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Termination Sites for Adenovirus Type 2 DNA Replication

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Termination sites for replication of adenovirus type 2 DNA have been demonstrated at both ends of the viral chromosome by the procedure of Danna and Nathans (1972). Single-stranded DNA from replicating intermediates was also characterized by hybridization with separated strands of viral DNA. The results indicate that both strands are exposed during replication.

Adenovirus type 2 (Ad2) DNA is a nonpermuted linear duplex molecule with no single-strand discontinuities (8, 25). The molecular weight is about 23×10^6 (4, 8, 25) and there are inverted terminal repetitions at the ends (7, 26). Ad2 DNA labeled for short time periods was observed to exhibit some properties not typical for mature molecules: it sediments with a fast rate in sucrose gradients and bands at a high density in CsCl equilibrium gradients (12, 21, 24). Pulse chase experiments have shown that these properties are due to the presence of single-stranded branches on replicating molecules (10, 12, 24). Replicating DNA was also studied by electron microscopy: Y-shaped molecules with a single-stranded arm and linear molecules of unit length with single-stranded gaps of varying length were observed (19, 21, 24), suggesting a "strand displacement model" for replication of adenovirus DNA (19, 21). Replication starts at one end and the progeny strand, which is synthesized first, displaces one of the parental strands, thus leading to a Y-forked intermediate. After replication of one strand is completed, the replicating intermediate separates to give a linear duplex DNA molecule and a free single strand which is replicated separately. Sometimes replication initiates on the displaced strand before displacement synthesis is completed (5, 19).

The strand displacement model raises the question whether replication starts from one specific end of the molecule always displacing the same strand. Sussenbach et al. (19) studied replication of Ad5 DNA in vitro in isolated nuclei. Hybridization experiments and analysis of partially denatured replicating molecules by electron microscopy suggested that one specific strand always is displaced and that replication starts from the AT-rich end of Ad5 DNA. Experiments reported by Lavelle et al. (10) in contrast, showed that single-stranded DNA, which was isolated from cells infected with Ad2, hybridizes equally well with both strands of the viral DNA.

In the present study we have investigated the termination points for Ad2 DNA replication by the method of Danna and Nathans (3). Termination sites at both ends have been detected. We have also characterized the single-stranded branches that are exposed during replication.

MATERIALS AND METHODS

Cell cultures and virus. HeLa or KB cells were concentrated to 10^7 cells/ml in Eagle spinner medium with 2% calf serum and infected with Ad2 at a multiplicity of 10^4 particles/cell. After 30 min the cells were diluted to 2×10^5 cells/ml with warm spinner medium with 7% calf serum. At 13 to 14 h the cells were concentrated to 1 to 2×10^6 cells/ml and pulse labeled for 5, 10, 45, and 60 min. To label the newly synthesized DNA, 50 to 150 μCi of *methyl*-[^{3}H]thymidine (New England Nuclear; 40 to 60 mCi/mM) per ml was used. At the end of each pulse the cells were mixed with crushed frozen phosphate-buffered saline and the DNA was extracted.

^{32}P-labeled virus and DNA was made according to Pettersson and Sambrook (15).

DNA extraction. Cells were pelleted and resuspended in 0.65% Nonidet P-40 in phosphate-buffered saline at a concentration of 5×10^6 cells/ml or less. After incubation at 0 C for 10 min the nuclei were spun down, washed with phosphate-buffered saline, lysed with 0.5% sodium dodecyl sulfate, and digested with 1 mg of Pronase per ml in a buffer which contained 0.1 M EDTA, 0.1 M NaCl, and 0.01 M Tris-hydrochloride (pH 7.9) for 2 h at 37 C. The sample was then extracted twice with phenol (saturated with 0.5 M Tris-hydrochloride, 10 mM EDTA, pH 7.9) and once with chloroform:isoamylalcohol (24:1). All steps except the Pronase digestion were carried out at 4 C.

Restriction enzymes. EndoR·*Eco*RI was purified from *Escherichia coli* strain RY13 as described by Pettersson and Philipson (14). Digestions were made in 0.1 M NaCl, 0.01 M $MgCl_2$, and 0.01 M Tris-hydrochloride (pH 7.9).

EndoR·*Hpa*I was purified by the method of Sharp et al. (18) and was a kind gift from R. Kamen,

Imperial Cancer Research Fund, London. Incubation mixtures contained 0.1 M KCl, 0.006 M $MgCl_2$, 0.006 M β-mercaptoethanol, and 0.006 M Tris-hydrochloride (pH 7.5).

EndoR·*Hind*III was kindly supplied by Ernst Winnacker from the University of Cologne, West Germany. Digestions were carried out in 0.06 M NaCl, 0.007 M $MgCl_2$, 0.001 M β-mercaptoethanol, and 0.006 M Tris-hydrochloride (pH 7.9).

Polyacrylamide gel electrophoresis. After digestion was completed samples for electrophoresis were extracted with phenol, precipitated with ethanol, and dissolved in 0.1 ml of 0.01 M Tris-hydrochloride (pH 7.9) with 10^{-3} M EDTA (TE buffer).

Composite gels which contained 0.7% agarose (Sigma) and 2.2 or 1.1% acrylamide (Serva, Germany) were prepared as described by Pettersson et al. (13). Electrophoresis was carried out in cylindrical gels at 4.5 V/cm in a buffer which contained 5 mM sodium acetate, 1 mM EDTA, and 0.04 M Tris-hydrochloride (pH 7.9). Fragments obtained after cleavage with EndoR·*Hpa*I and EndoR·*Eco*RI were separated on 13-cm gels and fragments after cleavage with EndoR·*Hind*III were separated on 18-cm gels. Bands were visualized by staining with ethidium bromide (18) and DNA was eluted electrophoretically from gel slices as described by Pettersson et al. (13). To achieve separation between fragments *Hind*III-D and -E and between fragments *Hind*III-F and -G, the combined fragments were eluted from gel slices and subjected to a second cycle of electrophoresis for 36 to 48 h.

Hybridizations. Hybridizations of replicating DNA with isolated single strands of Ad2 DNA were carried out in 0.14 M phosphate buffer (equimolar amounts of Na_2HPO_4 and NaH_2PO_4) with 0.4% sodium dodecyl sulfate and 1 M NaCl at 65 C for 4 to 5 days. Samples were then assayed by chromatography on hydroxylapatite columns (Bio Gel HTP) as described by Sambrook et al. (16). Single-stranded DNA was eluted with 0.14 M phosphate buffer with 0.4% sodium dodecyl sulfate and double-stranded DNA with 0.4 M phosphate buffer with 0.4% sodium dodecyl sulfate.

^{32}P-labeled DNA was determined as Čerenkov radiation. Fractions with ^{3}H-labeled DNA were precipitated on membrane filter disks (Millipore Corp.) with 10% trichloroacetic acid before counting.

Separation of the complementary strands from Ad2 DNA. Unlabeled strands were prepared by equilibrium centrifugation in CsCl of denatured viral DNA which had been complexed with ribopoly(U,G) as described in detail by Tibbetts et al. (23). ^{32}P-labeled strands were prepared by incubation of denatured labeled DNA with an excess of one unlabeled DNA strand under annealing conditions. Labeled complement-specific DNA was isolated by fractionation of the incubation mixture on hydroxylapatite. The method for strand separation of ^{32}P-labeled DNA has been described in detail by Tibbetts and Pettersson (22).

The strand which has a higher buoyant density in CsCl when complexed with poly(U,G) is referred to as the H-strand. The strand with a lighter buoyant density when complexed with poly(U, G) is accordingly designated the L-strand.

Equilibrium centrifugation in CsCl. ^{3}H-labeled DNA and ^{32}P-labeled marker Ad2 DNA was mixed with TE buffer and the density was adjusted to 1.705 g/ml with solid CsCl. Centrifugation was performed in a Spinco Ti50 or Ty50 fixed angle rotor for at least 42 h at 40,000 rpm at 20 C. Gradients were fractionated from the bottom and aliquots were assayed for trichloroacetic acid-insoluble radioactivity.

In some experiments the DNA was sheared by passage twice through a 25-gauge syringe needle prior to equilibrium centrifugation.

Velocity sedimentation in sucrose. Samples (0.1 to 0.2 ml) were layered on preformed 5 to 25% sucrose gradients which contained 1 M NaCl, 1 mM EDTA, and 10 mM Tris-hydrochloride, pH 7.9. Separations were carried out in the Spinco SW56 rotor for 90 min at 50,000 rpm and 20 C. Gradients were fractionated from the bottom and aliquots were assayed for trichloroacetic acid-insoluble radioactivity.

BND-cellulose chromatography. Benzoylnaphthoyl-DEAE-cellulose (BND-cellulose) which had been purchased from Serva, Germany, was washed with ether as described by Sedat et al. (17). DNA in 0.3 M NaCl, 1 mM EDTA, and 0.01 M Tris-hydrochloride (pH 7.9) was loaded on columns (2 by 1.5 cm). Double-stranded DNA was eluted with a buffer which contained 1 M NaCl, 1 mM EDTA, and 10 mM Tris-hydrochloride (pH 7.9), and DNA containing single strands was eluted with 2% caffeine, 1 M NaCl, 1 mM EDTA, and 10 mM Tris-hydrochloride (pH 7.9). The column was finally washed with a buffer containing 0.1 M NaOH, 0.9 M NaCl, and 1 mM EDTA to eluate any remaining DNA. Fractions with double-stranded DNA were pooled and the density was adjusted by addition of solid CsCl before equilibrium gradient centrifugation.

RESULTS

Location of termination sites for replication of Ad2 DNA. Completed DNA molecules, which have been pulse labeled during a time period shorter than the time needed for one complete round of replication, will be preferentially labeled at the termination sites for replication. With the use of restriction endonucleases it is possible to isolate specific DNA fragments and thereby determine the specific radioactivity in different segments of a given DNA molecule. This experimental approach has been utilized to map the origin and termination point for replication on simian virus 40 and polyoma DNA (2, 3).

We have pulse labeled cells 14 h after infection for 5, 10, and 60 min. The total intracellular DNA was extracted as described above and the DNA was fractionated by chromatography on BND-cellulose. Since intermediates in adenovirus replication contain single-stranded branches, this procedure should efficiently sep-

arate replicating viral DNA molecules from mature DNA molecules. Fractions which were eluted with 2% caffeine from BND-cellulose columns contained, as expected, predominantly DNA with a higher buoyant density than mature viral DNA (not shown). About 20% of the labeled DNA after a 5-min pulse was eluted with 1 M NaCl and these fractions were collected and purified by equilibrium centrifugation to remove contaminating cellular DNA (Fig. 1). In some experiments the DNA was further purified by velocity sedimentation on a sucrose gradient to eliminate traces of replicating or degraded molecules. Purified mature DNA was precipitated with ethanol and digested with EndoR·*Eco*RI after addition of ^{32}P-labeled DNA which had been extracted from purified virus. Fragments were separated on composite 1.1% polyacrylamide, 0.7% agarose gels which were stained with ethidium bromide. Bands were eluted electrophoretically and counted after precipitation with trichloroacetic acid. The ratio between ^{3}H- and ^{32}P-labeled DNA was determined for all six fragments and the results are illustrated in Fig. 2. Corrections were made for differences in base composition between individual fragments by comparing the ^{3}H/^{32}P ratios for mixtures of DNA uniformly labeled with [^{3}H]thymidine and [^{32}P]phosphate. A clear increase in the ratio was observed towards the right hand end of the genome when DNA was analyzed after pulse labeling for 5 and 10 min. This suggests that one terminus for replication is located at the right hand end of Ad2 DNA. Possible termination points in the left hand half of the Ad2 DNA could not be resolved after cleavage with EndoR·*Eco*RI since the large *Eco*RI-A fragment which corresponds to 59% of Ad2 DNA is located in this part of the molecule (11). Therefore, pulse-labeled, mature molecules were also cleaved with EndoR·*Hpa*I after addition of ^{32}P-labeled DNA. This enzyme cuts Ad2 DNA into seven fragments, four of which are located within fragment *Eco*RI-A (6, 11). The fragments were separated on composite 1.1% polyacrylamide, 0.7% agarose gels. After staining with ethidium bromide, the fragments were eluted and analyzed as described above and the results are summarized in Fig. 3. An increasing ^{3}H/^{32}P ratio was observed also towards the left hand end of the Ad2 DNA when completed molecules were analyzed which had been pulse labeled for

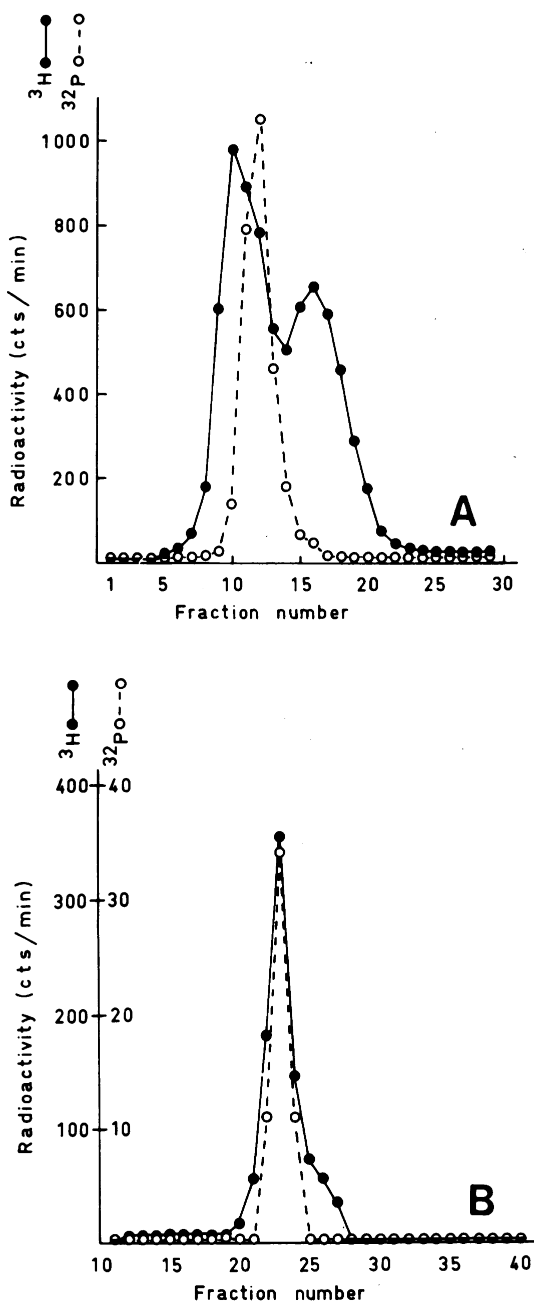

FIG. 1. *CsCl equilibrium gradient centrifugation of intracellular DNA before and after fractionation by BND-cellulose chromatography. Cells were pulse labeled for 10 min with [^{3}H]thymidine (120 µCi/ml) at 14 h after infection. The total intracellular DNA was extracted as described and fractionated by BND-cellulose chromatography. Centrifugation was performed at 20 C and 40,000 rpm for 42 h in the Spinco Ti50 rotor. The density increases towards the left. (A) Intracellular DNA before fractionation by BND-cellulose chromatography. (B) Double-stranded DNA eluted from BND-cellulose with a buffer containing 1 M NaCl, 1 mM EDTA, 0.01 M Tris-hydrochloride (pH 7.9). Symbols:* ●, *intracellular DNA;* ○, *^{32}P viral DNA marker.*

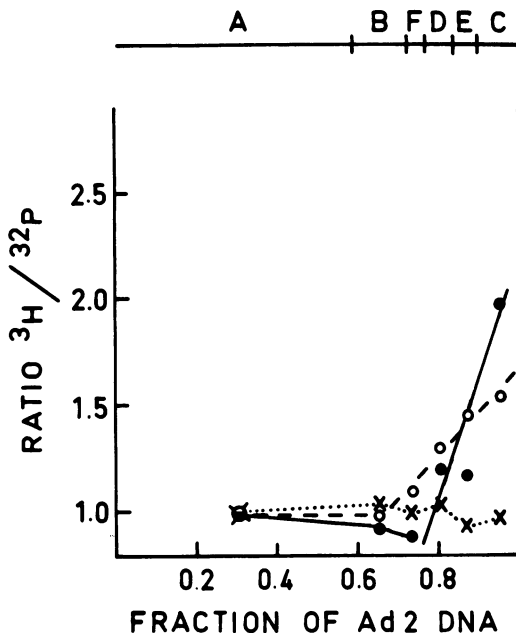

FIG. 2. *Relative amounts of [^{3}H]thymidine label in different fragments obtained by cleavage of newly completed molecules with EndoR·EcoRI. DNA which had been pulse labeled for 5 (●), 10 (○), and 60 min (×) was analyzed. ^{32}P-labeled DNA extracted from purified virions was used as a reference. The fragment order (11) is shown on top of the figure. Corrections have been made for differences in thymidine content in individual fragments by determination of the ratio between ^{3}H/^{32}P for fragments which had been derived from DNA uniformly labeled with [^{32}P]phosphate and [^{3}H]thymidine. The ratios in the figure are normalized to fragment RI-A.*

5 and 10 min. Thus there seems to be an additional termination site at the left hand end of the Ad2 genome.

To confirm these results and to improve the resolution of possible internal termination sites, mature DNA that had been pulse labeled for 10 min was also cleaved with EndoR·*Hind*III. This enzyme cuts Ad2 DNA in 12 fragments (R. Roberts, personal communication), the order of which is shown in Fig. 4. All fragments except the smallest (*Hind*III-L) were isolated from gels and the ratio between ^{3}H pulse-labeled and ^{32}P-labeled reference DNA was determined. The termination sites at the right and left hand ends were confirmed but no clear internal termination sites were observed. A minor increase in ratio near the middle of the Ad2 genome was observed but this increase was always much less prominant than the maxima at both ends. (Fig. 4)

Characterization of single-stranded DNA in cells infected with Ad2. Results from several laboratories indicate that replicating Ad2 DNA molecules contain an unusual amount of single-stranded branches (12, 21, 24). It was therefore proposed that adenovirus DNA replicates by a strand displacement mechanism. In this study we have designed experiments to determine whether one specific or both strands are displaced during replication. Since replicating molecules contain exposed single strands it should be possible to directly perform hybridization studies between replicating molecules and separated strands of Ad2 DNA. Two kinds of experiments have been performed.

Infected cells were pulse labeled 14 h after infection with [^{3}H]thymidine. The total intracellular DNA was extracted and fractionated by equilibrium centrifugation in CsCl. Most of the label banded at the position of the viral marker DNA and a small peak was observed at the density of cellular DNA (Fig. 5). DNA with a greater buoyant density than the marker (heavy DNA) was also observed.

This DNA has previously been found to contain replicating molecules and its increased buoyant density is due to the presence of single strands (12). Fractions corresponding to the heavy DNA were pooled as indicated in Fig. 5

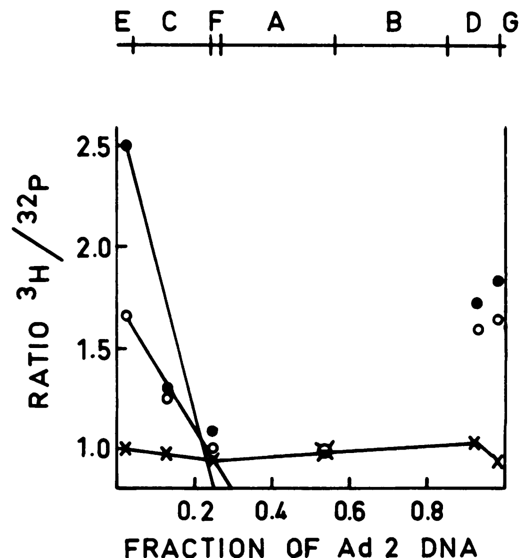

FIG. 3. *Relative amounts of [^{3}H]thymidine label in different fragments obtained by cleavage of newly completed molecules with EndoR·HpaI. The symbols are explained in the legend to Fig. 2. The fragment order (11) is shown on top of the figure. Fragments HpaI-A and HpaI-B were not separated. All values are adjusted for variations in thymidine content and normalized to a mixture of fragments HpaI-A and HpaI-B.*

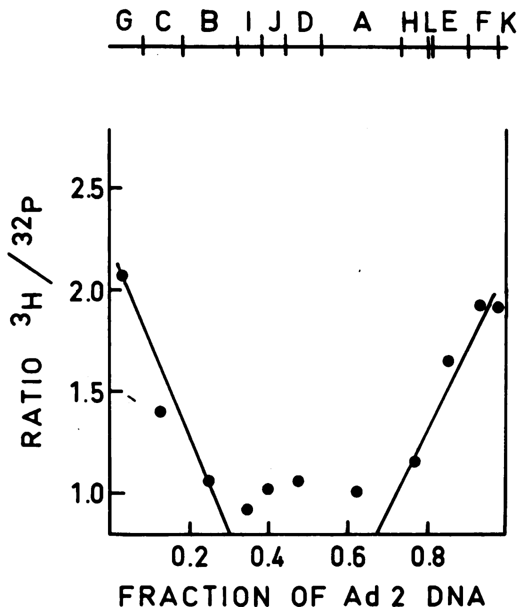

FIG. 4. *Relative amounts of [³H]thymidine label in different fragments obtained by cleavage of newly completed molecules with EndoR·HindIII. Only DNA pulse labeled for 10 min was analyzed. The fragment order which is shown on the top of the figure was kindly provided by R. Roberts, Cold Spring Harbor Laboratory. The fragment size was estimated by determination of the distribution of ³²P labeled in each fragment after cleavage and separation of DNA which was uniformly labeled with [³²P]phosphate (U. Pettersson unpublished data). Fragments are designated according to their mobility in agarose gels. It has been observed that fragments HindIII-D and -E and fragments HindIII-F and -G migrate in the reverse order in composite 0.7% agarose-2.2% acrylamide gels (R. Roberts, personal communication). The ratios in the figure are normalized to fragment HindIII-A.*

and dialyzed against TE buffer. Heavy DNA without further treatment was then incubated under annealing conditions with ³²P-labeled H- and L-strand probe DNA as described above. The fraction of the ³²P-labeled probe DNA which became double stranded after incubation was determined by chromatography on hydroxylapatite. Both labeled probes hybridized to a nearly equal extent in repeated experiments and about 50% of the ³²P-labeled DNA could be recovered in the double-stranded fraction regardless whether H- or L-strand DNA was used for hybridization (Table 1). Saturation of the strands was presumably not achieved because some regions of each strand are exposed with a low frequency and because of self-hybridization between replicating molecules. The results suggest that both strands are exposed during replication of Ad2 DNA.

Another type of experiment was performed to confirm that both the complementary strands are exposed in adenovirus-infected cells: cells were pulse labeled for 45 min, 14 h after infection as described above. The total intracellular DNA was extracted and fractionated by equilibrium centrifugation in CsCl. The fractions which corresponded to heavy DNA were pooled as indicated in Fig. 5. After removal of CsCl by chromatography on Sephadex G50 the DNA was degraded by sonication for 30 s in TE buffer with 0.01 M NaCl. The sonicated DNA was chromatographed on hydroxylapatite and fractions which eluted with 0.14 M phosphate buffer were collected. About 20% of the pulse-labeled heavy DNA was recovered in the single-stranded DNA fraction (Table 2) whereas control experiments showed that less than 3% of duplex adenovirus DNA elutes with 0.14 M phosphate buffer after sonication. Single-stranded DNA, labeled with [³H]thymidine and isolated by hydroxylapatite chromatography from sonicated heavy DNA, was then incubated under annealing conditions with an excess of unlabeled H- and L-strand DNA prepared from virions as described by Tibbetts et al. (23) The fraction of ³H-labeled DNA which became double stranded after incubation was determined by chromatography on hydroxylapatite. The

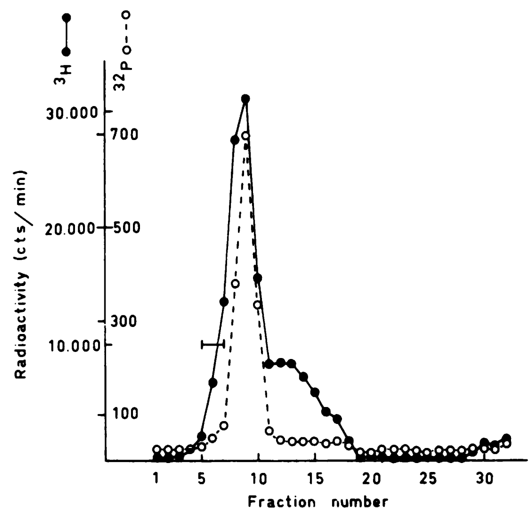

FIG. 5. *Equilibrium centrifugation in CsCl of intracellular DNA, extracted from cells which were pulse labeled with [³H]thymidine (50 µCi/ml) for 45 min at 13 h after infection. The initial density was 1.705 g/ml and centrifugation was at 40,000 rpm and 20 C for 43 h in a Spinco Ti50 rotor. Fractions containing heavy DNA were pooled as indicated by the bar. The density increases towards the left. Symbols: ●, intracellular DNA; O, ³²P-labeled viral DNA marker.*

TABLE 1. *Hybridization between ^{3}H-labeled heavy DNA and ^{32}P-labeled complement-specific Ad2 DNA*

Expt	Amount of heavy DNA[a] (^{3}H counts/min)	% ^{32}P-labeled L-strand DNA[b] in duplex	% ^{32}P-labeled H-strand DNA[b] in duplex
1	350	14[c]	13
	1,050	26	24
2	2,500	15	23
	6,250	27	34
3[d]	28,000	41	51
	94,500	45	58

[a] The amount of ^{3}H label was used to quantitate heavy DNA from different experiments. The DNA in all experiments was labeled under identical conditions and should thus have the same specific activity. Because of different amounts of contaminating completed molecules in preparations of heavy DNA from different experiments there may, however, be some variations in the ratio between concentration of single-stranded DNA and radioactivity.

[b] The probe DNA was degraded to an approximate size of 350 nucleotides by boiling for 20 min in 0.3 M NaOH before hybridization.

[c] Less than 4% of the probe DNA was recovered in the duplex fraction after incubation without heavy DNA.

[d] In this experiment the extracted DNA was sheared by passage twice through a 25-gauge syringe needle prior to equilibrium gradient centrifugation to increase the capacity of the gradient.

TABLE 2. *Percentage of ^{3}H-labeled sonicated heavy DNA which is eluted with 0.14 M phosphate buffer after chromatography on hydroxylapatite*[a]

Expt	% Heavy DNA eluted with 0.14 M phosphate buffer
1	24
2	20
3	20
4	18

[a] Infected cells which had been pulse labeled for 45 min were extracted and the total intracellular DNA was fractionated by equilibrium gradient centrifugation. Fractions corresponding to heavy DNA were pooled and sonicated before passage over hydroxylapatite columns.

results showed that 50 to 65% of the ^{3}H-labeled DNA became double stranded after incubation with both L- and H-strand DNA (Table 3). A significant fraction of the single-stranded DNA also self-hybridized when incubated under annealing conditions without addition of unlabeled single strands of Ad2 DNA (Table 3). This explains why more than 50% of the labeled DNA in most experiments was recovered as duplex DNA when hybridized with either one of the complementary strands.

Sussenbach and van der Vliet (20) have shown that single-stranded viral DNA accumulates in infected cells which have been grown in the presence of hydroxyurea. We have isolated DNA from infected cells which were treated with 10^{-2} M hydroxyurea at the time of infection. The cells were washed with warm medium 18 h after infection and then incubated in the presence of 4 μCi of [^{3}H]thymidine per ml for 30 min. The total intracellular DNA was extracted and fractionated on CsCl gradients. The labeled DNA from hydroxyurea-treated cultures contained considerably more single-stranded DNA as compared with cultures which were grown in absence of the drug (not shown). Heavy DNA was isolated, as described above, and degraded by sonication. Single-stranded DNA, labeled with [^{3}H]thymidine was collected after chromatography on hydroxylapatite and used in hybridizations with unlabeled L- and H-strand

TABLE 3. *Hybridization of ^{3}H-labeled single strands from heavy DNA with unlabeled L- and H-strand Ad2 DNA*

Expt	Unlabeled DNA	% ^{3}H-labeled DNA in duplex[a]
1	0	12[b]
	L-strand	66
	H-strand	59
2	0	38
	L-strand	59
	H-strand	66
3	0	17
	L-strand	57
	H-strand	68
4[c]	0	11
	L-strand	48
	H-strand	54
5[c]	0	24
	L-strand	50
	H-strand	56

[a] In experiments 2 and 3 two different concentrations of unlabeled L- and H-strands were used which gave identical results.

[b] Samples which were not incubated under annealing conditions contained less than 4% of DNA in duplex.

[c] DNA was isolated from cells which had been treated with hydroxyurea.

DNA. The results showed that single strands which were generated in the presence of hydroxyurea also hybridized with both strands of the viral DNA (Table 3).

DISCUSSION

Experiments performed according to the procedure of Danna and Nathans (3) reveal termination sites at both ends of the Ad2 genome. This finding is in agreement with the results of Horwitz (9) which showed that the two molecular halves of Ad2 DNA have the same specific activity when newly completed DNA molecules were analyzed after pulse labeling for short periods of time. For DNA molecules which are replicated as proposed by Cairns (1) this would suggest an internal origin of replication. However, available experimental data convincingly show that the two strands of adenovirus DNA replicate asynchronously (5, 12, 19, 21, 24) which makes the interpretation of the results more difficult. Sussenbach and his colleagues (5, 19, 21) have proposed that replication of Ad5 DNA always starts at the AT-rich end (the right hand end). Assuming that Ad2 DNA replicates in an identical way one would expect to find a termination site at the left hand end as we have demonstrated after cleavage of pulse-labeled DNA with EndoR·*HpaI* and EndoR·*HindIII*. From the Sussenbach model several additional termination sites would be expected from the so-called complementary strand synthesis. If displacement synthesis predominantly leads to duplex daughter molecules and free single strands, a strong termination site could be expected also at the right hand end if one assumes that replication only rarely initiates internally on the displaced single strand. However, we have found by hybridization experiments that both complementary strands of Ad2 DNA are exposed in cells which contain replicating molecules; a finding which is in agreement with the results reported by Lavelle et al. (10). The results are in conflict with those of Sussenbach et al. (19) and would be compatible with a mechanism of replication which allows initiation to take place at either end of the adenovirus DNA. It is noteworthy that the two ends of adenovirus DNA are identical because of the presence of an inverted terminal repetition (7, 26) and the ends may thus serve as recognition sites for the DNA polymerase. The discrepancy between our results and those of Sussenbach et al. (19) are puzzling. One possibility is that replication during the first round(s) initiates at one specific end, but later proceeds according to another scheme which leads to displacement of both strands. When comparing different studies on adenovirus DNA replication it is important to remember that all investigations so far, including our own, have been made under extraordinary conditions, such as growth in the presence of bromodeoxyuridine, infection at high multiplicities, or synthesis in isolated nuclei. Also, techniques used for selective extraction and purification of replicating DNA may preferentially lose molecules which are of particular importance for replication. Therefore the results should be interpreted with caution until experimental conditions are available which allow studies of replicating molecules under more natural conditions.

We have recently learned that E. Winnacker and co-workers (personal communication) have independently demonstrated termination sites for Ad 2 DNA replication near both ends of the viral DNA.

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