Isolation and Characterization of a Homogeneous DNA-Protein Complex from Adenovirus Type 2 Virion

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SUMMARY

Adenovirus DNA-protein complex purified by sedimentation on a sucrose gradient containing 4 M-guanidine hydrochloride was found to contain other virion proteins in addition to the terminal protein of mol. wt. 55000. In this report, we describe a simple and rapid method for the isolation of a homogeneous DNA-protein complex. The procedure involves gel electrophoresis of the complex on agarose in the presence of sodium dodecyl sulphate. DNA was found to migrate into the gel with a single protein of mol. wt. 55000 tightly attached to it. Restriction enzyme cleavage analysis of the DNA-protein complex shows that the protein is associated with the two terminal fragments.

Human adenovirus (Ad) 2 DNA extracted from the virion by treatment with pronase, sodium dodecyl sulphate (SDS) and phenol consists of linear, duplex molecules of mol. wt. $23 \times 10^6$ (van der Eb & van Kersteren, 1966; Green et al. 1967). Robinson et al. (1973) originally reported that when Ad 2 DNA was extracted from the virion with guanidine hydrochloride, circular molecules could be detected by electron microscopy. It was proposed that these structures were formed as a result of a protein binding tightly to the two ends of the DNA molecule. Subsequent studies (Rekosh et al. 1977) demonstrated a protein of mol. wt. 55000 (55 K) in the Ad 2 DNA-protein complex. These terminal protein-protein interactions could be inhibited by treatment with SDS or a protease, resulting in linear monomeric DNA. Similarly, the DNA–protein complexes have also been isolated by treatment of Ad 2 virion with the detergent sarkosyl (Doerfler et al. 1974; Brown et al. 1975). The DNA–protein complexes obtained from sarkosyl treatment of Ad 2 also gave rise to the circular form of the virus DNA. Brown et al. (1975) first demonstrated that these DNA–protein complexes did not migrate into agarose-polyacrylamide composite gels during electrophoresis. This observation allowed analysis of the protein-bound DNA by agarose gel electrophoresis of restriction enzyme fragments (Brown et al. 1975; Sharp et al. 1976; Padamabhan & Padmanabhan, 1977; Larsen & Nathans, 1977; Tibbetts, 1977). These data substantiated electron microscopic observations that the binding of protein(s) occurred at the termini of Ad DNA. It was not clear whether a single 55 K protein was involved or whether the interactions of other virion component(s) with the 55 K protein are required in causing circularization or in preventing the Ad DNA from migrating into the agarose gel. There was an apparent paradox which remained unsolved; that is, when the Ad DNA–protein complex was visualized under an electron microscope, only 30 to 60% of the molecules (Robinson et al. 1973) were in the circular forms, whereas 90 to 95% of the complex did not migrate into an agarose gel. We wish to report an alternative but simple procedure to isolate Ad 2 DNA–protein complex in which a single protein of 55 K was found to be associated with the DNA. By digesting the complex which was labelled with $^{125}$I in its protein moiety, with a restriction enzyme from E. coli (EcoR I), we were able to show that the 55 K protein was attached to the terminal fragments of Ad 2 DNA. This linkage between the protein and the DNA was stable to SDS.
Fig. 1. Characterization of proteins in the Ad 2 DNA–protein complex. Ad 2 DNA–protein complex was recovered from sucrose density gradients containing 4 M-guanidine hydrochloride and subsequently dialysed (Robinson et al. 1973). In (a) and (c), the dialysed complex was purified further by a Sepharose 4B column prior to iodination (in the presence of SDS) using the method of Bolton & Hunter (1973). Electrophoresis on agarose gel was carried out under the conditions in which both the gel and the HP buffer contained 0.1% SDS. In (a), 125I-labelled Ad 2 DNA–protein complex (6 µg of DNA containing 6 × 10⁶ cts/min) and in (b), 32P-labelled complex (0.8 µg of DNA containing 6500 cts/min) were electrophoresed. The autoradiography of the gel is shown. (c) This represents electrophoresis of another aliquot of the sample as in (a). After the run, the gel was washed three times with HP buffer containing 25% methanol to remove SDS and then incubated with HP buffer containing 0.5 µg/ml of ethidium bromide. The fluorescent DNA band was photographed (Sharp et al. 1973). (d) SDS–polyacrylamide gel electrophoresis of 125I-labelled protein associated with Ad 2 DNA. The DNA–protein complex from band 2 in (a) was eluted electrophoretically from the agarose gel and the DNA was digested as described in the text. Aliquots of the protein fraction were applied to an SDS–polyacrylamide gel. (e) 35S-methionine-labelled polypeptides synthesized by Ad 2+ND–infected KB cells (Jay et al. 1977) were used as mol. wt. markers. [see Maizel et al. (1968) and Anderson et al. (1973) for the nomenclature of virion proteins and their mol. wt.]. (f) The DNA–protein complex was recovered from the sucrose density gradients containing 4 M-guanidine hydrochloride and subsequently dialysed. Iodination was carried out in the absence of SDS. 125I-labelled DNA–protein complex was digested with deoxyribonuclease I (Worthington Biochemicals, Freehold, N.J., U.S.A.) and S1 nuclease. A portion of this sample was applied. In (d) to (f), a stacking gel of 5% and a separating gel of 10% polyacrylamide were prepared in the presence of 0.1% SDS. The samples were treated with urea, 2-mercaptoethanol and SDS as described in the text. O and I refer to the origin of the stacking gel and the interphase between the stacking and separating gels, respectively. Bovine serum albumin (BSA; 68 K) pepsin (35 K) and Cytochrome c (12 K) were used as internal protein standards.

The extraction and purification of the DNA–protein complex from the virus was carried out according to Robinson et al. (1973). The complex after dialysis against 0.01 M-tris, pH 7.4, 0.001 M-EDTA and 0.1 M-NaCl (TEN buffer) was further fractionated on Sepharose 4B (Pharmacia Fine Chemicals) columns (Brown et al. 1975). About 100 µg of DNA–protein complex were applied to a column (1 x 21 cm) of Sepharose 4B and eluted with 0.1 M-borate buffer, pH 8.5, containing 0.1% SDS. About 3 ml fractions were collected and the DNA–protein complex eluted in the first void volume (between fractions 11 to 20) was pooled and dialysed against the eluant buffer. The preparation of 32P-labelled complex has been described earlier (Padmanabhan & Padmanabhan, 1977). The DNA–protein complex (60 µg of DNA) in 50 µl of 0.1 M-borate buffer, pH 8.5, containing 0.1% SDS was iodinated, following the method described by Bolton & Hunter (1973). After iodination, the samples were diluted to 0.5 ml and dialysed against 11 of TEN buffer, with three changes of the dialysate. The 125I-labelled DNA–protein complex
was electrophoresed on 1.4% agarose gel using a buffer containing 0.036 M-tris, 0.03 M-NaH₂PO₄ (pH 7-8) and 1 mM-EDTA [Hayward-phosphate (HP) buffer], with the addition of 0.1% SDS. Figure 1a shows the autoradiograph of the agarose gel. About 25% of the ¹²⁵I radioactivity remained at the origin of the agarose gel (band 1 in Fig. 1a). Figure 1(b) shows the parallel experiment in which the DNA-protein complex, labelled uniformly with ³²P, was applied. Under conditions where very little DNA remained at the origin (Fig. 1b), a considerable amount of ¹²⁵I radioactivity (band 1) remained at the origin, presumably due to aggregation of some virion protein(s). The fact that there was very little DNA associated with band 1 was confirmed by identifying the DNA band in a similar experiment by its fluorescence in the presence of ethidium bromide (Fig. 1c). The fact that band 1 in Fig. 1(a) contained protein was confirmed by incubating the agarose gel in TEN buffer in the presence of 10 mM-mercaptoethanol and 100 μg of protease K. Under these conditions, about 60% of the ¹²⁵I radioactivity became acid-soluble in 1 h at 37 °C. The ¹²⁵I-labelled DNA-protein band (band 2 in Fig. 1a) indicates that the DNA was associated with protein and that this linkage is stable to 0.1% SDS. The mol. wt. of the protein associated with the DNA on a SDS agarose gel (band 2 in Fig. 1a) was determined as follows: 4 μg of Ad 2 DNA-protein complex labelled uniformly with ³²P were mixed with ¹²⁵I-labelled sample (20 μg as DNA). The mixture was heated for 5 min at 100 °C in an incubation mixture containing 30 mM-sodium acetate, pH 4.6, 4 mM-ZnCl₂ and 5% glycerol. The reaction mixture was cooled quickly in ice and 380 units of Aspergillus oryzae S₁ nuclease (Ando, 1966; Vogt, 1973) were added. The incubation was carried out at 37 °C for 1 h. The denaturation of DNA and the addition of 380 units of S₁ nuclease was repeated three times. The percentage of trichloroacetic acid-soluble cts/min (measured as Cerenkov radiation) was determined at the end of each incubation period. No further radioactivity was made acid soluble after the second S₁ nuclease treatment, indicating that the digestion of DNA moiety was complete.

A 32 μl amount of saturated urea was added to 10 μl of ¹²⁵I-labelled protein and the mixture was incubated at room temperature for 5 min. 2-Mercaptoethanol was added to the mixture (10%) and the incubation was continued for 30 min. SDS was then added (1% final concn) and the mixture was heated to 100 °C for 5 min. The sample was loaded on to the SDS-polyacrylamide gel (Weber & Osborn, 1967; Laemmli, 1970) after the addition of a mixture containing 10% sucrose and 0.003% bromophenol blue dye. ⁸⁵S-labelled, Ad 2⁺ND₂ coded proteins were used as mol. wt. markers. As shown in Fig. 1(d), there was essentially one major band of radioactivity with an apparent mol. wt. of 55 K. This value for the mol. wt. of protein linked to DNA is consistent with the report of Rekosh et al. (1977). Figure 1(f) shows the presence of other virion proteins in the DNA-protein complex (purified by sedimentation on sucrose gradients alone) before SDS-agarose electrophoresis. SDS-agarose gel electrophoresis dissociated these virion proteins (band 3 in Fig 1a) which were originally associated with the DNA-protein complex. The SDS-polyacrylamide gel electrophoresis of band 3 in Fig. 1(a) showed the presence of all the proteins shown in Fig. 1(f) expect the bands with the apparent mol. wt. of 18.5 K and 12 K. These proteins might be part of the aggregate that remained at the origin of the agarose gel (band 1). When band 4 in Fig. 1(a) was analysed for its protein content, it was found that no TCA-precipitable material could be recovered. It is most likely that this band essentially consisted of low mol. wt. ¹²⁵I-labelled compounds generated from the iodination reaction, still present due to the incomplete removal by dialysis.

Although previous studies by electron microscopy have supported the conclusion that a protein is bound at the two termini of adenovirus DNA, it has not been directly shown whether the linkage of the protein to DNA was stable to SDS at both termini. In a separate experiment, ¹²⁵I-labelled DNA-55 K protein complex corresponding to band 2 in Fig 1(a)
Fig. 2. Agarose gel electrophoresis of EcoR1-digested Ad 2 DNA–protein complex. The DNA–55 K protein complex (band 2 in Fig. 1a) was eluted from the SDS-agarose gel and further purified as described in the text. 125I-labelled Ad 2 DNA–protein complex (with a DNA content of about 10 μg) was digested with EcoR1 (Mulder et al. 1974) prior to agarose gel electrophoresis in the presence of SDS. After the electrophoresis, the gel was treated and the DNA bands were visualized as described in 1(c). (a) Autoradiography of the gel; (b) fluorescence due to ethidium bromide binding to DNA.

was eluted from the gel electrophoretically in the presence of 0.1% SDS. The solution was then concentrated to about 0.5 ml by extracting with n-butanol (this step also removed SDS from the aqueous phase). Guanidine hydrochloride was added to a final concentration of 4 M. The DNA–55 K protein complex was further purified by sedimentation on a gradient of sucrose (between 5 and 20%) in the presence of 4 M-guanidine hydrochloride. Fractions containing the DNA–protein complex were pooled and dialysed against TEN buffer. We tested the presence of this protein at the two termini by cleaving the 125I-labelled DNA–protein complex with EcoR1 prior to agarose gel electrophoresis in the presence of SDS. The DNA bands were visualized by ethidium bromide fluorescence (Fig. 2b), and the 125I-labelled protein attached to the DNA was detected by autoradiography (Fig. 2a). The data showed that the protein is attached to both terminal fragments of DNA and that the linkage is stable to 0.1% SDS.

In order to investigate the nature of the interaction between the 55 K protein and Ad 2 DNA at the termini or to analyse the comparative fingerprints of the terminal protein from different serotypes of adenovirus, it is necessary to prepare a homogeneous DNA–55 K protein complex. The existing methods to prepare the complex such as (i) sedimentation on a sucrose gradient containing 4 M-guanidine hydrochloride or (ii) chromatography on Sepharose 4B in the presence of 0.1% SDS were not satisfactory to prepare a homogeneous DNA–55 K protein complex. Figure 1(f) shows that the complex purified by the first method alone contained other virion proteins which co-sedimented with the DNA–55 K protein complex when the Ad 2 virions were denatured and sedimented on a sucrose gradient containing 4 M-guanidine hydrochloride. Sharp et al. (1976) reported that dialysis of the DNA–protein complex prepared by sedimentation on sucrose gradients against saline buffer to remove the guanidine hydrochloride renders the protein–protein interactions.
resistant to dissociation by further treatment with 4 M-guanidine hydrochloride, presumably due to aggregation. The authors suggested that the process might not be due to the simple affinity of two proteins for each other or of one protein for the two termini of the virus DNA. We observed that once the complex was aggregated during the dialysis step, the DNA–55 K protein complex could not be readily purified by Sepharose 4B column chromatography using 0.1 M-borate buffer, pH 8.5, containing 0.1% SDS. It might be necessary to boil the DNA–protein complex in a buffer containing SDS prior to Sepharose 4B column chromatography in the presence of SDS, in order to dissociate the aggregate of other virion proteins from the DNA–55 K protein complex.

Rekosh et al. (1977) described a procedure in which the DNA–protein complex was first purified by sedimentation on a CsCl gradient and, subsequent to a dialysis step, the protein moiety of the complex was labelled with $^{125}$I. The authors purified the labelled complex on a CsCl gradient containing 0.1% sarkosyl. Their data on the SDS-polyacrylamide gel analysis of the $^{125}$I-labelled proteins indicated the presence of additional virion proteins still contaminating the DNA–55 K protein complex even after purification through a CsCl gradient containing 0.1% sarkosyl. We found an aggregate of virion protein(s) at the origin of SDS-agarose gels (band 1 in Fig. 1 a). Although it was not completely dissociated into individual proteins forming the aggregate, it was dissociated from the DNA–55 K protein complex (band 2). The DNA band (band 2 in Fig. 1 a) was found to be associated with a single 55 K protein (Fig. 1 d). The resistance of the aggregated (denatured) virion proteins to 0.1% SDS (band 1 in Fig. 1 a) is not surprising and has been reported earlier by Maizel et al. (1968). These authors found that the virus polypeptides in SDS (1 to 2%) in the absence of virus DNA aggregated and essentially did not migrate into SDS-polyacrylamide gel unless the samples were heated to 100 °C for at least 1 min. Our samples of DNA–protein complex containing 0.1% SDS were not heated prior to either Sepharose 4B column chromatography or SDS-agarose gel electrophoresis. However, when the component polypeptides of the complex after the digestion of the virus DNA were heated to 100 °C for 2 min in the presence of 7 M-urea and 10% mercaptoethanol, they could be prevented from forming an aggregate and could be analysed by SDS-polyacrylamide gel electrophoresis. Keegstra et al. (1977) reported that the presence of the terminal protein(s) of the Ad 5 DNA–protein complex could be visualized as knobs under an electron microscope. Their data indicated that there were: (i) molecules without the knob (the terminal 55 K protein), (ii) molecules with knob at only one end of DNA and (iii) molecules with knobs at both ends. Their observation that the sizes of the knobs were different supported the possibility that the knob consisted of a complex of proteins rather than of a pure terminal protein. Our data showing the presence of additional virion proteins forming a tight complex with the 55 K protein at the termini of DNA are consistent with the electron microscopic observation of Keegstra et al. (1977).

After denaturation of the DNA, $S_1$ nuclease was used to digest the DNA. The protein linked to the DNA was analysed by SDS-polyacrylamide gel electrophoresis. The use of $S_1$ nuclease for this purpose has a definite advantage over DNase I because the DNA–55 K protein complex (band 2 of Fig. 1 a) eluted off the SDS-agarose gel can be used as substrate without prior removal of SDS. It has been reported earlier (Vogt, 1973) that $S_1$ nuclease is fairly active (about 60%) even in the presence of 0.3% SDS.

The function of the 55 K protein is unknown. It has been suggested that the protein may have a structural role by circularizing the DNA with the virus particles (Brown et al. 1975; Corden et al. 1976). It may protect the DNA from exonuclease digestion (Sharp et al. 1976). It has been proposed that it may be involved in DNA replication by allowing initiation or
completion of the 5' ends of the progeny strands (Rekosh et al. 1977). The 55 K protein has been found to be associated with the replicating molecules of Ad 5 and Ad 2 DNA (Girard et al. 1977; Kelly & Lechner, 1978). It is unknown at present whether the 55 K protein is virus-coded or of cellular origin. Further studies are required such as the comparative fingerprint analyses of the 55 K protein and other proteins coded by the virus genome, in order to elucidate its biological function.

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Note added in proof: Harter et al. (1979) have recently described a procedure for the purification of a homogeneous DNA–55 K protein complex from Ad 2 virions using exclusion chromatography on Sepharose in SDS. The authors disrupted the virions by heating in the presence of 2% SDS and 0.15 M-LiCl at 80 °C prior to exclusion chromatography. Our inability to obtain a homogeneous complex by a similar procedure described in the text might be due to the fact that the complex used by us was initially purified by centrifugation through gradients of sucrose and subsequently dialysed against TEN buffer. This dialysis step presumably results in aggregation of virion proteins. Under the conditions used, such an aggregate was found to be resistant to complete dissociation by exclusion chromatography in 0.1% SDS.

REFERENCES


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