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Studies on the Mechanism of Replication of Adenovirus DNA

IV. Discontinuous DNA Chain Propagation[1]

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The replication of adenovirus type 5 DNA occurs by discontinuous chain propagation via short pieces of DNA. These pieces accumulate if the infected cells are treated with hydroxyurea. They have a sedimentation coefficient of 11 S corresponding to a molecular weight of about 700,000, and they contain sequences derived from all parts of the adenovirus genome. Annealing and hybridization studies show that the short pieces represent sequences complementary to both strands of adenovirus type 5 DNA.

INTRODUCTION

Adenovirus type 5 (Ad5) DNA has a linear duplex structure and a molecular weight of 22.8×10^6 (van der Eb and van Kesteren, 1969). Evidence has been presented showing that the replication of Ad5 DNA proceeds in a semi-conservative fashion (van der Vliet and Sussenbach, 1972). Based on both biochemical and electron microscopic data a displacement model for the replication of Ad5 DNA has been proposed in which replication takes place via linear intermediates (Sussenbach *et al.*, 1972; Ellens *et al.*, 1974). According to this model, replication starts from the A-T-rich end of the molecule and proceeds by displacement of the H-strand, i.e., the strand with the higher equilibrium density in alkaline CsCl. This so-called displacement synthesis on the L-strand proceeds until a double-stranded daughter molecule of genome size is generated. Then synthesis of the complementary strand occurs on the displaced H-strand. Occasionally this synthesis of the complementary strand starts before the displacement synthesis has been completed. The question whether the synthesis of both complementary strands proceeds continuously or discontinuously has not yet been answered.

In a variety of organisms DNA is synthesized discontinuously. Chain propagation proceeds by the synthesis of small pieces of DNA linked to short stretches of RNA. These short pieces of DNA have different sizes ranging from 4–5 S in polyoma and SV40 up to 8–11 S in bacteria and bacterial viruses (Okazaki *et al.*, 1968; Magnusson *et al.*, 1973; Fareed and Salzman, 1972).

Replicative intermediates in the synthesis of DNA of the human adenoviruses types 2 and 5, upon alkaline denaturation, appear to contain pieces of DNA smaller than genome size (Horwitz, 1971; van der Eb, 1973). However, most of the pieces have sedimentation coefficients between 10 and 34 S, the latter being the coefficient of single strands of genome size in alkaline sucrose gradients. From these data it has been concluded that chain elongation occurs by sequential addition of single nucleotides to the growing DNA rather than by joining of short pieces. In contrast to the human adenoviruses it has been shown that the replication of CELO DNA in chicken cells proceeds in a discontinuous fashion (Bellett and Younghusband, 1972). Replicative intermediates from this type of DNA appear to contain short pieces of about 12 S.

[1] Part of this work has been presented at the 39th Cold Spring Harbor Symposium on Quantitative Biology 1974.

Magnusson (1973) and Laipis and Levine (1973) have reported that the existence of short pieces in replicative intermediates of polyoma and SV40 DNA can be readily demonstrated if DNA synthesis is inhibited by hydroxyurea (HU). This drug lowers the levels of the intracellular deoxyribonucleoside triphosphate pools (Skoog and Nordenskjöld, 1971) by inhibition of the enzyme ribonucleotide reductase (Krakoff *et al.*, 1968). The accumulation of short pieces in the presence of HU is probably due to an inhibition of the joining step (Laipis and Levine, 1973).

In our studies on the mechanism of Ad5 DNA replication we were able to show discontinuity in chain propagation by the use of HU.

MATERIALS AND METHODS

Cell culture and virus infection. The growth of KB cells, the purification of Ad5 and its DNA were performed as before (van der Vliet and Sussenbach, 1972). KB cells were infected at a concentration of 5×10^5 cells/ml medium (Sussenbach *et al.*, 1973). The infection with Ad5 was performed at a multiplicity of infection of 2000–3000 physical particles per cell.

Conditions of labeling and isolation of new viral DNA. HU (Sigma, St. Louis, MO) was added 18 hr after infection at a concentration of 10^{-2} *M*. After 30 min of incubation in the presence of HU the infected cells were concentrated in the same medium to 5×10^6 cells/ml and subsequently exposed to radioactive thymidine (methyl-[^{3}H]thymidine, 52 Ci/mmole, Radiochemical Centre, Amersham, England) for a period of 30 min. Incorporation of radioactivity was stopped by dropping the incubation mixture into ice-cold phosphate buffered saline containing 1 m*M* nonradioactive thymidine. Viral DNA synthesized in the presence of HU was selectively extracted according to a modified Hirt procedure (van der Vliet and Sussenbach, 1972). The Hirt supernatant fluid was fractionated by centrifugation through a neutral sucrose gradient. Fractions containing viral DNA were pooled. The viral DNA was then separated from residual cellular DNA by CsCl equilibrium density gradient centrifugation. The replicative viral DNA was analyzed on alkaline isokinetic sucrose gradients (van der Vliet and Sussenbach, 1972).

Hydroxyapatite chromatography. DNA was subjected to chromatography on hydroxyapatite (HAP) columns.(0.5 cm × 0.5 cm) as described by Flavell *et al.* (1972). HAP was obtained from BioRad (Richmond, VA). Sodium phosphate (NaP) buffers at pH 6.8 were used for elution. DNA in 0.05 *M* NaP was bound to the column at room temperature. Then the temperature was raised to 60° and single-stranded DNA molecules were eluted with 0.15 *M* NaP, while further elution with 0.4 *M* NaP yielded double-stranded DNA molecules.

Annealing conditions. After sonication single-stranded DNA was annealed at 80° for different periods of time in 1 *M* CsCl, 0.015 *M* trisodium citrate, 0.001 *M* EDTA, pH 7.5, in closed ampoules coated with bovine serum albumin. After annealing, the samples were diluted to a salt concentration of less than 0.05 *M* and further analyzed by HAP chromatography. Filter hybridization was carried out according to Aloni *et al.* (1969). Sonically treated and denatured Ad5 DNA was bound to Millipore filters and incubated with sonicated single stranded DNA in 0.04% bovine serum albumin, 0.3 *M* NaCl, 0.03 M trisodium citrate and 0.002 *M* EDTA, pH 7.5, at 70° for 16 hr.

Separation of complementary strands of Ad5 DNA. The complementary strands of Ad5 DNA were separated by buoyant density gradient centrifugation with poly(U, G) as described by Tibbetts *et al.* (1974). Poly(U, G), purchased from Schwarz/Mann (lot no. 7001, U/G ratio 3.3), was kindly supplied by Dr. L. Philipson. The strand (H-strand) with a 2–3 mg higher buoyant density than its complement (L-strand) in alkaline CsCl appeared in the less dense complex with poly(U, G). A similar inversion in the relative positions of the separated strands in poly(U, G) and alkaline CsCl gradients, respectively, has also been observed for Ad2 DNA (Tibbetts *et al.*, 1974). In this paper the H-strand corresponds to the strand with the higher

density in alkaline CsCl.

Fragmentation of Ad5 DNA. Ad5 DNA was digested as described by Sharp *et al.* (1973), with a restriction endonuclease from *Haemophilus parainfluenzae* (Hpa I). Hpa I was a generous gift from Dr. R. J. Roberts. Separation of the fragments was carried out by agarose gel electrophoresis, as described by Sharp et al (1973), in 0.7% agarose. For the isolation of the Hpa I fragments the gels were run in electrophoresis buffer containing 0.5 μg/ml ethidium bromide. Examination of the gels in direct illumination from a uv light source reveals the exact position of the DNA fragments. The fluorescent bands of DNA were cut from the gels and the DNA was recovered by electrophoresis into dialysis sacs.

RESULTS

HU was added 18 hr after infection to Ad5-infected KB cells at a concentration of 10^{-2} *M*. At this time after infection only viral DNA is synthesized as judged by CsCl density gradient centrifugation. At different times after administration of HU, DNA synthesis was measured by incorporation of [^{3}H]thymidine for periods of 10 min (Fig. 1). Within 30 min, DNA synthesis is reduced to less than 5% of the control value. The residual DNA synthesis remains constant for a period of 70 min.

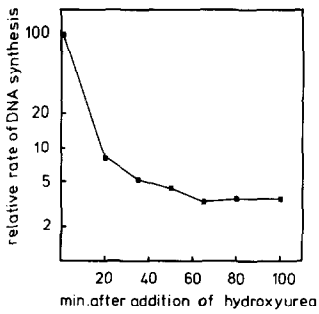

FIG. 1. Time course of viral DNA synthesis during inhibition with HU. At 18 hr p.i., HU was added to infected cells at a concentration of 10^{-2} *M*. At different times after administration of HU, DNA synthesis was measured by incorporation for periods of 10 min of [^{3}H]thymidine in acid-precipitable material. The relative rate of DNA synthesis in the presence of HU is expressed as the percentage of DNA synthesis in the absence of HU during a 10 min pulse with [^{3}H]thymidine.

The viral DNA synthesized in the presence of 10^{-2} *M* HU was further characterized. After 30 min of incubation in the presence of the drug, DNA synthesis was allowed for another 30 min in the presence of [^{3}H]thymidine. The pulse-labeled viral DNA was selectively extracted by a modified Hirt procedure (van der Vliet and Sussenbach, 1972) and subjected to the following purification steps. The pulse-labeled viral DNA was separated from low molecular weight material by isokinetic neutral sucrose gradient centrifugation (Fig. 2A). Fractions containing the replicating viral DNA, which sediments from 31 to about 60 S (van der Vliet and Sussenbach, 1972) were pooled. Subsequently this

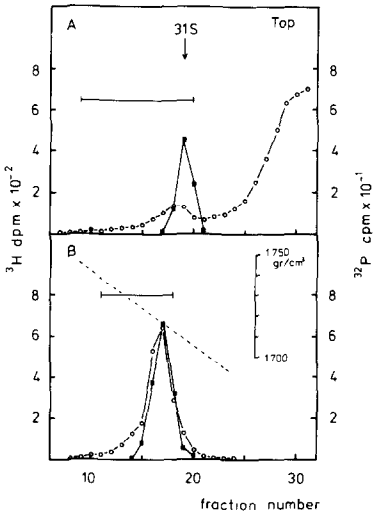

FIG. 2. (A) Neutral sucrose gradient centrifugation of viral DNA labeled with [^{3}H]thymidine from 30–60 min after HU administration. Labeling and isolation of viral DNA were performed as described in Materials and Methods. The viral DNA was centrifuged on an isokinetic sucrose gradient containing 0.2 *M* NaCl, 0.01 *M* Tris, pH 8.1, 0.001 *M* EDTA and 0.1% sarkosyl for 16 hr at 21,000 rpm and 5° in a Spinco SW 25.1 rotor. The fractions containing replicating DNA were pooled as indicated by the bar. (B) CsCl equilibrium centrifugation of replicating DNA. DNA of the pooled sucrose gradient fraction (A, above) was subjected to CsCl gradient centrifugation in 0.15 *M* NaCl, 0.015 *M* trisodium citrate, 0.1% sarkosyl, pH 7.5, at 38,000 rpm for 65 hr at 10° in a Spinco 50 Ti angle rotor. The fractions containing viral DNA were pooled as indicated by the bar. --O--, ^{3}H-labeled Ad5 DNA; --■--, ^{32}P-labeled Ad5 marker DNA.

DNA was centrifuged to equilibrium in CsCl to separate viral DNA from remaining host DNA (Fig. 2B). The fractions with densities between 1.716 g/cm^3 and 1.732 g/cm^3 containing the viral replicating DNA were pooled and further analyzed by alkaline sucrose gradient centrifugation.

A typical sedimentation pattern of pulse-labeled viral DNA synthesized in the presence of HU is shown in Fig. 3A. Most of the pulse-labeled DNA is present as short pieces sedimenting between 8 and 20 S. The peak found at 11 S corresponds to a molecular weight of about 700,000 (Studier, 1965). In different preparations the peak had sedimentation coefficients varying between 11 and 13 S. A small percentage of the labeled DNA sedimented near, but never exactly at the marker position (Fig. 3A). The pieces generated in the presence of HU can be chased into longer strands after removal of HU (Fig.3B). It is known from earlier observations that the inhibition of Ad5 DNA synthesis by HU is readily reversible (Sussenbach and van der Vliet, 1973 and Ellens *et al.*, 1974). Our results show that the short pieces are probably transient forms in the replication of Ad5 DNA. The appearance of these short pieces is not due to breakage of longer strands. We reached this conclusion from the observation that viral DNA prelabeled for 20 min with [^{14}C]thymine 2 hr before administration of HU remains of genome size during residual DNA synthesis in the presence of HU (data not shown).

The distribution of these short pieces along the adenovirus genome has been studied using a restriction endonuclease, Hpa I. Ad5 DNA is digested by Hpa I into 7 fragments, which are denoted a, b, c, d, e, f and g depending on their electrophoretic mobility on gels (Mulder *et al.*, 1974). In the following experiments only the fragments a, b, c and d, representing 89.7% of the Ad5 DNA, were considered. Replicating molecules synthesized in the presence of HU were isolated from a neutral sucrose gradient as indicated in Fig. 2A. These molecules were incubated with Hpa I during 16 hours at 37° and the digestion products were analyzed on agarose gels as described by Sharp *et al.* (1973). Fig. 4

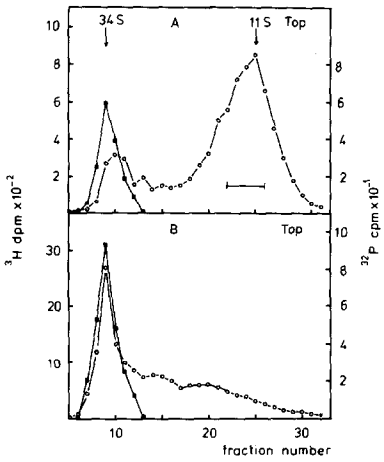

FIG. 3. Alkaline isokinetic sucrose gradient centrifugation of viral DNA synthesized in the presence of HU. Replicating viral DNA was centrifuged on an alkaline sucrose gradient containing 0.3 *M* NaOH, 0.9 *M* NaCl, 0.01 *M* Tris, 0.001 *M* EDTA and 0.1% sarkosyl for 16 hr at 36,000 rpm and 5° in a Spinco SW 41 rotor. Fractions containing DNA with a sedimentation coefficient of about 11 S were pooled as indicated by the bar and used for further analysis. (A) Replicating viral DNA labeled for 30 min with [^{3}H]thymidine in the presence of HU was obtained as described in Fig. 2. (B) Replicating viral DNA was labeled for 30 min with [^{3}H]thymidine in the presence of HU and then incubated for 45 min in the absence of HU in medium containing 2.5 × 10^{-4} *M* unlabeled thymidine. This DNA was isolated by neutral sucrose gradient and CsCl gradient centrifugation as described in Fig. 2. --O--, ^{3}H-labeled Ad5 DNA; --■-- ^{32}P-labeled Ad5 marker DNA (34S).

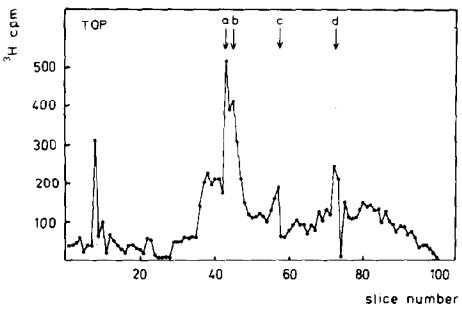

FIG. 4. Agarose gel electrophoresis of Hpa I-digested replicating viral DNA synthesized in the presence of HU. Replicating viral DNA was obtained as described in Fig. 2A. The arrows indicate the positions of Ad5 fragments obtained after digestion of ^{32}P-labeled mature Ad5 DNA. After electrophoresis the gels were cut into slices of 1.5 mm and radioactivity was measured.

shows the electrophoretic pattern; the fragments e, f and g have run off the gel. Obviously there is radioactivity comigrating with the Hpa I marker fragments a, b, c and d, indicating that the short pieces are derived from all parts of the adenovirus genome. A considerable part of the radioactivity does not comigrate with the marker fragments. This may be caused by the presence in the fragments of the single-stranded stretches from replicating molecules, which may affect the electrophoretic mobility. Furthermore, the Hpa I recognition sites may be in single-stranded form that prevents the endonuclease action. That the short pieces were related to all the Hpa I fragments considered could also be demonstrated in another way. Mature Ad5 DNA was digested and the fragments a, b, c and d were recovered in quantity from the gels. These fragments were sonicated, denatured and subsequently bound to filters (Aloni, 1969). Short pieces generated in the presence of HU were isolated from alkaline sucrose gradients as indicated in Fig. 3A. The isolated short pieces were hybridized with the filter-bound Hpa I fragments. It could be demonstrated that the short pieces hybridized with all Hpa I fragments considered. As shown in Table 1 the percentage of hybridization corresponds roughly to the relative size of the Hpa I fragments. These results indicate again that the short pieces generated in the presence of HU were derived from all parts of the genome.

To answer the question whether these short pieces originate from both strands or from one particular strand, the following experiments were performed. Short pieces were obtained as described in Table 1. They were mainly single stranded as shown by HAP chromatography. A small portion (about 15%) behaved as double-stranded DNA, probably due to the presence of internal loops (Tibbetts *et al.*, 1973). In order to determine whether the short pieces contain complementary sequences, annealing was allowed in the presence of 1 *M* CsCl, 0.015 *M* trisodium citrate, 0.001 *M* EDTA at 80° for different periods of time (Table 2). Repeated analysis showed that the short pieces do contain complementary sequences for about 65%, indicating that the short pieces originate from both strands of Ad5 DNA. The remaining 35% of

TABLE 1

HYBRIDIZATION OF SHORT PIECES WITH ISOLATED HPA I FRAGMENTS[a]

Hpa I fragments	Short pieces hybridized (%)	Relative size (%)
a	10.1	32.3
b	7.4	27.6
c	6.3	20.2
d	6.0	9.6

[a] Short pieces labeled with ^3H were pooled as indicated in Fig. 3A. The pooled short pieces were neutralized and dialyzed against 0.015 *M* trisodium citrate, 0.001 *M* EDTA, pH 7.5, for 48 hr and concentrated by ethanol precipitation. The short pieces were hybridized with Hpa I fragments. Two micrograms of Ad5 DNA were digested with Hpa I into 7 fragments. Each of the 4 larger fragments was isolated from agarose gels, and after sonication and denaturation immobilized on filters. The 4 types of fragments were hybridized separately with ^3H-labeled short pieces as described in Materials and Methods. The concentration of the short pieces was below detection level with uv spectrophotometry. The hybridization is expressed as the percentage of input radioactivity (5234 dpm) bound to the DNA on duplo filters after correction for aspecific binding. The data on the relative size of the Hpa I fragments were obtained from Mulder *et al.* (1974).

TABLE 2

ANNEALING OF ISOLATED SHORT PIECES[a]

Time of annealing (hr)	ss (%)	ds (%)
0	85	15
24	60	40
48	41	59
72	30	70
140	41	59

[a] Short pieces labeled with ^3H and isolated as described in Table 1 were allowed to anneal for different periods of time (see Materials and Methods). After annealing the DNA was analyzed by HAP chromatography. The results are expressed as the percentage of input radioactivity (500 dpm) in single-stranded (ss) or double-stranded (ds) DNA respectively. The total radioactivity recovered from the column was almost 100% of the radioactivity applied.

the small pieces may be derived from one particular strand.

Another approach to establish that the small pieces were derived from both complementary strands is to determine whether the fragments hybridize with the separated strands of Ad5 DNA. Separated strands of Ad5 DNA were obtained as described in Materials and Methods and bound to filters. The isolated short pieces (Table 1) were allowed to hybridize with the separated strands for 16 hr at 70° in 0.3 *M* trisodium citrate, 0.002 *M* EDTA, pH 7.5, and 0.4% bovine serum albumin. Table 3 shows that the short pieces hybridize for 18% with the L-strand and for 12% with the H-strand. These results support the previous conclusion that the short pieces originate from both strands and show in addition that sequences of the H-strand type are present in excess. Although the excess of H-strand sequences is found in isolated short pieces, this excess might be a general property of DNA synthesized in the presence of HU. To investigate this possibility the total replicative viral DNA generated in the prescence of HU (Fig. 2A) was hybridized with the L- and H-strand respectively (Table 3). Again there is a preference for hybridization with the L-strand showing that the excess of H-strand sequences is a general property of viral DNA synthesized in the presence of HU. The percentage of hybridization in this case is lower than with the isolated short pieces. This is probably due to the competition of labeled DNA with unlabeled DNA present in the total replicative viral DNA.

TABLE 3

HYBRIDIZATION OF REPLICATING VIRAL DNA WITH SEPARATED STRANDS OF Ad5 DNA[a]

	Hybridization with H-strand (%)	Hybridization with L-strand (%)	Relative hybridization L-strand/H-strand
Isolated short pieces	12	18	1.5
Total replicative viral DNA	8	11	1.4

[a] Viral DNA labeled with ^3H and synthesized in the presence of HU was hybridized with the separated strands from Ad5 DNA. Three-tenths of a microgram of DNA from each of the separated strands was immobilized on a filter. The separated strands were hybridized with short pieces isolated as described in Table 1 and with total replicative viral DNA (Fig. 2A). The hybridization is expressed as the percentage of input radioactivity (ca. 5000 dpm) bound to duplo filters after correction for aspecific binding.

DISCUSSION

In the present investigation HU has been used to show discontinuity in chain propagation during Ad5 replication. HU appears to be a potent inhibitor of viral DNA synthesis. Within 30 min after addition of HU, DNA synthesis has decreased to a level of 5% of the original value and thereafter remains constant for at least 70 min. The product of the residual DNA synthesis is of viral origin as could be concluded from CsCl density gradient centrifugation of pulse-labeled DNA. Analysis of this DNA under alkaline conditions showed the presence of short stretches of single-stranded DNA with a sedimentation coefficient of about 11 S.

The short pieces are transient forms in Ad5 DNA replication, since in the absence of HU they can be chased into longer pieces. This observation further indicates that the effect of HU is reversible. The possibility that the short pieces are generated by the breakage of pre-existing chains has been excluded.

The short pieces originate from all parts of the adenovirus genome as shown by digestion of replicating viral DNA with Hpa I and hybridization of short pieces with Hpa I fragments. Assuming that the short pieces have a molecular weight of 700,000, about 15 pieces can be aligned along the genome. The short pieces anneal for about 65% indicating that they are derived from both complementary strands of Ad5 DNA. About 35% of the short pieces does not anneal even after very long periods, which is probably caused by an unequal distribution of sequences from L- and H-strands. Hybridization of the short pieces with the separated complementary strands of Ad5 DNA shows a relative abundance of pieces of the H-strand type. It is

tempting to assume that the non-annealing fraction of the short pieces is of the H-strand type.

A relative abundance of newly synthesized H-strand sequences is also found in total replicative viral DNA synthesized in the presence of HU. The relative preponderance of pieces of the H-strand type probably reflects a difference in the rates of synthesis of the two complementary strands. This difference may be introduced by HU. Earlier observations (Sussenbach and van der Vliet, 1973) have shown that after removal of HU from cells, which were infected in the presence of HU, the synthesis of the two complementary strands is highly asynchronous. Only displacement synthesis takes place leading to an accumulation of H-strands. A preferential synthesis of H-strand sequences might also be induced upon addition of HU at later stages in viral DNA replication. On the other hand a difference in the rate of synthesis of the two complementary strands may be a normal feature of the replication of Ad5 DNA. This is suggested by a relative scarcity of L-strand sequences in replicative intermediates observed by Sussenbach et al. (1973).

Our studies show that chain propagation during Ad5 DNA replication proceeds via the synthesis of short pieces on both parental strands as has been shown for polyoma and SV 40 (Pigiet *et al.*, 1973; Fareed *et al.*, 1973). Such short pieces have also been demonstrated during the replication of CELO virus DNA (Bellett and Younghusband, 1972). In previous studies on the replication of human adenovirus DNA in which no HU was used, such small pieces could hardly be detected (Horwitz, 1971; van der Eb, 1973).

However, recent experiments in which Ad5-infected KB cells were pulse-labeled with [^{3}H]thymidine at 20° have shown an accumulation of short pieces in the absence of HU. These pieces could be chased into strands of genome size (full details will be published elsewhere). These data support our former conclusion that Ad5 DNA replication proceeds discontinuously.

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