SPECIFIC INTERACTION OF A PROTEIN(S) AT OR NEAR THE TERMINI OF ADENOVIRUS 2 DNA

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SUMMARY

When human adenovirus type 2 DNA was extracted from the virions, and the sedimentation profile of the DNA-protein complex in a linear sucrose gradient was analyzed by the nitrocellulose filter binding assay, it was found that 90-95% of adenovirus 2 DNA molecules was tightly associated with the protein(s). The analyses of the binding site of the protein(s) on the DNA by using the restriction endonucleases suggested that the protein(s) interact with the DNA at or near the termini of the viral genome. The stability of the linkage between the DNA and the protein(s) was tested in the presence of various reagents. From the available data, it is suggestive that the protein(s) is linked to the DNA covalently.

INTRODUCTION

Human adenovirus (Ad)* DNA extracted from the virions by treatment with pronase, SDS and phenol, consists of linear, nonpermuted duplex molecules of 23 x 10^6 daltons (1,2). Ad 2 DNA prepared from the virions by several methods that avoided the use of pronase and SDS, contained many molecules that by sedimentation and electron microscopy appeared to be in the circular form (3,4). The circular molecules were converted to the duplex linear monomers by treatment with pronase which suggested that the circularization of the genome was due to the protein - protein interaction at the termini of the DNA. We started the present studies on the location of the binding site of the protein(s) on Ad 2 DNA as a diversion from our many unsuccessful attempts to label the 5' termini of deproteinized Ad 2 DNA. The working hypothesis we made was that the proteolysis of Ad 2 DNA-protein complex might have left some residual peptide still attached at

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*Abbreviations used: Ad, adenovirus; SDS, sodium dodecyl sulfate; Eco Rl, Hae III and Hpa I - restriction endonucleases from E. coli carrying the RTF1 plasmid, Hemophilus aegyptius and Hemophilus parainfluenzae, respectively (see Ref. 5); TE buffer, 0.01 M Tris-HCl (pH 7.4) and 0.001 M EDTA; HEPES, N-2-Hydroxyethyl piperazine-N'-2 ethane sulfonic acid;

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or near the 5' termini to block the phosphorylation by the polynucleotide kinase. There are presently two methods for the isolation of Ad 2 DNA-protein complexes. One that was described by Robinson et al (3) involves the use of guanidine hydrochloride (at 4 M final concentration) for the extraction from the virions as well as during the purification by sucrose density gradient. Doerfler et al (4) described a method for the isolation of Ad 2 DNA - protein complex by treatment of the virions with sarkosyl, followed by centrifugation to equilibrium on a CsCl density gradient. We used the former method to analyze the binding site of the protein(s) on the DNA using the restriction enzymes, Eco RI and Hpa I (5). We found that the protein(s) was associated with the terminal fragments of the DNA cleaved by these enzymes. We also report here data regarding the stability of the linkage between the protein(s) and the DNA to the various reagents. These results suggest that the protein(s) might be covalently linked to the DNA.

MATERIALS AND METHODS

Enzymes Eco RI, Hpa I were purchased from Biolabs (Boston, Mass.).

Viral DNA-protein complex or DNA uniformly labeled with [^{32}P]. To prepare [^{32}P] labeled Ad 2, KB cells were infected at a MOI of 50 PFU/cell. Four hrs later, the cells were suspended in phosphate-free medium containing HEPES buffer, pH 7.4, 2% horse serum and 0.4 mci/ml [^{32}P] phosphoric acid. The virus was purified as described earlier (6,7). The extraction and the purification of the DNA-protein complex from the virus was according to Robinson et al (3) except that the sucrose density gradient centrifugation was carried out at 25,000 rpm at 4°C for 20 hours.

A portion of the complex (23 µg of DNA) after dialysis against TE buffer containing 0.05 M NaCl, was deproteinized with protease K for 30 min at 37°C in a reaction mixture (2.2 ml) containing 100 mM NaCl, 10 mM Tris-HCl (pH 7.4) 1 mM EDTA, 1 mM 2-mercaptoethanol and 0.1% SDS. The mixture was then extracted with TE buffer - saturated phenol, precipitated twice with 2 volumes of ethanol and the DNA dissolved in TE buffer (0.5 ml).

Nitrocellulose filter-binding assay Ad 2 DNA-protein complex (containing about 1 µg of DNA) after various treatments as described, was made 4 M in guanidine hydrochloride. The solution was filtered through a nitrocellulose membrane filter (0.45 µ; 25 mm, Millipore Corp.) presoaked in TE buffer containing 4 M guanidine hydrochloride. The filter was washed with two 0.5 ml aliquots of the above buffer. The combined filtrate as well as the filter were counted for Cerenkov radioactivity.

RESULTS

Ad 2 DNA-protein complex, uniformly labeled with [^{32}P] was purified through a sucrose density gradient centrifugation in the presence of 4 M guanidine hydrochloride as shown in Fig. 1. The protein(s) tightly associated with the DNA was located by using the millipore filter binding assay. In this system, the labeled DNA not bound to the protein(s) will pass through

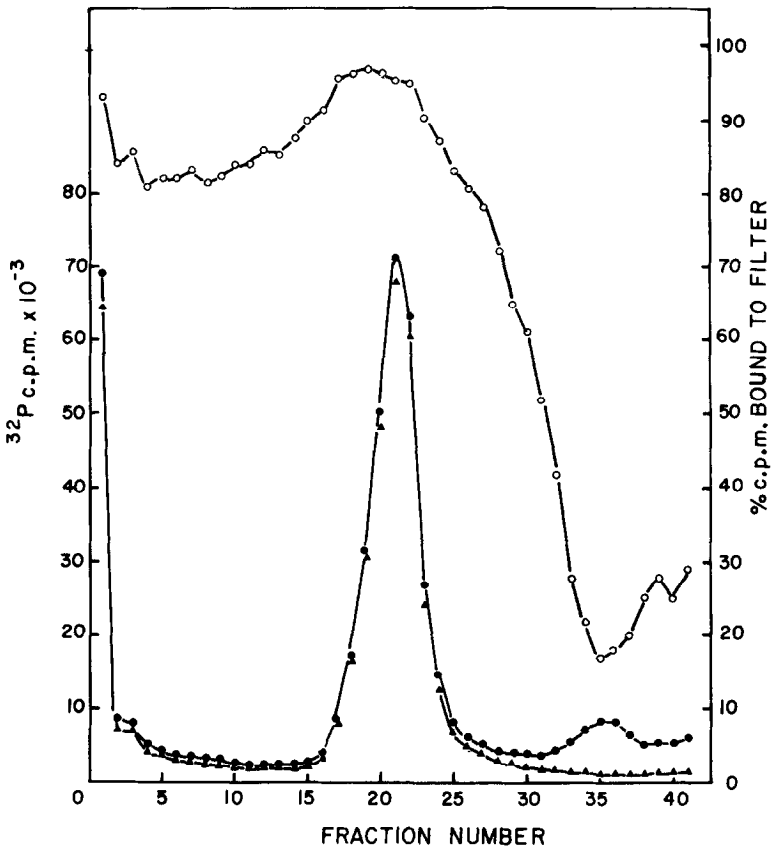

Fig. 1 Localization of Ad 2 DNA-protein complex in the sucrose density gradient fractions. The procedure used for the extraction and the purification of Ad 2 DNA-protein complex was the same as described (3). Fractions of 0.9 ml each were collected from the bottom. Aliquots of 10 µl were counted for total radioactivity as Cerenkov cpm. Each aliquot of the fractions was then mixed with 0.5 ml of 4 M guanidine hydrochloride in TE buffer (pH 7.4) and passed through a nitrocellulose filter as described under methods. The filter and the filtrate were counted for Cerenkov radioactivity. ● —— ●, Total cpm in 10 µl aliquots; ▲ —— ▲, Filter-bound cpm; o —— o, % cpm bound to the filter.

the filter, whereas the protein(s) - bound DNA will be retained (see Table I, Expts. 1 and 3). The material sedimenting at the bottom of the gradient had Ad 2 DNA sequences, as its Eco RI pattern after treating with pronase was identical to that of Ad 2 DNA. It was also associated with protein(s) as shown by the filter binding assay. The high sedimentation value of this complex is presumably due to the intermolecular protein - protein interaction between the monomer units resulting in the formation of oligomers. The major peak of radioactivity contained about 90-95% of the DNA molecules

TABLE I

Nature and the stability of the linkage between Ad 2 DNA and the protein(s)

Expt.	Treatment of DNA-protein complex	cpm bound to the filter (Cerenkov cpm)	cpm in the filtrate (Cerenkov cpm)	% bound to the filter
1	No treatment			100
2	+ 6.0 M urea	31238	6852	82
3	+ pronase	1443	31400	4
4	+ NaCl (4 M)	25111	937	96
5	+ guanidine hydrochloride (4 M)	28782	655	98
6	+ hydroxylamine hydrochloride (3.86 M, pH 4.23) 4 hrs at 37°C	28640	451	98
7	+ NaOH (0.35 N) 1 hr at room temperature; then neutralized	37282	4052	90
8	+ NaOH (0.35 N); 8 hrs at room temperature; then neutralized	47514	4642	91

The stability of the linkage between the DNA and the protein was tested by the nitrocellulose filter-binding assay as described under Materials and Methods. The stability of the linkage in the presence of 1% SDS could not be tested by this method because SDS is precipitated in the presence of 4 M guanidine hydrochloride. Hence, the electrophoretic method on agarose gel was used and it was found that the complex is stable under the conditions tested. The value in Expt. 1 is taken as 100% for the reason stated in the text.

bound to the protein(s) while the minor peak of radioactivity was almost free of any tightly associated protein(s). The plot of the percentage cpm bound to the filter (see Fig. 1) shows that although the fractions 2-15 had very little labeled DNA, 81-88% of the radioactivity in these fractions bound to the filter. This value drops from 97% for the fraction 19 to 17% for the fraction 35, where the minor peak of radioactivity sediments.

For the location of the binding site of the Ad 2 DNA-associated protein(s), we used *Eco* RI (8) and *Hpa* I (9,10) (Figs. 2 and 3, respectively),

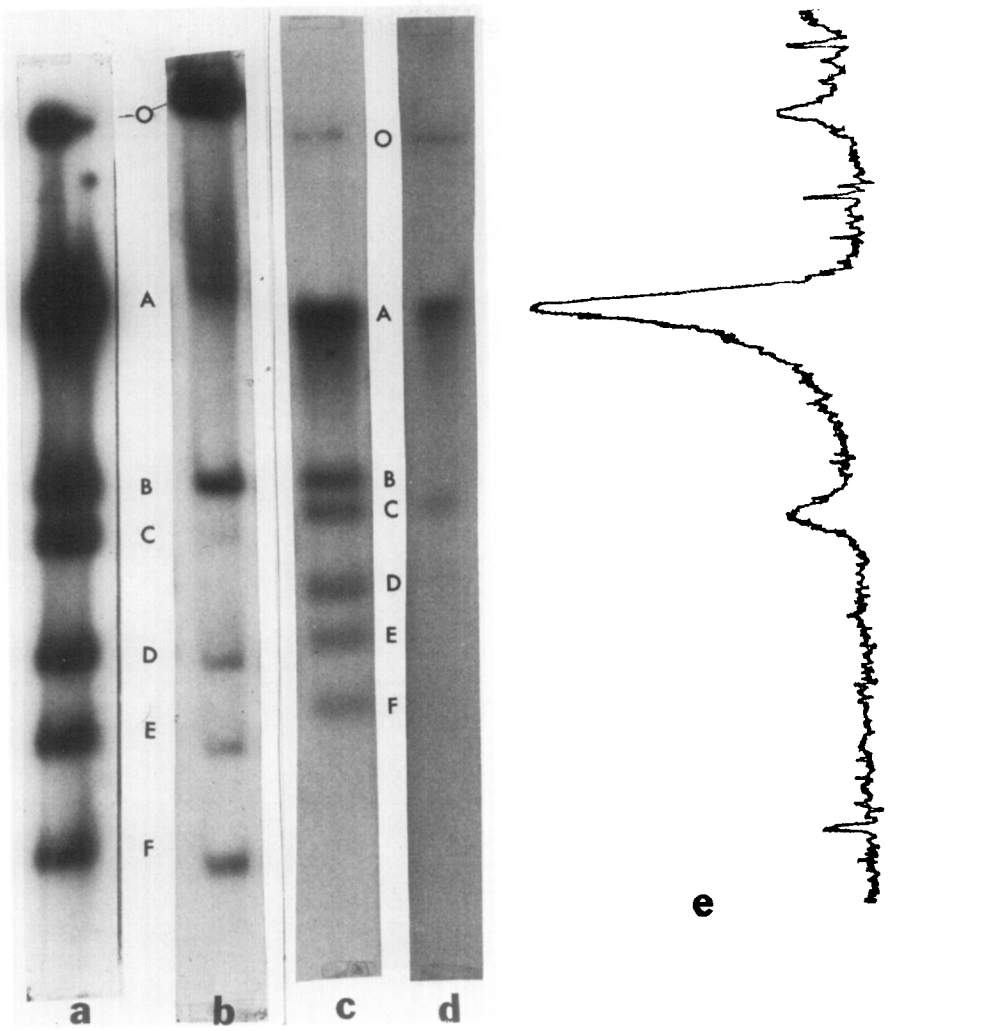

Fig. 2 Analysis of the protein-binding site on Ad 2 DNA using Eco RI restriction endonuclease. The specific activity of the DNA in the complex (the major peak of radioactivity in Fig. 1) was 7×10^5 cpm/µg. The digestion with Eco RI was carried out as described (8). A sample of Ad 2 DNA deproteinized with protease K was also digested with Eco RI. Eco RI digests of a) Ad 2 DNA (deproteinized) b) Ad 2 DNA-protein complex, c) same as a) except that about one sixth the amount of radioactivity was applied onto the gel and d) the material which stayed at the origin in b) after purification by hydroxylapatite chromatography and pronase treatment as described under Results, were fractionated on a 1% agarose slab gel; e) a microdensitometer (Joyce, Loebl Co.) scan of the autoradiograph from which d) was derived. The ratio of the relative densities of A and C bands, calculated from e) is 6:1. A similar scan of c) yielded the values of percent density of the bands A to F as 59.5, 12.1, 9.8, 7.4, 6.4 and 4.8, respectively, very close to the values obtained from their molecular weights (8).

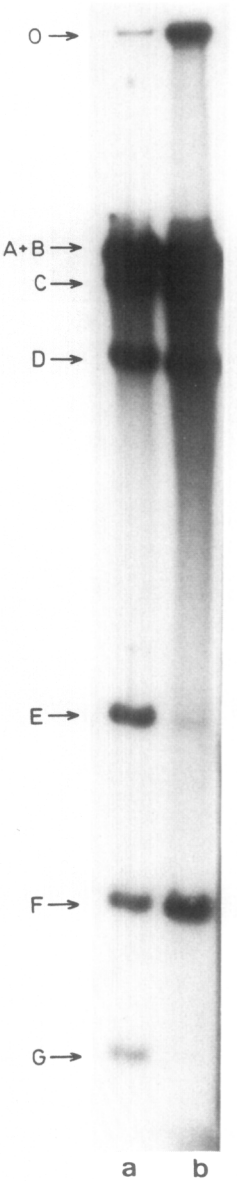

Fig. 3 Analysis of the protein binding site on Ad 2 DNA using Hpa I restriction endonuclease. Hpa I enzyme digestion was carried out at 37°C for 2 hours in an incubation volume of 50 µl containing 6 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM KCl, 25 mM NaCl and 1 unit of the enzyme for 1.5 µg of the labeled DNA. Hpa I digests of a) Ad 2 DNA (deproteinized) and b) Ad 2 DNA-protein complex, were fractionated on a 1.4% agarose slab gel.

since their cleavage maps on Ad 2 DNA are known. The restriction endonuclease digests of the DNA-protein complex or the DNA after the removal of the protein(s) were analyzed by agarose slab gel electrophoresis, making use of the observation that the protein - bound DNA fragments stayed at the origin of the gel (11). By comparing the restriction enzyme patterns of Ad 2 DNA (Fig. 2a, obtained after deproteinization of the DNA-protein complex, it is evident that the terminal fragments, A and C were involved with the b inding of the protein(s), as they were prevented from migrating into the gel. When this material was eluted from the origin of the agarose gel using hydroxyapatite chromatography (using HTP grade from Biorad Labs. and 0.3 M phosphate buffer in 4 M guanidine hydrochloride), followed by treatment with pronase, terminal A and C fragements were obtained (Fig. 2c and 2d). These data are very similar to those of Brown et al (11) using the sarkosyl cores of Ad 2 DNA-protein complex. A small percentage of radioactivity which migrated to the position of A and C bands in Fig. 2b, resulted from the DNA molecules devoid of protein(s), present in our preparation of DNA-protein complex (the amount of these molecules varies between 10-15%). Since these Eco RI terminal fragments of Ad 2 DNA, A and C are about 60% and 10% respectively, of the genome (8), it was necessary to narrow down the region involved with the binding of the protein(s). Therefore, the labeled Ad 2 DNA-protein complex or the DNA was treated with Hpa I enzyme. The terminal fragments E and G are missing in the Hpa I pattern of the DNA-protein complex, presumably retained at the origin of the agarose gel due to the binding with the protein(s) (see Fig. 3). These terminal fragments E and G are about 1400 base pairs (4%) and 493 base pairs (1.4%) long, respectively (10). These data show that the binding sites of the protein(s) are present within the Hpa I terminal fragments of Ad 2 DNA.

The stability of the linkage between Ad 2 DNA and the protein(s) was tested in the presence of various reagents using nitrocellulose filter binding assay. The preparation of DNA-protein complex contained about 10% of the DNA molecules not associated with the protein(s) as this fraction passed through the filter (see above). The value of % bound to the filter in the presence of various reagents was recalculated to take into consideration the presence of a small percentage of molecules devoid of protein(s). The presence of 4 M guanidine hydrochloride during the filtration through the nitrocellulose filters was necessary to avoid any non-specific adsorption of the complex to the filter and also to dissociate any loosely bound proteins to the complex. The data shown in Table I indicate that the linkage between the DNA and the protein(s) is stable in 6 M urea, 4 M NaCl, 4 M guanidine hydrochloride (pH 7.0), 3.86 M hydroxylamine hydrochloride (pH 4.2) and alkali (0.35 N) but sensitive to pronase.

DISCUSSION

We describe in this communication a useful method of the nitrocellulose filter binding assay, applicalbe specifically to analyze the stability of the linkage between Ad 2 DNA and the protein(s). This method has been used quite extensively for studying the lac (or λ) operator-repressor complexes (12,13,14), the E. coli RNA polymerase binding to the specific sites on T7 DNA and amino acyl-RNA synthetase-tRNA complexes (15,16).

In the sarcosyl core described by Brown et al (11), polypeptide VII, according to the nomenclature of Maizel et al (17), with a molecular weight of 18,500 (18) was the predominant protein component present, while other polypeptides were present in minor amounts. The authors had reported that polypeptide VII can be quantitatively separated from the viral DNA by treatment with alkali or boiling in SDS. Robinson et al (3) reported that treatment of Ad 2 DNA-protein complex with SDS or alkali converted circular molecules into linear monomers. These data suggest that the circularization of the adenovirus 2 genome due to protein - protein interaction at or near the termini of the DNA may be mediated through the polypeptide VII in conjunction with a linker protein(s) present in either sarcosyl core or in the DNA-protein complex prepared using guanidinium hydrochloride (11,3). Once this polypeptide VII is removed, Ad 2 DNA can presumably no longer remain in the circular form.

The resistance of the DNA-linker protein complex to alkali, 4 M guanidine hydrochloride, 4 M NaCl, 6 M urea and 1% SDS suggests that the linker-protein(s) is covalently attached to the DNA. However, our data can not rule out the possibility that a strong non-covalent bonding in the presence of urea, NaCl and guanidine hydrochloride which is then converted to a covalent bond in the presence of alkali or SDS. It is unlikely that such a conversion would take place quantitatively in vitro (see Ref. 19). The resistance to 3.86 M hydroxylamine hydrochloride at pH 4.23 does not rule out the absence of phosphamide linkage between the DNA and the protein(s) (20), as the reaction is also influenced by adjacent peptide and nucleotide sequences (21).

A salt-stable SV40 DNA-protein complex has been isolated and the binding site of the protein has been localized at about 0.67 map unit clockwise from the Eco RI cleavage site, proximal to the origin of replication (22,23). A covalent attachment of a protein presumably at a specific site but to staggered nicks near the origin of replication of SV40 DNA has also been described, when the virus was subjected to a more stringent SDS lysis (24,19). DNA-protein complexes have also been reported from Bacillus bacteriophages,

ϕ 29 and GA-1 and a protein has been shown to be involved in the circularization of phage DNA as well as in the transfection (25,26,27). A covalent attachment of a protein to the 5' terminus of the DNA has been described in the Col E_1 protein-DNA complexes (28). In the presnet system of Ad 2 DNA-protein complex, it is not known whether the same protein molecule is involved in binding to both ends of the DNA molecule. When this manuscript was under preparation, a similar finding by Sharp et al (27) that Ad 5 DNA is associated with protein(s) at or near its termini was reported. However, the use of the nitrocellulose filter-binding assay has allowed us for the first time to study the stability of the Ad 2 DNA-protein complex under various conditions. Further work is needed to determine the size of the protein, characterize the nature of the linkage between the DNA and the protein and elucidate the biological role of the protein, tightly associated with the viral genome.

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