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Adenovirus Type 2 DNA Replication

II. Termini of DNA Replication

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Complete, mature adenovirus type 2 DNA molecules were isolated from virus-infected HeLa cells, pulse-labeled at 20 h postinfection in [^{3}H]thymidine pulses shorter than the time necessary for one round of viral DNA replication. After digestion with the restriction endonucleases Eco RI, Hpa I, and Hind III, a temporal order of synthesis of different regions of the viral genome was established from the relative specific radioactivities in the restriction enzyme fragments. A comparison with the physical order of these fragments revealed the existence of two termini of DNA replication towards both the molecular right and left ends, respectively, of the viral chromosome.

Double-stranded, mature adenovirus type 2 (Ad2) DNA can be isolated from intact virions in a linear (5) or circular form (11) of molecular weight 23 × 10^6 (5). Its replication in nuclei from infected human cells in tissue culture proceeds discontinuously (18) through replicative intermediates which are characterized by their increased buoyant density (13, 16, 18) and their sensitivity to single-stranded DNA-specific nucleases as compared to mature DNA molecules (9). Electron microscopy analysis (3) of newly synthesized viral DNA revealed the presence of various types of branched and unbranched linear intermediates containing extended regions of single-stranded DNA. On the basis of these and other observations, a model was proposed (3, 14) for the replication of Ad2 DNA according to which DNA synthesis starts at one end of the linear duplex DNA by displacement of the complementary strand, whereas synthesis on this complementary strand is initiated only with a considerable delay.

Additional studies, which included synchronization of viral DNA synthesis after reversal of a hydroxyurea block and an analysis of partially denatured replicating molecules (3), suggested that replication starts from the molecular right end of Ad5 DNA through displacement of the viral H-strand.

We have used a different approach to determine the location of termini as well as of origins of Ad2 DNA replication. In our experiments the temporal order of synthesis of different regions of the viral chromosome was studied by an analysis of the distribution of radioactivity in restriction enzyme fragments from mature viral DNA, synthesized during [^{3}H]thymidine pulses of various length in infected cells. This method, which was applied earlier in a study of simian virus 40 DNA replication (2), revealed two termini of DNA replication at both ends of the viral chromosome. The results agree in part with those reported earlier (17) from a similar analysis of pulse-labeled Ad2 DNA synthesized in isolated nuclei.

MATERIALS AND METHODS

Chemicals and buffers. [^{3}H]thymidine (27 Ci/mmol) and [2-^{14}C]thymidine were obtained from Amersham Buchler GmbH, Braunschweig; proteinase K (18) was kindly provided by E. Lang, Merck, Darmstadt; agarose was obtained from L'Industrie biol., Française S.A.; and acrylamide, *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetramethylenediamide were from Serva, Heidelberg. The compositions of PBS-d and Tris-saline have been described (18).

Virus and cells. Methods for the propagation of HeLa cells in monolayer or suspension cultures as well as the preparation and titration of Ad2 virus stocks have been described (18). ^{14}C-labeled marker DNA was prepared as described previously (18).

Monolayers of HeLa cells on 60-mm (1.5 × 10^6 cells) petri dishes were infected with Ad2 from crude cell extracts in appropriate dilutions in Tris-saline at a multiplicity of 50 to 100 PFU/cell and kept at 37 C in Dulbecco medium containing 5% calf serum. Two methods for pulse labeling of viral DNA were employed. (Method i) At 18 to 20 h postinfection (p.i.), medium was removed from 60-mm petri dishes until 2 ml was left, and [^{3}H]thymidine was subsequently added at a concentration of 100 μCi/ml. After incubation at 37 C for 2 to 80 min, the pulses

were terminated by three consecutive washes each with ice-cold Tris-saline and PBS-d. (Method ii) At 18 to 20 h p.i., medium was removed from petri dishes by suction and, after one wash with ice-cold Tris-saline, replaced by 0.6 ml of a 1:1 mixture of Dulbecco medium and Tris-saline (Tris-Dulbecco) containing 150 μCi of [^{3}H]thymidine per ml. After 20 min at 4 C, 12 ml of Tris-Dulbecco preheated to 39 C was added to the monolayers, which subsequently were incubated for 2 to 80 min while floating on a 37 C waterbath. Under these conditions, the temperatures of the monolayers reached 36 to 37 C within 30 s to 1 min. The presence of 1:1 mixtures of Dulbecco medium and Tris-saline ensured a constant pH on the monolayers during the incubation periods at both 4 and 37 C. Pulses were stopped as described above. In some experiments, viral DNA was prelabeled from 12 to 20 h p.i. with [^{14}C]thymidine (1 to 2 μCi/ml) prior to the pulse-labeling period.

Isolation of viral DNA. Monolayers from infected, pulse-labeled cells were lysed by addition of 0.6 ml of 0.6% sodium dodecyl sulfate in 0.02 M Tris-hydrochloride and 0.005 M EDTA, pH 8.0, and by incubation at 37 C for 60 min in the presence of 100 μg of proteinase K per ml. After addition of 5 M NaCl to a final concentration of 1 M NaCl, the lysates were kept at 4 C for 4 h and centrifuged at 4 C in a Beckman SW56 rotor for 30 min at 35,000 rounds/min.

Supernatants were diluted with 2 volumes of 0.01 M Tris-hydrochloride and 0.005 M EDTA, pH 7.4, and were extracted once with 1 volume of phenol, equilibrated with 0.05 M sodium borate, pH 9.5 (19). After low-speed centrifugation at 5,000 rounds/min, the aqueous phase was dialyzed against two changes of 200 volumes of 0.25 M NaCl, 0.01 M Tris-hydrochloride, 0.005 M EDTA, pH 8.0, and subjected to chromatography on benzoylated-naphthoylated DEAE-cellulose (BND-cellulose). In an alternative method, crude lysates from incubation with 0.6% sodium dodecyl sulfate and proteinase K were diluted with 2 volumes of 0.010 M Tris-hydrochloride and 0.005 M EDTA, pH 8.0, and extracted with phenol as described above. The aqueous phases were subsequently subjected to equilibrium centrifugation in neutral cesium chloride under conditions described previously (18). Aliquots of gradient fractions were analyzed for radioactivity and the fractions containing mature viral DNA were pooled and dialyzed against appropriate buffers for BND-cellulose chromatography or restriction endonuclease digestion.

Yields of mature viral DNA were comparable in both methods.

Restriction endonuclease digestion of viral DNA. The restriction endonucleases Eco RI and Hpa I were obtained from R. Greenberg, Cologne, and L. Crawford, London, as mentioned previously (1, 17, 18). The enzyme endo Hind III was prepared as described (12). Digests of viral DNA with all three restriction endonucleases were performed in 0.02 M Tris-hydrochloride, 0.01 M $MgCl_2$, 0.1 M NaCl, pH 7.4, for 60 min at 37 C. Incubation mixtures of 200 μl contained up to 5 μg of viral DNA and 5 to 20 μl of the appropriate enzyme solutions.

Reactions were stopped by addition of 1 volume of chloroform/isoamyl alcohol (24:1). After low-speed centrifugation, the aqueous phase was dialyzed against 1/10 concentrated electrophoresis buffer (18).

Gel electrophoresis. Gel electrophoresis was performed on composite polyacrylamide-agarose gels, containing 1.5% polyacrylamide-0.7% agarose for separation of Eco RI or Hpa I fragments or 2.2% polyacrylamide-0.8% agarose in the case of Hind III fragments. Buffer compositions have been described previously (18). Cylindrical gels (5-mm diameter, 15 cm long) were run at room temperature for 10 to 12 or 22 h (2.2% polyacrylamide-0.8% agarose) at 2 to 3 V/cm. Up to 300 μl of sample, in 10% sucrose containing 0.001% bromophenol blue, was applied and prerun for 30 min at 1 V/cm. Gels were cut into 0.8-mm slices and incubated in scintillation vials for 18 h at 37 C in 0.3 ml of 0.1% sodium dodecyl sulfate (18). After addition of Whatman GF/C filters, vials were dried at 80 C for 6 h prior to counting in a toluene based scintillator (18).

DNA fragments were eluted from gel slices in preparation of additional digests by incubation of gel slices in 0.3 ml of Nonidet P-40, 0.01 M Tris-hydrochloride, 0.1 M NaCl, pH 8.0, for 18 h at 37 C. Solid cesium chloride was added to pooled eluates to a final density of 1.710 g/cm^3, and the solutions were centrifuged to equilibrium at 15 C in a Beckman SW56 rotor for 48 h at 30,000 rounds/min. Aliquots of gradient fractions were analyzed for radioactivity on Whatman GF/C filters, and the appropriate gradient fractions were pooled and dialyzed against 0.02 M Tris-hydrochloride, 0.1 M NaCl, pH 7.4. Endonuclease digests were performed after addition of $MgCl_2$ from a 0.2 M stock solution and the appropriate enzyme under conditions described above. The equilibrium centrifugation served both as a concentration and a purification step since it was found difficult, if not impossible, to obtain complete digest of DNA in gel eluates with the Hind III restriction endonuclease.

Chromatography on BND-cellulose. BND-cellulose was prepared as described previously (19). Viral DNA in 0.25 M NaCl, 0.01 M Tris-hydrochloride, 0.005 M EDTA, pH 7.8, was loaded at room temperature onto columns (5 by 10 mm). Elution was carried out with five 1-ml portions of 1 M NaCl, 0.01 M Tris-hydrochloride, 0.005 M EDTA, pH 7.8, for double-stranded DNA and with five 1-ml portions of 2% caffeine in 1 M NaCl, 0.01 M Tris-hydrochloride, 0.005 M EDTA, pH 7.8, for single-stranded DNA. Analysis of viral DNA in various BND-eluates by velocity centrifugation in neutral and alkaline sucrose gradients and by equilibrium centrifugation in neutral cesium chloride was performed under conditions described previously (18).

RESULTS

A gradient of radioactivity is generated in viral DNA molecules, synthesized, and completed from radioactive DNA precursors in pulse periods, shorter than the time necessary for completion of one round of viral DNA replication (1, 2). Analysis of this gradient by

the use of appropriate restriction enzymes permits identification of loci with highest or lowest specific activities as termini and origins, respectively, of viral DNA replication. Two prerequisites had to be fulfilled to apply this approach, which has been used successfully in studies of simian virus 40 (2) and polyoma (1) DNA replication to the replication of Ad2 DNA: (i) sufficient radioactivity had to be incorporated from [^{3}H]thymidine into mature viral DNA during pulse periods shorter than 20 min, the estimated replication time for Ad2 DNA (16); (ii) methods had to be employed which ensured a clean separation of newly synthesized pulse-labeled viral DNA, extracted from infected cells, into mature and replicating forms. Experimental results, relating to both of these problems, will be discussed subsequently prior to the results on the identification of termini of viral DNA replication.

(i) Preparation of pulse-labeled viral DNA. The kinetics of [^{3}H]thymidine incorporation into total nuclear DNA was studied 20 h p.i. in HeLa cells, infected with a multiplicity of 50 PFU/cell. Under these conditions, i.e., late in the infection cycle when viral DNA synthesis occurs at optimal rate (18), acid-insoluble radioactivity in total nuclear DNA is a measure for viral DNA synthesis since cellular DNA synthesis is turned off and contributes only 5 to 12% to the total incorporation (18). Figure 1 summarizes the results on [^{3}H]thymidine pulses ranging from 2 to 60 min under conditions described in the method section. According to method i, the observed increase in acid-insoluble radioactivity is nonlinear for the first 10 to 15 min and reaches a linear time course only after pulses longer than the estimated replication time. In addition, the amount of radioactivity in newly synthesized viral DNA after short pulse periods is relatively modest. The labeling procedure was then modified to include a preincubation of the infected cells for 20 min at 4 C in the presence of [^{3}H]thymidine prior to the pulse experiments at 37 C (method ii). Under these conditions (Fig. 1) the kinetics of [^{3}H]thymidine incorporation are linear even at the shortest pulse periods tested. Moreover, specific incorporation of radioactivity increases by at least one order of magnitude and thus facilitates considerably the experiments to be described subsequently.

In control experiments it was found that infected cells produce normal amounts of infective virus particles at 48 h p.i. after being exposed at 20 h p.i. for a 20-min incubation period at 4 C. Moreover, as shown subsequently (see Fig. 3), the properties of viral DNA synthesized under this modified schedule are indistinguishable from controls upon analysis by sedimentation in neutral and alkaline sucrose gradients.

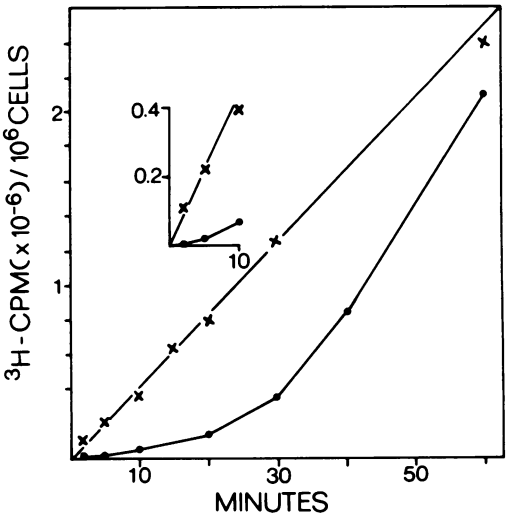

FIG. 1. *Incorporation of [^{3}H]thymidine into viral DNA during pulses of virus-infected HeLa cells ranging from 2 to 60 min. Pulses were performed under different conditions as described. Symbols: ●, pulses performed according to method i by addition of [^{3}H]thymidine to infected monolayers at 37 C; ×, pulses performed according to method ii, which involves a preincubation of infected monolayers in the presence of [^{3}H]thymidine at 4 C for 20 min prior to the pulse experiments at 37 C. The insert shows the extent of incorporation during short pulse periods at an enlarged scale.*

(ii) Purification of mature viral DNA. Pulse-labeled viral DNA consists of a mixture of mature, completed DNA molecules and a spectrum of replicating DNA molecules (9, 18). These have previously been shown to contain extensive single-stranded regions (9, 13, 16). Equilibrium centrifugation in neutral cesium chloride of viral DNA, synthesized in 5-, 10-, and 30-min pulses (Fig. 2), confirms this conclusion and can be used as a method for the separation of mature viral DNA (peak II) from replicating forms (peak III) as well as from cellular DNA (peak I; 9, 18). Since, however, this method is not expected to separate mature viral DNA from replicating molecules whose replication has progressed only moderately, we have used chromatography on BND-cellulose (9, 16) and gel electrophoresis on composite polyacrylamide-agarose gels to isolate completed DNA molecules.

Table 1 summarizes the proportions of radioactivity in the 1 M sodium chloride eluates, representing mature viral DNA, and the 1 M sodium chloride-2% caffeine eluates, containing

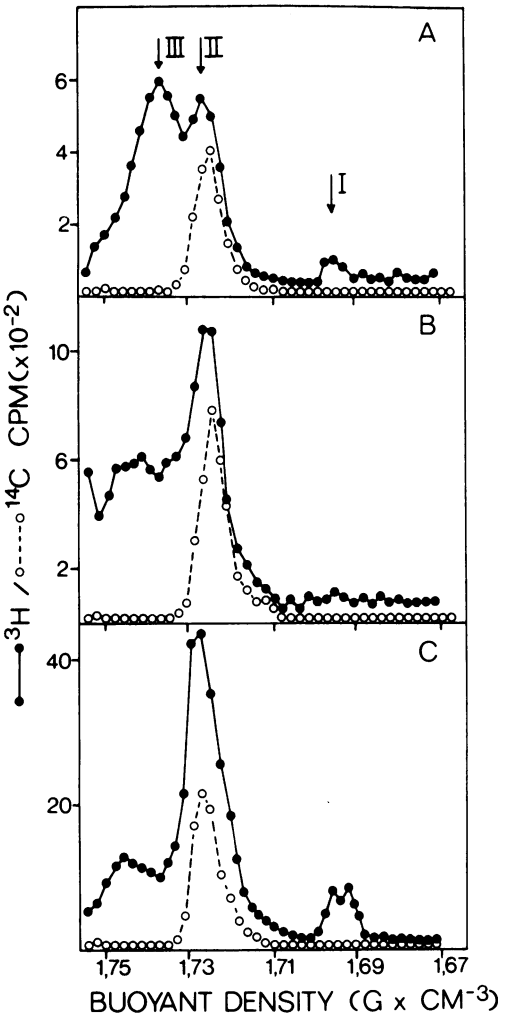

FIG. 2. *Equilibrium centrifugation in neutral cesium chloride of intracellular DNA pulse labeled with [³H]thymidine (150 μCi/ml) for 5, 10, and 30 min at 20 h p.i. Total DNA was extracted after completion of the pulses and centrifuged as described. (A, B, C) Banding pattern of newly synthesized DNA from 5-, 10-, and 30-min pulses, respectively. Arrows with roman numbers indicate the banding positions of cellular DNA (I), mature viral DNA (II), and replicative form DNA (III). Symbols: ●, ³H pulse-labeled DNA; ○, ¹⁴C-labeled viral marker DNA.*

replicative forms, from various pulse-labeling periods. As expected, the proportion of radioactivity in mature DNA increases with increasing pulse length.

The sedimentation behavior of viral DNA from a 60-min pulse in both eluates was analyzed in neutral and alkaline sucrose gradients (Fig. 3). At neutral conditions, DNA from the 1 M sodium chloride extracts (Fig. 3, panel A) co-sediments with marker DNA, isolated from intact virions (18), whereas the radioactivity eluted in the presence of caffeine sediments nonhomogeneously (Fig. 3, panel C) with *S* values ranging from 34S, the marker position, up to 50 to 60*S* (13, 16). In alkaline gradients, the radioactivity from the 1 M sodium chloride eluate (Fig. 3, panel B) again co-sediments with marker DNA, indicating that only very few if any breaks occur in completed viral DNA molecules obtained under the modified labeling schedule. In contrast, the sedimentation behavior of acid-insoluble radioactivity in the caffeine eluate (Fig. 3, panel D) is quite heterogeneous, indicating the absence of fast-sedimenting forms and the presence of a whole spectrum of single-stranded DNA molecules of variable length. The method thus provides a convenient and clean separation of mature viral DNA from replicating forms.

TABLE 1. *BND-cellulose chromatography of pulse-labeled viral DNA*[a]

Solution	Pulse time (min)				
	2	5	10	20	60
1 M NaCl	26.5	35.8	50.2	61.3	81.5
1 M NaCl plus caffeine	73.5	64.2	49.8	38.7	18.5

[a] Numbers indicate proportions of acid-insoluble radioactivity in 1 M NaCl and 2% caffeine-1 M NaCl eluates of BND-cellulose chromatograms of newly synthesized DNA.

Additional support for this conclusion was obtained from gel electrophoresis experiments. Unfractionated viral DNA, obtained in different pulse experiments, was subjected to gel electrophoresis on composite polyacrylamide (1.5%)-agarose (0.7%) gels. It was observed that a certain portion of the applied radioactivity remained on top of the gels whereas the other portion entered the gels, moving together with marker ¹⁴C-labeled Ad2 DNA. Moreover, the proportions of radioactivity retained on top of the gels as compared to the amounts entering the gels were shown to be identical with the relative amounts of radioactivity found after BND-cellulose chromatography of unfractionated viral DNA from different pulse-labeling experiments in the 1 M NaCl-2% caffeine and the 1 M NaCl eluates (Table 1). Our conclusion, that replicative forms of Ad2 DNA are completely retained upon gel electrophoresis on composite gels, was confirmed when mature and replicative form DNA in different BND-cel-

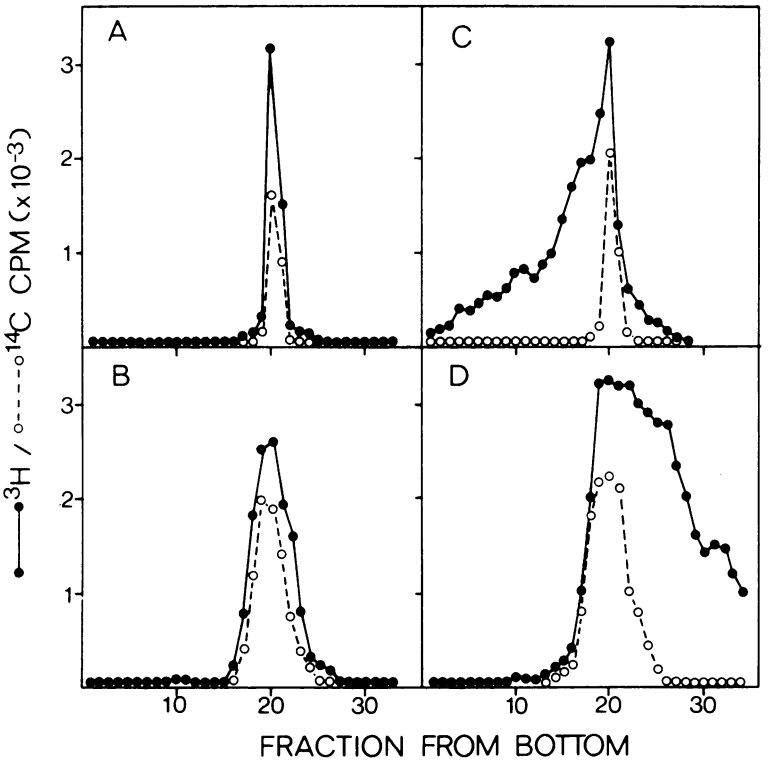

FIG. 3. *Analysis of pulse-labeled viral DNA on neutral and alkaline sucrose gradients. Infected HeLa cells were pulse labeled at 20 h p.i. for 60 min according to method ii. Viral DNA was extracted and subjected to BND-cellulose chromatography (Table 1). (A and B) Neutral and alkaline sucrose gradients of viral DNA eluted with 1 M sodium chloride; (C and D) neutral and alkaline sucrose gradients of viral DNA eluted with 1 M sodium chloride-2% caffeine. Symbols: ●, ^3H pulse-labeled viral DNA; ○, ^{14}C-labeled marker DNA.*

lulose eluates were analyzed separately by gel electrophoresis. More than 99% of the radioactivity from 1 M NaCl eluates, expected from sedimentation analysis (Fig. 3) to represent mature viral DNA, consistently entered the gels and co-migrated with ^{14}C-labeled marker DNA whereas none of the radioactivity from caffeine eluates ever entered the gels. BND-cellulose chromatography thus appears to provide a satisfactory purification of completed, mature Ad2 DNA molecules from its replicative forms.

(iii) Termini of Ad2 DNA replication. In Ad2 DNA molecules, completed during pulses shorter than the time necessary for one round of viral DNA replication, regions of the DNA near the terminus of DNA replication are expected to contain more radioactivity than regions near the origin of replication (1, 2). This unequal distribution of newly incorporated radioactivity along the viral genome was analyzed in the present paper by determination of relative specific radioactivities in different sets of restriction enzyme fragments. Newly completed Ad2 DNA molecules were isolated from Ad2-infected HeLa cells, pulse-labeled with [^3H]thymidine for 5, 10, 20, and 40 min. The viral DNA had been prelabeled with [^{14}C]thymidine from 12 to 20 h p.i. prior to the [^3H]thymidine pulses. After digestion of these Ad2 DNA molecules, containing a ^3H pulse label and a uniform ^{14}C label (both in thymidine), with the restriction endonucleases Eco RI and Hpa I and separation of the resulting fragments by gel electrophoresis, the $^3H/^{14}C$ ratios were determined for each of the fragments. These ratios were compared in Fig. 4, 5, and 6 with the physical order of the various fragments on the viral genome.

From the plot of the relative specific activities and genome locations of the Eco RI fragments (Fig. 4), a gradient of radioactivity becomes apparent which increases from fragment B towards fragment C, i.e., towards the molecular right end of the genome. This gradient is most pronounced in the set of fragments corresponding to the DNA from the 5-min pulse and decreases with increasing pulse length. It is thus concluded that fragment C, since it consistently

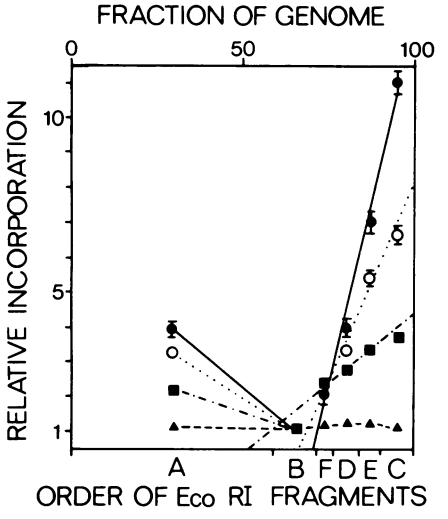

FIG. 4. *Relative specific activities ($^3H/^{14}C$ ratios) of DNA fragments obtained after digestion of completed viral DNA molecules with the restriction endonuclease Eco RI from Escherichia coli. Infected cells were prelabeled with [^{14}C]thymidine (2 μCi/ml) from 12 to 20 h p.i. prior to [^3H]-thymidine (150 μCi/ml) pulses for 5, 10, 20 and 40 min. Mature viral DNA was extracted and the amounts of ^3H and ^{14}C label were determined in each of the Eco RI DNA fragments. The location (8) of the fragments A to F is given on the abscissa. No corrections for thymidine content have been made since both newly synthesized and marker DNA are labeled in thymidine only. All ratios are normalized for fragment B. Symbols: ●, 5-min pulse; ○, 10-min pulse; ■, 20-min pulse; ▲, 40-min pulse. Bars indicate deviations observed in different electrophoresis experiments. Data of the 5-min pulse were obtained from a digest of 22,000 ^3H counts/min and 29,000 ^{14}C counts/min.*

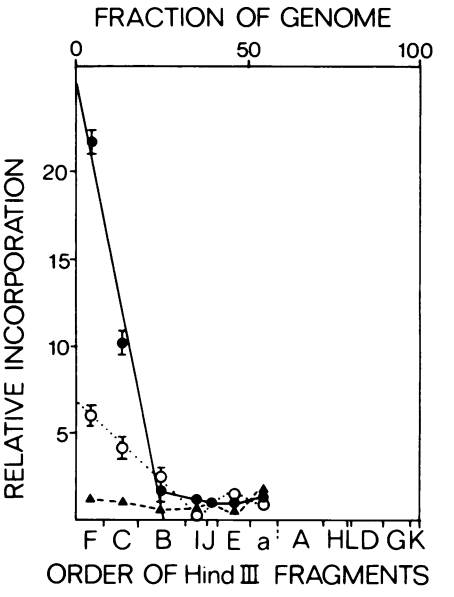

FIG. 5. *Relative specific activities of DNA fragments obtained after digestion of Eco RI fragment A from pulse-labeled completed Ad2 DNA molecules with the restriction endonuclease Hind III. Eco RI fragment A was isolated from gels of Eco RI digests of mature pulse-labeled DNA, obtained as described in the legend to Fig. 4. After concentration and purification through cesium chloride equilibrium centrifugation, the fragments were digested with the enzyme Hind III. The fragment order, determined in this laboratory, is shown on the abscissa. Big letters indicate Hind III fragments, the small letter a points to the Hind III fragment from the molecular right end of the Eco RI fragment A. All ratios are normalized for fragment I. Symbols are explained in the legend to Fig. 4.*

shows a higher specific activity than any of the other fragments, contains a terminus of Ad2 DNA replication.

In Fig. 4, an additional, minor increase of specific radioactivity is observed from fragment B towards fragment A, representing the molecular left end of the Ad2 molecule. Since fragment A corresponds to nearly 60% of the viral genome, this gradient is only poorly resolved. To increase the resolution in this region of the viral DNA, fragment A of Eco RI digests of completed Ad2 DNA molecules from 5-, 10-, and 40-min pulses was isolated from gels and further digested with the endonuclease Hind III. The corresponding plot of relative specific activities versus the physical order of the fragments (Fig. 5) reveals an increase of newly incorporated radioactivity from the center of the molecule towards fragment F, i.e., the molecular left end of the viral genome. Again, the gradient is observed in different sets of fragments corresponding to viral DNA from 5- and 10-min pulses whereas the distribution in viral DNA from a 40-min pulse is nearly uniform.

The data from the Eco RI and Hind III digests on the temporal order of Ad2 DNA synthesis were confirmed after digestion with the endonuclease Hpa I (Fig. 6). Although this digest permits no resolution in the center of the viral genome, it reveals maxima of specific radioactivity both in fragments E and G, representing the molecular left and right ends, respectively, of Ad2 DNA. Since, again, these maxima are observed in different sets of fragments from DNA of different pulse periods, it is concluded that Ad2 DNA replication terminates at both molecular ends of Ad2 DNA.

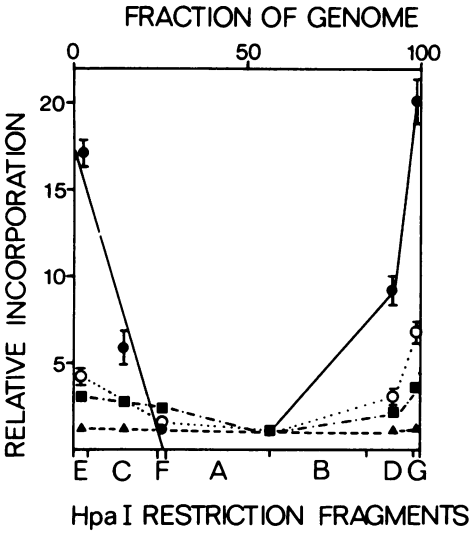

FIG. 6. *Relative specific activities of DNA fragments obtained after digestion of complete viral DNA molecules with the restriction endonuclease Hpa I from Haemophilus parainfluenzae. Mature DNA molecules were obtained after pulse-label experiments as described in the legend to Fig. 4. The fragment order (8) is given on the abscissa. All ratios are normalized to fragments A and B which are not separated under our electrophoresis conditions. Symbols are explained in the legend to Fig. 4.*

DISCUSSION

Analysis of the distribution of radioactivity along the viral genome in newly synthesized, completed viral DNA molecules reveals the existence of two termini of DNA replication at the molecular right and left ends of the viral DNA molecule. Ad2 DNA replication thus occurs bidirectionally. Since the increase in relative specific activity per unit length of viral DNA is similar in both the left and the right gradient, the rates of replication in both directions must be very similar.

The data agree with those presented by Tolun and Pettersson in the accompanying paper (15) and with a similar analysis presented earlier (17) on viral DNA synthesized in a system of isolated nuclei. The nuclei system, however, revealed one additional terminus in the center of the Ad2 DNA molecule. The reason for this discrepancy is not known. In isolated nuclei, the overall rate of replication is reduced and the relationships between the rates of the different reactions involved in the elongation of daughter strands appear to be changed as compared to in vivo replication (18). Such a system thus might resolve intermediates which cannot be detected under in vivo conditions. At present it is not clear whether the small increases of specific radioactivities detected in the center of completed Ad2 molecules after Hind III digests (Fig. 5) are related to the previous observations in isolated nuclei.

The data presented in this communication leave two basic possibilities for the location of origins of DNA replication. They are consistent with bidirectional replication starting either internally or at both ends of the Ad2 DNA molecule. In addition, internal initiation sites could be located at random or at specific sites along the viral genome. These different possibilities cannot be distinguished as yet by our results on the temporal order of DNA synthesis along various regions of the viral genome. We hope, however, that a forthcoming analysis of the strand specificity of newly incorporated radioactivity in pulse-labeled viral DNA will permit a decision in favor of one of these replication models.

At present, a number of lines of evidence seem to favor the physical ends of the Ad2 DNA molecule as initiation sites for viral DNA synthesis. Horwitz (6) studied the mechanism of Ad2 ND_1 replication in HeLa cells. In this system, the kinetics of appearance of newly synthesized adenovirus- and simian virus 40-specific DNA appear to be consistent with initiation at both molecular ends of the viral DNA. Moreover, replicating Ad2 DNA has been shown to contain extended single-stranded regions (9, 13, 16, 18) whereas detailed electron microscopy analyses (3) never appeared to reveal the presence of either circular or linear concatomeric intermediates, typically observed in replication of other circular and linear DNA molecules.

Based on these and other observations on replicating Ad5 DNA molecules, synthesized after release from a hydroxyurea block, Sussenbach et al. (3, 14) have suggested that adenovirus replication occurs by displacement synthesis, starting on the AT-rich molecular right end of the molecule by displacement of the parental H-strand. Initiation of DNA replication on this complementary strand was proposed to occur with considerable delay through initiation at different sites of the genome (3). Although this model clearly predicts one terminus of DNA replication at the molecular left end, it would have to be modified in its analysis of complementary strand synthesis to permit initiation of DNA synthesis only at the parental 3′ end.

We have previously shown that Ad2 DNA

synthesis occurs discontinuously (18) and that the observed 10S DNA intermediates in discontinuous Ad2 DNA synthesis occur on both strands of the viral genome. In addition, Tolun and Pettersson (15) and Lavelle et al. (7) have demonstrated that both complementary strands are exposed in replicating Ad2 DNA. It is thus possible that Ad2 DNA synthesis occurs by asynchronous displacement synthesis starting with equal probability from either end of the genome. This does not appear unreasonable, since both ends are identical at least up to the first 70 base pairs (10) because of the presence of an inverted terminal repetition (4, 20). Whether initiation, however, occurs simultaneously at both ends in a given, single molecule or randomly at one or the other end in different molecules cannot as yet be decided.

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LITERATURE CITED

1. Crawford, L. V., A. K. Robbins, and P. M. Nicklin. 1974. Location of the origin and terminus of replication in polyoma virus DNA. J. Gen. Virol. **25:** 133-142.
2. Danna, K., and D. Nathans. 1972. Bidirectional replication of simian virus 40 DNA. Proc. Natl. Acad. Sci. U.S.A. **69:**3097-3100.
3. Ellens, D. J., J. S. Sussenbach, and H. S. Jansz. 1974. Studies on the mechanism of replication of adenovirus DNA. III. Electron microscopy of replicating DNA. Virology **61:**427-442.
4. Garon, C. F., K. W. Barry, and J. Rose. 1972. A unique form of terminal redundancy in adenovirus DNA molecules. Proc. Natl. Acad. Sci. U.S.A. **69:**2391-2395.
5. Green, M., M. Piña, R. Kimes, P. C. Wensink, L. A. McHattie, and C. A. Thomas, Jr. 1967. Adenovirus DNA. I. Molecular weight and conformation. Proc. Natl. Acad. Sci. U.S.A. **57:**1302-1309.
6. Horwitz, M. S. 1974. Location of the origin of DNA replication in adenovirus type 2. J. Virol. **13:**1046-1054.
7. Lavelle, G., C. Patch, G. Khoury, and J. Rose. 1975. Isolation and partial characterization of single-stranded adenovirus DNA produced during synthesis of adenovirus type 2 DNA. J. Virol. **16:**775-782.
8. Mulder, C., J. R. Arrand, H. Delius, W. Keller, U. Pettersson, R. J. Roberts, and P. A. Sharp. 1974. Cleavage maps of DNA from adenovirus types 2 and 5 by restriction endonucleases Eco RI and Hpa I. Cold Spring Harbor Symp. Quant. Biol. **39:**397-400.
9. Pettersson, U. 1973. Some unusual properties of replicating adenovirus type 2 DNA. J. Mol. Biol. **81:**521-527.
10. Roberts, R. J., J. R. Arrand, and W. Keller. 1974. The length of the terminal repetition in adenovirus 2 DNA. Proc. Natl. Acad. Sci. U.S.A. **71:**3829-3833.
11. Robinson, A. J., H. B. Younghusband, and A. J. D. Bellet. 1973. A circular DNA-protein complex from adenoviruses. Virology **56:**54-69.
12. Smith, H. O., and K. W. Wilcox. 1970. A restriction enzyme from Hemophilus influenzae. I. Purification and general properties. J. Mol. Biol. **51:**379-391.
13. Sussenbach, J. S., D. J. Ellens, and H. S. Jansz. 1973. Studies on the mechanism of replication of adenovirus DNA. II. The nature of single-stranded DNA in replicative intermediates. J. Virol. **12:**1131-1138.
14. Sussenbach, J. S., P. C. van der Vlieth, D. J. Ellens, and J. S. Jansz. 1972. Linear intermediates in the replication of adenovirus DNA. Nature (London) New Biol. **239:**47-49.
15. Tolun, A., and U. Pettersson. 1975. Termination sites for adenovirus type 2 DNA replication. J. Virol. **16:**759-766.
16. van der Eb, A. J. 1973. Intermediates in type 5 adenovirus replication. Virology **51:**11-23.
17. Winnacker, E. L. 1974. Origins and termini of adenovirus type 2 DNA replication. Cold Spring Harbor Symp. Quant. Biol. **39:**547-550.
18. Winnacker, E. L. 1975. Adenovirus type 2 DNA replication. I. Evidence for discontinuous DNA synthesis. J. Virol. **15:**744-758.
19. Winnacker, E. L., G. Magnusson, and P. Reichard. 1972. Replication of polyoma DNA in isolated nuclei. I. Characterization of the system from mouse fibroblast 3T6 cells. J. Mol. Biol. **72:**523-537.
20. Wolfson, J., and D. Dressler. 1972. Adenovirus 2 DNA contains an inverted terminal repetition. Proc. Natl. Acad. Sci. U.S.A. **69:**3054-3057.