RESTRICTION ENDONUCLEASE MAPPING OF DNA USING IN SITU DIGESTION IN TWO-DIMENSIONAL GELS

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1. Introduction

Physical mapping of DNA with restriction endonucleases is an important preliminary step for many studies on plasmids, viruses and isolated fragments of complex genomes (reviewed [1]). However, the difficulties encountered in such mapping greatly increase with larger molecules.

The approach most used for ascertaining which fragments of an endonuclease digest are adjacent in the intact molecule has been to isolate partial digest products, and to determine the fragments that they contain. The experiments described below show that this approach can be simplified by dispensing with the isolation of the partial digest products. Instead, after electrophoresis of the partial digest, the DNA is digested to completion with the same or another enzyme within the agarose gel, and the products are then resolved by electrophoresis in the second dimension.

2. Experimental

Plasmid pWWO-84 was isolated from *Pseudomonas putida* as in [2,3]. Digestion of phage λcI857 DNA and plasmid DNA with endonucleases *Eco*R1 and/or *Hin*dIII was carried out in 0.01 M Tris–HCl buffer, pH 7.5, containing 0.01 M $MgCl_2$, 0.05 M NaCl and 0.01 M 2-mercaptoethanol, at 37°C for complete cleavage and at 20°C for partial cleavage. The enzymes were inactivated by heating at 70°C for 10 min followed by cooling on ice. Samples were concentrated in a vacuum desiccator and mixed with loading buffer as in [2].

Definition: 1 unit of endonuclease is the amount of enzyme which cleaves 1 μg of λ DNA in 1 h at 37°C.

Electrophoresis in the first dimension was carried out in 0.7% agarose rod gels, (3 × 200 mm) in 0.036 M Tris, 0.03 M NaH_2PO_4 and 0.001 M EDTA, pH 7.7 [4], at constant voltage (80 V) until the bromophenol blue marker had migrated 12 cm.

Digestion of DNA within the gel was carried out after the piece of rod gel containing the DNA was excised and dialysed against enzyme buffer for 90 min. The gel was then placed in a tube of internal diameter 1 mm greater than the diameter of the rod, incubated for 10 h at 37°C in buffer containing 10 units *Eco*R1/0.1 ml and afterwards dialysed against the electrophoresis buffer of the first dimension. For electrophoresis in the second dimension, the rod gel was placed between two glass plates (200 mm square) held apart by 3 mm perspex strips at the sides. It was then overlayered with a 2 mm layer of the first dimension agarose gel such that the third (bottom) edge of the gel-casting block was sealed. When set, the whole was filled with either 1% or 1.5% agarose gel in 0.085 M Tris–borate buffer, pH 8.3 containing 0.025 M EDTA [5]. This combination of buffer systems has been used in [6]. Either upward or downward vertical electrophoresis of such gels was carried out at constant voltage (150 V). Horizontal electrophoresis of gels that had been set onto a single glass plate, using buffer-saturated wicks of J-cloth (Jeyes Group Ltd, Thetford, Norfolk) laid upon the surface of the gel, was also performed in several cases.

3. Results and discussion

The utility of this approach depends upon the restriction endonuclease being able to enter the agarose gel and to cleave the DNA within it. To develop this method we used phage λ DNA and *Eco*R1 endonuclease, since the cleavage sites have been well characterised [7].

Electrophoresis of intact phage λ DNA was carried out in rod gels and the section containing DNA (located by staining a rod gel run in parallel) was digested with endonuclease *Eco*R1 (see section 2). Good resolution of the six known digest products in the second dimension (fig.1) shows that complete digestion of λ DNA within the gel is achieved under these conditions, and that there is insignificant diffusion of DNA within or out of the gel rod during the various manipulations. In further experiments we found that most DNA was cleaved to completion within 2 h. Although small amounts of uncleaved DNA remained after 8 h digestion, for most purposes an incubation of from 4–6 h should suffice. Under similar conditions, endonuclease *Hin*dIII also cleaves λ DNA within the gel.

It is obvious that when partially digested DNA is separated by electrophoresis and then redigested within the gel, more complex patterns may result. Since the conditions of electrophoresis in both dimensions are similar, those fragments unaffected by the second digestion will lie on the diagonal. In contrast, two or more fragments formed during the second digestion will move relatively further in the second dimension than did their parent molecule in the first. Such fragments will then lie in a vertical line below the diagonal. The knowledge of which fragments came from a particular partial digest product can then be used as the basis for map construction. Two-dimensional electrophoretograms of the complete and partial digests of λ DNA with endonuclease *Eco*R1 are shown in fig.2a and fig.2b respectively. The singlet fragments (lettered according to their size) lie on the diagonal, whereas those arising from in situ digestion lie below it (fig.2b). By comparing their positions with those of the products of complete initial digestion it can be inferred that the partial digest contained the following complex fragments: EDB(a), DB(b), DE(c) and CF(d). Close examination also revealed a partial digest product AEDB and BCF. The composition of those fragments allows us to arrive unambiguously at the order for the six fragments first determined [7] (fig.3).

In a case where the pattern of endonuclease *Eco*R1 fragments was too complex for direct two-dimensional analysis, a different approach was used. A derivative of the *Pseudomonas putida* degradative plasmid TOL,

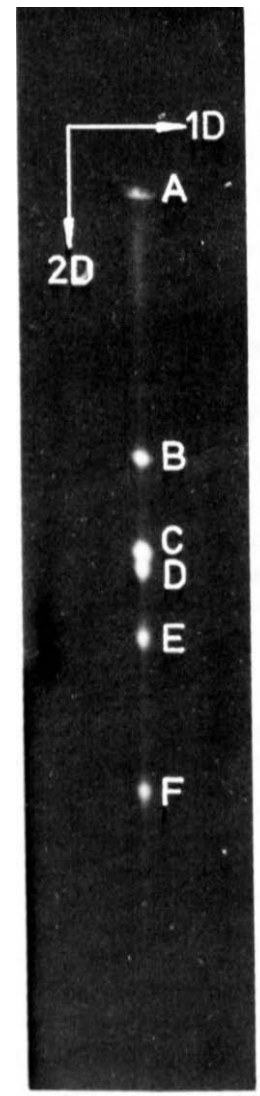

Fig.1. Digestion of λ DNA within an agarose gel. 0.3 μg λ DNA was electrophoresed in an 0.7% agarose rod gel. The section containing DNA was cut out from the gel rod, dialysed, digested with endonuclease *Eco*R1 and set within the second-dimension agarose gell (1%) as described in the text.

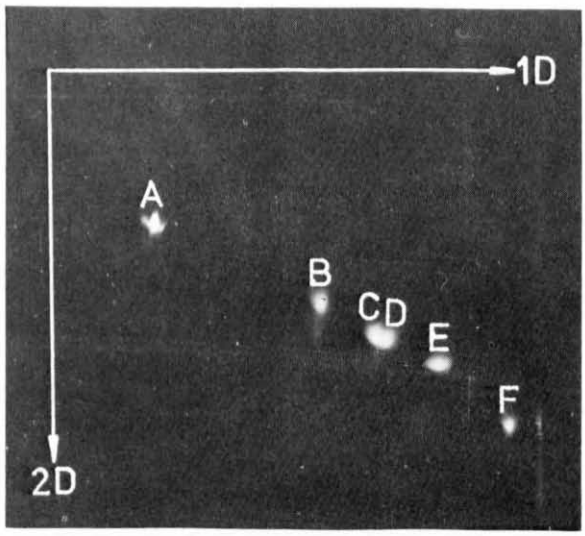

Fig.2a

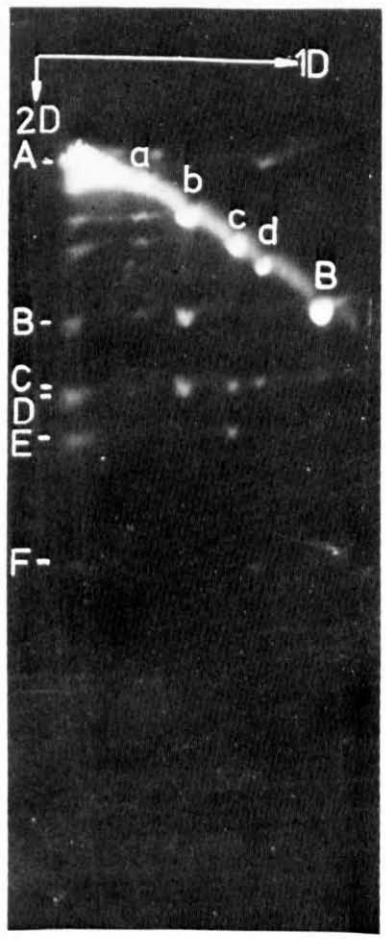

Fig.2b

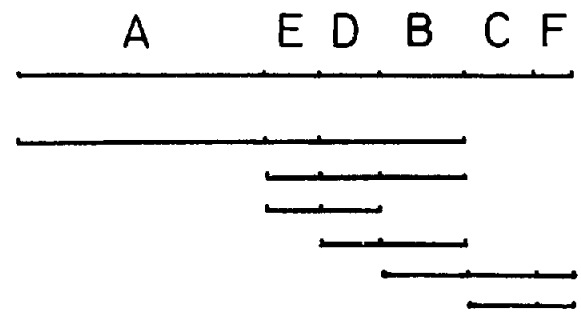

Fig.3. Schematic representation of λcI857 DNA restriction map with endonuclease *Eco*R1 [7] together with a map of λ DNA partial digest products as deduced from two-dimensional gel electrophoresis data.

pWWO-84 [3], was first completely digested with endonuclease *Hin*dIII, giving a comparatively simple set of fragments, which were then redigested by endonuclease *Eco*R1 within the gel. Two *Hin*dIII fragments, F and H, do not have *Eco*R1 sites, whereas the other fragments each give a characteristic pattern of secondary products (fig.4b). We are currently using this approach to map pWWO-84 and the parental TOL plasmid.

An extension of this technique could be used to establish whether different sized plasmids, harboured by a single bacterial strain contain similar sequences of DNA. Here, resolution of the intact plasmids in the first dimension would be followed by in situ digestion and comparison of the fragmentation patterns given by the different size species in the second dimension.

Fig. 2a. Two-dimensional diagonal gel electrophoresis of a complete endonuclease *Eco*R1 digest of λ DNA using 1 μg DNA. Electrophoresis was carried out as described in the text.
Fig. 2b. Two-dimensional diagonal gel electrophoresis of a partial endonuclease *Eco*R1 digest of λ DNA in the first dimension followed by complete digestion within the gel before the second dimension, as described in the text. (a–d) various partial digest products not digested to completion during the secondary digestion. Only a part of the gel containing partial digest products is represented.

Table 1

Characteristics of oxygen reduction by ferrocytochrome *c* oxidase and by an interfering protonmotive redox system actuated during ferrocyanide oxidation in rat liver mitochondria

Medium	[red] (mM)	ΔO	→H^+ (val)	ΔH_0^+ (FCCP)	excess O	→H^+/(excess O)
Reductant-pulse experiments						
	$K_4Fe(CN)_6$					
A	0.5	4.8	0	− 9.5	0	−
B	0.3	2.9	0	− 6.0	−0.1	−
B + NEM	0.3	3.0	2.5	− 4.8	0.6	4.1
C	0.3	4.0	4.0	− 6.0	1.0	4.0
C + NEM	0.3	4.0	4.2	− 5.8	1.1	3.8
O_2-pulse experiments						
	$K_4Fe(CN)_6$					
B	0.3	5.3 (19.8)	4.3	− 8.3	1.1	3.9
B + NEM	0.3	5.3 (19.8)	4.2	− 8.4	1.1	3.8
C	0.3	5.2 (49.8)	5.7	− 7.4	1.5	3.8
C + NEM	0.3	5.1 (49.8)	5.4	− 7.4	1.4	3.9
D	0.3	10.9 (47.6)	10.8	−16.6	2.6	4.1
D	0.9	28.8 (47.6)	28.3	−44	6.8	4.1
B	0.9	12.1 (24.9)	10.0	−19.4	2.4	4.2
B	0.9	12.1 (49.8)	8.8	−18.4	2.9	3.1
	Ferrocyto-chrome *c*					
B	0.025	23.2 (24.9)	0	−46	0.2	−
B	0.05	27.4 (24.9)	0	−54	0.4	−
B	0.05	27.4 (49.8)	0	−54	0.4	−
B + dig	0.025	28.5 (24.9)	0	−57	0	−
B + NEM	0.025	25.8 (24.9)	0	−52	−0.2	−
B + NEM	0.05	30 (24.9)	0	−61	−0.5	−
E	0.05	24.6 (24.9)	0	−49	0.1	−

Main components of the suspension media were: A, 150 mM KCl; B, 150 mM KCl, 1 mM EDTA (K salt); C, 150 mM choline chloride, 1 mM EDTA (K salt); D, 230 mM sucrose, 10 mM KCl, 10 mM $MgCl_2$; E, 150 mM KCl, 1 mM EGTA, 5 mM $MgCl_2$. In addition, all media (3.3 ml) contained 3.3 mM glycylglycine, carbonic anhydrase (30 μg/ml), rat liver mitochondria (about 6 mg protein/ml), 0.4 μM rotenone and antimycin (36 μg/g mitochondrial protein). Where indicated, 0.2 mM NEM and digitonin (0.05 mg/mg mitochondrial protein) were added 5 min before the reductant or O_2 pulses. Experiments were done at 25°C at pH_o 7.0–7.1, and either valinomycin was present (10 μg valinomycin/g mitochondrial protein in media A, B and E or 100 μg valinomycin/g mitochondrial protein in media C and D), or 1 μM FCCP. In each set of experiments, O_2 reduction occurred at the same rate in the presence of valinomycin as in the presence of FCCP.

In reductant-pulse experiments, reductant was injected into the aerobic suspension to give the concentration indicated.

In O_2-pulse experiments, the anaerobic suspension containing the reductant was preincubated for 20 min before injection of air-saturated saline (150 mM KCl, 150 mM choline chloride or 10 mM KCl in 230 mM sucrose, as appropriate). The rate of the antimycin-insensitive O_2 reduction in the absence of added reductant was measured in separate experiments.

The values for ΔO, →H^+ (val), ΔH_0^+ (FCCP) and excess O are expressed as μg atoms O or μg ions H^+ min^{-1} per g mitochondrial protein, corrected for the antimycin-insensitive respiration. The values in brackets in the ΔO column represent the quantity of O_2 (ng atoms O) injected. Values for excess O that are less than 2% of the corresponding ΔO value are not experimentally significant. We express the data as quantities per unit time in the O_2-pulse experiments for comparison with the reductant-pulse experiments. This is done simply by dividing the measured total changes by the times taken for oxygen reduction, because the oxygen reduction rates are virtually constant during these experiments