CLEAVAGE OF ADENOVIRUS-5 DNA BY AN ENDONUCLEASE FROM *HAEMOPHILUS PARAINFLUENZAE*

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1. Introduction

Adenovirus type 5 is able to transform cells in vitro, and it has been shown that only about 6% of the genome is necessary for transformation [1]. In attempts to determine which part of the genome is involved in the transformation process, and in studies of the control of transcription in transformed cells, well characterized DNA fragments with known localization in the genome are very valuable. Mapping of the fragments obtained by cleavage of virus DNA with various restriction enzymes is therefore an important approach in such studies.

The DNA of adenovirus type 5 (Ad-5) is a linear duplex with molecular weight of about 23×10^6 [2]. It was shown that the restriction endonuclease EcoRI cleaves Ad-5 DNA into three fragments denoted A, B and C in order of decreasing size [3]. In the intact virus-DNA the fragments occur in the sequence: A-C-B [4]. In the present paper the fragments obtained by cleavage of adenovirus type 5 DNA with endonuclease HpaI have been mapped.

2. Materials and methods

2.1. *Cell culture and virus preparation*

Adenovirus type 5 was propagated in human amnion monolayer cell cultures, strain FL [5]. The virus was extracted from the cells by 4 times freezing at −60°C and thawing at 35°C. The extracted virus was concentrated and purified twice in CsCl gradients [6,7]. ^{3}H-labelled virus was obtained by including [5-^{3}H] thymidine (spec. act. 4.6 Ci/mmol) in the medium 10 hr after the infection. DNA was isolated according to Bello and Ginsberg [8]. The specific activity of ^{3}H-labelled DNA was 0.2 – 2.3 $\times 10^5$ counts per min/μg.

2.2. *Isolation of enzymes*

Endonuclease EcoRI was isolated and purified according to Yoshimoi [9]. The standard incubation mixture for the cleavage of DNA contained 10 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.5 μl of EcoRI per μg of DNA. The mixture was incubated for 2 hr at 37°C and the digestion was stopped by addition of EDTA to a final concentration of 50 mM.

Endonuclease HpaI was isolated and purified as described by Sharp et al. [10]. The amount of enzyme needed for complete cleavage was measured by titration against Ad-5 DNA. The standard incubation mixture for cleavage of DNA contained 10 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 6 mM NaCl, 1 mM dithiothreitol, and 10 μl of enzyme per μg of DNA. The mixture was incubated for 4 hr at 37°C and the reaction was terminated by the addition of EDTA.

2.3. *Extraction of digested fragments*

Samples of endonuclease-treated DNA were adjusted to 1 × SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0), extracted with equal volumes of phenol and chloroform-isoamyl alchohol (24 : 1) and precipitated with 2 volumes of ethanol chilled to −20°C.

2.4. *Gel electrophoresis*

Agarose gels were prepared as described by Sharp et al. [10], except that different concentrations of

agarose were used as described in legends to figures. The gels were prepared in cylindrical glass tubes (0.5 × 12 cm). The voltage used was 4 V/cm and the electrophoresis lasted for 2–3 hr. The gels were stained in electrophoresis buffer (10 mM Tris–HCl, pH 7.8, 5 mM sodium acetate, 1 mM EDTA), containing 0.5 μg/ml ethidium bromide. In order to determine the position of radioactively labelled fragments the gels were cut into 0.9 mm slices and solubilized in Soluene-350 before the addition of scintillation solution.

Elution of DNA fragments was carried out in polyethylene tubes, to which were attached dialysis bags. The eluted samples were concentrated by aquacide, or by evaporation and then the DNA fragments were precipitated with ethanol.

2.5. *Labelling of terminal fragments*

Terminal deoxynucleotidyl transferase (spec. act. 6.250 units/mg) was obtained from P-L Biochemicals, Inc. The standard incubation mixture (40 μl) contained 40 mM cacodylate (pH 6.8), 8 mM $MgCl_2$, 0.5 mM [^{3}H]dATP (spec. act. 60 mCi/mmol), and 20 μg Ad-5 DNA. The incubation was carried out at 27°C for 40 min. The samples were then extacted with chloroform–isoamyl alcohol (24 : 1) and labelled DNA was separated from [^{3}H]dATP on a Sephadex G-100 column, equilibrated with 1 × SSC and 5 mM EDTA. The ^{3}H-labelled Ad-5 DNA was concentrated and precipitated with ethanol.

2.6. *Electron microscopy*

DNA preparations were made with the formamide/cytochrome *c* spreading technique of Westmoreland et al. [11]. The grids were rotary shadowed with platinum/carbon at an angle of 7°C. Electron micrographs were taken at 10 000 × in a Siemens Elmiskop I. Tracings of the DNA molecules were made from enlarged prints (3 ×), and the length was measured with the aid of an X-Y measuring stage connected to a Wang calculator (computer programme developed by Dr Olav Kaalhus).

3. Results and discussion

3.1. *Cleavage of adenovirus 5 DNA with endonuclease HpaI*

When Ad-5 DNA was treated with HpaI endonuclease, and the electrophoresis was carried out in gels containing 1 and 2% agarose as in fig.1, 6 distinct fragments appeared (fig.1a). When the electrophoresis was carried out in gels containing 0.7% agarose, the slowest moving peak was shown to consist of two fragments. Accordingly, the fragments were designated alphabetically in order of decreasing size as shown in fig.1a. The molecular weight of fragments D-G were estimated on the basis of the electrophoretic mobil-

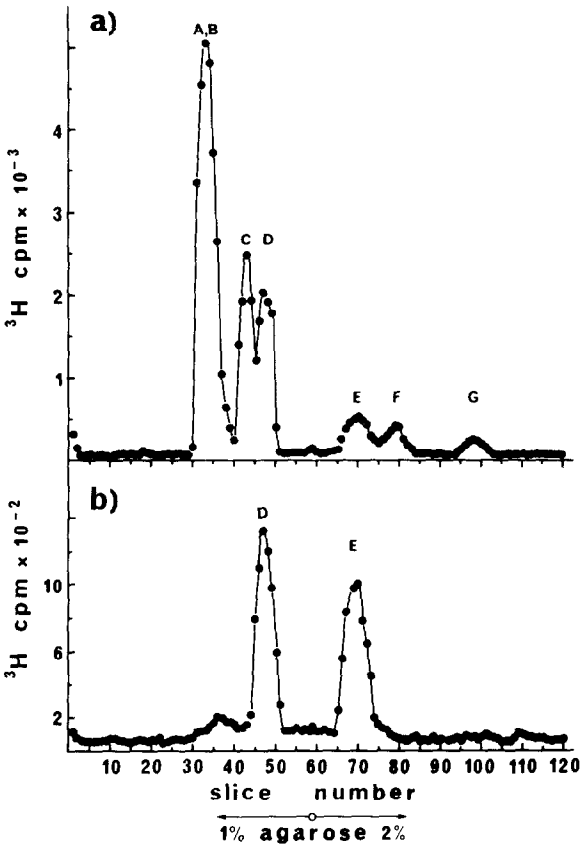

Fig.1. (a) Agarose gel electrophoresis of ^{3}H-labelled HpaI fragments of Ad-5 DNA. Ad-5 DNA (2.7 μg) was cleaved with HpaI, as described in Materials and methods and submitted to electrophoresis in agarose gels for 2 hr. The first half of the gel contained 1% agarose and the second half 2%.(b) Distribution of ^{3}H-labelled terminal fragments in agarose gels. Unlabelled Ad-5 DNA (3 μg) was labelled in the terminal position, using terminal deoxynucleotidyl transferase as described in Materials and methods. Then the DNA was cleaved with HpaI endonuclease and analyzed by agarose gel electrophoresis under the same conditions as above.

Table 1
Molecular weights of fragments obtained by cleavage of Ad-5 DNA with endonuclease HpaI

Fragment	Molecular weight ($\times 10^{-6}$)		
	Measured by electron microscopy[a]	Measured according to electrophoretic mobility	Calculated according to fraction of radioactivity in peak[b]
A + B[c]			13.6 (± 0.1)
A	8.3		6.9
B	6.1		
C	4.1		4.7 (± 0.1)
D		2.8	2.8 (± 0.05)
E		0.82	0.92(± 0.07)
F		0.72	0.71(± 0.03)
G		0.41	0.37(± 0.03)

[a] With polysterene spheres, diameter 0.312 μm as marker
[b] Average and range in 3 experiments
[c] When the electrophoresis was run in gels containing more than 0.7% agarose, fragments A and B were not separated.

ity. Due to the difficulty involved in measuring molecular weights of large fragments by this method, fragments A–C were collected after electrophoresis and examined in the electron microscope, using polysterene spheres with diameter 0.312 μm as a reference. The results are shown in table 1.

If all peaks contain unique fragments of DNA and, if the label is fairly evenly distributed throughout, the amount of radioactivity recovered in each peak should be proportional to the molecular weight of the corresponding DNA fragments. Since the molecular weight of intact Ad-5 DNA is known, it should be possible to calculate the size of the different fragments from the fraction of the total radioactivity present in the corresponding peaks. The data in table 1 show that the molecular weights calculated by this method are close to those determined by electron microscopy and by electrophoretic mobility.

3.2. *Mapping of the HpaI fragments*

To determine which of the fragments are derived from the ends of the intact Ad-5 DNA the 3′-terminal ends of the DNA duplex were labelled by attaching radioactive dATP residues by the aid of terminal nucleotidyl transferase [12]. Then the DNA was cleaved with HpaI and analyzed by gel electrophoresis. The results (fig.1b) showed that two peaks of radioactivity were found in positions corresponding to fragments D and E. Furthermore, the total amount of radioactivity in the two peaks were similar. This indicates that the fragments D and E are the terminal fragments.

Endonuclease EcoRI cleaves Ad-5 DNA into three distinct fragments with positions as shown in fig.3 [4]. These fragments were used for further localization of the HpaI fragments in the intact DNA. When EcoRI fragment A was cleaved with HpaI four peaks were obtained by electrophoresis in 1.4% agarose gels (fig. 2a). These peaks migrated at rates corresponding to the fragments A+C*, E and G obtained when intact Ad-5 DNA was treated with HpaI and run in gel electrophoresis under the same conditions. Furthermore, in fig.2a an additional fragment (X) appeared, which moved somewhat faster than fragment C.

EcoRI fragment B was cleaved by HpaI into three different fragments (fig.2b), two of which moved at the same rate as fragments D and F obtained by treat-

* Fragments A and C were not separated under the conditions of electrophoresis in fig.2a (i.e. 1.4% agarose).

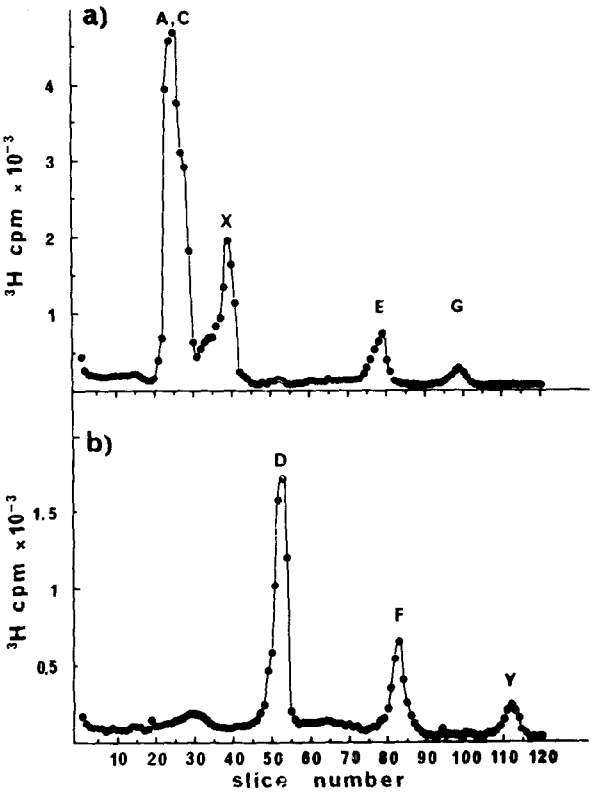

Fig.2. Agarose gel electrophoresis of fragments obtained by cleavage of EcoRI fragments of ^{3}H-labelled Ad-5 DNA with endonuclease HpaI. (a) EcoRI fragment A (3 μg) was treated with HpaI endonuclease as described in Materials and methods, and analyzed by electrophoresis in gels containing 1.4% agarose for 3 hr. (b) EcoRI fragment B (0.9 μg) was treated with HpaI endonuclease and analyzed by agarose gel electrophoresis under conditions as above.

ing intact Ad-5 DNA with HpaI endonuclease. The third peak (Y in fig.2b) moved somewhat faster than fragment G. Thus, this is a peak not appearing in the original HpaI digest of the intact DNA. Since fragment D is a terminal fragment it is clear that the material in peak Y must be derived from DNA to the left of fragment F in fig.3.

EcoRI fragment C was not cleaved by the HpaI endonuclease. Since the terminal fragment E must be derived from the left end of EcoRI fragment A (fig.3) it follows that fragment X in fig.2a must be derived from the right end of EcoRI fragment A. The molecular weights of fragments X and Y, appear-

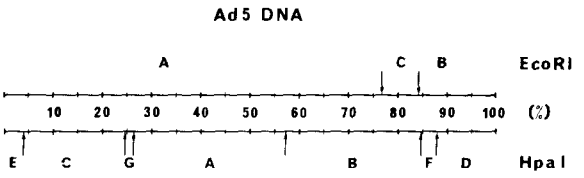

Fig.3. Cleavage map of HpaI and EcoRI-fragments of adenovirus-5 DNA. The size of the fragments is given in per cent of the molecular weight of intact Ad-5 DNA.

ing in fig.2a and b, and the molecular weight of EcoRI fragment C add up to the molecular weight of HpaI fragment B. Furthermore, cleavage of HpaI fragment B with EcoRI gives EcoRI fragment C, and two fragments with molecular weights corresponding to fragments X and Y. Since the data in fig.2b indicate that fragment Y is adjacent to fragment F it follows that also HpaI fragment B is adjacent to fragment F in the intact DNA.

In order to determine the position of HpaI fragment C and G in relation to E and A, partially digested with HpaI endonuclease. By this method DNA pieces consisting of fragments E + C and C + G were found. Since fragment E is derived from the terminal part of the intact Ad-5 DNA (fig.1b), it follows that the sequence of the 3 fragments is E–C–G. Furthermore, since fragment B is connected with fragment F (fig. 2a) it follows that fragment A is located between fragments G and B. Thus, the total sequence is E–C–G–A–B–F–D.

According to the data of Graham et al. [1] the minimal part of the adenovirus-5 genome capable of inducing transformation was between 1% and 6% from the left end of the molecule. The terminal fragment obtained from the left end of Ad-5 DNA by cleavage with HpaI (fragment E) was 4.2% of the genome. It will be interesting to see if this fragment has the ability to induce transformation.

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