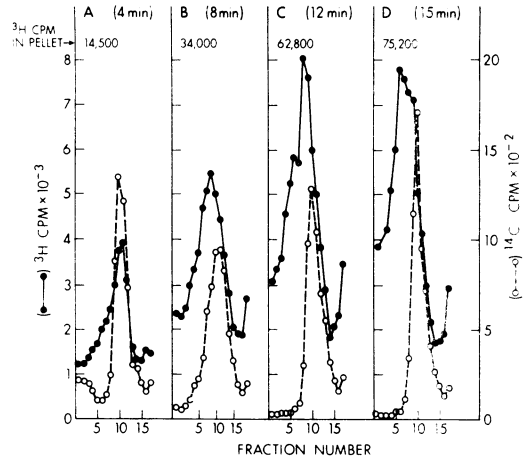

FIG. 4. *HeLa cell DNA intermediates. HeLa cells (4 ml, 2 × 10^6/ml) were exposed to 225 μCi of ^{3}H-thymidine, and 1-ml samples were removed and diluted into ice-cold spinner salts at the indicated times. After resuspension of the cells in 0.5 ml of 0.15 M NaCl and addition of ^{14}C virus marker, the samples were centrifuged for 14.5 hr at 18,000 rev/min. The size of the HeLa cell DNA intermediates (●——●) is compared to the size of intact viral DNA (○- - -○).*

sedimenting DNA: (i) the residual host DNA synthesis was reduced by pulse-labeling viral DNA during the G1 and G2 phase of the cell cycle and (ii) DNA-DNA hybridization was performed on the small intermediates.

Viral DNA synthesis in synchronized HeLa cells. HeLa cells were synchronized by thymidine arrest as described in Materials and Methods. Mock-infected cells served as controls. DNA synthesis was monitored by labeling 2-ml samples with 0.5 μCi of ^{14}C-thymidine for 30 min.

Figure 5 shows the results for both virus-infected and uninfected cells. At 7 hr after infection, 0.1 μg of colchicine per ml was added to a 20-ml portion of each culture to assess the time of mitosis and the degree of synchrony. After the thymidine release, both cultures were actively synthesizing DNA. A marked decrease of DNA synthesis followed as the cells entered the G2 phase of the cell cycle. Seventy-eight per cent of the uninfected cells underwent mitosis by 14 hr and continued to show low levels of DNA synthesis. Mitosis did not occur in the infected cells.

Between 10.5 and 12.5 hr after infection, DNA synthesis began to increase in the infected cells. At 12 hr after infection and release of the thymidine block, infected and uninfected cells were concentrated to 2×10^6/ml and radioactively labeled for 10 to 240 sec. Thus, DNA synthesis in the G1 phase of uninfected cells was compared with the G2 phase of infected cells. HeLa cell DNA synthesis should not occur at either of these times.

Figure 6 demonstrates that DNA smaller than the viral genome still appeared in the infected synchronized HeLa cells supporting the viral origin of the DNA pieces. Even with labeling

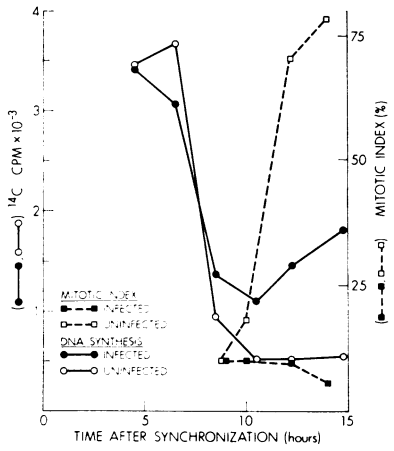

FIG. 5. *DNA synthesis and mitotic index in infected and control cultures. DNA synthesis in HeLa cells was synchronized by thymidine arrest (Materials and Methods), and at the time of release from the second thymidine block the cells were infected with adenovirus type 2. Similarly synchronized and processed uninfected HeLa cells served as controls. To measure DNA synthesis at the indicated times after infection, 2-ml amounts of infected and uninfected cells were labeled with 0.5 μCi of ^{14}C-thymidine for 30 min. At 7 hr, 20-ml samples were removed from each culture, and 0.1 μg of colchicine per ml was added. For the next 7 hr the mitotic index (mitotic cells/total cells × 100) was followed by examining the cells with a phase-contrast microscope.*

times as short as 10 sec, ^{3}H-thymidine appeared in large (fractions 10 to 20) as well as small (fractions 21 to 26) pieces of DNA, suggesting that the addition of new radioactivity was not exclusively via small DNA species. In the uninfected HeLa cells, the earliest acid-precipitable radioactivity was found near the tops of the gradients, suggesting that the polymerization of ^{3}H-thymidine in these cells may only be into small fragments of DNA.

DNA-DNA hybridization of fractions of alkaline sucrose gradients with viral or HeLa DNA. Another method to assess the viral origins of the small DNA pieces is DNA-DNA hybridization with adenovirus DNA immobilized on nitrocellulose filters. Virus-infected cells were pulse-labeled for 2, 4, and 6 min with ^{3}H-thymidine at 16 hr after infection. The results of alkaline sucrose gradient centrifugation and the determination of acid-precipitable radioactivity from a portion of each fraction are shown in Fig. 2. Fractions of small DNA pieces (fractions 15 to 17, approximate molecular weight 2.5×10^6 daltons) were pooled and dialyzed against 0.01 × SSC. The DNA was hybridized against either 3 μg of purified adenovirus DNA, 10 μg of HeLa cell DNA, or blank filters. The results (Table 2) demonstrate that the small pieces of DNA (fractions 15 to 17) hybridized to viral DNA with at least the same efficiency as virus controls. Some host DNA probably contaminated the viral intermediates. Thus, by hybridization, the small DNA pieces were predominantly of viral origin, confirming the results from synchronized cell experiments.

When the pulse-labeling experiments were done 16 to 18 hr into the infection cycle, the pellets contained about 10 to 15% of the radioactivity that would be present in the pellets of uninfected controls. The radioactivity of the pellet was presumed to represent residual host synthesis. An alternate possibility was that this DNA represented a large viral intermediate. The pellets of the alkaline sucrose gradients were examined under conditions identical to those employed for pulse-labeling and hybridization of small viral DNA intermediates (Fig. 2 and Table 2). Table 1 shows that only 4 to 6% of the radioactivity in the pellet did hybridize with purified adenovirus DNA after short pulse-labeling conditions. This amount did not change significantly with longer labeling times. Thus, most of the radioactivity in the pellet was not viral-specific.

Elongation of viral DNA intermediates. If the small DNA pieces in the infected cells are intermediates in the normal pathway of viral DNA synthesis, they should elongate to the size of full viral genomes. This hypothesis was tested in cells which were infected for 19 hr and labeled with

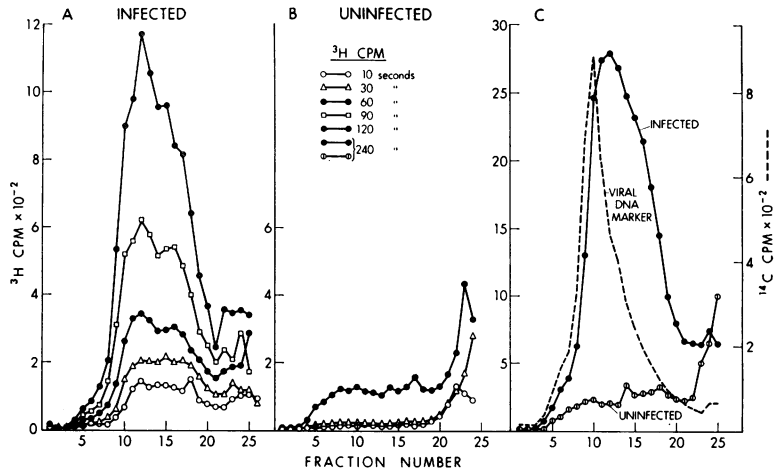

FIG. 6. *DNA intermediates in synchronized uninfected HeLa cells (G_1) vs adenovirus-infected cells (G_2). HeLa cells were synchronized by thymidine arrest and infected as described in Materials and Methods. Similarly synchronized but uninfected HeLa cells served as controls. At 12 hr after infection, 6-ml amounts of each culture at a concentration of 2 × 10^6 cells/ml were labeled with 40 μCi of ^{3}H-thymidine per ml for 10 to 240 sec. All pulses were terminated by dilution in spinner salts at 0 C. The cells were centrifuged at 200 × g for 5 min and resuspended in 0.5 ml of NaCl. Virus marker containing ^{14}C-labeled DNA was added to the cells, and separation of DNA was achieved on alkaline sucrose gradients as described in Materials and Methods. The cells were centrifuged in an SW 27 rotor at 24,000 rev/min for 15.5 hr at 4 C. Gradients were fractionated and analyzed for acid-insoluble radioactivity as described in Materials and Methods. Panel A shows the profile for infected cells between 10 and 120 sec. Panel B describes similar results for the uninfected cell. Panel C compares the 240-sec pulse of infected, uninfected, and marker DNA from purified whole virus. Pellets of gradients of infected cells contained from the 10- to 240-sec pulse 195, 307, 430, 772, 1,233, and 3,753 counts per min, respectively. For the uninfected cells these values were 60, 66, 287, and 760, respectively.*

^{3}H-thymidine for 4 min. The radioactivity was removed by washing the cells in medium containing excess unlabeled thymidine in an attempt to reduce the incorporation of radioactivity. The results are shown in Fig. 7, which compares alkaline sucrose gradient profiles of cells labeled for 4 min with ^{3}H-thymidine and those of cells "chased" for an additional 4 or 12 min. The shift of the distribution of radioactivity to sedimentation values consistent with whole viral DNA is apparent. However, there was little absolute decrease of radioactivity in small DNA pieces to account for the increase of counts in the whole genome. Since the washout of ^{3}H-thymidine did not completely stop the continued incorporation of new radioactivity, the counts in small DNA pieces probably represent new synthesis.

Other pulse-chase experiments were undertaken in an attempt to show the disappearance of small viral DNA pieces. The effective elongation of small DNA could be shown in the presence of very high thymidine concentrations which effectively inhibited new radioactively labeled DNA synthesis. Concentrations of thymidine 10^5 times the amount of ^{3}H-thymidine used during the pulse-labeling were employed. The high concentrations of thymidine slowed but did not prevent elongation of DNA to full genome size. The disappearance of small DNA pieces, which are mostly elongated by 22 min, can be followed. Even with such large excesses of thymidine the incorporation of radioactive label is not immediately shut off but continues for approximately 8 min (Fig. 8).

A similar pulse-chase experiment was done on the uninfected cells (Fig. 9). The host intermediate is much smaller than intact HeLa cell DNA. The host intermediate clearly disappears and elongates to a size that places it into the pellet of the gradient.

DISCUSSION

After brief periods of labeling with ^{3}H-thymidine, the predominant species of DNA synthesized in the infected cell sediments slower than mature viral DNA. There were no DNA intermediates sedimenting slightly faster than viral DNA even though this was a region rich in host cell DNA intermediates in the uninfected cell. According to the formula of Studier for determination of molecular weights (23), the pellets of the gradients contain single-stranded DNA of a

TABLE 2. *Hybridization of small deoxyribonucleic acid (DNA) intermediates to viral or HeLa DNA*

Source of DNA	CPM hybridized to 3 μg of adenovirus DNA[b]	CPM hybridized to 10 μg of HeLa DNA[b]	Total acid-precipitable radioactivity applied to filter
Fractions 15–17[a]			
2-min pulse	314	0	482
4-min pulse	667	49	1,102
6-min pulse	1,098	124	1,587
Adenovirus type 2	2,982	38	5,520
HeLa	42	1,493	13,066

[a] ^{3}H-thymidine-labeled DNA extracted from alkaline sucrose gradients (fractions 15–17) was hybridized to viral or HeLa cell DNA. The DNA from gradients was obtained as described in Fig. 2. For comparison, either purified ^{14}C-labeled adenovirus or ^{3}H-labeled HeLa cell DNA was used. Hybridization was performed as described in Materials and Methods.

[b] Nonspecific counts attached to blank filters were subtracted. CPM, counts per minute.

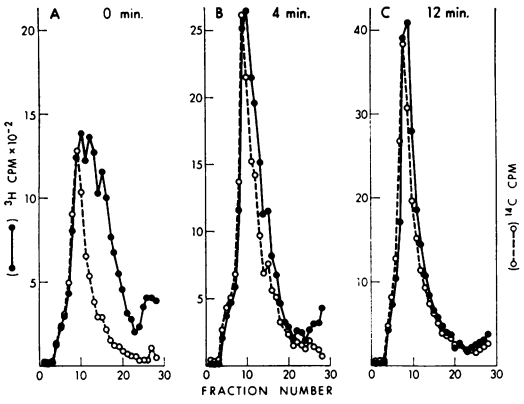

FIG. 7. *Elongation of viral DNA intermediates. Adenovirus-infected HeLa cells were suspended at 1.5 × 10^6 cells/ml at 19 hr after infection and pulse-labeled with ^{3}H-thymidine at a concentration of 40 μCi/ml. After 4 min the cells were chilled in 5 volumes of cold medium containing 2.5 × 10^{-4} M unlabeled thymidine. One sample (A) was prepared for centrifugation on alkaline sucrose gradients, and the remainder was washed free of thymidine and suspended in regular Eagle's medium. Additional samples were taken 4 (B) and 12 (C) min later and prepared for alkaline sucrose gradient centrifugation. Marker DNA was added to each gradient. Centrifugation was at 22,000 rev/min for 19 hr. The gradients were fractionated, and acid-precipitable radioactivity was determined. ^{3}H counts per minute in pellets: A, 3,817; B, 5,998; C, 8,577.*

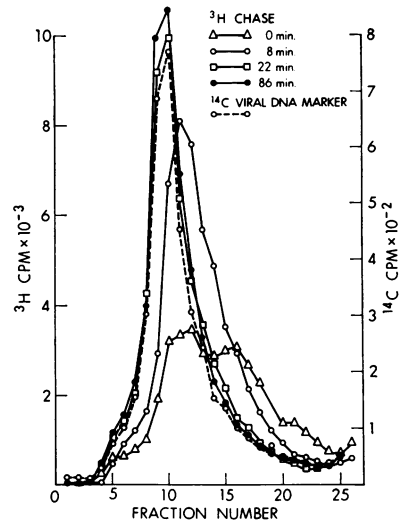

FIG. 8. *Elongation of DNA intermediates in the presence of a high concentration of thymidine. At 17 hr after infection, HeLa cells were concentrated to 2 × 10^6 cells/ml and pulse labeled with ^{3}H-thymidine at a concentration of 30 μCi/ml. After 4 min of pulse-labeling, the cells were diluted into medium containing 2 × 10^{-3} M unlabeled thymidine, a 10^5-fold excess over the amount of radioactive thymidine present. These concentrations of thymidine inhibit DNA synthesis by 76% as measured by the incorporation of deoxyadenosine into DNA (alkali-resistant, trichloroacetic acid-precipitable radioactivity). Samples were taken at the times designated, chilled in ice-cold medium, and prepared for alkaline sucrose gradients to separate the DNA fragments. There was some incorporation of new radioactivity during the chase period, but this had terminated by 8 min after the chase was begun. The size distribution of the precursors is shown at various times during the chase together with ^{14}C intact virus DNA. ^{3}H counts per minute in pellet at chase time: 0 min, 4,164; 8 min, 7,427; 22 min, 5,292; 86 min, 7,390. ^{14}C counts per minute, 125.*

molecular weight larger than 35 × 10^6 daltons. Pellet DNA is predominantly host DNA; however, a few per cent of its radioactivity was hybridizable to viral DNA. Because a small amount of DNA from purified virions also sediments in the pellet (Fig. 2), it is hard to distinguish between a minor but authentic large molecule containing viral DNA and some nonspecific aggregation of a few per cent of viral DNA. The percentage of viral DNA in the "pellet" does not change after pulse or pulse-chase experiments, indicating that it is not a dynamic intermediate in the viral DNA cycle. The viral specific radioactivity in the pellet may represent the integration of a small amount of viral DNA into the host genome as shown for Ad 12 in nonlytic infections of baby hamster kidney cell cultures (4).

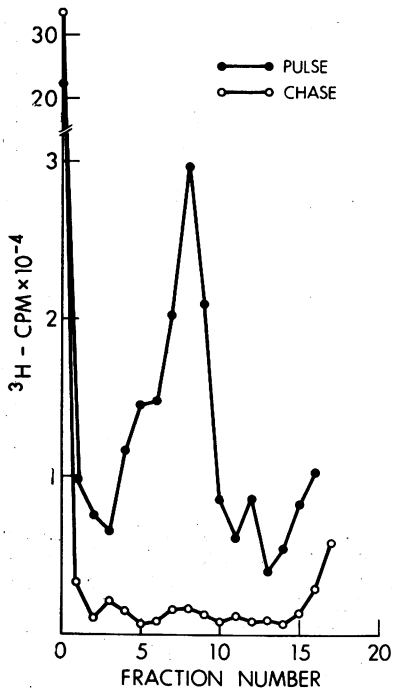

FIG. 9. *Elongation of HeLa cell DNA intermediates. HeLa cells at a concentration of 2 × 10⁶/ml were labeled for 15 min with 25 μCi of ³H-thymidine per ml. The cells were chilled and divided. One half was placed onto alkaline sucrose gradients; the other was washed free of radioactivity, incubated for an additional 60 min, and placed on an alkaline sucrose gradient. The cells were centrifuged 15.5 hr at 17,000 rev/min. Radioactivity was determined after fractionation of the gradients. (Note that the numbers in fraction "0," representing the pellets of the gradients, are expressed on a different scale.)*

There are numerous models of DNA replication to explain the observations of DNA intermediates both larger and smaller than the parent genome. Since there are no intermediates just larger than genome size, the models of nucleotide addition to parental molecules do not apply to adenovirus type 2 replication. The adenovirus data may be explained by the model of Okazaki (19) in which both parental strands are replicated discontinuously and exclusively from small pieces with subsequent joining of the pieces by the enzyme, ligase. However, in the adenovirus-infected HeLa cell after short pulse times (10 to 30 sec) 77% of the radioactive thymidine appeared in pieces of DNA larger than 10*S*. This suggests that the addition of nucleotides does not occur exclusively via small pieces but by the sequential addition of single nucleotides to the growing DNA.

The small adenovirus DNA intermediates are clearly not produced by mechanical shearing during manipulation, since intact cells were placed onto alkaline sucrose gradients and the lysis was achieved by contact with the alkaline buffers. Similarly the pieces do not appear to be the product of a nuclease breakdown of DNA, although recently endonucleolytic properties of the penton, a structural protein of the virion, have been demonstrated (B. T. Burlingham et al., Bacteriol. Proc., p. 172, 1970).

The data in the present report are at variance with the interpretation of other workers (2) who have shown that small pieces of adenovirus type 2 DNA in KB cells arise from nuclease activity. In our experiments, progeny DNA which has appeared as genome-size is not subsequently broken down either in 2 hr (Fig. 3) or when followed until 35 hr after injection (Fig. 1). Possible explanations for the discrepancy may be the lower viral doses (3 to 5 plaque-forming units per cell) or the minimum processing of the DNA before centrifugation employed in our experiments.

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