SPECIFIC CLEAVAGE OF THE TERMINAL PROTEIN FROM THE ADENOVIRUS 5 DNA UNDER THE CONDITION OF SINGLE-STRAND SCISSION BY NUCLEASE S1

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Received 21 September 1979

1. Introduction

The structural features of a linear duplex DNA of adenovirus are an association of a protein at the 5′-termini of either strand and the presence of an inverted terminal repetition (reviewed [1,2]). Recently, the nucleotide sequence of the 5′-terminal fragment of adenovirus 5 DNA [3] and 2 DNA [4,5] were reported. However, the precise structure of the 5′-terminal part is still obscure, because the presence of the terminal protein prevents the phosphorylation of the 5′-termini with polynucleotide kinase and digestion with phage λ 5′ → 3′ exonuclease [6].

Here we show that the terminal protein of adenovirus 5 DNA was cleaved out from DNA by nuclease S1 under the conditions for single-strand cleavage, suggesting a single-stranded structure in the region adjacent to the 5′-termini.

2. Materials and methods

Adenovirus type 5 (Ad5) DNA–protein complex was extracted from the Ad5 virions by 4 M guanidine–HCl [7] and purified by the sucrose gradient [8] and exclusion chromatography of Sepharose 4B [9]. In the case of the iodination of the Ad5 DNA–protein described below, CsCl equilibrium centrifugation [7] was added to the above two purification steps. Guanidine–HCl (4 M) and 0.02% Triton X-100 were included in all the purification steps.

The protein associated with the Ad5 DNA termini was assayed by the filter binding method [10] and BND-cellulose column chromatography [11,12].

Iodination of the terminal protein was performed with Bolton-Hunter reagent containing ^{125}I [13,14].

Identification of the cleavage of the terminal protein from the Ad5 DNA was carried out as follows: Ad5 DNA–[^{125}I]protein complex (~500 cpm) and Ad5 [^{3}H]DNA–protein complex (~1000 cpm) were mixed together and digested with the various enzymes. After digestion, the reaction mixture was mixed with blue dextran and phenol red, loaded onto the Bio-gel A 1.5 m column, 0.7 × 12 cm, equilibrated with 4 M guanidine–HCl, 10 mM Tris–HCl (pH 8.1), 1 mM EDTA, 0.02% Triton X-100, and eluted with the same buffer. Fractions (0.25 ml) were collected and the radioactivity of each fraction was counted.

The conditions for enzymatic digestion are in the footnote of table 1.

3. Results

The [^{3}H]thymidine-labeled Ad5 DNA–protein complex was extracted from the Ad5 virions and purified with sucrose gradient centrifugation and exclusion chromatography with Sepharose 4B containing 4 M guanidine–HCl. After digestion of the Ad5 [^{3}H]DNA–protein with the various enzymes, the association of the terminal protein with DNA was investigated by two methods (table 1). In the filter

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Table 1
Effect of the various enzymes on the presence of the terminal protein in Ad5 DNA

Treatment	Binding assay	BND-cellulose
Control	97.0%	100%
+ Pancreatic DNase I (200 μg/ml)	11.0%	5.2%
+ Micrococcal nuclease (500 unit/ml)	0	n.d.
+ Nuclease S1 (400 unit/ml)	7.7%	0.3%
+ Pancreatic RNase IA (100 μg/ml)	100%	n.d.
+ Proteinase K (200 μg/ml)	3.5%	0.5%
+ Pronase (1 mg/ml)	5.2%	n.d.

Ad5 [^{3}H]DNA–protein (~2000 cpm/assay) was treated with the various enzymes. The conditions of enzyme digestion were as follows: DNase I, 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$; micrococcal nuclease, 30 mM sodium borate (pH 8.8), 1.4 mM $CaCl_2$; RNase IA, 10 mM Tris–HCl (pH 8.1); proteinase K, 10 mM Tris–HCl (pH 8.1); pronase, 10 mM Tris–HCl (pH 8.1); nuclease S1, 30 mM sodium acetate (pH 4.5), 100 mM NaCl, 0.3 mM $ZnSO_4$. All the reactions were at 37°C for 1 h. After the reaction, the mixtures were diluted with 10 vol. (1 ml) buffer for the filter binding method (1 M NaCl, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA) or BND-cellulose column chromatography (0.3 M NaCl, 10 mM Tris–HCl (pH 8.1), 10 mM EDTA). The presence of the terminal protein was assayed as in section 2. n.d., not done

binding assay, protein-free DNA or DNA without covalently associated proteins can pass through the membrane, while DNA associated covalently with proteins can be retained on the membrane [10]. In the BND-cellulose column chromatography assay, protein-free DNA can be eluted with 1 M NaCl, while DNA associated covalently with proteins is eluted with 8 M urea and 1% SDS [11,12]. The results were similar in both the assays. Deoxyribonuclease (DNase) and micrococcal nuclease degraded [^{3}H]DNA, therefore, the association of the terminal protein with the yielded nucleotide or oligonucleotide could not be detected. Ribonuclease (RNase) showed no effect. This implies that ribonucleotide may not be contained near the 5′-termini. Proteinase K and pronase degraded the terminal protein, therefore protein-free DNA was filtered through the membrane. When the Ad5 [^{3}H]-DNA–protein was digested with nuclease S1 under the conditions for single-strand scission [15], the percentage retained on the membrane decreased as with DNase digestion. This is also confirmed by gel electrophoresis as shown in fig.1, in which the intact DNA-carrying protein hardly enters into the gel column, whereas the S1-treated DNA moved in the gel as with proteinase K-treated DNA. The possibility, that nuclease S1 degraded DNA to pieces too small for retention on the membrane, was excluded because the size of DNA before and after the S1 digestion was the same judging by centrifugation experiments in alkaline sucrose gradient (data not shown) and electrophoresis in agarose gel (fig.1: the position of a faint DNA band in the lane 1 gel is almost the same as those in the lanes 2, 3). This result implies that a nuclease S1-sensitive structure exists; that is, single-stranded regions or the regions without base pairings.

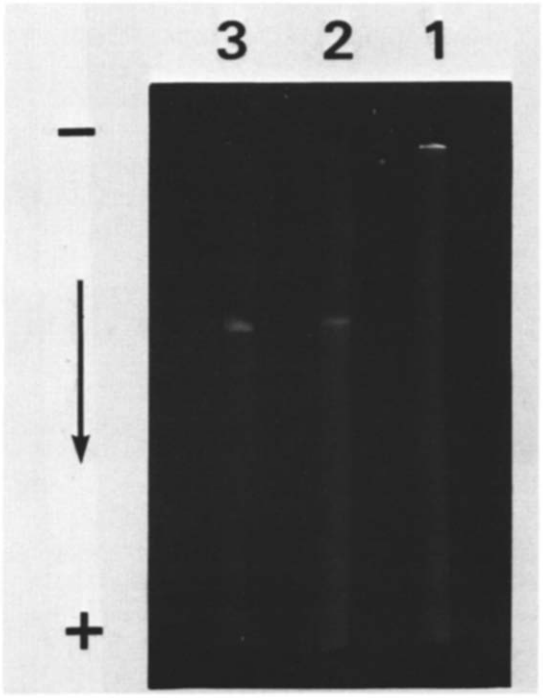

Fig.1. Gel electrophoresis of Ad5 DNA. Ad5 DNA was electrophoresed in 0.6% agarose gel, Sigma type 2, using 36 mM Tris–phosphate buffer (pH 7.8) containing 1 mM EDTA. After the gel was stained with ethidium bromide, photograph was taken under ultraviolet light. Enzymatic treatment was as in table 1. (1) Not treated; (2) treated with nuclease S1; (3) treated with proteinase K.

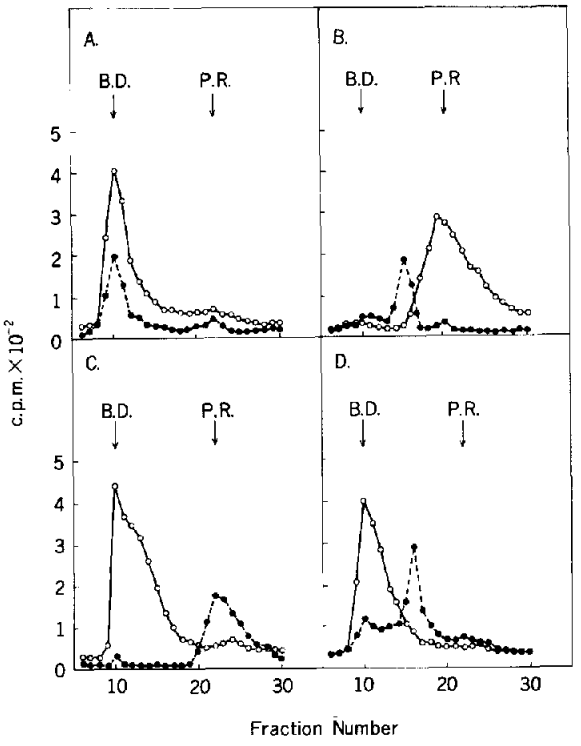

Fig.2. Gel filtration of Ad5 DNA. Ad5 DNA–[^{125}I]protein and Ad5[^{3}H]DNA–protein were mixed together and were digested with the DNase I, proteinase K, and nuclease S1 in conditions of table 1. After the digestion, the reaction mixture was added with blue dextran (BD) and phenol red (PR) and eluted through Bio-gel A 1.5 m column as in section 2. (○——○) [^{3}H]DNA; (●– – –●) [^{125}I]protein. (A) not treated; (B) treated with DNase I; (C) treated with proteinase K; (D) treated with nuclease S1.

Further, the terminal protein was iodinated with Bolton-Hunter reagent of ^{125}I [13,14]. Ad5 DNA–[^{125}I]protein was mixed with Ad5 [^{3}H]DNA–protein and digested with DNase, proteinase K, and nuclease S1, respectively. After digestion, [^{125}I]protein and [^{3}H]DNA were separated by the Bio-gel A 1.5 m column chromatography (fig.2). Non-treated Ad5 [^{3}H]DNA–[^{125}I]protein was eluted in the void volume (fig.2A). After DNase digestion, [^{3}H]DNA was eluted in the position of the low molecular weight and [^{125}I]protein was between the void and the low molecular weight position (fig.2B). After proteinase K digestion, in the opposite way, [^{3}H]DNA was in the void and [^{125}I]protein was in the low molecular weight position (fig.2C). With nuclease S1 digestion, [^{3}H]DNA was eluted in the void and [^{125}I]-protein was between the void and the low molecular weight without any degraded low molecular weight material (fig.2D). This result shows that nuclease S1 must split the junction between DNA and protein without degradation of either material. This result implies that the protein links to the short single-stranded stretch from the 5′-termini of the double-stranded Ad5 DNA.

4. Discussion

The replication of adenovirus DNA proceeds through a displacement mechanism [1,2]. The origin and terminus of replication has been localized near the molecular termini [16–21]. However, the mechanism of the start of replication at the 5′-termini remains unclear. From these points, the structure of the region near the 5′-termini is required to be clarified. The results reported here show the presence of the nuclease S1-sensitive structures near the 5′-terminal region.

Two possibilities are considered for the structure near the 5′-termini of Ad5 DNA:

(1) The presence of an short single-stranded stretch at the 5′-termini, to which protein is linked covalently;

(2) Two strands near the termini are not paired as in an ordinary way in DNA duplex.

Possibility (1) is preferable for many reasons, to be discussed. It is also reasonable to explain the binding to DNA of protein or primer for the initiation of DNA synthesis. If the terminal protein were linked to the DNA through tyrosine-phosphate linkage like that of poliovirus [22,23], it could be necessary for DNA to be single-stranded due to the chemical energy [24]. Although the length of single-stranded part has not been clarified yet, it would be several nucleotides long at most, because it can be discriminated in electron microscopic examination if it is ⩾10 nucleotides. It was observed that *Escherichia coli* DNA gyrase made a staggered cut, creating DNA termini with a free 3′-OH and 5′-extension, consisting of 4 bases, where DNA gyrase was linked covalently [25]. That structural feature seems to be very similar to the situation of Ad5 DNA.

The mechanism of the replication of the 5′-termini of Ad DNA remains unclear at present. A model with a terminal protein playing a role for initiation as a primer was proposed [14]. It was observed, however, that the initiation site was a short distance from the exact 5′-end of Ad12 DNA [26] and that the initiation step is sensitive to ribonuclease in Ad5 DNA replication in vitro [27]. The explanation for these could not be done only by the terminal protein-primed model. Thus, the nuclease S1-sensitive structure near the 5′-terminal might be a key to clarify the initiation mechanism of DNA synthesis. The presence of the similar single-stranded stretch at the 5′-terminus was also reported for *B. subtilis* phages, which carries a protein at the 5′-terminus of DNA also [28]. These specific structures may play a common role for the initiation of replication of this DNA.

Following our finding here, the terminal fragment (*Hin*dIII-G fragment) of Ad12 DNA was sequenced [29] and it was found that 5 nucleotides at the 5′-terminus did not encounter the complementary nucleotides at the 3′-terminal region in an opposite strand. This supports our results with Ad5 DNA.

Acknowledgements

The authors thank Dr T. Ando for his kind supply of nuclease S1. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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