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Initiation of adenovirus DNA replication does not occur via a hairpin mechanism

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ABSTRACT

Models have been proposed suggesting that initiation of adenovirus DNA replication might occur via a hairpin mechanism. A consequence of the models is a covalent linkage of progeny and parental DNA in newly completed molecules. Analysis of mature molecules from KB cells infected with adenovirus type 5 indicates that, if a hairpin mechanism is involved, the length of the hairpin must be shorter than 50 basepairs long. Recent nucleotide sequence analysis of the termini of adenovirus type 5 DNA (P.H.Steenbergh et al.(1977) Nucl. Acids Res.4, 4371-4389) has shown that a hairpin of this size does not exist and that therefore a hairpin mechanism is unlikely.

INTRODUCTION

Human adenovirus DNA has a linear duplex structure and replicates in the nucleus of human cells. The mechanism of replication has been studied extensively by biochemical and electron microscopical techniques (for a review see (1)). The structure of the replicative intermediates indicates that replication proceeds via a displacement mechanism (2,3). Origins and termini of replication have been localized at both molecular termini (4,5,6,7,8).

One of the major unanswered questions in adenovirus DNA replication concerns the mechanism of initiation of replication. Comparative studies in other DNA synthesizing systems have revealed that initiation always requires a free 3'-OH group as a primer which might be provided by an RNA primer or by a nick in one of the strands of double-stranded DNA. Furthermore, replicative intermediates in general have a circular form or are able to form concatenated structures (9). However, the presence of an inverted terminal repetition of about 100-140 base pairs long in adenovirus DNA prohibits the formation of circles and concatemers (10).

Recently, it has been proposed that initiation of adenovirus DNA replication might occur via a hairpin mechanism (11, R.J.Roberts, personal communication, see also 12) (Fig. 1). According to this model the termini of adenovirus DNA should contain a palindromic sequence which should permit the

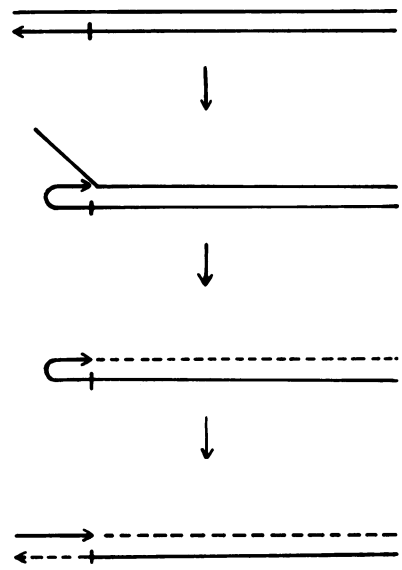

Fig.1. Hypothetical model for the initiation of adenovirus DNA replication (11, R.J.Roberts, personal communication). The terminus contains a short palindromic structure which allows the formation of a hairpin providing a free 3'-OH group. This group is used as primer for progeny strand synthesis (dashed lines). For completion of the molecular ends in the final stage the parental strand (solid lines) is cleaved just outside the palindrome releasing a new primer which is used to replicate the hairpin. The arrow indicates the 3'-end of a strand.

formation of a self-complementary structure providing a 3'-OH group suitable for DNA priming. More details of the model are shown in Fig.1. A consequence of the model is that in the final stage a tiny piece of progeny DNA is covalently linked to the 3'-end of the parental strand and that a similar piece of parental DNA is linked to the 5'-end of the progeny strand (Fig.1).

To determine whether a covalent linkage of parental and progeny DNA indeed exists the following strategy was used. Infected KB cells were pulse-labeled with 5-bromodeoxyuridine to produce a pool of heavy-heavy viral DNA molecules. Subsequently the cells were transferred to medium without 5-bromodeoxyuridine containing ^{3}H-thymidine. Prolonged incubation for many rounds of DNA replication will yield hybrid molecules containing one radioactive light strand and one heavy unlabeled strand. These molecules were tested for the presence of a tiny piece of ^{3}H-labeled DNA covalently linked to the heavy strand by alkaline CsCl gradient centrifugation of DNA fragments.

MATERIALS AND METHODS

The growth of KB cells, the infection with adenovirus type 5 (Ad5) and

the isolation of viral DNA have been described previously (13). Benzoylated naphtoylated DEAE (BND)-cellulose chromatography was performed as described by Kiger and Sinsheimer (14). Sucrose gradient centrifugation was performed in a 5-27% isokinetic sucrose gradient in a Spinco SW25 rotor at 20,000 rpm for 16 h at 4 ^{o}C (13). Neutral and alkaline CsCl gradient centrifugations were performed in a Spinco R50 rotor at 38,000 rpm for 64 h at 10 ^{o}C (13). Restriction enzyme analysis has been described previously (8).

RESULTS AND DISCUSSION

KB cells were infected with Ad5 and grown for 16 hours at 37 ^{o}C in the presence of 2 x 10^{-5} M 5-bromodeoxyuridine and 5 x 10^{-6} M 5-fluorodeoxyuridine. Under these conditions all viral DNA synthesized contains bromouracil instead of thymine resulting in a buoyant density of the DNA in CsCl of 1.800 g/cm^{3}, the density of HH viral DNA (13) (results not shown). Then, the cells were collected by low-speed centrifugation,washed with fresh medium and resuspended at a cell concentration of 10^{7} cells/ml. The cells were further incubated in fresh medium without 5-bromodeoxyuridine but in the presence of ^{3}H-thymidine (50 µCi/ml; specific activity 50 Ci/mmole) for a period of 8 hours. Then viral DNA was isolated according to a modified Hirt procedure (13) and the isolated DNA was subjected to sucrose gradient centrifugation (Fig. 2 a). The sedimentation profile is characteristic for replicating

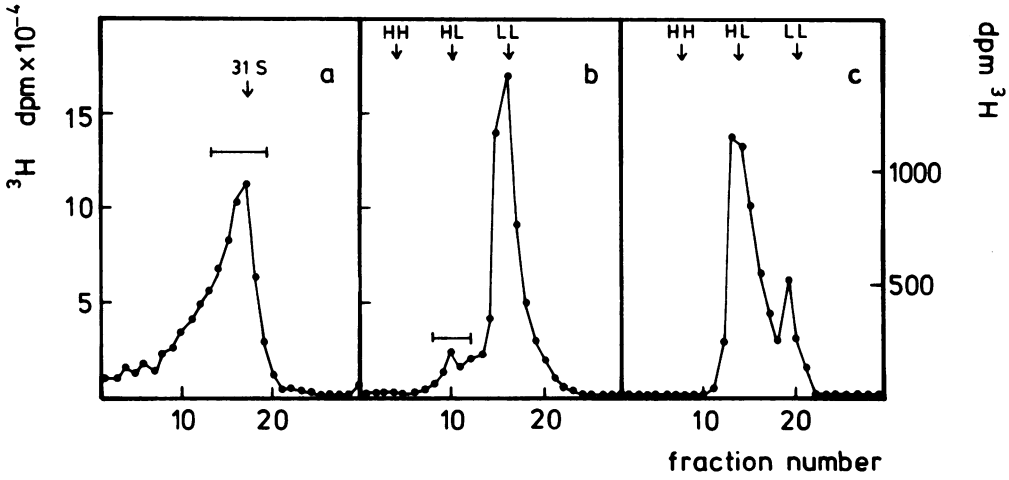

Fig. 2. (a) Sucrose gradient centrifugation of ^{3}H-labeled DNA isolated from cells as indicated in the text. Sedimentation is from right to left. The fractions indicated by the bar were pooled and analyzed by BND-cellulose chromatography. The purely double-stranded fraction from the BND-cellulose colmn was subjected to CsCl gradient centrifugation (panel b). The fractions containing HL mature molecules were pooled as indicated by the bar. A part was centrifuged in a neutral CsCl gradient (panel c).

adenovirus DNA showing a peak at 31 S (mature DNA). Mature molecules of genome size were selcted by pooling of the peak fractions,followed by BND-cellulose chromatography of this material. Purely double-stranded DNA was eluted with 1 M NaCl from the BND-cellulose column and was subjected to neutral CsCl gradient centrifugation (Fig. 2 b). Most of the radioactive DNA bands at the position of normal (LL) viral DNA (1.716 g/cm^3). However, a small portion of the material bands at the position of hybrid (HL) molecules (1.759 g/cm^3). The fractions around the HL position were pooled and a part of the DNA was recentrifuged in a neutral CsCl gradient (Fig. 2 c). Indeed most of the material bands at the HL position, while also some LL DNA is present. The ^{3}H-DNA banding at the HL position should contain parental heavy (H) strands with 3'-terminal sequences containing ^{3}H-radioactivity if the above model is correct. The possible linkage of parental and progeny DNA was tested by addition of mature ^{32}P-DNA to the isolated ^{3}H-DNA and subsequent cleavage of this DNA with the restriction enzymes Eco RI and Hsu I, respectively. Eco RI cleaves Ad5 DNA into three fragments which map in the order A-C-B, while the nine fragments produced by Hsu I map in the order G-E-C-H-D-A-B-F-I (8). The terminal Hsu I fragments G (2500 base pairs) and I (1000 base pairs) and the Eco RI fragments C (2500 base pairs) and B (6000 base pairs) were isolated after electrophoresis of the fragmented DNA, and were then subjected to alkaline CsCl gradient centrifugation (Figs 3 and 4).

If covalent linkage of the ^{3}H-labeled progeny DNA and bromodeoxyuridine-labeled parental DNA occurs, it is expected for the terminal fragments that at the heavy (H) position in the gradient there should exist an excess of ^{3}H-label as compared to the ^{32}P-radioactivity at this position. Figs 3 and 4 indicate that such an abundancy of ^{3}H-label at the H position is not found for the Eco RI-B, the Hsu I-G and the Hsu I-I fragments. The presence of ^{3}H- and ^{32}P-label at the H position in all gradients is probably due to statistical broadening of the L peak, since recentrifugation of the material from the H position of the I fragment shows that this material bands mainly at the L position (Fig. 4 c).

To estimate the maximal length of a possible hairpin the relative amounts of ^{3}H- and ^{32}P-label banding in the H region of the Hsu I-I fragment (Fig. 4 b and c) were determined and expressed as percentages of the total amount of radioactivity in the gradient. Since heavy strands with a tiny piece of ^{3}H-DNA will band in the H region, the maximal length of this piece of ^{3}H-DNA can be calculated from these percentages.

Examination of the distribution of the ^{3}H-radioactivity in Fig. 4 b and

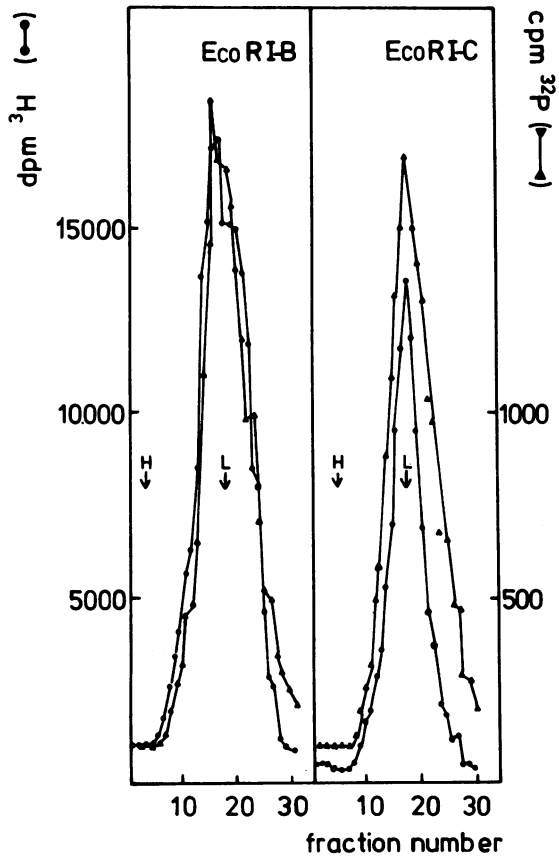

Fig. 3. Alkaline CsCl gradient centrifugation of Eco RI-B and C fragments obtained by cleavage with Eco RI of the tritiated HL fraction of Fig. 2 b mixed with ^{32}P-labeled DNA from virions. The fragments were separated by electrophoresis on 1.4% agarose gels, eluted from the gels (8) and subjected to alkaline CsCl gradient centrifugation.

c reveals that in Fig. 4 b 18.5% of the radioactivity in the gradient bands in the H region (fractions 1-9), while in Fig. 4 c 29% of the ^{3}H-label bands in the H region (fractions 1-11). For the ^{32}P-radioactivity similar calculations reveal that 19.5% of the label in Fig 4 b bands in the H region (fractions 1-9) and 15% of the ^{32}P-label in Fig. 4 c (fractions 1-11).
Combi nation of the data from Fig. 4 b and c shows that the maximal value of the relative amount of ^{3}H-label banding in the H region is 5.3% (0.185 x 0.29 x 100%) of the original amount of label, while for the ^{32}P-label this percentage is 2.9% (0.195 x 0.15 x 100%). Since the Hsu I-I fragment is about 1000 basepairs long (8), these results indicate that the maximal length of the hairpin is about 53 basepairs long (5.3% of 1000) without correction

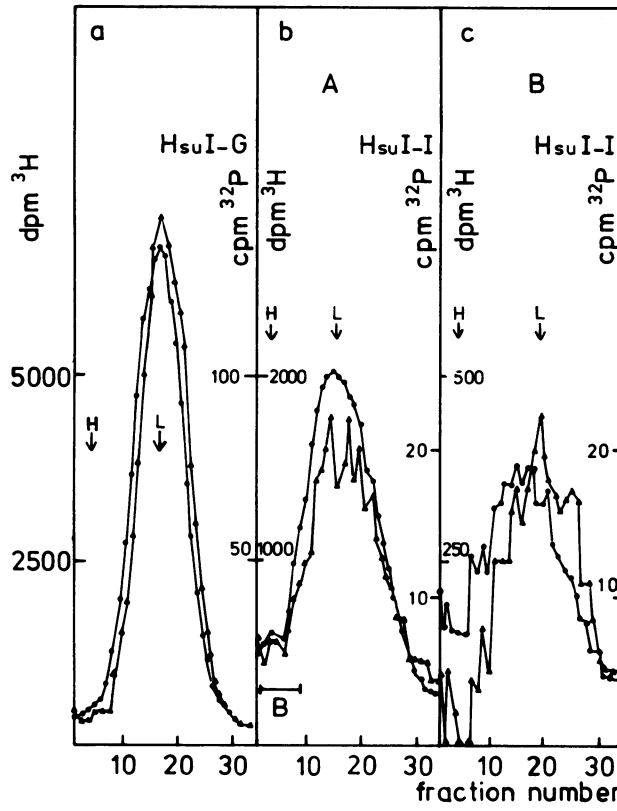

Fig. 4. Alkaline CsCl gradient centrifugation of Hsu I-G (a) and Hsu I-I (b) fragments obtained after cleavage with Hsu I of the tritiated HL fraction of Fig. 2 b mixed with ^{32}P-labeled DNA from virions. After cleavage the fragments were separated by electrophoresis in 1.4% agarose gels and subjected to alkaline CsCl gradient centrifugation. ^{3}H-label is indicated by ●—● and ^{32}P-label by ▲—▲. The first nine fractions of the gradient containing the HsuI-I fragment (panel b) were pooled as indicated by B and recentrifuged as shown in panel c.

for the background, or 24 basepairs long (2.4% of 1000) with a correction for the background (2.9% according to the ^{32}P-label in the H region).

The above described considerations indicate that if a hairpin is involved in initiation the maximal length of the palindromic sequence must be shorter than 20-50 basepairs long, and thus must be located within the terminal repetition. However, recent nucleotide sequence analysis of the termini of Ad5 DNA has shown that such a palindromic sequence is not present within the terminalrepetition (15). In conclusion, combination of the results of the present study and sequence data indicates that initiation of adenovirus DNA replication via a hairpin mechanism as shown in Fig. 1 is very unlikely.

During the course of this investigation Stillman et al. (16) communicated data which also exclude the involvement of a hairpin in initiation.

Recently, another model for initiation has been proposed suggesting that initiation occurs via a protein which binds deoxycytidine as primer and sticks to the termini of adenovirus DNA (17). However, until now no evidence is available which supports this model.

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