Biochimica et Biophysica Acta, 563 (1979) 89—99

BBA 99473

PRESENCE OF PROTEIN AT THE TERMINI OF INTRACELLULAR ADENOVIRUS TYPE 5 DNA

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(Received October 3rd, 1978)

Key words: Adenovirus DNA; Replication; DNA protein complex

Summary

Adenovirus type 5 contains linear double-stranded DNA with protein covalently attached to the ends of the molecules. The presence of protein at the termini of intracellular viral DNA in adenovirus type 5-infected cells was investigated at different stages during the replication process. The intracellular viral DNA was isolated from the nuclei by lysis in 4 M guanidine hydrochloride. Electrophoresis on agarose gels of *Hsu*I restriction enzyme fragments and sucrose gradient centrifugation were used to detect protein on intracellular viral DNA. After uncoating parental DNA still contains protein attached to the termini of the viral genome. Replicating and mature progeny viral DNA can also be isolated in the form of DNA-protein complexes. These complexes exhibit the same properties as the DNA-protein complex isolated from purified virions. These results suggest that the protein at the termini of intracellular viral DNA is identical to the protein attached to the 5′-ends of the DNA extracted from virions and that it is possibly involved in the replication of viral DNA.

Introduction

Human adenoviruses contain a linear double-stranded DNA molecule with a mol. wt. of $20—25 \cdot 10^6$ [1,2]. The DNA has an inverted terminal repetition [3,4]. Recently, Steenbergh et al. [5] determined the terminal sequences of adenovirus type 5 DNA; the repetition appears to be perfect and extends from

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Abbreviation: BND-cellulose chromatography, benzoylnapthoyl-DEAE-cellulose chromatography.

the ends of the genome to 103 base pairs inwards. Isolation of the DNA from virions with 4 M guanidine hydrochloride, avoiding proteolytic enzymes, releases a DNA-protein complex in which a protein with a mol.wt. of 55 000 is covalently linked to the termini of the DNA [6—8]. Electron microscopy of the DNA-protein complex reveals molecules with a linear as well as a circular structure [6,7,9] in which the terminal protein can be visualized [10]. Robinson et al. [6] and Robinson and Bellett [7] have suggested that the protein might play a role in DNA replication or in virus assembly.

The replication mechanism of the adenovirus DNA has been investigated extensively (for a review see Ref. 11). Biochemical and electron microscopical analysis of replicating adenovirus DNA indicates that replication starts at the right-hand end of the genome and proceeds to the left displacing the parental r-strand or starts at the left-hand end and proceeds to the right displacing the parental l-strand.

Recently, Rekosh et al. [8] proposed a model in which the terminal protein functions as a primer for DNA synthesis. There is, however, no direct evidence that the terminal protein is actually involved in the replication of adenovirus DNA. We present evidence that after infection of cells with adenovirus type 5 the intranuclear parental DNA contains protein attached to both ends of the genome before the onset of DNA replication. We also show that replicating and mature progeny DNA contains proteins as well.

Materials and Methods

Cells and virus. Primary African Green Monkey kidney cells and Cynomolgus Monkey (*Macaca fascicularis*) kidney cells were cultivated in Dulbecco's modified Eagle's medium. KB cells in monolayer or suspension were grown as described before [12]. Purified adenovirus type 5 was obtained according to Green et al. [1]. Virus containing ^{3}H-labeled DNA was obtained as described by Sussenbach [13]. Before isolation of the DNA-protein complex the virus, stored in CsCl, was recentrifuged in a performed CsCl gradient at 35 000 rev./min for 20 h at 5°C using a Spinco SW41 rotor.

Conditions of infection. KB monolayer cells were brought in suspension by scraping the cells from the culture flasks or by trypsinization. $3 \cdot 10^7$ cells were suspended into 50 ml of Earle's balanced salt medium containing 0.5% lactalbumin, 5% calf serum, 100 U of penicillin G/ml and 100 μg streptomycin-sulphate/ml. Cynomolgus and African Green Monkey kidney cells were infected in monolayer. For the investigation of viral progeny DNA a multiplicity of infection of 500—1000 particles/cell was used, while 2000—10 000 particles/cell were used for the characterization of the parental DNA. In the latter case 0.01 M hydroxyurea was added to the cells to prevent DNA replication [14]. At 18 h post-infection cells were harvested using a rubber policeman. The cells were washed extensively to remove non-absorbed input viruses and nuclei were prepared by douncing in hypotonic medium as described before [15].

Isolation of the DNA-protein complex from purified virions. The DNA-protein complex was isolated by a modification of the method described by Robinson et al. [6]. An equal volume of 8 M guanidine hydrochloride in 0.1 M

NaCl, 0.05 M Tris-HCl, 1 mM EDTA, pH 7.2, was added to the virus suspension in the same buffer. The extraction with chloroform/isoamylalcohol (24 : 1) and the following dialysis step as described in the original procedure were omitted. The lysed virus preparation was directly layered on top of a 5—20% sucrose gradient containing 4 M guanidine hydrochloride and centrifuged or purified by centrifugation in CsCl containing 4 M guanidine hydrochloride.

Isolation of DNA-protein complexes from infected cells. The washed nuclei were lysed after addition of an equal volume of 8 M guanidine hydrochloride to the resuspended pellet. Sometimes 0.1% sarcosyl was added. After a few hours at 4°C the lysate was purified by centrifugation through a sucrose gradient containing 4 M guanidine hydrochloride, and analyzed without further purification unless otherwise stated.

Other procedures. Digestions with the restriction enzymes *Eco* RI and *Hsu*I were carried out as described by Sussenbach and Kuijk [16]. The *Eco* RI and *Hsu*I restriction maps of adenovirus type 5 DNA have been established by Mulder et al. [17] and Sussenbach and Kuijk [16], respectively. Gel electrophoresis of double-stranded DNA fragments was performed on 1.4% agarose (BioRad, Richmond, CA) as described before [16]. The bands were visualized with ethidiumbromide and the gel was sliced and counted for radioactivity in a liquid scintillation counter.

Separation of the strands of *Eco* RI and *Hsu*I fragments was performed on 1.4% agarose (Calbiochem, San Diego, CA) according to a procedure which was originally described by Hayward [18]. The ethanol-precipitated fragments were dried and dissolved in 10 μl 0.3 M NaOH, 10% glycerol and 1 mM EDTA and kept at room temperature for about 15 min. After electrophoresis in 0.04 M Tris-HCl, 0.005 M sodium acetate, 1 mM EDTA, pH 7.8, without ethidiumbromide the gels were soaked in the same buffer containing ethidiumbromide (0.5 μg/ml) to visualize the positions of the DNA fragments. Benzoylnaphthoyl-DEAE-cellulose chromatography was carried out according to Kiger and Sinsheimer [19].

Results

Properties of the DNA-protein complex isolated from purified virions

The presence of protein at both ends of the genome of adenovirus DNA can be detected in several ways: (a) In neutral sucrose gradients linear adenovirus type 5 DNA molecules of genome length sediment at 31 S, whereas circular DNA-protein complexes of genome length sediment at about 36 S. Higher sedimentation coefficients are obtained when multimeric forms are present [6,10]. (b) The presence of circular and more complex forms can also be detected in the electron microscope [6,9,10]. (c) The most simple way is to use the retainment of the terminal restriction enzyme fragments containing a terminal protein on top of agarose gels upon electrophoresis [7—9]. In this communication we have mainly employed restriction enzyme analysis to detect the presence of a protein at the termini of viral DNA. Restriction enzyme analysis of the DNA-protein complex isolated from adenovirus type 5 as described in Materials and Methods reveals that employing the restriction endonuclease *Hsu*I the two terminal fragments G and I (7.2 and 3.0% of the

whole genome, respectively) remain on top of the gel, while they do appear in the gel after pronase incubation (Fig. 1). Similar results were obtained if the restriction endonuclease *Eco* RI was used. Digestion of adenovirus 5 DNA with the restriction enzyme *Eco* RI produces three fragments which map in the order ACB. After electrophoresis of the *Eco* RI-digested complex only the internal C fragment enters the gel, whereas the two terminal fragments A and B remain on top of the gel. However, after pronase treatment the fragments A and B do migrate into the gel (results not shown).

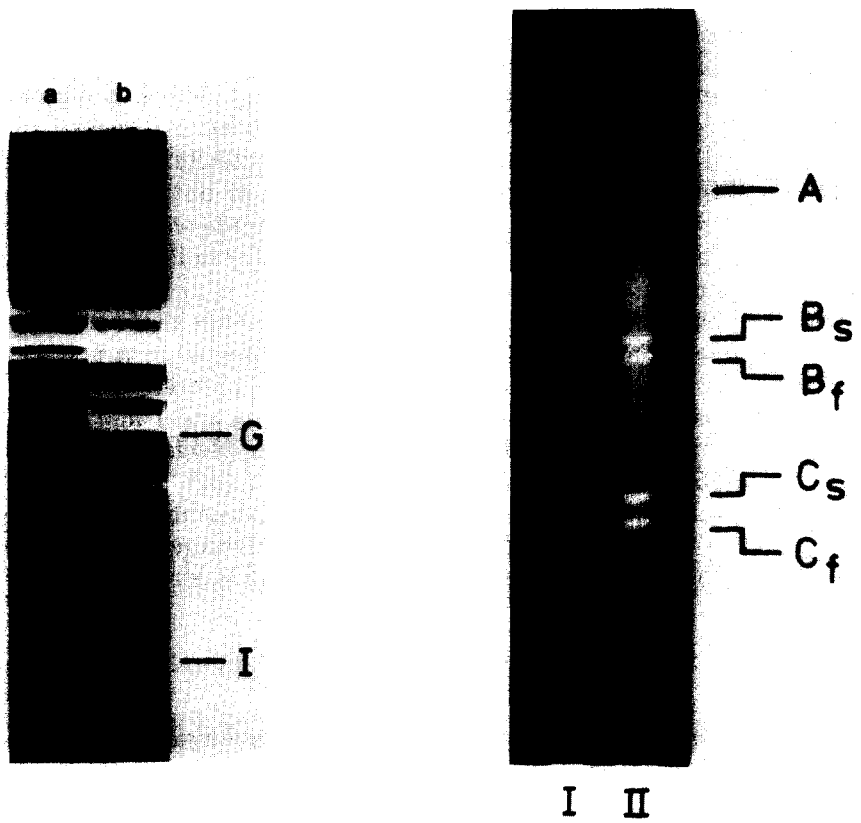

Fig. 1. Electrophoresis on agarose gels of DNA-protein complexes isolated from purified adenovirus type 5 virions after digestion with *Hsu*I. The DNA-protein complex isolated as described in Materials and Methods was digested with *Hsu*I and electrophoresis was carried out in 1.4% agarose gels. (a) DNA-protein complex digested with *Hsu*I. (b) As (a) but also incubated with pronase. The terminal *Hsu*I fragments G and I of adenovirus type 5 DNA are indicated.

Fig. 2.Gel electrophoresis of denatured *Eco* RI restriction enzyme fragments of adenovirus type 5 DNA-protein complexes isolated from virions. The DNA-protein complex from adenovirus type 5 lysed with 4 M guanidine hydrochloride was purified by CsCl centrifugation in the presence of 4 M guanidine hydrochloride and 0.1% sarkosyl. 3 μg of the complex were digested with the restriction enzyme *Eco* RI. Part of the digest was further indicated with pronase. The DNA fragments were treated as described in Materials and Methods and electrophoresis was performed in neutral 1.4% agarose gels. The gels were stained with ethidiumbromide in 0.04 M Tris-HCl, 0.005 M sodium acetate, 1 mM EDTA, pH 7.8. (I) DNA protein complex digested with *Eco* RI. (II) As (I) but also incubated with pronase. The slow and fast moving strands are indicated by the index s and f, respectively.

To detect whether the terminal protein is attached to the 5'-ends or the 3'-ends of the adenovirus type 5 genome, *Eco* RI restriction enzyme fragments were denatured in 0.3 M NaOH and the strands were separated by electrophoresis in a neutral 1.4% agarose gel. The two strands of both the *Eco* RI B and C fragments from adenovirus type 5 DNA can be separated, while the separation of the two strands of the A fragment is not always succesfull. The fast moving strands of the *Eco* RI B and C fragments originate from the viral r-strand [16], while the fast moving strand of the terminal B fragment contains the 5'-end of the intact r-strand. Analysis in this way of the DNA-protein complex isolated from adenovirus type 5 virions shows that the fast moving B strand is absent in the gel, due to the presence of the terminal protein, while incubation with pronase leads to appearance of the fast moving B strand (Fig. 2). A similar experiment was performed with the denatured right terminal *Hsu*I G restriction enzyme fragment (result not shown). In the gel of the denatured G fragment of the complex the fast moving G strand, containing the 5'-end of the intact l-strand [20], was absent. However, after pronase incubation both complementary strands of the G fragment were present.

These results present evidence that the DNA isolated from purified virions with 4 M guanidine hydrochloride contains protein at both ends of the genome and that the protein is attached to the 5'-termini of the DNA strands.

Characterization of the parental DNA in infected cells

After infection of cells with adenovirus the virions are gradually uncoated and reach the nucleus after 30 min—2 h post-infection. The amount of parental adenovirus type 5 DNA in the nucleus does not increase after 5 h post-infection [13]. We have investigated whether the parental DNA in the nucleus is still in the form of a DNA-protein complex or looses its terminal protein during the process of transport to and penetration into the nucleus. Cynomolgus cells were infected at a high multiplicty of infection with adenovirus type 5 containing ^{3}H-labeled DNA. 0.01 M hydroxyurea was added 2.5 h post-infection to prevent DNA replication [14]. At 18 h post-infection cells were washed and nuclei were isolated and lysed in 4 M guanidine hydrochloride as described in Materials and Methods. The crude lysate was layered on top of a neutral sucrose gradient containing 4 M guanidine hydrochloride and centrifuged. The DNA sediments mainly at the 31 S position. The recovery of radioactivity after sucrose gradient centrifugation in the presence of 4 M guanidine hydrochloride is about 80%, the remainder of the material is lost on the walls of the centrifugation tubes. Further purification of the viral DNA was carried out by equilibrium centrifugation in CsCl containing 4 M guanidine hydrochloride. The viral DNA bands as a single homogeneous peak (not shown). After dialysis of the viral DNA-containing fractions against 0.1 M NaCl, 0.05 M Tris-HCl, 1 mM EDTA, pH 7.2, the DNA was analyzed in neutral sucrose gradients as shown in Fig. 3. The parental intranuclear viral DNA sediments at the 31 S and 35—36 S positions, indicating the presence of circular DNA molecules of genome length in the gradient. Upon incubation with pronase the DNA is converted to 31 S protein-free DNA.

For a further analysis of the parental viral DNA KB cells were infected with adenovirus type 5 containing ^{3}H-labeled DNA. Hydroxyurea was added and the

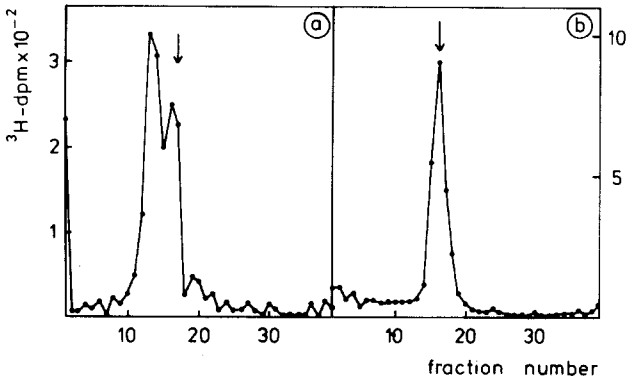

Fig. 3. Sucrose gradient centrifugation of the parental DNA-protein complex isolated from infected cells. KB monolayer cells were infected at a multiplicity of infection of 10 000 particles/cell with adenovirus type 5 containing ^{3}H-labeled DNA with a specific activity of 100 000 dpm ^{3}H/μg DNA. At 2.5 h post-infection 0.01 M hydroxyurea was added and at 18 h post-infection nuclei were isolated and lysed as described in Materials and Methods. The lysate was purified by centrifugation in a sucrose gradient with 4 M guanidine hydrochloride and the DNA-containing fractions were dialyzed against 0.01 M Tris-HCl, pH 7.5, 1 mM phenylmethylsulphonyl fluoride. The DNA was centrifuged in a neutral 5—20% isokinetic sucrose gradient for 16 h at 24 000 rev./min and 5°C in a Spinco SW27 rotor. Fractions were collected and radioactivity was determined. (a) Untreated parental DNA-protein complex. (b) The complex incubated with pronase. The arrows indicate the 31 S positions. Sedimentation is from right to left.

DNA extracted from the nuclei as described before. After dialysis of the DNA-containing fractions of a sucrose gradient with 4 M guanidine hydrochloride against 0.01 M Tris-HCl, pH 7.5, the DNA was digested with the restriction enzyme *Hsu*I. As shown in Fig. 4 the terminal G and I fragments are absent in the agarose gel, but are present after pronase treatment. The missing radioactivity of the G and I fragments in Fig. 4 was found quantitatively attached to the incubation tube as was shown by treatment of the tube with pronase followed by gel electrophoresis of the released radioactivity.

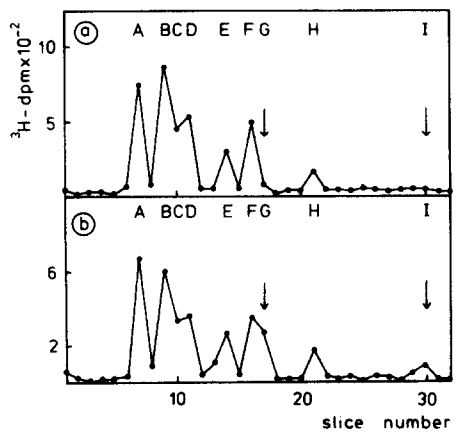

Fig. 4. Electrophoresis in agarose gels of the parental DNA-protein complex digested with *Hsu*I. Tritiated parental DNA-protein complex isolated as indicated in Fig. 3 was subjected to restriction enzyme analysis employing *Hsu*I. After digestion with *Hsu*I in the presence of protein-free marker DNA and electrophoresis on 1.4% agarose gels the bands were visualized, sliced and counted for radioactivity. (a) Parental intracellular DNA-protein complex digested with *Hsu*I. (b) As (a) but also digested with pronase. The terminal fragments are indicated by arrows.

In conclusion, the parental adenovirus type 5 DNA in the nucleus has the same properties as the DNA-protein complex isolated from purified virions. The above experiments show that a protein is attached to the ends of the parental DNA and represents probably the terminal protein present in the virion, which is not lost during the process of intracellular uncoating. However, it cannot be excluded that new terminal protein has been synthesized early in the lytic cycle and may be substituted for the parental protein.

Characterization of replicating and mature progeny DNA in infected cells

To detect at which stage in the infection process protein is attached to the ends of the progeny DNA the following experiments were performed. KB cells infected with adenovirus type 5 were labeled 17 h post-infection for 12 min with [^{3}H]thymidine. Nuclei were isolated, lysed in 4 M guanine hydrochloride and layered on top of a sucrose gradient with 4 M guanidine hydrochloride. After centrifugation the gradient was divided into three regions as indicated in Fig. 5. Pools A and B represent the fast sedimenting replicative intermediates, while pool C contains viral DNA sedimenting around the 31 S position. The three pools of viral DNA were purified in a CsCl gradient with 4 M guanidine hydrochloride and 0.1% sarkosyl (not shown). Restriction enzyme analysis of replicating DNA is hampered by the presence of extensive amounts of single-stranded DNA [21,22], as is shown in Fig. 6a. To overcome this difficulty, the pools A, B and C were first digested with *Hsu*I and the digest was subsequently subjected to BND-cellulose chromatography. In this way the fragments containing the replication forks plus single-stranded tails were removed. The purely double-stranded fractions were used for electrophoresis in agarose gels. The results of these experiments are shown for pools A and C of the sucrose

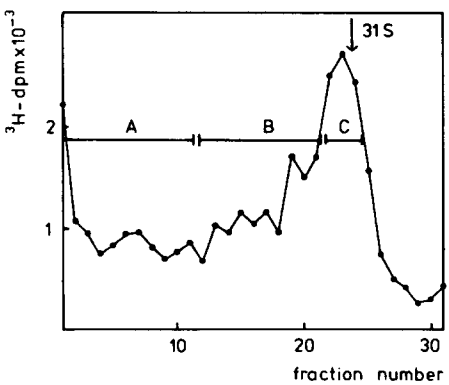

Fig. 5. Sucrose gradient centrifugation of intracellular adenovirus type 5 progeny DNA in the presence of 4 M guanidine hydrochloride. Adenovirus type 5-infected cells were labeled for 12 min with [^{3}H]-thymidine at 17 h post-infection. Nuclei were prepared as described in Materials and Methods and lysed with 4 M guanidine hydrochloride. Most of the cellular DNA was removed by centrifugation in a Spinco SW 65 rotor for 30 min at 20 000 rev./min in the presence of 0.1% sodium dodecylsulphate. The supernatant containing 95% of the labeled DNA was layered on top of a 10—30% linear neutral sucrose gradient with 4 M guanidine hydrochloride and centrifuged for 21 h at 25 000 rev./min and 5°C in a Spinco rotor. 5-µl samples of each fraction were counted for radioactivity. The position of the ^{32}P-labeled protein-free adenovirus 5 DNA is indicated by the arrow. Sedimentation is from right to left. The fractions of the gradient were pooled as indicated.

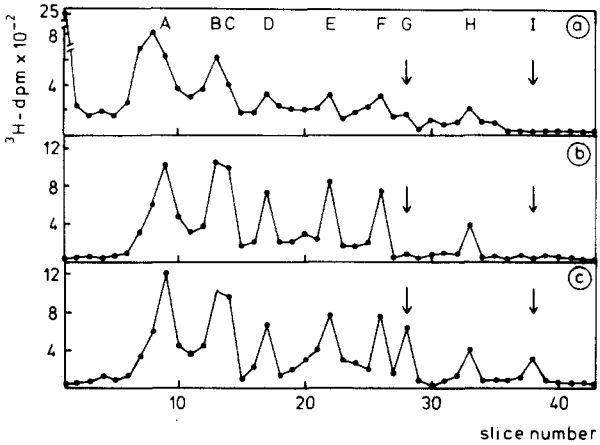

Fig. 6. *Hsu*I restriction enzyme analysis of 31 S intracellular adenovirus type 5 DNA. The DNA of pool C (Fig. 5) was recentrifuged in a CsCl gradient containing 4 M guanidine hydrochloride and 0.1% sarkosyl. Peak fractions banding at the position of ^{32}P-labeled protein-free adenovirus type 5 marker DNA were dialyzed against 0.01 M Tris-HCl, 1 mM EDTA, pH 7.8, mixed with unlabeled protein-free adenovirus type 5 DNA and digested with *Hsu*I. Part of the digest was incubated with pronase and the pronase treated and untreated samples were subjected to BND-column chromatography. The 1 M NaCl fractions containing the purely double-stranded DNA were precipitated with ethanol. The pellets were resuspended in 0.01 M Tris-HCl, 1 mM EDTA, pH 7.8, and electrophoresis was performed on 1.4% agarose gels. The DNA was visualized with ethidiumbromide and the gels were sliced and counted for radioactivity. (a) 31 S adenovirus type 5 DNA-protein complex digested with *Hsu*I. BND-cellulose chromatography was omitted in this sample. (b) As (a) but subjected to BND-cellulose chromatography after digestion with *Hsu*I. (c) As (b) but also incubated with pronase. The terminal fragments are indicated by the arrows.

gradient (Figs. 6b, c and 7). In these gels the larger fragments contain more often single-stranded DNA and are lost on the BND-cellulose column. Figs. 6 and 7 show that in slow as well as fast sedimenting replicative intermediates the terminal G and I fragments after *Hsu*I digestion are absent in the gel, but do appear after further incubation with pronase.

The presence of protein on intracellular viral DNA was also studied in African Green Monkey kidney cells. These cells are non-permissive for adenoviruses and late proteins are synthesized at a very reduced level, although viral

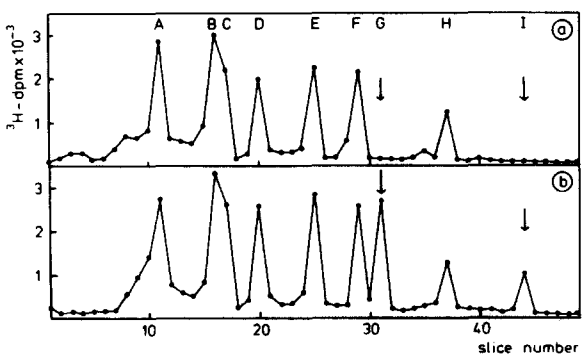

Fig. 7. *Hsu*I restriction enzyme analysis of fast sedimenting replicative intermediates of adenovirus type 5 DNA. The DNA of pool A (Fig. 5) was analyzed. The experimental procedures was the same as described in Fig. 6. (a) Fast sedimenting replicative intermediates of adenovirus type 5 DNA digested with *Hsu*I. (b) As (a) but also incubated with pronase. The terminal fragments are indicated by the arrows.

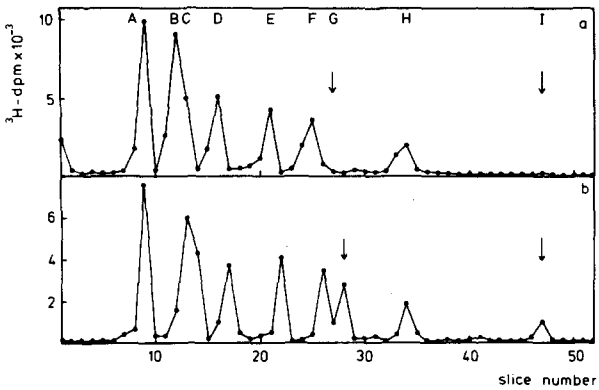

Fig. 8. Gel electrophoresis of DNA-protein complex isolated from adenovirus type 5-infected African Green Monkey kidney cells after digestion with *Hsu*I. African Green Monkey kidney cells were infected with adenovirus type 5 and labeled with [^{3}H]thymidine for 2 h, starting at 17 h post-infection. Viral DNA was isolated from lysed nuclei and purified on a sucrose gradient with 4 M guanidine hydrochloride. DNA sedimenting at the 31 S position (mature progeny DNA) was dialyzed against 0.1 M NaCl, 0.05 M Tris-HCl, 1 mM EDTA, pH 7.2, digested with *Hsu*I, and electrophoresis was performed on 1.4% agarose gels. (a) DNA-protein complex digested with *Hsu*I. (b) As (a) but also digested with pronase. The arrows indicate the positions of the terminal fragments.

DNA replication takes place [23]. African Green Monkey kidney cells were infected with adenovirus type 5 and the viral progeny DNA was labeled with [^{3}H]thymidine for 2 h starting at 17 h post-infection. After purification of the nuclear lysate on a sucrose gradient with 4 M guanidine hydrochloride, the intact 31 S mature viral DNA was analyzed on 1.4% agarose gels. The DNA stays on top of the gel, but after incubation with pronase it enters the gel almost completely. Further the DNA was digested with *Hsu*I followed by an incubation in the presence or absence of pronase and analyzed by agarose gel electrophoresis (Fig. 8). Without pronase treatment the terminal fragments G and I are almost quantitatively absent, whereas in this case at the top of the gel some radioactivity can be detected. The remainder of the terminal fragments was found in the incubation tube. After incubation with pronase, however, the terminal fragments do migrate into the gel, and the recovery in the gel was 100%.

These results indicate that a terminal protein is present in progeny DNA synthesized in African Green Monkey kidney cells.

Discussion

The DNA from adenovirions can be isolated as a DNA-protein complex if non-proteolytic isolation procedures are employed. We have demonstrated by agarose gel electrophoresis of denatured restriction enzyme fragments that in the virion the terminal protein is attached to the 5′-termini of the genome. This in agreement with Carusi's observations [24] that the DNA is resistant to λ-exonuclease, but sensitive to exonuclease III, indicating a block at the 5′-ends.

It has been suggested that the protein might play a role in DNA replication or in virus assembly [6—8]. This study shows that after infection of cells with

adenovirus type 5 parental as well as the replicating and mature progeny DNA can be isolated from the nuclei by lysis in 4 M guanidine hydrochloride as a DNA-protein complex. The intracellular complex shows the same properties as the DNA-protein complex isolated from purified virions viz.: (a) the complex is stable in 4 M guanidine hydrochloride upon sucrose as well as CsCl centrifugation, and therefore protein is firmly attached to the DNA, and might be covalently bound as in the virion. (b) The complex is detectable as circular and more complex forms in sucrose gradients indicating end to end interactions caused by protein. (c) Protein is located at the termini of the intracellular viral genome as is shown by gel electrophoresis. (d) The intracellular intact DNA-protein complex and the terminal fragments of the complex digested with *Hsu*I are very stickly, also caused by protein at the termini. It is therefore likely that the protein at the ends of the intracellular viral DNA is the same protein as the terminal protein present in virions, although the presence of another viral protein or a host protein cannot be excluded.

The presence of a protein at the termini of all types of replicative intermediates suggests that this protein is involved in DNA replications and that attachment occurs in an early stage of DNA replication. An early function for the terminal protein is also suggested by experiments with African Green Monkey kidney cells. These cells are non-permissive for adenovirus type 5 and late proteins are synthesized in reduced amounts [23]. However, in these cells adenovirus type 5 DNA replicates normally and our analysis of the progeny viral DNA reveals the presence of protein on both end of the genome. This suggests that this terminal protein is probably an early protein, although the involvement of late proteins cannot be excluded. The low levels of late viral proteins in African Green Monkey kidney cells might be sufficient for the formation of DNA-protein complexes.

Rekosh et al. [8] proposed a model for DNA replication in which the terminal protein functions as a primer for DNA synthesis. In this model circularization or palindromic sequences are not required for the initiation of DNA replication. Recently, Steenbergh et al. [5] determined the terminal sequences of adenovirus type 5 DNA and found no palindromic sequence in the first 190 base pairs at the left or right-hand end. Therefore hairpin-primed initiation of replication is unlikely. Recently direct evidence against hairpin-primed initiation of DNA replication has been presented [25,26]. The results presented in this communication are consistent with a priming function of the terminal proteins as indicated in the model presented by Rekosh et al. [8]. A function in packaging of the DNA into the virion is not excluded, although the presence of the terminal protein early in replication makes it unlikely.

During the preparation of this manuscript Girard et al. [27] also showed the presence of a terminal protein on replicating and mature DNA of adenovirus types 2 and 5-infected HeLa cells.

Acknowledgements

The authors thank R. Schiphof for the isolation of the restriction enzymes and Dr. H.S. Jansz for the stimulating discussions. This investigation was supported in part by the Netherlands Foundation for Chemical Research

(SON), with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

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