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STUDIES ON THE NATURE OF THE LINKAGE BETWEEN THE TERMINAL PROTEIN AND THE ADENOVIRUS DNA

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Summary: The termini of human adenovirus types 7 and 2 DNA extracted from the virions using pronase and protease K are covalently linked to peptides that contain tyrosine residue(s). The peptide attached to each terminus can be labeled with [^{125}I] in vitro. The linkage between the peptide moiety and the terminal nucleotide is sensitive to snake venom phosphodiesterase, Aspergillus nuclease S_1 and micrococcal nuclease. The available data suggest that the peptide is linked to the terminal nucleotide of these DNA molecules through a phosphodiester bond.

Human Ad* DNA extracted from the virion using guanidine hydrochloride is a linear, duplex molecule containing a protein of 55,000 daltons tightly associated with its termini (1-8). Treatment of the Ad virion or the DNA-protein complex with a protease results in a linear DNA whose 5' ends were blocked to digestion with 5' exonucleases and phosphorylation by phosphatase, polynucleotide kinase and [γ-^{32}P] ATP (9). However, the 3'-specific E. coli exonuclease III acts on such viral DNA molecules (10,11,12). These observations suggested that the 55K protein bound (presumably covalently) to the 5' termini leaves a residual amino acid or peptide attached to the 5' terminal nucleotide upon treatment with protease K or pronase. The 5' terminal nucleotide has been shown to be a dC residue (9,13-15). In this communication, we wish to report that the viral DNA isolated from Ad 2 or Ad 7 serotype by treatment with protease K and/or pronase can be specifically labeled at the 5' termini with [^{125}I] using chloramine-T as the oxidizing agent (16). Although under some circumstances iodine may react with sulf-

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*Abbreviations used: Ad, Adenovirus

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hydryl groups or with tryptophan, the chemistry of iodination is essentially the chemistry of substitution of iodine into tyrosine groups (17). We also present evidence that a peptide containing tyrosine residue is most likely bound to the 5' phosphate of DNA through a phosphodiester bond.

MATERIALS AND METHODS: Ad2 and Grider Strain of Ad7 were grown in KB cells, and virions purified as described (18). The dialyzed virus suspension (10 ml) was treated with pronase (100 μg/ml; Calbiochem) in an incubation mixture containing 0.2 mM mercaptoethanol, 10 mM Tris-HCl, pH 7.2 and 5 mM EDTA for 30 min at 37°C. Subsequently the reaction mixture was treated with 500 μg of protease K (EM Laboratories) in the presence of 0.1% SDS at 37°C for 30 min. The DNA was phenol extracted and dialyzed against 0.01 M Tris-HCl, pH 7.4 containing 1 mM EDTA and precipitated with ethanol before labeling with [^{125}I]. Iodination was carried out in the absence of SDS.

[^{125}I] labeling of Ad DNA: Ad7 DNA (78 μg) or Ad2 DNA (60 μg) was dissolved in 100 μl of 0.05 M sodium phosphate, pH 7.5 in an Eppendorf tube (1.5 ml capacity). Chloramine-T (2 μl of 1 mg/ml) and 1 mC of carrier-free Na ^{125}I (Amersham Corp.) were then added and the contents of the tube were mixed well. The reaction mixture was incubated in ice for 30 min. The reaction was stopped by the addition of 5 μl of sodium metabisulfite (1 mg/ml) and 10 μl of KI (10 mg/ml) and the mixture was diluted to 300 μl with H_2O. A 50 μl aliquot of 0.02% bromophenol blue and 50% glycerol was added and the mixture was applied to an agarose column (1.5 M from BioRad Corp.) packed in a plastic disposable pipet of 10 ml capacity. The column was eluted with 0.01 M Tris-HCl, pH 7.5, 0.001 M EDTA and 0.1 M NaCl. The [^{125}I] labeled DNA, was precipitated with two volumes of ethanol. The precipitate was dissolved in 100 μl of 0.01 M Tris-HCl, pH 7.4, containing 1 mM EDTA.

Enzymatic Digestion: Ad7 DNA was digested with pancreatic DNAse I in an incubation mixture (50 μl) containing 7.8 μg of DNA, 6 mM $MgCl_2$, 60 μg of DNAse I and 1 mM phenyl methyl sulfonyl fluoride. Incubation was carried out at 37°C for 60 min. At the end of the reaction, 25 μl of the reaction mixture was treated at 37°C for 2 h with 37.5 μg of snake venom phosphodiesterase (Worthington Biochemical Corp.) in an incubation mixture containing 50 mM Tris-HCl, pH 8.0 and 10 mM $MgCl_2$. An aliquot of [^{125}I] labeled Ad7 DNA (7.8 μg) was denatured by heating in a boiling water bath for 5 min. and quickly cooled in ice. It was subsequently treated with single-strand specific S_1 nuclease (20 units) at 37°C for 1 h in an incubation mixture (20 μl) containing 2.5 μg of denatured calf thymus DNA, 5 mM $ZnSO_4$, 30 mM sodium acetate, pH 4.6 and 5% glycerol.

Staphylococcus aureus (micrococcal) nuclease digestion of [^{125}I] labeled Ad7 DNA (2 μg) was carried out in an incubation mixture (50 μl) containing 10 μg of calf thymus DNA, 20 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$ and 300 units of the enzyme at 37°C for 1 h.

[^{125}I] labeled Ad7 DNA-protein complex: The DNA terminal protein complex of Ad7 was purified by the method described by Robinson et al. (1) and labeled with [^{125}I] as described above. The labeled complex was dialyzed to remove unbound [^{125}I], digested with protease K, extracted with phenol and precipitated with ethanol.

Acid hydrolysis of [^{125}I] labeled Ad DNA: The labeled DNA was hydrolyzed in sealed glass ampules under nitrogen in the presence of 20 μg of bovine serum albumin carrier protein in 200 μl of 2 M HCl at 110°C for 24 h.

3MM Paper Ionophoresis: The enzyme digests were spotted on a 34 cm length of Whatman No. 3MM paper and subjected to electrophoresis in pH 1.9 formic/acetic acid buffer system as described (19).

RESULTS AND DISCUSSION: The [^{125}I] labeled Ad DNA was analyzed by cleaving with restriction enzymes. Fig. 1a,c,e and g show the ethidium bromide fluorescence (20) and fig. 1b,d,f and h show the autoradiography of 1.4% agarose gel. Fig. 1a and c show the

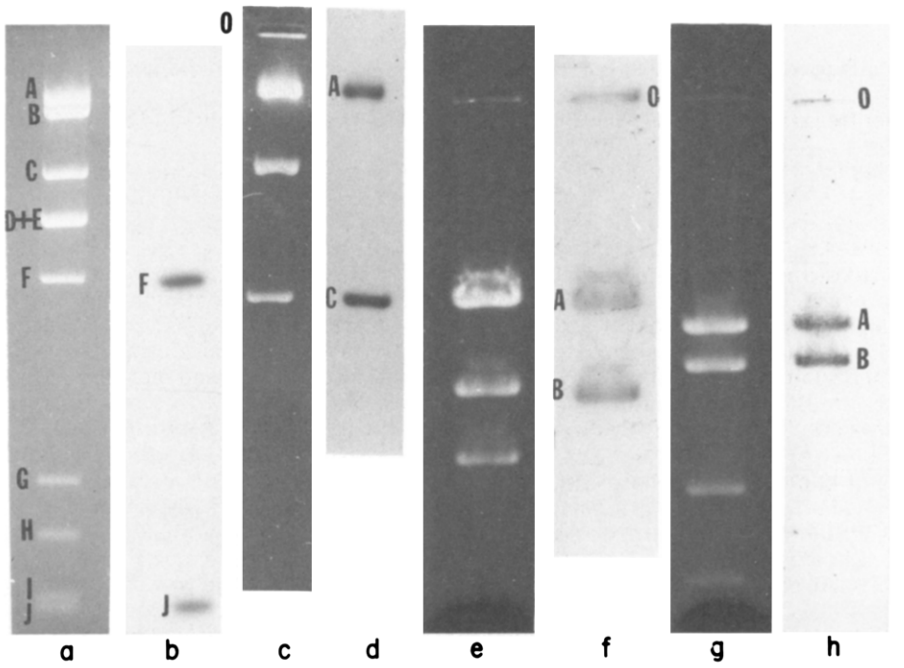


Fig. 1 Cleavage of [^{125}I]-labeled Ad7 and Ad2 DNA with restriction enzymes. a-d. [^{125}I]-Ad7 DNA (1 μg) was digested with BamHI (31) (a & b) and HpaI (20) (c & d). e-h. [^{125}I]-Ad2 DNA (4 μg) was digested with SalI (23) (e & f) and BamHI (g,h). One unit of enzyme was added in each case and the incubation was for 1 h at 37°C. The DNA fragments were fractionated on a 1.4% agarose gel and the ethidium bromide fluorescence was photographed (20) (a,c,e and g). b,d,f and h represent the autoradiography of [^{125}I] labeled DNA fragments, fractionated on gels a,c, e and g, respectively.

cleavage pattern of BamHI and HpaI of [^{125}I] labeled Ad7 DNA, respectively. Fig. 1b and d show the autoradiography of the dried gel from Fig. 1a and c. Only the terminal fragments BamHI-F and J and HpaI-A and C (21, our unpublished observations) were labeled. Fig. 1e and g show the cleavage pattern of SalI and BamHI of [^{125}I] labeled Ad2 DNA and Fig. 1f and h show the autoradiography of the same gel. All the label was incorporated into the terminal fragments, SalI-A and B and BamHI-A and B (22,23). The internal SalI-D fragment which maps between 24.7 and 25.6 map units was too faint and is not seen in this gel. The results in Fig. 1 indicate that the label was specifically bound to terminal fragments of both Ad7 and Ad2 DNA.

Ad7 DNA-protein complex was isolated, labeled with [^{125}I] and subsequently treated with protease K. A protease resistant peptide containing a portion of the total [^{125}I] label still remained associated with the DNA and the restriction enzyme analysis demon-

strated that the label was also associated with the terminal fragments identical to the results shown in Fig. 1a-d. Moreover, the specific activity of the labeled DNA was approximately the same (3×10^5 cpm of [^{125}I] per μg of DNA) because the number of tyrosine residues that were susceptible to labeling and resistant to protease was the same whether labelling preceded or followed protease treatment. We do not know, in the present studies, exactly how much of the 55K terminal protein is resistant to protease, since the complex purified by velocity sedimentation on sucrose gradients containing guanidine hydrochloride was found to contain a number of other virion proteins associated with the terminal 55K protein (24). These data indicate that the iodinatable moiety at the termini of Ad DNA probably originates from the terminal protein.

The linkage between DNA and [^{125}I] labeled moiety was stable to treatment with 4M hydroxylamine, pH 4.2, at 37°C for 2 h. Under these conditions hydroxylamine is known to cleave phosphamide bonds while phosphodiester bonds are hydroxylamine-resistant (25). On the other hand, over 80% of the label was rendered acid-soluble after incubation with 0.1 N NaOH at 37°C for 17 h (Fig. 2). This observation can serve as an indication for presence of a phosphodiester bond between the terminal phosphate and a hydroxyl group of serine, threonine, or probably also tyrosine since these bonds are particularly sensitive to

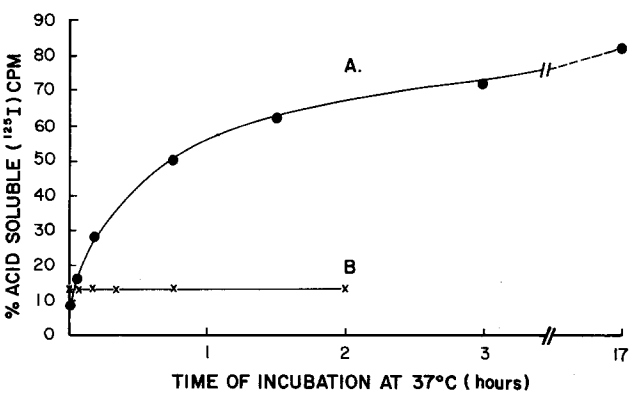
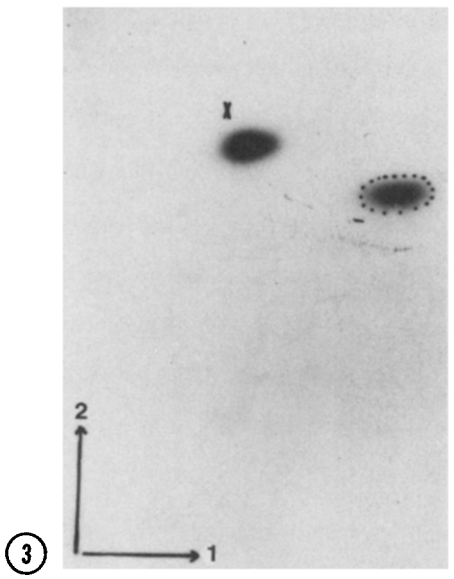


Fig. 2 Effects of alkali (—●—●—) and hydroxylamine (—X—X—) treatments on the linkage of [^{125}I] tyrosine-peptides to Ad7 DNA. [^{125}I] labeled Ad7 DNA (1.5 μg) was incubated with 0.1 N NaOH or with 4M hydroxylamine at pH 4.2 at 37°C. The aliquots (2.5 μl) were removed at indicated intervals of time and the acid-soluble radioactivity was measured (32) using a Packard gamma counter. The background for acid-soluble cpm was 10-13% due to the presence of [^{125}I]$^-$ incompletely removed by agarose exclusion chromatography.



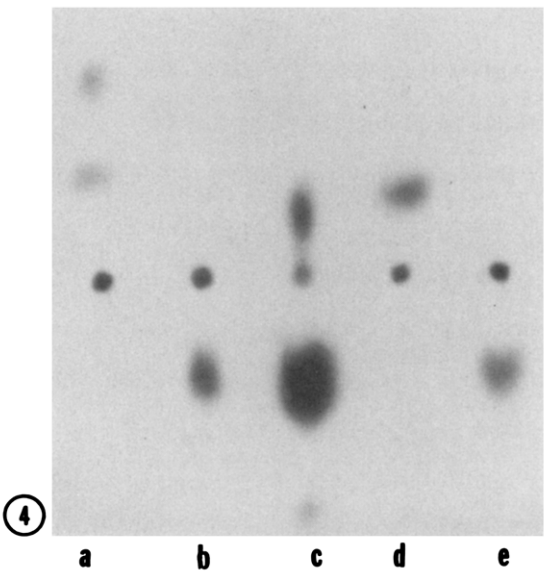


Fig. 3 Two-dimensional thin layer chromatography. [^{125}I] labeled Ad7 DNA was hydrolyzed in the presence of 2N HCl as described in the text. The hydrolysate was chromatographed in two dimensions in cellulose-coated thin layer plate as described (26). The position of unlabeled monoiodotyrosine marker was determined by ninhydrin staining and is indicated by the dashed outline. The relative amount of spot X was variable, increasing with the time of acid hydrolysis. In this experiment, the monoiodotyrosine spot contained 37% of the input.

Fig. 4 Ionophoretic separation at pH 1.9 of [^{125}I] labeled Ad7 DNA. Ad7 DNA was extracted from the virion after proteinase K treatment and labeled with [^{125}I] as described in the text. a. DNAseI (Worthington Biochemical Corp.) digestion of [^{125}I] Ad7 DNA. b. DNAseI, followed by snake venom phosphodiesterase. c. Denatured [^{125}I]-Ad7 DNA (7.8 μg) after treatment with S_1 nuclease. d. Micrococcal nuclease digestion of [^{125}I]-Ad7 DNA. The reaction was terminated by heating to 90^{o}C for 5 min. One half of the sample was treated with E. coli alkaline phosphatase (1 unit) at 37^{o}C for 1 h and the sample was then applied to Whatman 3MM paper (e). Electrophoresis was carried out using formic/acetic acid buffer (pH 1.9) system (19) at 2400 volts. At the end of electrophoresis, the paper was dried and autoradiographed.

alkali treatment (25). This result is consistent with the observation of Carusi (9) who showed that alkali treatment of the viral DNA permitted labeling of the 5' end by alkaline phosphatase, polynucleotide kinase and [γ-^{32}P] ATP. [^{125}I] labeled DNA was acid hydrolyzed and analyzed by two dimensional thin layer chromatogrphy. Autoradiography (Fig. 3) revealed two spots. One of these spots comigrated with the monoiodotyrosine standard and the other (spot X) migrated as free [^{125}I]$^-$ control (not shown). [^{125}I]$^-$ or [^{125}I]Cl appeared due to partial decomposition of monoiodotyrosine during acid hydrolysis (16). Prior to acid hydrolysis, the material contained no free monoiodotyrosine and a small amount of unbound [^{125}I] (data not shown). These results indicate that the [^{125}I] label was incorporated into a tyrosine residue(s) present in the terminal peptide.

In order to further investigate the nature of the linkage between the [^{125}I]-tyrosine-containing moiety and Ad7 DNA, the labeled DNA was digested either with DNAseI and snake venom phosphodiesterase or denatured and subsequently treated with S_1 nuclease. The products were analyzed by paper electrophoresis at pH 1.9. Fig. 4 (a-c) shows the autoradiogram of such an experiment. The DNAseI digestion generated a series of oligonucleotides bound to the labeled moiety which moved towards the anode (Fig. 4a). However, when the DNAseI digest was treated with snake venom phosphodiesterase, a 3'→5' exonuclease, the mobility towards the anode was reversed, and the radioactivity migrated towards the cathode (Fig. 4b). When the denatured [^{125}I]-Ad7 DNA was treated with S_1 nuclease (Fig. 4c), material migrating towards the cathode was produced, indicating that the linkage between the labeled moiety and the terminal nucleotide is also sensitive to S_1 nuclease. The most plausible explanation of these results is that the terminal peptide is linked to the 5'-terminal nucleotide via a phosphodiester bond and venom phosphodiesterase and S_1 nuclease cut the bond between the peptide and the 5' phosphate.

In order to confirm this explanation, the labeled DNA was treated with micrococcal nuclease, which cleaves the DNA, generating 3'-phosphate and 5'-OH terminated oligonucleotides. The [^{125}I] labeled moiety which migrated towards the anode was produced (Fig. 4d). When this material was subsequently treated with E. coli alkaline phosphatase, it migrated towards the cathode as in the case of venom phosphodiesterase or S_1 treatments (Fig. 4e). This observation can be explained if we assume that micrococcal nuclease generates peptides linked to a terminal phosphate moiety which originally represented the 5' terminal phosphate of DNA. This phosphate was removed by phosphatase treatment. Identical results were obtained when [^{125}I]-Ad2 DNA was used in these studies. The data presented above indicate that tyrosine-containing peptide moiety is bound to the 5' ends of Ad DNA most likely through a phosphodiester bond. Ambros and Baltimore and Rothberg et al. have shown that the 5' end of poliovirus RNA is linked to the terminal VPg protein via a phosphodiester bond which is formed by the hydroxyl group of a tyrosine residue (26,27). In Ad system, we do not yet have any evidence indicating which amino acid residue participates in the formation of the linkage. The tyrosine residue which was iodinatable is probably not linked to the terminal phosphate of Ad DNA. It was reported

that a free hydroxyl group is necessary for the incorporation of I atom to the phenyl ring of tyrosine (27,28). Further experiments are in progress to identify the amino acid that is linked to the terminal nucleotide of Ad DNA. We are also investigating whether iodination of the terminal peptide of Ad7 and Ad2 can be extented to Ad DNA of other groups. This new method of terminal labeling can be useful in physical mapping of Ad DNA molecules with several restriction endonucleases. The fact that two Ad DNAs belonging to different groups sharing less than 15% homology (29) have their nucleic acids linked to the terminal protein by the same type of linkage is consistent with the results of Green et al. (30) who have shown a remarkable similarity in the peptide maps of Ad2 and Ad7 terminal proteins. Future research will throw light on the biological function of the terminal protein.

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