Analysis of the DNA-Terminal Protein from Different Serotypes of Human Adenovirus

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The DNA-terminal proteins from adenovirus type 12, type 3, and type 5 were analyzed, after labeling *in vitro* with ^{125}I by the chloramine T method, and were shown to be serotype specific. We also studied the kinetics of cleavage by alkali of the terminal protein-DNA linkage and showed that the half-time of cleavage with either 0.1 M NaOH or 0.5 M piperidine at 37°C is about 15 to 30 min. The substitution of the volatile base piperidine for NaOH in this procedure provided a useful tool for rapid analysis of the labeled protein.

The DNA present within the adenovirus virion exists as a DNA-protein complex (12). Attached covalently to each 5' end of the DNA is a protein of apparent molecular weight of 55,000 in molecules isolated from adenovirus type 2 (Ad2) or Ad5 (9-11). Attempts to correlate this protein with a specific adenovirus gene product were until recently unsuccessful (9, 10), and indeed there has been speculation that the protein may be cellular (8, 9). Recently, however, a precursor form of the protein was identified (2, 3, 13), and comparison of this precursor with a polypeptide of identical molecular weight (87K), synthesized from RNA encoded by an early transcription region of adenovirus DNA, also only newly discovered (6, 14), finally clearly demonstrated that the terminal protein is indeed a bona fide adenovirus early gene product (13; D. Rekosh, M. H. Binger, S. J. Flint, unpublished data).

In this paper we examine in detail the mature form of the terminal protein from different serotypes of human adenoviruses of the A, B, or C subgroups, and demonstrate definitively that the protein of each serotype examined is distinct, although very similar to ones from the others, consistent with the notion that the protein is viral coded and highly conserved. We also examine in detail the susceptibility of the DNA-terminal protein linkage to cleavage with alkali and characterize the kinetics of such cleavage.

Alkaline cleavage of the DNA-protein linkage. Adenovirus DNA-protein complex normally is refractile to digestion with 5' exonuclease and will not function as a substrate for polynucleotide kinase (1). Pretreatment with 0.1 M NaOH, however, overcomes these blocks and indicates that the DNA-terminal protein linkage is susceptible to cleavage by NaOH. To study

this cleavage directly, radioactive DNA-terminal protein complex from Ad5 labeled *in vivo* with $^{32}\text{PO}_4^{2-}$ was prepared and treated with DNase as previously described (11). This material was then incubated with 0.1 M NaOH for various lengths of time before being analyzed on sodium dodecyl sulfate gels. In addition, a portion of the sample was not treated with alkali, and a portion was treated with RNase A. An autoradiogram of the resulting gel is shown in Fig. 1A.

Figure 1A (slot 7) contained the starting material which received no treatment other than DNase. A dark band was seen at the 55K position. This band represents the terminal protein attached to a few nucleotides (the limit digest of the DNase) (11). Slot 8 contained the sample treated with 1 mg of RNase per ml for 4 h as well as DNase. As there was little difference between slots 7 and 8, these data suggest that ribonucleotides were not connected to the protein. Slots 1 to 6 contained the DNase-treated samples incubated with NaOH for varying lengths of time. Analysis of the autoradiogram indicates that the band present at 55K disappeared as a function of incubation time. The half-time of hydrolysis appears to be about 15 to 30 min. These particular samples also contained a band at around 120K (the size of polypeptide II) which also disappeared with increasing incubation time. It has not appeared in other ^{32}P preparations and was not investigated further.

To confirm that alkaline hydrolysis removes the terminal protein from the DNA and that the protein remains intact after treatment, *in vitro* ^{125}I -labeled DNA-terminal protein complex was prepared and subjected to treatment with alkali. In the experiment shown here, 0.5 M piperidine was substituted for 0.1 M NaOH. We found that the use of piperidine, which can be removed by

FIG. 1. Cleavage of the linkage between terminal protein and DNA with alkali. (A) A 6.0- μ g amount of $^{32}\text{PO}_4$ -labeled DNA-protein complex (4×10^6 cpm/ μ g) was treated with 20 μ g of DNase I in 200 μ l of solution containing 0.005 M Tris (pH 7.5)-0.005 M MgCl_2 -0.001 M phenylmethylsulfonyl fluoride (PMSF) for 3 h at 37°C. After treatment with DNase, 150 μ l of the sample was made 0.1 M in NaOH (using 10 M NaOH) and incubated for a further period at 37°C. Samples of 25 μ l were taken at various times, neutralized, and dialyzed against 0.01 M Tris (pH 6.7)-0.001 M PMSF to remove the salt. (Slot 1) 5 min; (slot 2) 15 min; (slot 3) 30 min; (slot 4) 45 min; (slot 5) 60 min; (slot 6) 90 min; (slot 7) no NaOH, only DNase treatment (25- μ l sample); (slot 8) 1 mg of RNase added per ml for 4 h to 25 μ l of sample. The slot marked M contains [^{35}S]methionine-labeled Ad5-infected cell extract. The figure shown is the resulting fluorogram using Kodak XRP-1 film and a Cronex-Lighting Plus intensifying screen. (B) DNA-terminal protein complex was labeled *in vitro* with ^{125}I using chloramine T. In a typical iodination, 10 μ g of DNA-terminal protein complex in 0.5 ml of 0.05 M Tris-0.001 M EDTA (pH 7.5)-0.001 M PMSF was iodinated with 1 to 2 mCi of ^{125}I . Usually the iodine was added in a small volume (10 to 20 μ l), followed immediately by 10 μ l of a 5-mg/ml chloramine T solution, freshly prepared. The reaction was allowed to proceed for 3 min at room temperature, at which time 20 μ l of a 5-mg/ml sodium metabisulfite solution was added to stop the reaction. The radioactive DNA-terminal protein complex was then separated from free iodine by passage over a column of BioGel A-15 (100 to 200 mesh), made up in 0.05 M Tris-0.001 M EDTA-0.001 M PMSF-0.1% sodium dodecyl sulfate, in a 10-ml disposable pipette (approximate size, 1 by 15 cm). The peak of radioactivity which eluted at the void volume was collected and dialyzed extensively against several changes of 0.05 M Tris-0.001 M EDTA-0.001 M PMSF to remove the sodium dodecyl sulfate. A portion of this material was then made 0.5 M in piperidine (using 10 M piperidine) and incubated at 37°C for various lengths of time. At each time point, a 10- μ l sample was taken, neutralized with ammonium acetate (1.1 M, pH 5.5), and lyophilized. The lyophilized sample was taken up in sodium dodecyl sulfate sample buffer and applied directly to a slab gel which was then dried and fluorographed. (slot 1) 0 min; (slot 2) 5 min; (slot 3) 15 min; (slot 4) 30 min; (slot 5) 45 min; (slot 6) 60 min.

lyophilization, gave higher yields of protein and a more quantitative recovery than treatment with NaOH.

Figure 1B (slot 1) demonstrated that, without treatment, the starting material did not contain an ^{125}I -labeled band present on the gel at the 55K position (position of the arrow). In this particular experiment, the radioactive material did not remain directly on the top of the gel, as was usually the case, but was seen as a high-molecular-weight smear. The reason for this is not known, but may be due to degradation of

the DNA. Incubation in the presence of piperidine (Fig. 1B, slots 2 to 5), for increasing lengths of time, resulted in the disappearance of this smear and the appearance of a band at approximately 55K, with a half-time of about 15 min, comparable to the half-time obtained for the removal of nucleotides from ^{32}P -labeled DNA (Fig. 1A). This half-time is consistent with what would be expected to be the half-time of cleavage of the known bond—a phosphodiester linkage between the first nucleotide of the DNA and a β -OH on a serine (5).

Interestingly, the actual apparent molecular weight of the protein after treatment with either piperidine or NaOH is 53K rather than 55K as it appeared after DNase treatment (data not shown). The most likely explanation for the slight mobility difference of the protein is that the nucleotides remaining after DNase treatment are removed by the alkali and that these affect the mobility of the terminal protein. However, we cannot at present exclude the possibility that in addition to removing the nucleotides, treatment with alkali also cleaves or alters the protein itself, causing it to migrate differently. These findings recently were confirmed by others (13).

Size of the terminal protein from different serotypes. Green et al. (8) previously examined the terminal proteins from different serotypes of human adenoviruses. The proteins which they examined were derived from Ad12, Ad7, Ad2, Ad19, and Ad4, representing the adenovirus groups A to E, respectively. In that study, they concluded that the apparent molecular weights of the protein derived from each serotype were identical (i.e., 55K) and that the primary sequences of these proteins were either identical or highly conserved.

We reexamined this question. In our hands, in contrast to those of Green et al. (8), clear differences in the electrophoretic mobility of the protein serotypes of different subgroups could be demonstrated. For example, Fig. 2A shows the 125 I-labeled protein derived from Ad12 (slot 1) compared to the 125 I-labeled protein derived from Ad5 (slot 2), and Fig. 2B (slot 1) shows the 125 I-labeled protein derived from Ad5 (slot 1) compared to the 125 I-labeled protein derived from Ad3 (slot 2). In each case a marker of 125 I-labeled Ad5 virions was also run (slot M). In this experiment the terminal proteins were labeled in vitro with 125 I and were released from the DNA by treatment with DNase. The mobility differences seen here, however, were also maintained in proteins released from the DNA after treatment with piperidine (Fig. 2C). Thus, these mobility differences cannot be attributed simply to different lengths of nucleotides remaining attached to the different proteins after DNase treatment, as has been suggested (8), and must reflect distinct structural differences of the three proteins. The actual apparent molecular weights of the three proteins without nucleotides attached (after piperidine treatment) are 53, 52, and 50K for Ad5, Ad3, and Ad12, respectively. The same mobility differences have been seen in proteins from virus grown in 293 cells (7) (data not shown).

Partial proteolysis peptide maps. To fur-

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of terminal proteins from different serotypes. (A and B) 125 I-labeled DNA-protein complex was prepared, treated with DNase, and subjected to sodium dodecyl sulfate-polyacrylamide gel analysis as previously described (11). The dried gels were then fluorographed. (A) Slot 1, Ad12 protein; slot 2, Ad5 protein; M, 125 I-labeled Ad5 virions. (B) Slot 1, Ad5 protein; slot 2, Ad3 protein; M, 125 I-labeled Ad5 virions. (C) 125 I-labeled DNA-protein complex was prepared from the different serotypes, and half of each preparation was treated with 0.5 M piperidine as described in the legend to Fig. 1. Half was not treated. The samples were then applied to the gel which was dried and fluorographed. (Slot 1) Ad5 protein complex, piperidine treated; (slot 2) Ad3 protein complex, piperidine treated; (slot 3) Ad12 protein complex, piperidine treated; (slot 4) Ad5 protein complex, no treatment; (slot 5) Ad3 protein complex, no treatment; (slot 6) Ad12 protein complex, no treatment; (slot M) 125 I-labeled Ad5 virions.

ther characterize the structural differences between the terminal proteins of the different serotypes, the 125 I-labeled proteins from each se-

rotype were subjected to partial proteolysis peptide mapping with V8 protease using essentially the method first described by Cleveland et al. (4). In one experiment (Fig. 3), ^{125}I -labeled samples of Ad12 and Ad5 DNA-protein complex were treated with increasing concentrations of V8 protease and incubated at 37°C for a given length of time. After digestion, half of each DNA-protein complex sample was then treated with piperidine and lyophilized, and half was not treated. The samples were then applied to slab gels and analyzed.

Figure 3A shows the products of the Ad12

FIG. 3. V8 protease digestion of the Ad5 and Ad12 terminal proteins. ^{125}I -labeled DNA-terminal protein complexes from Ad5 or Ad12 were treated with varying concentrations of V8 protease. Each 50- μl reaction contained 0.006 optical density unit (at 260 nm) of DNA-protein complex and 5 μg of bovine serum albumin. Incubation was carried out at 37°C for 1 h. The digestion was then terminated by addition of phenylmethylsulfonyl fluoride to 1 mM and boiling, and half of each incubation was treated with 0.5 M piperidine for 2 h. (A) Piperidine-treated half; (B) untreated half. (Slots 1, 3, 5, 7) Ad5; (slots 2, 4, 6, 8) Ad12, treated with 25, 2.5, 0.25, and 0 μg of V8 protease per reaction mix, respectively. Slot M contains ^{125}I -labeled Ad5 virions as marker. The gel was dried and fluorographed.

complex (even slots) or Ad5 (odd slots) after digestion and piperidine treatment. Figure 3B shows the products only after digestion with protease but without piperidine treatment. As expected, the pattern in Fig. 3B is a subset of the pattern of Fig. 3A, because partials which remain attached to the DNA would not be expected to enter the gel unless released with piperidine. Distinct differences between the partial products of the Ad12 and Ad5 proteins can be seen. The most prominent difference is indicated by the arrow. In addition, it is clear from a direct comparison of each slot in Fig. 3A (piperidine treated) to those in Fig. 3B (not treated) that the partials differ in the way they are connected to the DNA. In particular, of the three partials seen in slot 6 (Ad12), only the largest two are attached to the DNA, whereas of the three similar partials seen in slot 5 (Ad5), only the largest is attached to the DNA. Thus, these proteins clearly must have different primary sequences.

Figure 4 shows the same sort of analysis carried out for the protein derived from Ad3 (odd slots) and Ad5 (even slots). In this experiment, the samples were not treated with piperidine so that only partials not attached to the DNA are

FIG. 4. V8 protease digestion of the Ad3 and Ad5 terminal proteins. ^{125}I -labeled DNA protein complexes from Ad3 and Ad5 were incubated for 1.5 h at 37°C with increasing amounts of V8 protease as described in the legend to Fig. 3. (Slots 1, 3, 5, 7) Ad3; (slots 2, 4, 6, 8) Ad5 containing 0, 0.25, 2.5, and 25 μg of protease, respectively. Slot M contains ^{125}I -labeled Ad5 virions added as marker. Samples were applied directly to the gel after treatment. The gel was dried and fluorographed.

displayed. Although the patterns of proteolysis of the Ad3 and Ad5 proteins appear very similar, slight differences in the size of the products definitely can be seen (the Ad3 partials migrated slightly faster), and a few bands are distinct to one serotype or another. Thus, as in the case of the protein derived from Ad12 and Ad5, we can conclude that there are distinct structural differences between the Ad3 protein and the Ad5 protein. Partial proteolysis maps of proteins derived from Ad2 and Ad5 showed no detectable differences (data not shown).

It should be noted that the method of analysis utilized in this study, i.e., partial proteolysis mapping, accentuate the differences between the polypeptides, because of the rather large effect that a single amino acid change can have on the pattern generated. Although the molecular weight and peptide map of each protein analyzed are very similar, the data do not allow us to comment quantitatively on the actual degree of similarity between the different proteins. The study of Green et al. (8), however, involved analysis of the terminal proteins by two-dimensional tryptic and chymotryptic mapping. This method does allow one to make an estimate of the degree of similarity, and in that study each peptide map appeared virtually identical to each of the others, leading the authors to speculate that the proteins might be identical. However, because small differences in single peptides can go undetected against the background of identical spots and not all peptides can be labeled with iodine, the authors could not truly distinguish identity from similarity. If the present study and that of Green et al. (8) are taken together, the conclusion one is led to is that the terminal protein must be a highly conserved virus-specified entity. It seems likely that this high degree of conservation is reflective of the highly specialized and similar role that the protein seems likely to have in DNA replication in each serotype.

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