Location of the Origin of DNA Replication in Adenovirus Type 2

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Utilizing the isolated left and right halves of both adenovirus type 2 and the nondefective adenovirus simian virus 40 hybrid (Ad2⁺ND₁), studies were undertaken to find the site on the DNA molecules at which replication begins. The data are consistent with several models which include an initiation event at both ends and bidirectional growth.

Adenovirus DNA molecules are linear, duplex, nonpermuted sequences (5) containing termini not usually found in other DNA species (6, 24). Electron microscope studies, which show that circular molecules are formed upon renaturation of single strands, have led to the conclusion that there are short complementary sequences at the end of each strand. This form of terminal redundancy does not lead to double-stranded circles such as those demonstrated with a variety of other DNAs including lambda bacteriophage (8). The molecular weight of adenovirus type 2 (Ad2) DNA is 23×10^6 (8). Previous studies have shown that the single-strand size of replicating intermediates does not exceed the length of the genome, suggesting that mechanisms involving the rolling circle model or covalent addition of newly replicated DNA to parental molecules does not occur (10). No Okazaki fragments (17) have been demonstrated in the replication of adenovirus type 2 (10); however, a recent report on the replication of CELO, an avian adenovirus, suggested that Okazaki fragments may play a role during replication of that DNA (1). The replicating intermediates contain extensive single-stranded regions as demonstrated both by their increased density in CsCl and by their ability to bind to benzoylated-naphthoylated cellulose (BND cellulose) in the presence of 1 M sodium chloride (18, 22, 23). Estimates of the synthesis time vary from 5 to 15 min (10, 18).

The present studies were undertaken to find the site on the DNA molecule at which replication initiates. The data are inconsistent with replication beginning at one end and progressing simultaneously on both strands to the other end. Internal initiation with bidirectional growth, as has recently been shown for the

linear T7 bacteriophage DNA, has been eliminated by the present experiments. The data are consistent with several models which include an initiation event at both ends and bidirectional growth.

MATERIALS AND METHODS

Cells and viruses. The source of HeLa cells, adenovirus type 2, and conditions of infection were previously described (14). Simian virus 40 (SV40) and Ad2⁺ND₁ (adenovirus-SV40 nondefective hybrid) originally isolated by Lewis et al. (13) were obtained from Stephen Baum. All experiments in suspension cultures of HeLa cells were done at an input multiplicity of 4,000 virions per cell.

Characterization of DNA. Band sedimentation of DNA on 5 to 20% alkaline sucrose gradients, onto which whole cells were layered to avoid fragmenting DNA, was done as previously described (10). In experiments in which host cell DNA was not to be further examined, the cesium chloride "cushion" was omitted from the bottom of the gradients. DNA-DNA hybridization on nitrocellulose membranes, radioactive labeling of DNA, and the determination of acid-precipitable radioactivity were done by methods described previously (7, 10). SV40 DNA used in the hybridization reactions was purified from lysates of CV1 monolayer cells infected at a multiplicity of less than 1 SV40 virus per cell. The SV40 DNA was extracted from Hirt supernatants (9) of the lysed culture and sedimented through neutral sucrose gradients (9). The 21S SV40 DNA was used for the DNA-DNA hybridizations.

Molecular halves of Ad2 DNA. Ad2, grown in 3 liters of KB cells, was purified by isopycnic banding on two consecutive cesium chloride gradients, and viral DNA was isolated as previously described (10). Shearing of the Ad2 DNA and separation of the half molecules was done by the procedure of Cohen et al. (2). Ad2 DNA (12.5 optical density units measured at 260 nm [OD₂₆₀]) was dialyzed against three 500-ml changes of sodium borate (0.005 M)-sodium sulfate

(0.1 M) buffer at pH 9. The DNA at a concentration of 0.50 OD₂₆₀ units/ml was sheared in a Fisher Stead-Speed Mixer set at 2,100 rpm for 35 min at 0°C. After the controlled shearing, 0.55 ml of mercuric chloride (10^{-3} M) and 24 g of cesium sulfate were added. The density was adjusted to 1.527 (refractive index of 1.3760). The DNA-mercury complex was centrifuged to equilibrium in four tubes covered with paraffin oil in the angle 40 rotor of the Spinco ultracentrifuge for 62 h at 38,000 rpm and 4°C. The gradients were collected by piercing the bottom of the centrifuge tube, pumping oil into the top, and collecting 70 to 80 fractions for subsequent analysis.

Reagents. [^3H]thymidine (14 to 23 Ci/mmol) and [^{14}C]thymidine (57 mCi/mmol) were purchased from Schwartz BioResearch, Inc.; [^3H]thymidine (40 Ci/mmol) was from New England Nuclear Corp.; CsCl and Cs₂SO₄ (optical grade) were from Harshaw Chemical Co.; and cycloheximide was from Nutritional Biochemicals Co.

RESULTS

Ad2 DNA half molecules. Adenovirus DNA half molecules were prepared as reagents to locate both the origin and terminus of DNA replication. After controlled shearing of the Ad2 genome and separation on cesium sulfate gradients containing mercury, the DNA was quantitated in each of the fractions of the gradient as shown in Fig. 1. There was a clear separation of the radioactivity into two peaks which corresponded to the optical density profiles. Since the sedimentation values of the DNA shown in Fig. 1 were consistent with molecules of 42 to 48% of the length of the whole adenovirus DNA, it was postulated that the two peaks on the cesium sulfate gradients are left and right half genomes. To show that each of the two radioactive peaks was different, the heavy (Fr 27 to 35) and the light (Fr 39 to 47) fractions were hybridized with similar preparations of unlabeled DNA (Table 1). All of the [^{14}C]DNA samples were extensively sheared by sonic treatment just prior to hybridization. As can be seen, the hybridization of [^{14}C]DNA from the light peak (Fr 39 to 47) is primarily to the unlabeled, similarly processed, light adenovirus DNA half. A similar preference of the heavy [^{14}C]DNA peak for the unlabeled heavy molecules is shown. In contrast, when the entire adenovirus DNA genome is extensively sheared and hybridized, there is equal hybridization to both the light and the heavy DNA peaks. These data prove that the two peaks resolved on cesium sulfate gradients were not identical and represented left and right half genomes.

Isolation of origin and terminus of adenovirus DNA replicating molecules. Methods to identify either the origin of DNA replication or

the termination site for such replication were similar to those used either by Dintzis (4) for the determination of origin and termination sites in the growth of polypeptides or more recently by Danna and Nathans for SV40 DNA (3). At 18 h postinfection (PI) when Ad2 DNA replication was proceeding at a maximum rate and host DNA synthesis was reduced to minimal values, infected HeLa cells were labeled with high-specific-activity, tritiated thymidine for times ranging between 2 and 120 min. DNA was purified from the cells and centrifuged on alkaline sucrose gradients. After short incubation periods, completed molecules (34S) should be preferentially labeled at those sites of the DNA molecule which are synthesized last, namely the termination site(s) (Fig. 2A). With longer incubation, the initial preferential radioactivity in the terminus of recently synthesized molecules will be followed with more uniform labeling of the entire DNA molecule. A complementary argument can be proposed to isolate the origins of replication. For this determination, pieces were again isolated from alkaline

FIG. 1. Separation of half molecules of Ad2 DNA. Ad2 DNA was radioactively labeled by adding 50 μCi of [^{14}C]thymidine to 3 liters of infected KB cells at 9 h PI. The cells were harvested at 48 h, and the virus was purified. The DNA was isolated, dialyzed against borate-sulfate buffer, sheared in a controlled manner, and subjected to equilibrium centrifugation on cesium sulfate gradients in the presence of mercuric ions. The gradient was fractionated, and the radioactivity in a portion of each fraction was quantitated by scintillation counting.

TABLE 1. Hybridization of Ad2 DNA half molecules^a

Unlabeled Ad2 DNA on filter (1.2 µg):	Counts/min hybridized from DNA in solution		
	Light ¹⁴ C (0.15 µg)	Heavy ¹⁴ C (0.15 µg)	Whole ¹⁴ C (0.30 µg)
Light (GC)	1,190	474	1,440
Heavy (AT) ^b	474	1,280	1,570
Whole (AT + GC)	1,260	1,300	2,360
Ratio L/H	2.60	0.369	0.916
Total counts/min in solution	1,760	1,730	3,520

^a [¹⁴C]Ad2 DNA sheared to half molecules and separated as described in Fig. 1 was hybridized to unlabeled Ad2 DNA similarly treated. The hybridization between the various species, namely light halves (GC-rich), heavy halves (AT-rich), and whole molecules were done on nitrocellulose filters. All DNAs in solution were extensively sheared by sonic treatment prior to hybridization and the results are presented with the counts per minute bound to calf thymus DNA subtracted.

^b Hg binds preferentially to AT-rich regions; therefore, the DNA banding "heavy" on CsSO₄ gradients with Hg is AT-rich in contrast to the usually "heavy" GC-rich regions on CsCl gradients without added Hg (16).

sucrose gradients. These DNA molecules were less than 25% of the length of the genome and were taken to be enriched with the origin(s) of replication. It is important to re-emphasize that there do not appear to be Okazaki fragments necessary for the replication of adenovirus type 2 (10). According to the model of Okazaki, all parts of at least one and probably both strands of the DNA must be replicated initially through a small intermediate; hence, origins could not be sought in the small DNA pieces in any replicating system in which Okazaki pieces play a role as replicating intermediates. The origin or terminus of replication isolated in this way (see footnote, Table 2) was hybridized both to the "light half" and "heavy half" of the Ad2 DNA. Table 2 shows the results of these hybridizations. The recently completed pulse-labeled molecules hybridized essentially as well to the light half as to the heavy half of the Ad2 genome. The ratio of light to heavy hybridizable counts was approximately 0.8 at all times from 2 to 120 min. The 120-min point was taken as a long-labeled equilibrium point and was similar to the hybridization data for whole molecules as previously shown in Table 1. Similar data were obtained when DNA of molecular weight less than a quarter of the length of the genome was hybridized to the isolated halves. These experi-

ments were repeated at various ratios of DNA in solution to DNA on filters with similar results. Thus, after short times of labeling, both the completed molecules taken as representative of preferential labeling of the terminus and the small DNA intermediates, representative of the origin of replication, hybridized equally well to both halves of the isolated Ad2 genome. These results indicate that neither the terminus nor the origin of DNA replication for Ad2 is uniquely placed on one end of the DNA molecule and eliminates model A (Fig. 2).

Two models which would be consistent with the data include initiation internally on the DNA molecule with bidirectional replication towards both ends (model B, Fig. 2) or initiation at both ends of the DNA template with replication simultaneously towards the center (model C, Fig. 2). In order to distinguish between these two models of replication, it is necessary to have a further marker on the DNA molecule. Recent reports show that the Ad2⁺ND₁ DNA has the insertion of a small segment of SV40 at a site 13.6 to 20% from one end of the molecule (15). Such an insertion of SV40 DNA could be used as a marker near the end of the adenovirus genome. However, it was first necessary to prove that the Ad2⁺ND₁, nondefective hybrid, replicated essentially as adenovirus DNA rather than under the control of the SV40 integrated segment.

Characterization of Ad2⁺ND₁ DNA replication. We previously showed that, at 16 to 18 h PI, Ad2 DNA synthesis was resistant to inhibitors of protein synthesis. In contrast, SV40 DNA synthesis (11) like other mammalian cell or viral DNAs is very rapidly terminated by protein inhibitors. The following experiment was done to determine whether the Ad2⁺ND₁ genome would replicate in the absence of new protein synthesis. Ad2⁺ND₁-infected HeLa cells were labeled at 18 h with tritiated thymidine either in the presence of cycloheximide (30 µg/ml) or in the absence of this inhibitor. After 1 h the cells were centrifuged free of radioactivity and layered onto alkaline sucrose gradients for separation of viral from host DNA. Figure 3 demonstrates that the radioactivity in the control and cycloheximide-treated cells are identical in the area of the Ad2⁺ND₁ DNA peak as shown by the bar (fraction 6 to 10). Thus, both the sedimentation values of Ad2⁺ND₁ DNA as well as the total radioactivity incorporated were equivalent in the presence or absence of new protein synthesis. Further confirmation, that the SV40 DNA segment had been synthesized in the cycloheximide-treated cells, was obtained

by hybridizing the radioactive molecules isolated from the viral peak of the alkaline sucrose gradients to SV40 DNA. A percentage of the radioactivity of the viral DNA (2.6%) made either in the presence of cycloheximide or in the control-infected cells hybridized to SV40 DNA. These hybridization data clearly show that replication of the SV40 fragment of the Ad2⁺ND₁ genome had occurred. Thus, the replication of the Ad2⁺ND₁ resembled adenovirus replication rather than that of SV40 DNA.

Electron microscopy clearly showed a single SV40 insertion site on Ad2⁺ND₁ DNA but did not exclude the possibility that the SV40 could be integrated either on the right or left half of the molecule (15). The following experiments were done to prove that the insertion of SV40 DNA in the Ad2⁺ND₁ hybrid was on one half of the molecule and to locate the specific half. The Ad2⁺ND₁ genome was purified from infected cells and sheared as previously described for Ad2. Separation on cesium sulfate gradients

was monitored by optical density readings at 260 nm. Refractive indices were obtained on every tenth fraction. The results of this separation are shown in Fig. 4 and demonstrate that the sheared Ad2⁺ND₁ DNA could be separated into two peaks. Table 3 demonstrates that the peak in fraction 20 is different from the peak in fraction 25 and that the peak which banded heavy on the cesium sulfate gradients containing Ad2⁺ND₁ halves is equivalent to the peak which banded heavy in the previous experiments utilizing Ad2.

Similar experiments to separate the molecular halves of Ad2⁺ND₁ were done with tritiated, labeled Ad2⁺ND₁ DNA molecules. After initial separation on mercury-cesium sulfate gradients similar to that shown in Fig. 4, the DNA from each of the two peaks was pooled, dialyzed against 0.1 M EDTA to remove the mercury from the samples, and subsequently centrifuged to equilibrium on cesium chloride gradients in the presence of [¹⁴C]Ad2 whole DNA. Figure 5

FIG. 2. Models of replication for Ad2 DNA. All models are drawn to show progeny strands completed after the addition of radioactive thymidine for times much shorter than the synthesis time for the whole molecule. Arrows imply overall direction of chain growth rather than direction of polymerization of individual nucleotides. Symbols: —, parental DNA; ---, progeny DNA; ●●●●, radioactively labeled DNA; ·, the SV40 site of insertion (B-E) in Ad2⁺ND₁ DNA.

TABLE 2. Hybridization of completed Ad2 DNA and small replicating molecules to the isolated light (GC) and heavy (AT) halves of the adenovirus genome^a

DNA	Counts/min hybridized at min ^b :					
	2	5	10	20	30	120
Completed DNA molecules hybridized to:						
Light half	204 ^c	1,202	4,958	13,487	19,406	48,118
Heavy half	248	1,671	5,547	15,915	25,774	57,760
Ratio L/H	0.82	0.72	0.91	0.85	0.75	0.83
Total counts/min in solution	1,830	12,864	46,295	120,138	170,869	389,534
DNA of mol wt less than 25% of genome hybridized to:						
Light half	140	312	1,027	2,116	3,064	6,339
Heavy half	103	397	1,242	2,652	2,757	7,531
Ratio L/H	1.36	0.79	0.83	0.80	1.11	0.84
Total counts/min in solution	494	1,408	3,184	6,840	8,814	20,045

^a Cells (2×10^6) infected with Ad2 for 18 h were concentrated to 33 ml in Eagle medium without serum and equilibrated at 37°C. A 2-mCi amount of tritiated thymidine (40 Ci/mmol) was added and 5-ml portions were removed into DNA purification buffer solution with 0.1% sodium dodecyl sulfate detergent (10) at 2 min and subsequent times as shown above. After purification and precipitation of the DNA, it was redissolved in 1 ml of $0.01 \times$ SSC and centrifuged at 24,000 rpm for 16 h. The completed DNA molecules (34S) as well as molecules with *S* values of 16 and less, representing DNA smaller than 25% of the genome, were separately pooled. For example, six alkaline sucrose gradients, each representing a pulse labeling time, were run under conditions identical to those shown in Fig. 3. As representative of whole molecules, fractions 6, 7, and 8, from the leading edge of the 34S peak, were used. For molecules under 25% of the genome length, fractions 16 to 24 were pooled. The actual distribution of radioactivity after pulse labeling of Ad2-infected HeLa cells, has been previously reported (10). The DNAs were dialyzed free of sucrose and hybridized to either the light or heavy half of the Ad2 DNA molecule. Each filter contained 0.5 μ g of DNA, and the DNA in solution was approximately 5 μ g for "completed molecules".

^b After [³H]thymidine addition.

^c Results are presented as counts per minute bound to filters containing adenovirus DNA minus the radioactivity bound to filters containing calf thymus DNA.

shows further separation of the Ad2⁺ND₁ half molecules. Since mercury binding to DNA is preferential to the AT-rich sequences (16), bands which were heavy on the cesium sulfate gradients banded on the lighter side of the marker DNA in cesium chloride gradients without mercury. The opposite relation for GC-rich DNA is also apparent. If one harvests the heavy side of the Ad2⁺ND₁ GC-rich half molecules after cesium chloride equilibrium centrifugation and the light half of the Ad2⁺ND₁ AT-rich molecules, it can be shown that the SV40 radioactivity is inserted on the AT-rich half molecule. Table 4 demonstrates the hybridization of the AT-rich half and the GC-rich half of the Ad2⁺ND₁ DNA to either SV40 or Ad2 DNA bound to membrane filters. Thus, the SV40 insertion in the Ad2⁺ND₁ molecule is on the AT-rich half of the DNA.

Termination sequence of Ad2⁺ND₁ DNA. From the Ad2 experiments described above

there is evidence for bidirectional DNA replication. The presence of SV40 DNA between 13.6 and 20% from the AT-rich end of the Ad2⁺ND₁ molecule allows one to differentiate between internal initiation near the center of the molecule with termination at both ends, and initiation at the ends of the DNA molecule with termination at an internal point. If initiation should be internal, SV40 DNA would be near the terminus of replication (Fig. 2B). After short periods of radioactive labeling, SV40 DNA in completed molecules would be radioactively labeled and, by approximately 1/3 of the synthesis time, the increase in radioactive SV40 DNA would be linear (Fig. 6A). In contrast, if the origin of replication were at both ends of the molecule, the linear increase of SV40 label would be delayed in appearing in finished molecules until nearly the duration of the synthesis time (Fig. 2C and Fig. 6A). In either of these models, if there are no rate-limiting delays

FIG. 3. Ad2+ND₁ DNA synthesis in the absence of protein synthesis. HeLa cells were infected with Ad2+ND₁. At 18 h after infection, two 10-ml fractions (2×10^8 cells/ml) were each labeled with 250 μ Ci of tritiated thymidine for 1 h. One sample contained cycloheximide (30 μ g/ml) which was added 15 min before the radioactivity and remained during the entire labeling period. At the end of the labeling, the cells were harvested by centrifugation in ice-cold medium and portions were taken for determination of acid-precipitable counts. The remainder of the sample was suspended in 0.15 M sodium chloride and centrifuged through a 5 to 20% sucrose gradient containing 0.2 N NaOH. Sedimentation in the SW27 rotor of the Beckman centrifuge was continued for 16 h at 24,000 rpm. Acid-precipitable radioactivity of a portion of each fraction was obtained. The bar between fraction 6 to 10 designates the fractions that were pooled for subsequent hybridization against both adenovirus and SV40 DNA as described in the text. Host DNA sedimented to the bottom of the gradient.

of polymerization at any point in the molecule, the Ad2 radioactive sequences would increase exponentially until the linear increase began at the end of one synthesis time. The results of such an experiment are shown in Fig. 6B. At 18 h after infection of HeLa cells with Ad2+ND₁, cells were labeled with tritiated thymidine and harvested at 5, 10, 15, 20, and 30 min after the addition of the radioactivity. The DNA was purified and sedimented on alkaline sucrose

FIG. 4. Separation of half molecules of Ad2+ND₁ DNA. Ad2+ND₁ DNA was processed similarly to Ad2 DNA described both in Fig. 1 and in Materials and Methods. This unlabeled DNA was monitored by pumping the effluent of the equilibrium gradient through a Gilford flow cell with continuous absorption readings at 260 nm. Densities were determined by removing small portions from every tenth fraction and measuring the refractive index. Fractions 15 to 20 and 25 to 30 were pooled and dialyzed against three 1-liter changes of 0.1 M EDTA, pH 7.4, to rid the samples of mercury and cesium sulfate. These portions were used for the hybridization experiments described in Table 3.

TABLE 3. Hybridization between Ad2 isolated half molecules and Ad2+ND₁ half molecules^a

Unlabeled Ad2+ND ₁ DNA on filter:	Counts/min hybridized from DNA in solution		
	[¹⁴ C]Ad2 (GC)	[¹⁴ C]Ad2 (AT)	[¹⁴ C]Ad2 (AT + GC)
Ad2+ND ₁ (GC)	2,901	1,256	2,137
Ad2+ND ₁ (AT)	1,228	2,662	2,117
Ratio GC/AT	2.36	0.48	1.01
Total counts/min in solution	3,488	3,705	3,180

^a The Ad2 DNAs shown in Table 1 were utilized in hybridization reactions with the isolated halves of the Ad2+ND₁ genome. The Ad2+ND₁ unlabeled DNA was placed on nitrocellulose filters and the [¹⁴C]Ad2 DNA fractions, as designated, were sheared, denatured, and added in solution for the hybridization reaction. The AT halves banded "heavy," and the GC halves banded "light" on the Cs₂SO₄ gradients which contained Hg (Fig. 1). The results are expressed as counts per minute bound to filters containing Ad2+ND₁ half molecules minus the counts per minute bound to filters containing calf thymus DNA.

gradients. The molecules in the position of completed viral DNA (34S) were pooled,

FIG. 5. Co-sedimentation of whole Ad2 with Ad2⁺ND₁ half molecules. Tritium-labeled Ad2⁺ND₁ DNA was processed as described in Fig. 4. After separating and pooling the appropriate portions of the heavy fractions (AT-rich) of Ad2⁺ND₁ DNA and the light fractions (GC-rich) of DNA, the samples were dialyzed against 0.1 M EDTA to remove the bound mercury and cesium sulfate. The DNA was then equilibrated with 0.01 × SSC and rerun on cesium chloride equilibrium gradients in the presence of marker ¹⁴C-labeled adenovirus whole DNA. After 62 h at 38,000 rpm at 25 C, the gradients were fractionated as described in Materials and Methods. Radioactivity in each of the fractions was quantitated, and the densities of the isolated Ad2⁺ND₁ AT- and GC-rich halves were compared to the Ad2 DNA marker. Symbols: ○, Ad2 DNA; ●, Ad2⁺ND₁ half molecules.

TABLE 4. Hybridization of Ad2⁺ND₁ half molecules to SV40 and Ad2 DNA^a

DNA on filter:	Counts/min hybridized from DNA in solution	
	[³ H]Ad2 ⁺ ND ₁ (AT)	[³ H]Ad2 ⁺ ND ₁ (GC)
SV40 (1 μg)	297	0
Ad2 (3 μg)	13,236	11,502
Total counts/min in solution	18,300	13,900

^a SV40 and Ad2 DNA were immobilized on nitrocellulose filters and hybridized to the isolated halves of Ad2⁺ND₁ DNA in solution. The Ad2⁺ND₁ halves had been purified extensively through cesium sulfate and cesium chloride equilibrium gradients as shown in Fig. 4 and 5. The results are expressed as counts per minute bound either to filters containing SV40 or Ad2 DNA minus that bound to filters containing calf thymus DNA.

dialyzed free of sucrose, denatured in alkali, and sheared. Portions of each DNA were hybridized either to Ad2 or to SV40 DNA on nitrocellulose membrane filters. The kinetics of appearance of adeno-specific DNA and SV40-specific DNA are plotted as a function of time of labeling. Figure 6B demonstrates that the linear increase in radioactive incorporation into SV40 DNA was delayed. These results are inconsistent with internal initiation which would place the SV40 DNA near the terminus (Fig. 2B). It is more consistent with initiation at both ends and bidirectional growth (Fig. 2C). The difference in the kinetics of labeling between adenovirus- and SV40-specific sequences likewise rules out random but simultaneous initiation at a variety of points on the DNA molecule.

DISCUSSION

Half molecules of Ad2 DNA have been previously separated both in the analytic ultracentrifuge (5) and preparatively on CsCl gradients (12). In this communication isolated adenovirus half molecules prepared in 150- to 200-μg amounts have been used to show that the origin and terminus are not uniquely placed at opposite ends of the DNA molecules. Similarly, internal initiation with bidirectional replication seems ruled out by the data obtained from studies on the replication of Ad2⁺ND₁ molecules. The data are most consistent with bidirectional replication starting at both ends of the molecule, and a possible model to explain this data has been proposed (Fig. 2C). However, in

FIG. 6. Kinetics of labeling of the SV40 and Ad2 DNA sequences in newly completed Ad2⁺ND₁ molecules. (A) In Ad2⁺ND₁ molecules recently completed after the addition of [³H]thymidine, the theoretical increase of radioactivity hybridizable either to Ad2 or SV40 DNA is shown. Ad2 DNA sequences are represented in relation to two possible models of synthesis which would place SV40 DNA either at the origin or terminus of DNA replication. Numbers on the abscissa refer to complete synthesis times. Numbers on the ordinate are corrected for differences in the amount of adenovirus and SV40 sequences in Ad2⁺ND₁. (B) HeLa cells (1.6×10^6) infected with Ad2⁺ND₁ were labeled at 18 h in a volume of 38 ml of Eagle medium with 2 mCi of tritiated thymidine (40 Ci/mmol). Equal samples were obtained at 5, 10, 15, 25, and 30 min, and the incorporation was stopped by the addition of sodium dodecyl sulfate. The DNA was purified and placed onto alkaline sucrose gradients for separation of molecules by size. The radioactivity which corresponded to full-size single-stranded DNA molecules (34S) was pooled and dialyzed free of sucrose prior to shearing by sonic treatment. Such DNA from each of the five time points was hybridized either to Ad2 DNA (3 μ g per nitrocellulose filter) or SV40 DNA (1 μ g per filter). Calf thymus DNA filters were included as controls for the subtraction of nonspecific counts bound. After the hybridization reaction the radioactivity present on each filter was plotted as a function of time after the addition of tritiated thymidine.

such a model one would expect an absolute delay in appearance of SV40 hybridizable counts in completed molecules until nearly one synthesis time was completed (Fig. 6A). The appearance of labeled SV40 sequences by 5 min and the increase in rate of accumulation of such radioactivity after 15 min suggests that some of the SV40 sequences may not only be at the origin but also at the terminus of DNA replica-

tion. Such kinetic data suggest two alternate models (Fig. 2D and E).

Since both the left and right ends of the molecule are identical, any system initiating polymerization may be unable to recognize the left from the right end. The possibility that some molecules replicate both strands simultaneously starting at the left end and other molecules have the equivalent replication starting at the right end has not been ruled out by the present data (Fig. 2D). Recently, Sussenbach et al. (20-22) have suggested that adenovirus replication proceeds through a mechanism of strand displacement and have proposed several models to incorporate this concept. In favor of a displacement model is the observation that during Ad2 DNA replication there is a lot of single-stranded DNA in the replication complex. In addition, data obtained after the reversal of hydroxyurea inhibition of DNA synthesis suggests that only one strand (heavy) is synthesized within the first 60 min after reversal and that the parental heavy strand is displaced (20, 21). In such a model, replication could begin on one strand on the AT-rich right side (20), progressing in the 5' to 3' direction. Replication of the second strand is delayed and could begin on the left hand after its displacement from the replicating intermediate. In such a case, replication of the second strand may also begin on the parental 3' end of the template and progress on the progeny strand in the 5' to 3' direction. Such replication in effect would have origins at both ends of the DNA, and bidirectional growth would be necessary for completion of the molecules. The displacement model presented in this form would not require Okazaki pieces to replicate DNA in the 5' to 3' direction on both strands. Since no Okazaki pieces were found in our previous investigations of Ad2 DNA replication (10), the latter hypothesis seems attractive and can explain all of the kinetic data presented in this manuscript, the data on the size of the intermediates previously presented, the structural data on the similarity of the ends of the adenovirus DNA, and the presence of large amounts of single-stranded DNA in the replication complex.

An exact quantitation of the purity of the isolated half molecules of Ad2 is not possible, but an estimate can be made from the studies of Ad2⁺ND₁. Ideally, there should not be any hybridization between left and right half molecules, but the inverted terminal redundancy recently shown for the adenovirus will cause a 1 to 3% homology between the halves. An additional source of homology may be a small

difference in the mechanical cleavage site near the middle of the molecule. "Middle sequences" may appear in some molecules on the left half and in others on the right. A third possibility is that similar sequences are incorporated to a limited extent on both right and left halves. Lastly, any inadequacy of the separation will contribute to contamination of the halves. In the Ad2+ND₁ half molecule experiments, the purity of separation can be assessed using the SV40 sequences which are inserted only on one half of the genome. The SV40 insertion site is separated from the inverted terminal redundancy as well as the middle of the molecule. After both Cs₂SO₄ and CsCl separation, the SV40 DNA hybridizes only with the AT-rich half of the Ad2+ND₁; however, after the Cs₂SO₄ equilibrium run alone, the ratio of SV40 hybridized to the Ad2+ND₁ AT-rich half compared to the GC-rich half was 3:1 (personal observation). Since the separation of Ad2 half molecules on Cs₂SO₄ (Fig. 1) seemed better than that of the Ad2+ND₁ halves (Fig. 4), it would appear that the contamination of the Ad2 halves would be less than that for the Ad2+ND₁.

Recently, using DNA purification without sodium dodecyl sulfate or proteolytic enzymes, circular forms of adenovirus DNA have been found and are presumably linked by protein molecules which hold the ends of DNA together in a noncovalent bond (19). None of the essential features of the linear models of replication presented in Fig. 2 would be altered by this type of circular structure.

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