VIROLOGY 61, 427–442 (1974)

Studies on the Mechanism of Replication of Adenovirus DNA

III. Electron Microscopy of Replicating DNA

D. J. ELLENS

Ultra Structure Research Unit, State University of Utrecht, Utrecht, The Netherlands

AND

J. S. SUSSENBACH,[1] AND H. S. JANSZ

Laboratory for Physiological Chemistry, State University of Utrecht, Utrecht, The Netherlands

Accepted June 18, 1974

Replicating Ad5 DNA was isolated from nuclei of infected KB cells and studied by electron microscopy. Branched as well as unbranched linear intermediates were observed containing extended regions of single-stranded DNA. The relationship between the branched and unbranched structures was studied during synchronized synthesis in the first round of replication after release of hydroxyurea inhibition. The branched intermediates represented early and the unbranched intermediates late stages in the replication cycle. Digestion of the branched intermediates with Eco RI endonuclease revealed that replication had started at the molecular right end (the A-T-rich end). This was confirmed by partial denaturation mapping of branched intermediates.

These results confirm and extend a tentative model on the mechanism of Ad5 DNA replication.

INTRODUCTION

The genome of adenovirus type 5 (Ad5) has a linear duplex structure of DNA and a molecular weight of 22.8×10^6 (van der Eb and van Kesteren, 1966). Replicating Ad5 DNA has been isolated from nuclei of infected KB cells as well as from intact cells (van der Vliet and Sussenbach, 1972; van der Eb, 1973). Electron microscopy of this DNA has revealed the existence of two classes of replicating intermediates, i.e., branched Y-shaped molecules containing one single-stranded arm, and unbranched linear intermediates containing single- and double-stranded stretches, respectively (Sussenbach, van der Vliet, Ellens and Jansz, 1972; van der Eb, 1973).

The relation between the different types of replicative intermediates is still a matter of speculation. It is most likely that the branched Y-shaped intermediates arise by DNA synthesis which starts at the end of the linear duplex, copying one parental strand and displacing the other. This so-called displacement synthesis generally leads to release of a complete single-strand. The unbranched intermediates may arise during the conversion of the displaced single-strand into a duplex daughter molecule by so-called complementary strand synthesis. The initiation of the complementary strand synthesis may also occur before displacement synthesis has been completed as suggested by the occurrence of Y-shaped molecules which contain a single-stranded arm with double-stranded stretches, although these molecules were observed less frequently.

This paper deals with a more detailed electron microscopic study of the different types of the replicative intermediates, their relationship and the location of the origin of the replication of Ad5 DNA. Some

[1] To whom requests for reprints should be addressed.

preliminary results have been presented earlier (Sussenbach *et al.*, 1973).

MATERIALS AND METHODS

Materials

5-Bromodeoxyuridine and hydroxyurea were obtained from Sigma, St. Louis, MO. 5-Bromodeoxyuridine triphosphate was prepared from deoxyuridine triphosphate according to Chamberlin and Berg (1964).

Tritiated deoxyadenosine triphosphate and deoxycytidine were supplied by the Radiochemical Centre, Amersham. Formamide (Merck, Darmstadt) was purified by extraction with ethyl ether and flash evaporation after removal of the ether by bubbling through of nitrogen gas. All solutions used for electron microscopy were filtered through a 100 nm membrane filter (Sartorius).

Cultivation of Cells and Viruses, Infection, Conditions and Purification of Replicating DNA

The growth of the KB cells, the purification of Ad5 and its DNA, the infection conditions of cells, the incubation of isolated nuclei, as well as the extraction of viral DNA and centrifugation in neutral sucrose and CsCl gradients were performed as before (van der Vliet and Sussenbach, 1972; Sussenbach *et al.*, 1972). The incubation and infection in the presence of hydroxyurea have been described (Sussenbach and van der Vliet, 1973).

Partial Denaturation of DNA

Spontaneous adsorption as described by Lang and Mitani (1970), combined with formamide as denaturating agent, was used for preparation of partially denatured DNA. Fifty-microliter droplets of a solution containing 0.05 μg/ml DNA, 85% formamide, 10 m*M* Tris-HCl, 1 m*M* Na_2-EDTA and 2 μg/ml cytochrome c (pH 8.5) were placed on a Teflon surface. After about 10 min of adsorption, at 21° in a water saturated atmosphere, copper grids with a carbon coated formfar supporting film were lightly touched with the surface.

Formamide Spreading of the Replicative Intermediates

After ethanol precipitation the DNA was dissolved in a solution containing 100 m*M* Tris-HCl, 10 m*M* Na_2-EDTA (pH 8.5). Spreading was performed according to Davis *et al.*, (1971). The spreading solutions contained 100 m*M* Tris-HCl, 10 m*M* Na_2-EDTA, pH 8.5, 0.5-1 μg/ml DNA, 0.2 mg/ml cytochrome c and formamide with final concentrations of 40 or 85% (v/v), corresponding to Tm-52° and Tm-25°, respectively. These solutions were spread under isodenaturating conditions onto a hypophase of 10 or 55% (v/v) formamide, respectively, buffered with 10 m*M* Tris-HCl, 1 m*M* Na_2-EDTA, pH 8.5 in a Teflon trough. Copper grids with carbon coated formfar supporting films were touched with the surface.

Electron Microscopy

Immediately after touching the grids with the cytochrome c surface, they were stained for 30 sec with 50 μ*M* uranyl acetate in 90% ethanol immersed in 2-methylbutane for 10 sec and air dried. The grids were rotary shadowed at an angle of 6° with platinum-palladium (80-20). Electron micrographs were obtained in a "Philips EM 200" electron microscope at a magnification of about 5000 (80 kV, 30 μm objective aperture). Magnification was calibrated with a carbon replica of a diffraction grating (2160 lines per mm, E. F. Fulham). To normalize the length of double- and single-stranded Ad5 DNA the ratio of the lengths of double-stranded to single-stranded Φ*X*174 DNA (Φ*X* RF/Φ*X* SS) was determined. The negatives (Kodalith LR 2572) were enlarged ten times, and the filaments were measured by tracing them with a map measurer, or with a Pencil Follower (D-Mac, Glasgow).

RESULTS

Morphology of Replicating Ad5 DNA

Purified replicating Ad5 DNA for electron microscopy was obtained from nuclei isolated from infected KB cells. These nuclei were incubated in the presence of

the four deoxyribonucleoside triphosphates, while thymidine triphosphate was replaced by 5-bromodeoxyuridine triphosphate (van der Vliet and Sussenbach, 1972). Replicating DNA from this system sedimented in neutral sucrose gradients at 31–80 S and banded in neutral CsCl gradients at higher buoyant densities (1.716–1.753 g/cm^3) than cellular DNA (1.700 g/cm^3) (Sussenbach *et al.*, 1972). Velocity centrifugation of replicating DNA from the nuclear system followed by CsCl equilibrium density centrifugation yielded preparations of replicating Ad5 DNA, which, although not completely free of cellular contaminations, were suitable for electron microscopic investigation (Fig. 1).

The preparations contained the different types of replicating Ad5 DNA as shown below. A heterogeneous class of linear duplex molecules of varying length and generally longer than mature Ad5 DNA was also observed. These molecules showed partial denaturation in a random fashion under conditions more than 20° below the temperature where denaturation of Ad5 DNA starts, which indicates that the heterogeneous material represents contaminating cellular DNA. This material was not further investigated.

About half of the molecules in the preparations consisted of branched and unbranched linear molecules of the same length as mature Ad5 DNA (11 μm) containing double- and single-stranded regions. These properties and their characteristic denaturation pattern (see below) identify these molecules as replicating Ad5 DNA. The branched intermediates accounted for 55% of the replicating Ad5 DNA and displayed a double-stranded stem, and two branches, one single- and the other double-stranded (Fig. 2). The length of the single-stranded branch varied in size from 0.02 up to 0.95 unit length and equaled the length of one of the double-stranded arms in 85% of the molecules (Fig. 3). In the remaining 15% of the molecules the single-stranded branch was shorter than either one of the double-stranded branches which is probably due to shear breakage. No single-stranded

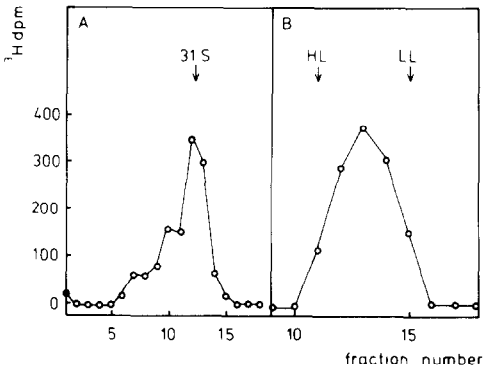


FIG. 1. (A) Isokinetic sucrose gradient centrifugation of new viral DNA synthesized for 2 hr in isolated nuclei in the presence of deoxyribonucleotide triphosphates including [^{3}H]dATP while dBUTP replaced TTP. Further details on the infection conditions and the procedures for DNA synthesis and extraction are indicated in Materials and Methods. Centrifugation was performed 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 19,000 rpm at 5° in the Spinco SW25 rotor. The 31 S position was determined with purified Ad5 DNA as marker. Centrifugation is from right to left.

(B) CsCl density equilibrium centrifugation of new viral DNA present in fractions 5–13 of A. The gradient consisted of CsCl in 0.15 *M* NaCl, 0.015 *M* trisodiumcitrate, 0.01 *M* EDTA pH 7.5, and 0.1% sarkosyl. Centrifugation was performed for 76 hr at 38,000 rpm at 10° in a Spinco R50 rotor. The positions of LL and HL molecules are indicated.

stretches were observed in the double-stranded arms. In a number of Y-shaped molecules the single-stranded branch was partially double-stranded (up to 75% of the length of the branch) (Fig. 3). This duplex region was generally observed at the free end of the single-stranded branch. The distribution of the branching points over the genome showed no distinct maxima (Fig. 3). The faint maximum around 0.6 unit genome length might be a statistical fluctuation due to the restricted number of molecules.

Besides the branched intermediates unbranched linear molecules containing single- and double-stranded regions were found representing 45% of the replicating Ad5 DNA. The percentage of single-strandedness varied strongly in this class of molecules (Figs. 4 and 5). Several molecules were almost completely double-

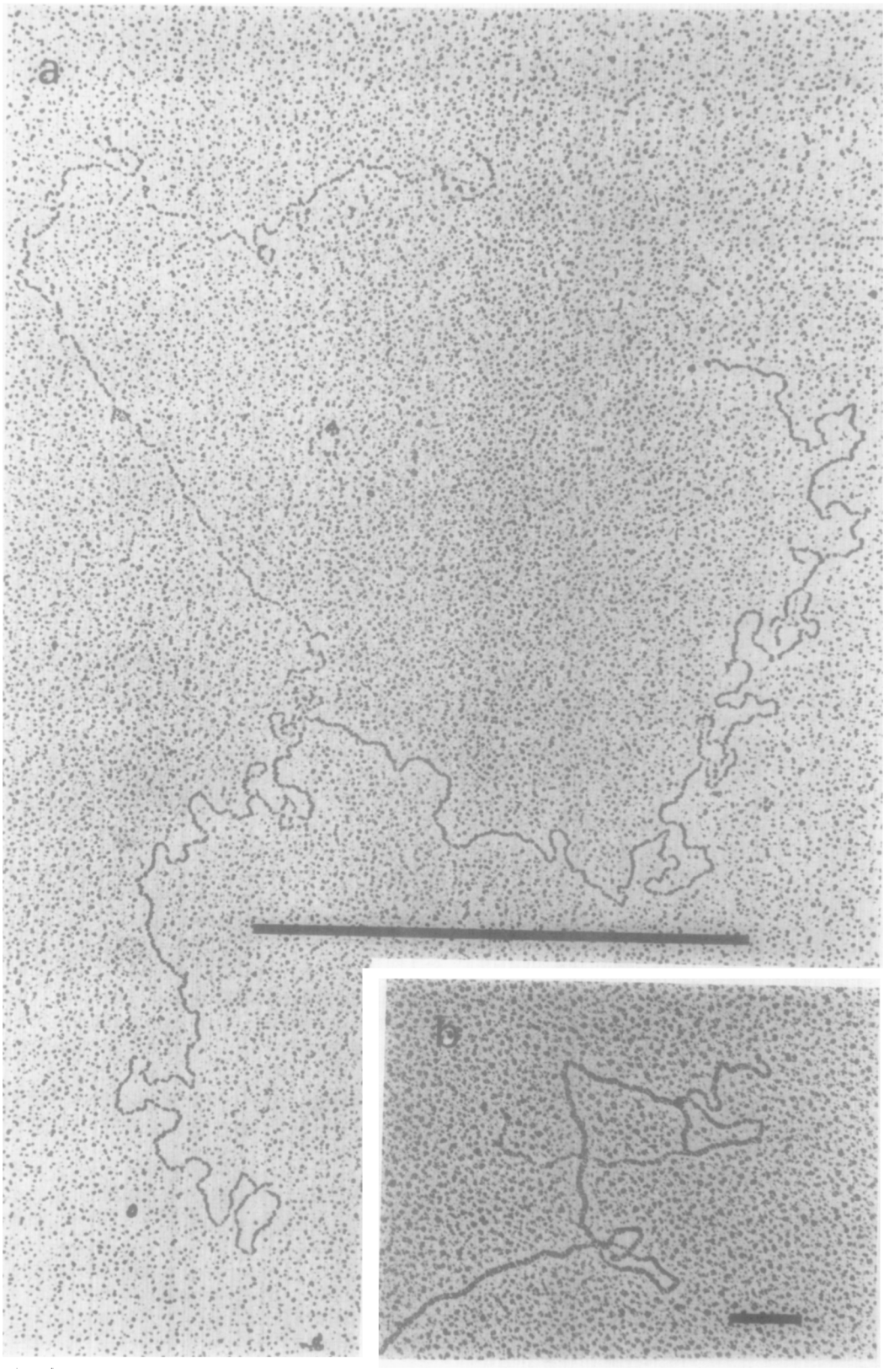


FIG. 2 (a and b)

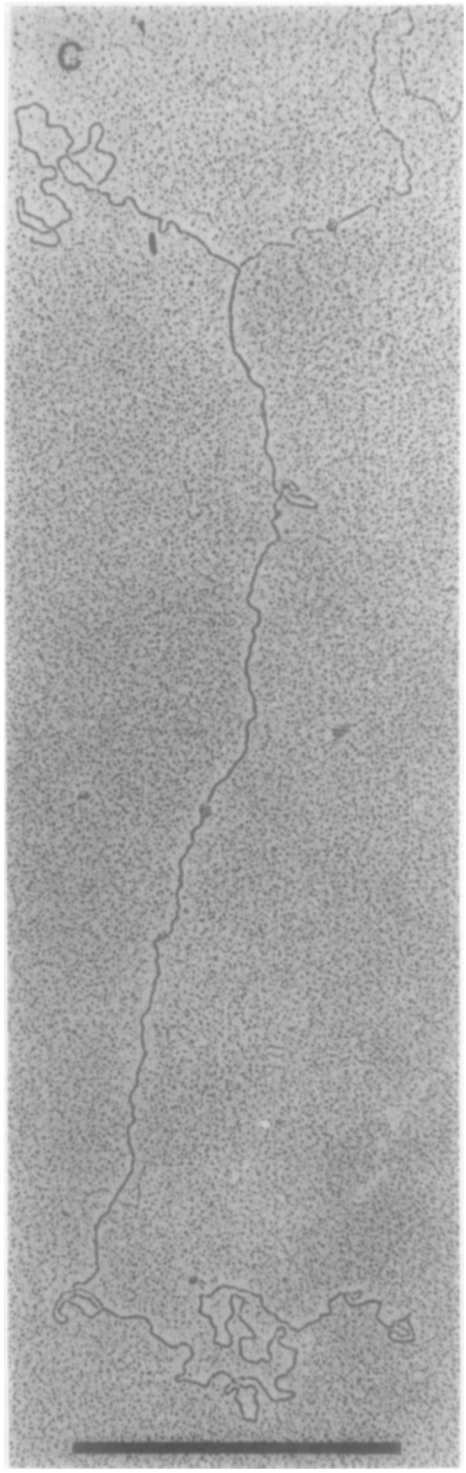


FIG. 2. Branched replicative intermediates of Ad5 DNA, isolated from purified infected nuclei. The DNA was mounted from a 40% formamide solution and layered onto a 10% formamide solution. The length ratio ΦX RF/ΦX SS equals 1.07. (a) Y-shaped molecule; displacement synthesis has progressed as far as 42% of the genome. Bar denotes 1 μm. (b) Detail of a Y-shaped molecule in which 3.5% of the genome has undergone displacement synthesis. Bar denotes 0.1 μm. (c) Y-shaped molecule; the displaced single strand equals 23% of the genome.

stranded and contained only small single-stranded gaps of about 0.3 μm length (Figs. 4c and 5c). Other molecules contained two or more extended single- or double-stranded regions (Fig. 5) indicating internal starts of complementary strand synthesis.

The majority of the unbranched intermediates shows only one duplex and one

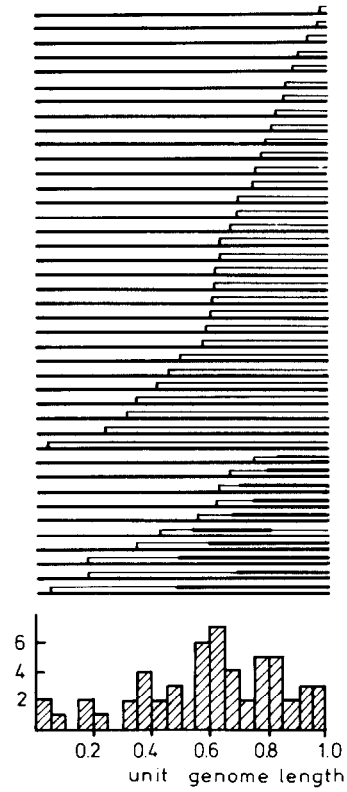
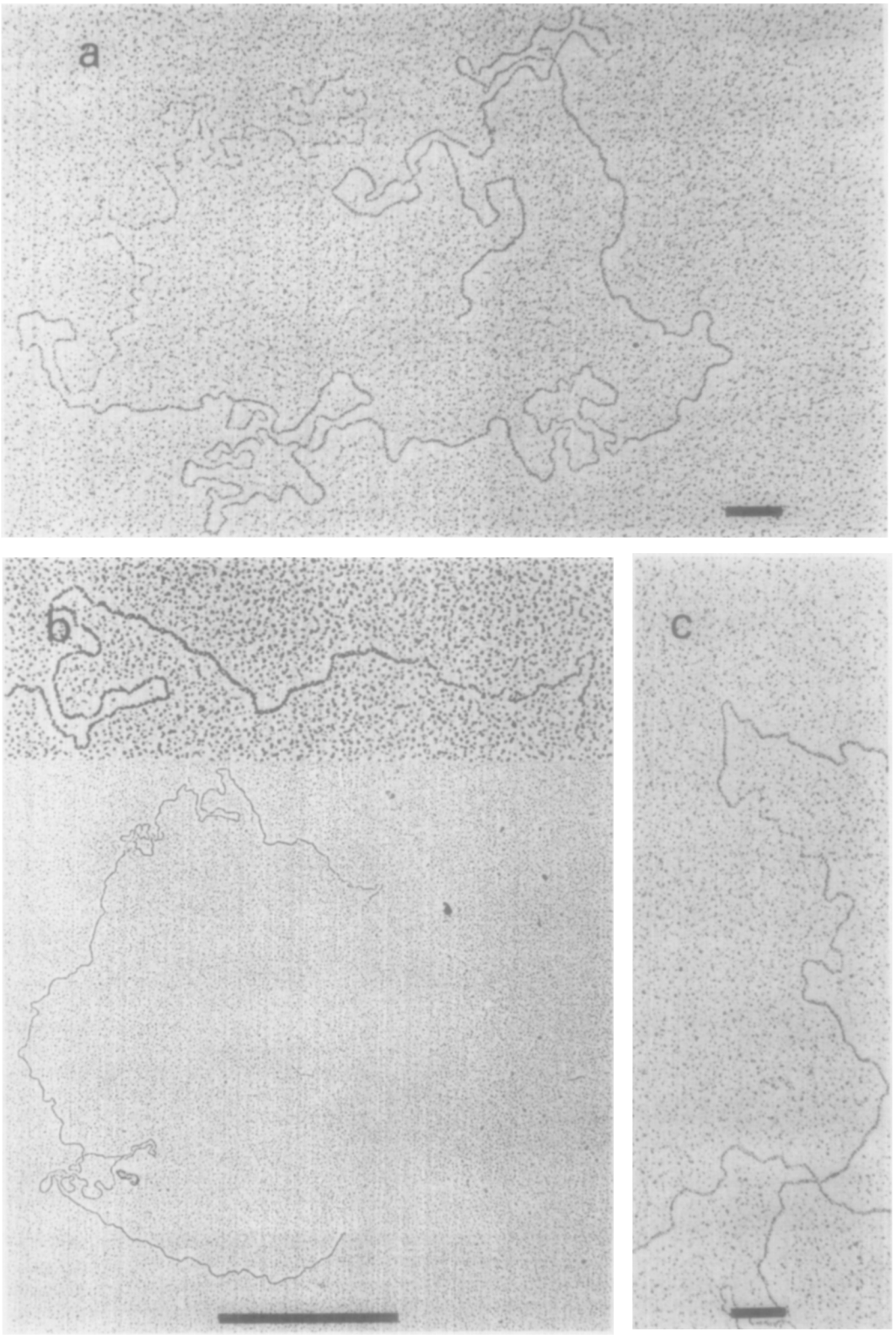


FIG. 3. Array of branched replicative intermediates of Ad5 DNA. Molecules have been normalized to unit genome length. Thick and thin lines represent double- and single-stranded DNA, respectively. All forks have been oriented to the right. In the lower part of the array branched molecules have been displayed with a duplex segment on their single-stranded branch. All normalized distances from the fork to the left end of the duplex have been summarized in the histogram.


a
b
c

single-stranded region (Figs. 4 and 5). The percentage of single-strandedness varied between 3 and 83%, but the double-stranded character dominated in 85% of these molecules. A distribution of the single-strand/double-strand transitions is presented in the histogram of Fig. 5. Two faint maxima can be observed around 0.1 and 0.35 unit length, respectively. Further, also pure single-stranded molecules of 11 μm were observed (not shown here). Several types of these molecules have also been observed in replicating Ad5 DNA isolated from intact infected KB cells (van der Eb, 1973).

Characterization of Replicating Ad5 DNA From Hydroxyurea-Treated Infected Cells

Experiments to determine in which stage of the replication cycle the different types of intermediates were involved, were highly facilitated by synchronization of the DNA replication. We have used hydroxyurea (HU), an inhibitor of DNA synthesis, to obtain a synchronous initiation of replication of Ad5 DNA (Sussenbach and van der Vliet, 1973). Cells were infected in the presence of 10^{-2} *M* HU. After 18 hr of incubation, HU was removed by washing and the cells were further incubated in the presence of [^{3}H]deoxycytidine and 5-bromodeoxyuridine. Under these conditions the first round of replication of Ad5 DNA starts immediately after removal of HU and takes about 15–20 min, (Sussenbach and van der Vliet, 1973). Cells were collected at 7 and 30 min after removal of HU, respectively. Viral DNA was extracted and purified by subsequent sucrose gradient and CsCl gradient centrifugations (Fig. 6). The viral DNA binding in neutral CsCl between the light and hybrid densities was studied by electron microscopy and the frequency of branched and unbranched intermediates was determined (Table 1). Early in replication preferentially branched

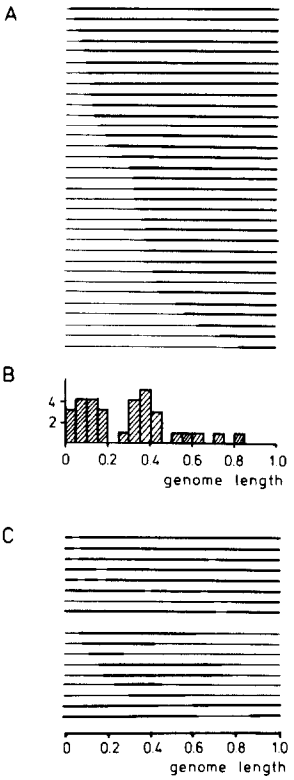


FIG. 5. Array of unbranched replicative intermediates of Ad5 DNA molecules which have been normalized to unit genome length. Thick and thin lines represent double- and single-stranded DNA, respectively. (a) Intermediates with a single-stranded molecular end. All single-stranded ends are oriented to the left. (b) A histogram of all normalized distances from the simplex-duplex transition to the single-stranded molecular end. (c) Linear intermediates with several single- or double-stranded regions. The upper half of the array represents gapped molecules.

intermediates were found, while the unbranched intermediates appeared later in replication. This suggested that replication started at an end of the linear duplex and proceeded by displacing one of the parental strands leading to the appearance of branched molecules. Later, the displaced strand might be completed into a duplex molecule via unbranched intermediates. Due to the limitations of the method of

FIG. 4. Linear single-strand containing replicative intermediates of Ad5 DNA from purified infected nuclei. The DNA was mounted as in Fig. 2. (a) Linear molecule with a single-stranded end representing 27% of the genome. Bar denotes 0.1 μm. (b) Linear molecule with a single-stranded end representing 7% of the genome. Bar denotes 1 μm. The inset shows an enlargement of the single-stranded end. (c) Detail of a linear molecule with a single-stranded gap of 3% of the molecular length. Bar denotes 0.1 μm.

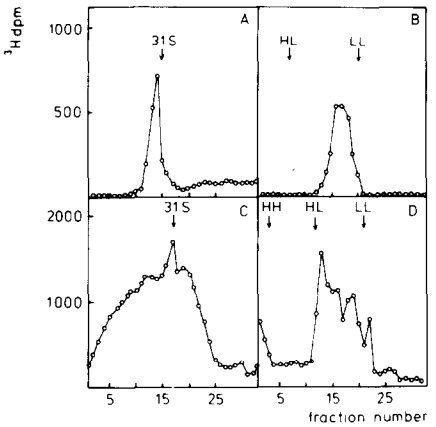


FIG. 6. Sucrose gradient centrifugations of new viral DNA synthesized in infected cells in the presence of [^{3}H]deoxycytidine and 5-bromodeoxyuridine after release of a HU-block during 7 min (A) and 30 min (C), respectively. Fractions 11–17 of A and 1–17 of C were pooled and centrifuged on CsCl density gradients (B and D, respectively). The conditions of centrifugation were as described in Fig. 1.

synchronization a more detailed analysis of the relationship between the different types of unbranched intermediates was not possible.

Analysis of the replicating Ad5 DNA synthesized during 30 min after removal of HU revealed the presence of some branched molecules with two single-stranded branches, which were not observed in DNA preparations from untreated cells (Fig. 7). The lengths of both single-stranded branches in a particular branched intermediate always lined up with one and the same double-stranded arm (Fig. 8). This suggested that under the conditions mentioned above, initiation of replication in a particular molecule always occurred at the same molecular end. Further, it could be concluded that new rounds of replication might start before termination of the previous round.

Spreading of replicating Ad5 DNA from 85% formamide sometimes revealed molecules with a second short single-stranded stretch protruding out of the branching point (Fig. 9). The length of the short stretch varied between 0.1 and 0.6 μm. These structures were not observed when preparations were spread from 50% formamide. The protruding ends were probably caused by so-called branch migration (Fig. 9). This phenomenon has first been described by Lee *et al.* (1970) and occurred preferentially just below the melting temperature of the DNA involved. Backflapping of a part of the displaced strand, removing the displacing end of the new strand, has been proposed as an explanation for this observation. Thanks to this phenomenon it was possible in some branched intermediates to determine which double-stranded arm the single-stranded branch was connected to (Fig. 9). Independently of length measurements it could be determined in this way that in double-initiated molecules both extended single-stranded branches originated from the same double-stranded end, indicating that in these molecules both initiations had occurred at the same molecular end.

The above observations raised the question of which particular molecular end was involved in initiation. The preferential appearance of branched intermediates early after removal of HU offered the opportunity to solve this question using Eco RI endonuclease. This restriction enzyme cuts Ad5 DNA in three fragments, called A, B, and C with a MW of 17.6×10^6, 3.7×10^6, and 1.7×10^6, respectively. Fragment A represents the molecular left end, and fragment B the molecular right end on the physical map of Ad5 DNA as defined below

TABLE 1

FREQUENCY DISTRIBUTION OF REPLICATIVE INTERMEDIATES[a]

Sample	% Branched molecules	% Unbranched molecules
DNA from untreated cells	54 ± 10	46 ± 10
DNA synthesized in HU-treated cells for 7 min after removal of HU	87 ± 12	13 ± 12
DNA synthesized in HU-treated cells for 30 min after removal of HU	40 ± 9	60 ± 9

[a] DNA from untreated and HU-treated infected cells was isolated as described in Figs. 1 and 6 and investigated by electron microscopy as described in Materials and Methods. The ranges indicate the 95% limits of confidence.

(C. Mulder and H. Delius, personal communication). To determine from which molecular end replication started, replicating Ad5 DNA isolated 7 min after removal of HU (Fig. 6) was digested with the Eco RI enzyme. Under these conditions replication had proceeded only for 25% as concluded from the density shift in CsCl gradients. Centrifugation on a neutral sucrose gradient of digested replicating DNA revealed that preferentially fragments B and C were labeled indicating that in the first round of replication after HU treatment, replication preferentially started at the molecular right end (Fig. 10).

Partial Denaturation of Ad5 DNA

In order to demonstrate that initiation of replication in untreated cells proceeded in the same manner as in HU-treated cells, the orientation of the branched intermediates from untreated cells was studied by partial denaturation mapping.

Adsorption of formamide-denatured DNA onto a monolayer of basic protein was used to investigate partial denaturation of small amounts of DNA. It is known that 1% formamide lowers the melting temperature of DNA by 0.6° (Blüthmann *et al.*, 1973). When Ad5 mature DNA was adsorbed from 80% formamide in 10 m*M* Tris–HCl, 1 m*M* Na_2-EDTA, pH 8.5 onto the cytochrome c surface, about 10% of the molecules showed small denatured regions. At 85% formamide, more than 90% of the 95 Ad5 DNA molecules inspected were partially denatured and showed a recognizable left and right end. The left side of the molecule was defined as that half which showed the least and smallest denaturation sites, according to Inman (1966). The lengths of the denatured molecules were normalized to unit length while the lengths of the denatured and undenatured regions were adjusted proportionally.

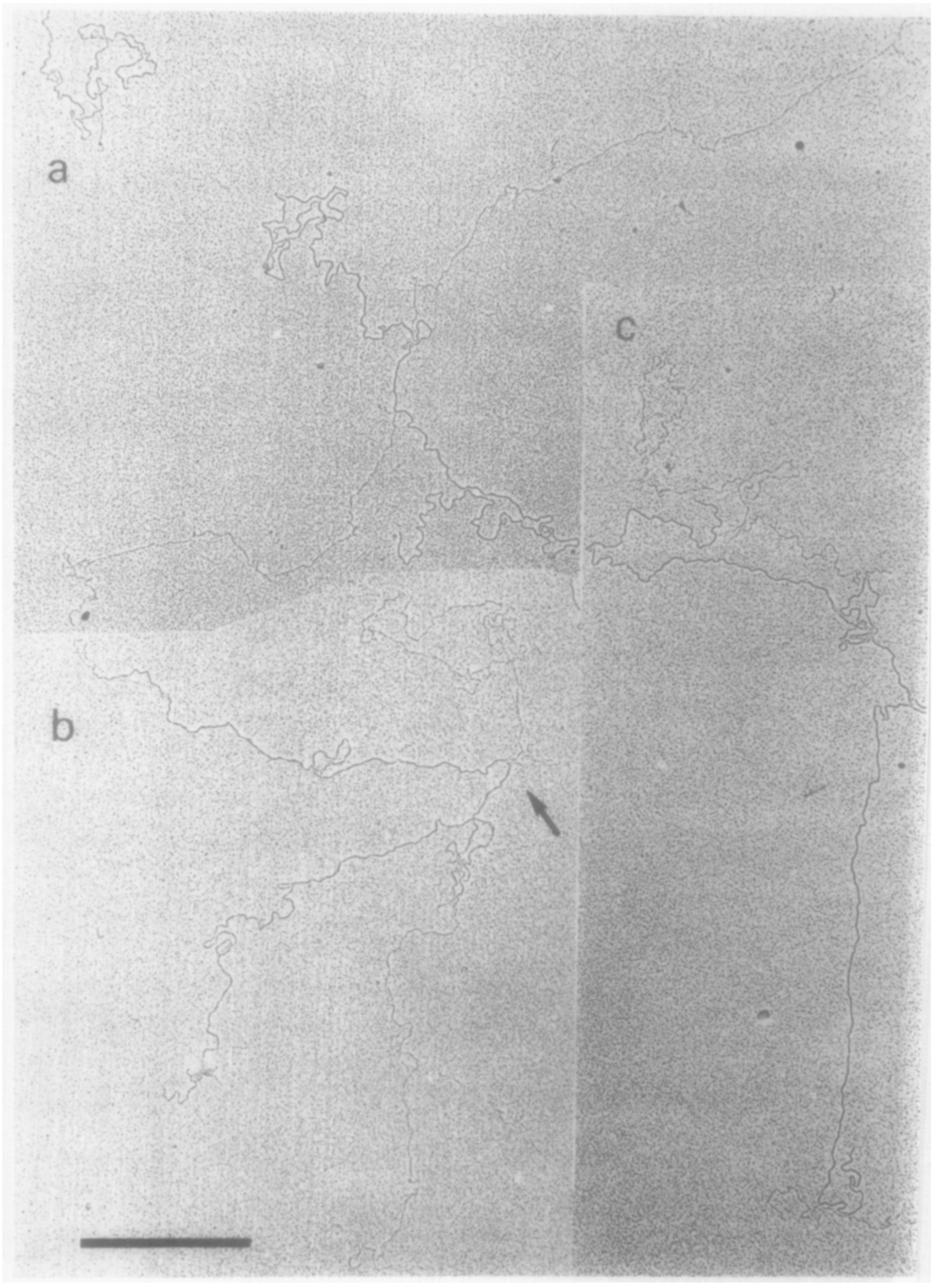
Figure 11 represents 24 partially denatured molecules normalized to unit length. The fraction of molecules denatured was determined at 200 sites across the genome. The resulting frequency distribution revealed three early melting regions. One early melting area was located at the left side of the molecule between 0.0 and 0.1 on the scale of fractional length, while two other regions were found at the right side of the molecule between 0.5 and 0.6, and between 0.8 and 1.0, respectively. The latter could be subdivided into three smaller early melting regions. The degree of denaturation (25 $\pm$ 2%) was not time-dependent, however, more DNA adhered to the grid when the adsorption time was increased. At pH values lower than 8.5 aggregation of Ad5 DNA in flowerlike structures was observed. Partial denaturation of Ad5 DNA by alkali revealed a denaturation pattern indistinguishable from the pattern obtained with formamide (unpublished results).

The denaturation pattern of mature Ad5 DNA resembles the pattern of Ad2 DNA (Doerfler and Kleinschmidt, 1970). Garon *et al.*, (1973) have mapped the nonhomologous regions between Ad2 and Ad5 DNA by electron microscopy of heteroduplexes. The nonhomologous regions were found at 0.50–0.65 and 0.78–0.92 on the fractional scale of unit genome length in which 1.00 represents the A-T-rich of molecular right end. Since the difference between Ad2 and Ad5 DNA do not show up in the denaturation patterns of the homologous DNA, the overall base composition of the heterologous regions must almost be identical.

Replicative intermediates obtained as indicated in Fig. 1 were subjected to the same denaturing conditions as mature Ad5 DNA. Within the duplex regions of the branched molecules an asymmetric distribution of early melting regions could be observed. A number of partially denatured normalized molecules is represented in Fig. 12.

The obtained frequency distribution of denatured sites is similar to the distribution obtained with mature Ad5 DNA (Fig. 11). Again, three early melting regions can be observed at the same positions as for mature DNA. This is direct proof that the branched molecules are of adenovirus origin. The branches are randomly distributed over the genome, as found for the nondenatured branched intermediates (Fig. 3).

The length of the single-stranded branch in 22 forked molecules is equal, within a


a
b
c

range of 10%, to the length of that double-stranded arm which displays the denaturation pattern characteristics of the right side of the genome. No molecules were observed which contained a single-stranded branch with a length equal to the left side of the genome. These results indicate that within 95% confidence the displacement synthesis starts at the right end of the genome in at least 84 out of 100 events. In a restricted number of partially denatured Y-shaped molecules the single-stranded arm is more than 10% shorter than both double-stranded arms. These molecules probably arise by breakage of the single-stranded arm during isolation and preparation.

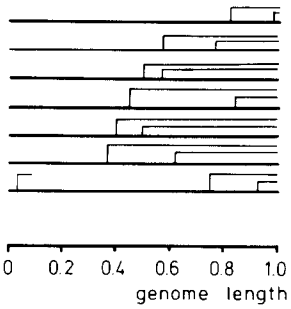


FIG. 8. Schematic representation of double-branched replicative intermediates isolated from cells 30 min after release of the HU-block. The small branching at the left side of the lowest drawing has presumably originated by breakage. The length of the branch does not correspond to the left double-stranded arm.

DISCUSSION

Electron microscopy of replicating Ad5 DNA isolated from nuclei of infected KB cells by a Hirt procedure displayed linear branched and unbranched intermediates with single-stranded regions. No circular structures were observed. The branched intermediates represented an early form of replicating Ad5 DNA and appeared before the unbranched intermediates as shown by analysis of replicating DNA synthesized after synchronization of the replication cycle with HU. Digestion of branched intermediates, which had replicated synchronously for about 25%, with Eco RI endonuclease showed that in these intermediates synthesis had started at the molecular right end (the A-T rich end). These data confirm and extend a model on the replication of Ad5 DNA which has been presented previously (Sussenbach *et al.*, 1972) (Fig. 13). According to this model branched forms arise by DNA synthesis which starts at the molecular right end and proceeds displacing the parental H-strand (the strand with the higher density in alkaline CsCl) (Sussenbach *et al.*, 1973).

Although the Eco RI digestion of branched intermediates from HU-treated cells indicates initiation of replication at the molecular right end, the question remains whether initiation in untreated cells proceeds in an identical fashion. Partial denaturation mapping of branched intermediates was performed with replicating DNA isolated from untreated infected nuclei which had been incubated in the presence of 5-bromodeoxyuridine triphosphate. It is known that A-BU rich regions melt out earlier than A-T rich regions (Inman and Schnös, 1970). However, we were not able to notice any difference in denaturation characteristics between branched intermediates and mature Ad5 DNA, probably by the fact that thymine was replaced by bromouracil in only one of the strands.

Partial denaturating mapping showed that also in untreated infected cells the initiation of replication of Ad5 DNA occurs at the molecular right end. However, whether synthesis starts at the very end, or internally in close proximity to this end is still unknown. The presence of branched intermediates with short displaced strands down to 0.02 unit genome length indicates that the origin of displacement synthesis is

FIG. 7. Double-branched replicative intermediates of Ad5 DNA isolated from HU-treated infected cells 30 min after release of the HU-block. The DNA was mounted from a 80% formamide solution and layered onto a 50% formamide solution. The length ratio ΦX RF/ΦX SS equals 0.98. Branch migration can be observed in all molecules presented. Bar denotes 1 μm. (a) Displacement synthesis has progressed as far as 44 and 51% of the genome. (b) Displacement synthesis has progressed as far as 51 and 61% of the genome. The arrow points to the fork enlarged in Fig. 9. (c) Displacement synthesis has progressed as far as 17 and 56% of the genome.

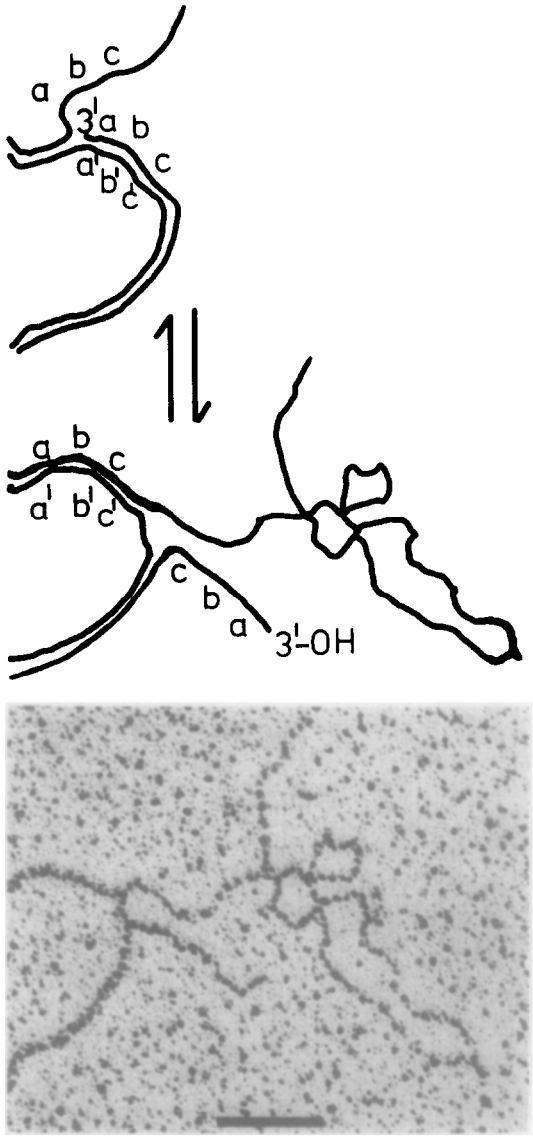


FIG. 9. Enlargement of the branch in Fig. 7 denoted by an arrow. The micrograph clearly shows that the long single strand is an elongation of the upper duplex strand. The process of branch migration which leads to the protruding short single strand is displayed in the drawing. Bar denotes 0.1 μm.

positioned within 700 base pairs from the molecular right end. We have never observed D-loops or eyes in replicating Ad5 DNA as found for mitochondrial DNA (Ter Schegget and Borst, 1971; Robberson *et al.*, 1972; Wolstenholme *et al.*, 1973). It can not be totally ruled out that DNA molecules initiated at the molecular left end or in the middle of the genome are preferentially lost in the extraction procedure for some structural reason, although this is rather unlikely.

The GC content of the molecular right end of Ad5 DNA is relatively high (Figs. 11 and 12). The origin of the first rounds of replication of the lambdoid phages λ, P2 and 186 (Schnös and Inman, 1970, 1971; Chattoraj and Inman, 1973) is also situated on a GC-rich stretch of the AT-rich half of the genome. However, the replication of T7 DNA does not start at a GC-rich cluster, though the origin is positioned on the AT-rich half (Dressler *et al.*, 1972). So, a general statement on the base composition of the origin of DNA replication cannot be made yet. The location of the origin of replication of Ad5 DNA at the molecular right end raises the problem for an initiation molecule to recognize a unique end of the molecule, while both ends contain an inverted terminal repetition of about 100 nucleotides with identical sequences (Garon *et al.*, 1972; Wolfson and Dressler, 1972b). However, the inverted terminal repetition of Ad5 DNA may be not as perfect as supposed and still may contain unique base sequences at the right end, while it is also possible that the unique origin is located adjacent to the inverted terminal repetition at the molecular right end.

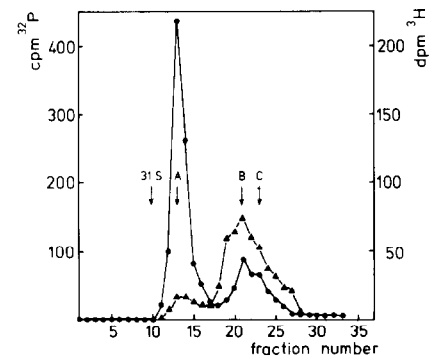


FIG. 10. Sucrose gradient centrifugation of new viral [^{3}H]DNA synthesized during 7 min after release of a HU-block (Fig. 6A and B) which has been digested by Eco RI endonuclease (▲). Digested mature [^{32}P]Ad5 DNA (●) was cocentrifuged to indicate the positions of the A, B, and C fragments, respectively. Centrifugation is from right to left, and was performed as described in Fig. 1.

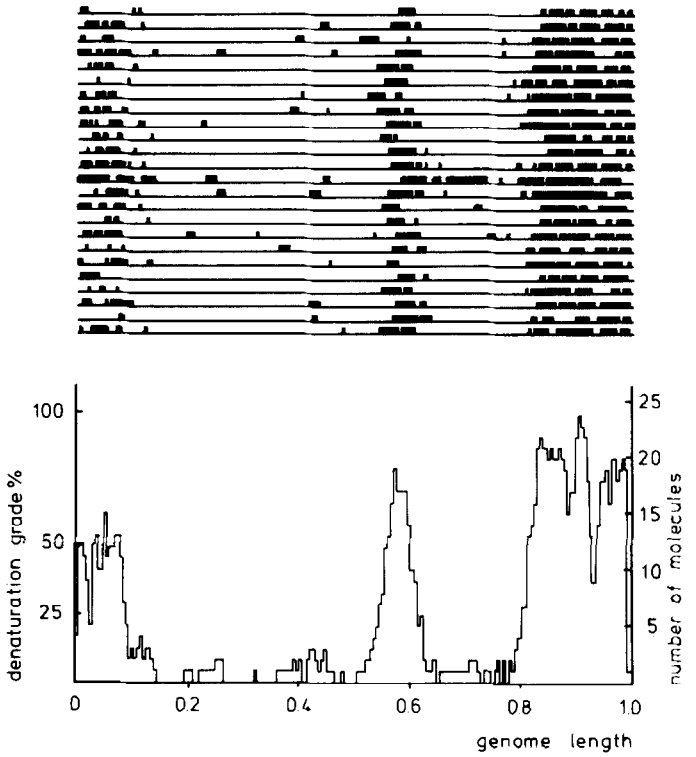


FIG. 11. Formamide denaturation of Ad5 DNA, represented by a set of normalized denaturation maps; the boxes correspond to denaturation loops. The histogram shows the frequency distribution of the melted regions. The denaturation grade (ordinate) is plotted vs the unit genome length of the DNA molecules (abscissa). The average denaturation grade in the presence of 85% formamide at 21° is 25 (± 2)%.

The extended single-stranded branches in the forked intermediates suggest that the synthesis of a new strand on the displaced strand, the so-called complementary strand synthesis is considerably delayed with respect to the displacement synthesis. The complementary strand synthesis occasionally starts on a branched intermediate leading to a double-stranded region in the displaced strand. However, the observed pattern of intermediates suggests that in general the complementary strand synthesis does not start until the viral H-strand is completely displaced. Single-stranded regions have been observed in replication forks of various types of DNA, i.e., λ, 186, T7, T4, SV40, polyoma and Drosophila (Schnös and Inman, 1971; Chattoraj and Inman, 1973; Wolfson and Dressler, 1972a; Delius *et al.*, 1971; Kriegstein and Hogness, 1974; Fareed, 1972; Bourgaux and Bourgaux-Ramoisy, 1971). However, the asynchrony in synthesis of both strands in these cases is relatively small and causes single-stranded stretches of 500–2000 nucleotides. A more pronounced asynchrony as for Ad5 has only been observed for mitochondrial DNA (Robberson *et al.*, 1972).

Ad5 replicative intermediates isolated from HU-treated cells contain a higher amount of single-stranded DNA than replicating Ad5 DNA from untreated cells (Sussenbach and van der Vliet, 1973). Biochemical and electron microscopical analysis of replicating Ad5 DNA from HU-treated cells indicates that shortly after HU-treatment the complementary strand synthesis does not take place. On the other hand under these conditions, frequently reinitiation of the displacement synthesis occurs before the first round is terminated. These observations indicate that changes in growth conditions have different effects

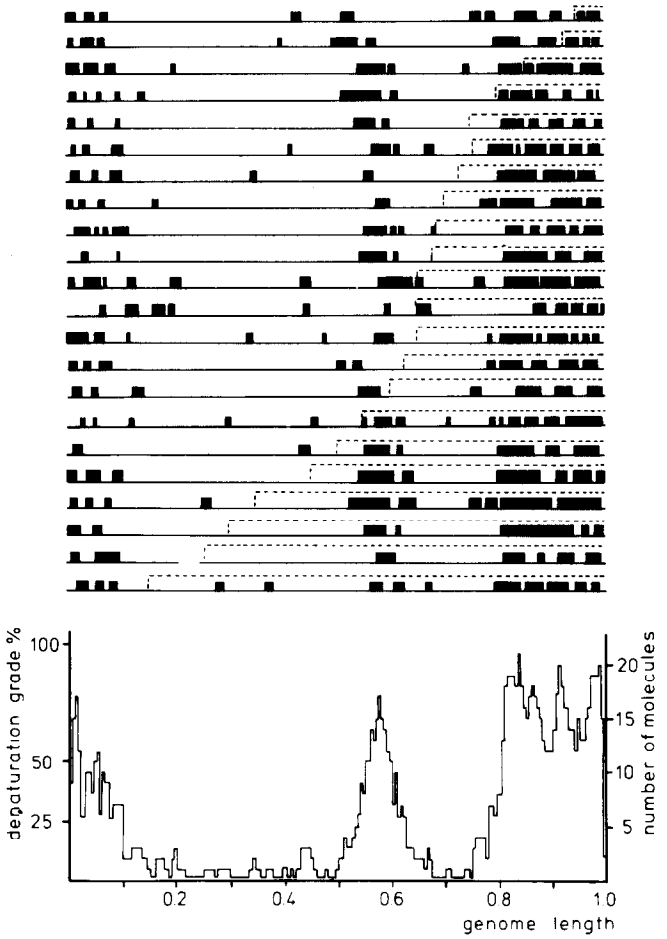


FIG. 12. Formamide denaturation of branched replicative intermediates, isolated from purified, infected nuclei. In the normalized denaturation maps the dashed lines correspond to single-stranded branches. The histogram plotted in the same way as in Fig. 8, shows the frequency distribution of the single-stranded loops. The average denaturation grade in the presence of 85% formamide at 21° is 27 (± 2)%.

on the initiations of the displacement and complementary strand synthesis and that both processes are not stringently linked.

In 70% of the branched intermediates the length of the displaced strand is shorter than 50% of the genome length (Fig. 3). This may indicate that the rate of displacement synthesis increases at the end of the genome; however, preferential shear of the extensively displaced molecules during isolation will lead to the same result. The rather flat distribution of branched molecules excludes the presence of specific holding sites during replication.

In our model the unbranched intermediates are involved in the complementary strand synthesis which may proceed discontinuously. Their role as late replication intermediates is confirmed by the HU synchronization study. However, a more detailed interpretation of the different classes of unbranched structures in terms of the origins and the direction of the complementary strand synthesis is not feasible from the presented data, since it is not known whether single strand/double strand transitions represent growing points or initiation points. The presence of linear single-stranded replicative intermediates with two duplex regions strongly suggests that initiation of the complementary strand synthesis can take place at different sites on the genome. This implies that the complementary strands are synthesized in fragments which are later joined.

A major problem of a mechanism of

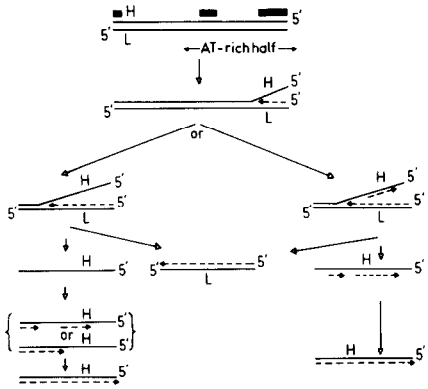


FIG. 13. Model for replication of Ad5 DNA. Parental strands have been drawn as solid lines and new daughter DNA has been indicated with broken lines. The black bars in the upper part represent the three prominent denaturations sites. The complementary strands with the lower and higher equilibrium density in alkaline CsCl have been indicated with L and H, respectively.

replication involving linear intermediates is the conservation of the genetic information of the 5′ ends. Circularization or polymerization of linear genomes has already been proposed as a general possibility to overcome this problem (Watson, 1972). In the case of Ad5 DNA the molecular ends may be linked by the inverted terminal redundant sequences (Garon *et al.*, 1972; Wolfson and Dressler, 1972b), a protein complex (Robinson *et al.*, 1973), or a combination of both. The absence of circular structures in our preparations may be caused by the isolation procedure or the short life time of circular intermediates. On the other hand Ad5 DNA might have extraordinary molecular ends which permit replication via linear intermediates. A study on the structure of the molecular ends is in progress.

ACKNOWLEDGMENTS

The authors thank Miss T. van Dam and Miss M. G. Kuijk for their expert technical assistance. A gift of Eco RI endonuclease from Dr. F. A. J. de Vries is gratefully acknowledged. This investigation was supported in part by the Netherlands Foundation for Chemical Research with financial aid from the Netherlands Organization for the Advancement of Pure Reearch.

REFERENCES

BLÜTHMANN, H., BRÜCK, D., HÜBNER, C., and SCHOFFSKI, A. (1973). Reassociation of nucleic acids in solutions containing formamide. *Biochem. Biophys. Res. Commun.* **50,** 91-98.

BOURGAUX, P., and BOURGAUX-RAMOISY, D. (1971). Asymmetrical model for polyoma virus DNA-replication. *J. Mol. Biol.* **62,** 513-524.

CHAMBERLIN, M., and BERG, P. (1964). Mechanism of RNA polymerase action: Formation of DNA-RNA hybrids with single-stranded templates. *J. Mol. Biol.* **8,** 297-313.

CHATTORAJ, D. K., and INMAN, R. B. (1973). Origin and direction of replication of bacteriophage 186 DNA. *Proc. Nat. Acad. Sci. USA* **70,** 1768-1771.

DAVIS, R. W., SIMON, M., and DAVIDSON, N. (1971). Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. *In* "Methods in Enzymology" (L. Grossman and K. Moldave, eds.), Vol. 21, pp. 413-428. Academic Press, New York.

DELIUS, H., HOWE, C., and KOZINSKI, A. W. (1971). Structure of replicating DNA from bacteriophage T4. *Proc. Nat. Acad. Sci. USA* **68,** 3049-3053.

DRESSLER, D., WOLFSON, J., and MAGAZIN, M. (1972). Initiation and reinitiation of DNA synthesis during replication of bacteriophage T7. *Proc. Nat. Acad. Sci. USA* **69,** 998-1002.

DOERFLER, W., and KLEINSCHMIDT, A. K. (1970). Denaturation pattern of the DNA of adenovirus type 2 as determined by electron microscopy. *J. Mol. Biol.* **50,** 579-593.

FAREED, G. C., SEBRING, E. D., and SALZMAN, N. P. (1972). Cleavage of replicative intermediates of SV40 DNA by the restriction endonuclease of *E. coli* B. *J. Biol. Chem.* **247,** 5872-5879.

GARON, C. F., BERRY, K. W., HIERHOLZER, J. C., and ROSE, J. A. (1973). Mapping of base sequence heterologies between genomes from different adenovirus serotypes. *Virology* **54,** 414-426.

GARON, C. F., BERRY, K. W., and ROSE, J. A. (1972). A unique form of terminal redundancy in adenovirus DNA molecules. *Proc. Nat. Acad. Sci. USA* **69,** 2391-2395.

INMAN, R. B. (1966). A denaturation map of the λ phage DNA molecules determined by electron microscopy. *J. Mol. Biol.* **18,** 464-476.

INMAN, R. B., and SCHNÖS, M. (1970). Partial denaturation of thymine- and 5-bromouracil containing λ DNA in alkali. *J. Mol. Biol.* **49,** 93-98.

INMAN, R. B., and SCHNÖS, M. (1971). Structure of branch points in replicating DNA: Presence of single-stranded connections in λ DNA branch points. *J. Mol. Biol.* **56,** 319-325.

KRIEGSTEIN, H. J., and HOGNESS, D. S. (1974). Mechanism of DNA replication in Drosophila chromosomes: Structure of replication forks and evidence for bidirectionality. *Proc. Nat. Acad. Sci. USA* **71,** 135-139.

LANG, D., and MITANI, M. (1970). Simplified quantitative electron microscopy of biopolymers. *Biopolymers* **9,** 373-379.

LEE, C. S., DAVIS, R. W., and DAVIDSON, N. (1970). A physical study by electron microscopy of the terminally repetitious, circulary permuted DNA from the coliphage particles of *Escherichia coli* 15. *J. Mol. Biol.* **48,** 1-22.

ROBBERSON, D. L., KASAMATSU, H., and VINOGRAD, J. (1972). Replication of mitochondrial DNA. Circular replicative intermediates in mouse L cells. *Proc. Nat. Acad. Sci. USA* **69,** 737-741.

ROBINSON, A. J., YOUNGHUSBAND, H. B., and BELLETT, A. J. D. (1973). A circular DNA-protein complex from adenoviruses. *Virology* **56,** 54-69.

SCHNÖS, M., and INMAN, R. B. (1970). Positions of branch points in replicating DNA. *J. Mol. Biol.* **51,** 61-73.

SCHNÖS, M., and INMAN, R. B. (1971). The starting point and direction of replication in P2 DNA. *J. Mol. Biol.* **55,** 31-38.

SUSSENBACH, J. S., VAN DER VLIET, P. C., ELLENS, D. J., and JANSZ, H. S. (1972). Linear intermediates in the replication of adenovirus DNA. *Nature New Biol.* **239,** 47-49.

SUSSENBACH, J. S., and VAN DER VLIET, P. C. (1973). Studies on the mechanism of replication of adenovirus DNA. I. The effect of hydroxyurea. *Virology* **54,** 299-303.

SUSSENBACH, J. S., ELLENS, D. J., and JANSZ, H. S. (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.

TER SCHEGGET, J., and BORST, P. (1971). DNA synthesis by isolated mitochondria. II. Detection of product DNA hydrogen-bonded to closed duplex circles. *Biochim. Biophys. Acta* **246,** 249-257.

VAN DER EB, A. J. (1973). Intermediates in type 5 adenovirus DNA replication. *Virology* **51,** 11-23.

VAN DER EB, A. J., and VAN KESTEREN, L. W. (1966). Structure and molecular weight of the DNA of adenovirus type 5. *Biochim. Biophys. Acta* **129,** 441-444.

VAN DER VLIET, P. C., and SUSSENBACH, J. S. (1972). The mechanism of adenovirus DNA synthesis in isolated nuclei. *Eur. J. Biochem.* **30,** 584-592.

WATSON, J. D. (1972). Origin of concatemeric T7 DNA. *Nature New Biol.* **239,** 197-201.

WOLFSON, J., and DRESSLER, D. (1972a). Regions of single-stranded DNA in the growing points of replicating bacteriophage T7 chromosomes. *Proc. Nat. Acad. Sci. USA* **69,** 2682-2686.

WOLFSON, J., and DRESSLER, D. (1972b). Adenovirus-2 DNA contains an inverted terminal repetition. *Proc. Nat. Acad. Sci. USA* **69,** 3054-3057.

WOLSTENHOLME, D. R., KOIKE, K., and COCHRAN-FOUTS, P. (1973). Single-strand containing replicating molecules of circular mitochondrial DNA. *J. Cell Biol.* **56,** 230-245.