# PARTIAL DIGESTION OF $^{32}$P-fd DNA WITH $T_4$ ENDONUCLEASE IV

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Received 2 September 1971

## 1. Introduction

Studies on the nucleotide sequence of DNA have been hindered by a lack of available specific enzymes for cleaving DNA, as well as the lack of small DNA molecules on which methods of DNA sequencing may be developed. Techniques for the rapid sequencing of RNA on the other hand have been developed with small RNA molecules such as tRNA and 5 S RNA [1–3]. That these techniques could be used to determine sequences in bacteriophage RNA (more than 3,000 nucleotides in length) was demonstrated by Adams et al. [4] when they first showed that small specific fragments fractionated on polyacrylamide gel may be obtained from R17 RNA by a partial enzymatic digestion of the RNA. Subsequent isolation and analysis of various R17 RNA fragments have yielded detailed chemical structures of a number of regions on the phage genome [4–7]. Since bacteriophage DNA are the smallest DNA molecules readily available and since these are at least several thousand nucleotides in length, an enzyme capable of creating specific fragments from phage DNA would clearly be a valuable tool for DNA sequencing.

In this report is described conditions of partial digestion of $^{32}$P-labelled fd DNA with the enzyme $T_4$ endonuclease IV [8]. This enzyme is found in *E. coli* B infected with bacteriophage $T_4$ and appears to be specific for single-stranded DNA, cleaving adjacent to cytosine residues [8]. fd is one of the smallest DNA-containing bacteriophages known closely related to f1 and M13. Its DNA is a single-stranded circle with a molecular weight of about $2 \times 10^6$ daltons and in these respects, the DNA of fd is similar to the DNA of the bacteriophages ØX174 and S13. Specific fragments of various sizes are produced by the digestion of fd DNA with endonuclease IV and these fragments are separated by polyacrylamide gel electrophoresis. Some of the fragments are characterized by "fingerprinting" their depurination products [10].

## 2. Materials and methods

$T_4$ endonuclease IV was prepared from $T_4$ phage infected *E. coli* B in the laboratory of Dr. Paul D. Sadowski by a method modified from that previously described [8]. Details of this procedure will be communicated by P.D. Sadowski. The enzyme had a specific activity of 240,000 units per mg protein where one unit of activity is equivalent to the amount of enzyme required to release 1 nmole of acid-soluble nucleotide from fd DNA in the presence of exonuclease I in the assay originally defined by Sadowski and Hurwitz [8]. The enzyme was stored in 50% glycerol at -20°.

Uniformly labelled $^{32}$P-fd DNA with a specific activity of $1–2 \times 10^6$ cpm per μg DNA was prepared as previously described [10].

Digestion of $^{32}$P-fd DNA was performed in a small volume (20–100 μl) containing: 0.05 M Tris-HCl pH 8.4, 0.01 M $MgCl_2$, 0.01 M β-mercaptoethanol, 60 μg per ml of *E. coli* tRNA, 0.1–1.0 mg per ml

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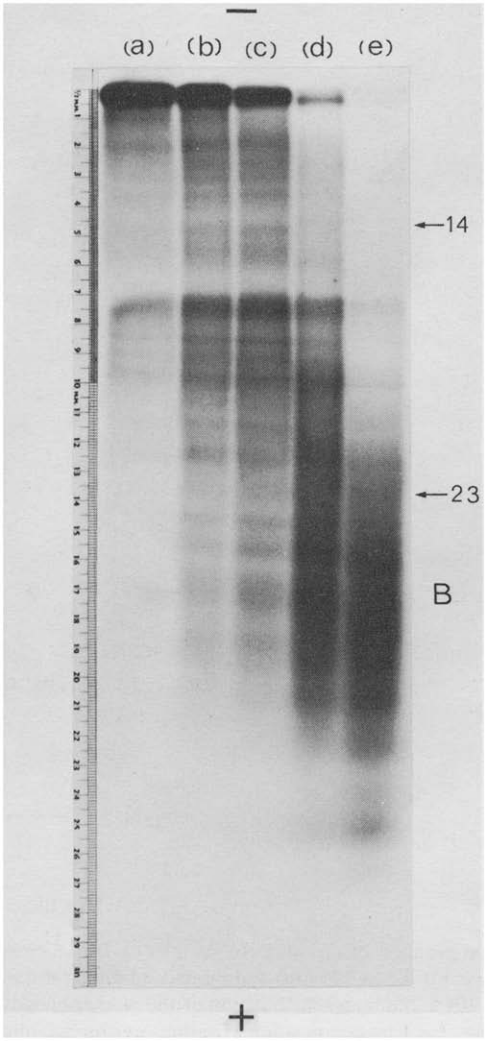


Fig. 1. Radioautograph of the polyacrylamide gel fractionation of $^{32}$P-fd DNA digested with endonuclease IV under various conditions. The digestion conditions are described in Materials and methods. Incubation volume was 20 μl containing 2 μg of $^{32}$P-fd DNA and incubation temperature was at 40°. Digestions (a), (b) and (d) contained 2, 5 and 10 units of enzymes, respectively, and incubation was for 30 min. Digestions (c) and (e) contained 10 units of enzyme and incubation was for 10 and 75 min, respectively. Digestions were terminated by heating in a boiling water bath for 3 min. These digestions were then applied to a 12.5% polyacrylamide gel slab and electrophoresis was performed as previously described [4]. B denotes the position to which the bromphenol blue dye had migrated. Bands 14 and 23 denote the approximate positions of the fragments, isolated from a large scale preparation, whose depurination fingerprints are shown in fig. 2(b) and 2(c), respectively.

of DNA and an appropriate amount of endonuclease IV enzyme. Generally, it was found that 5 units of enzyme per μg of DNA gave a suitable range of fragments of different sizes. Incubation was at 40° for 30 min. Digestion was terminated either by heating in a boiling water bath for 3 min or by extracting with phenol.

Fractionation of DNA fragments was performed by electrophoresis on a large polyacrylamide gel slab as described by Adams et al. [4]. The separated $^{32}$P-labelled fragments were visualized by radioautography as bands and these were cut out and eluted for analysis.

Isolated DNA fragments were characterized by fingerprinting their depurination products [11] on a two-dimensional thin-layer system [12]. Details of this procedure has previously been described [10].

## 3. Results and discussion

Fig. 1 shows the radioautography of a polyacrylamide gel electrophoresis fractionation of $^{32}$P-labelled fd DNA digested with endonuclease IV under various conditions. A series of about 25 bands is produced. This is the only enzyme we tested which gave DNA fragments that migrated as discrete bands on polyacrylamide gel; partial digestion with pancreatic DNase for example, gave a streak with no discrete bands. A mild digestion of fd DNA with endonuclease IV, fig. 1(a), produced fragments which remained near the origin on the polyacrylamide gel while a severe digestion, fig. 1(e), produced fragments which migrated near the bromphenol blue marker dye. Observations in this laboratory of the separation of $^{32}$P-labelled R17 RNA fragments on the same system have indicated that RNA fragments of 30–50 nucleotides in length migrated in the region of the bromphenol blue dye while fragments remaining near the origin were 200–300 nucleotides in length (Dr. U. Rensing, personal communication). It is likely that the DNA fragments observed in fig. 1 are in these size ranges.

To further determine whether the observed bands created by endonuclease IV were unique DNA fragments, a large scale digestion of 100 μg of $^{32}$P-labelled fd DNA was performed in a volume of 0.1 ml using condition (b) of fig. 1. The resulting fragments

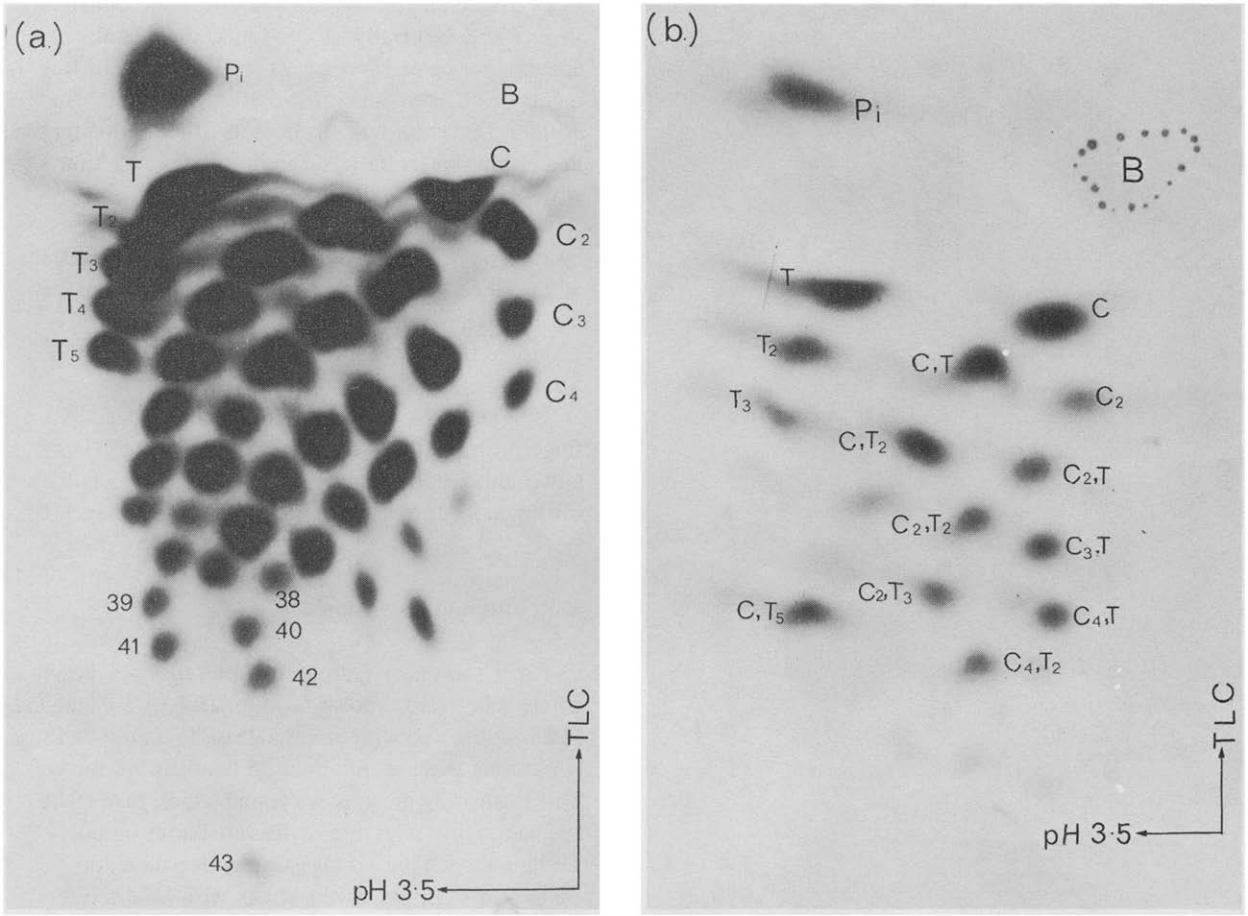


Fig. 2. Radioautographs of the two-dimensional fractionation of depurination products of various DNA samples on DEAE-cellulose thin-layer [10]. The first dimension was electrophoresis on cellulose acetate at pH 3.5 in 7 M urea and the second dimension was ascending chromatography with a partially hydrolyzed solution of 3% yeast RNA. B denotes the position of the marker blue dye Xylene Cyanol F.F. $P_j$ is free phosphate released by the depurination reaction. The base composition of various pyrimidine oligonucleotides is indicated. (a) whole fd DNA, (b) endonuclease IV fragment band 14, see fig. 1.

were fractionated as in fig. 1, and a number of bands were cut out, eluted and fingerprinted.

Fig. 2(a) shows the two-dimensional fingerprint of the depurination products of whole fd DNA. A regular pattern of 43 nucleotide spots is observed and each of these nucleotides has previously been characterized [10]. While most of the nucleotide spots occurred in many molar yields and are hence composed of isomeric pyrimidine oligonucleotides of different sequences, some of the larger oligonucleotides greater than 9 residues in length (nucleotides 38 to 43) occur only once in the DNA molecule and their sequences have been determined [10].

Fig. 2(b) is a depurination fingerprint of band 14, a fragment which migrated on polyacrylamide gel about half way between the origin and the bromphenol blue dye (fig. 1). It may be observed that the pattern of pyrimidine oligonucleotides obtained in fig. 2(b) is quite different and simpler than that of whole phage DNA, fig. 2(a). The compositions of the more prominent spots are deduced from their positions on the thin-layer plate [10] and are indicated. The largest pyrimidine oligonucleotides observed in this band are hexamers of compositions ($C, T_5$) and ($C_4, T_2$). The large oligonucleotides 38–43 of fig. 2(a) were not observed. Some of these

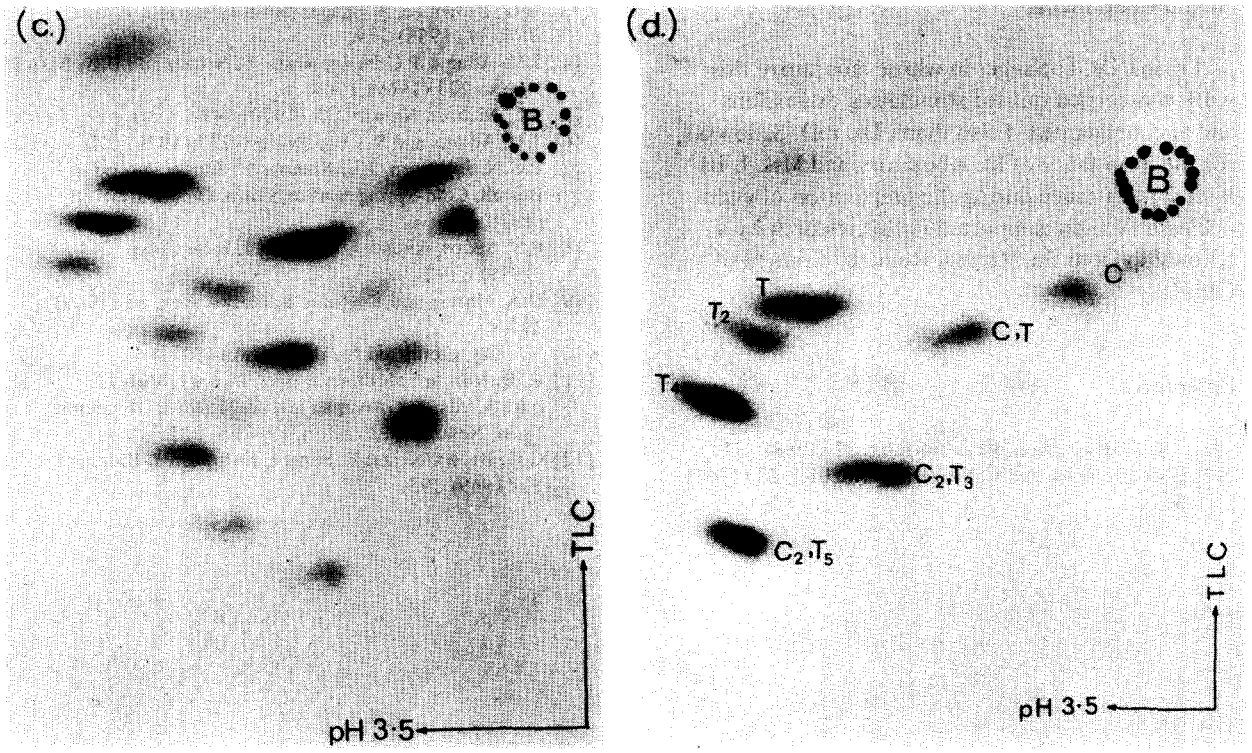


Fig. 2 (continued). (c) endonuclease IV fragment band 23, see fig. 1, (d) a purified component of band 23, see text.

were found only in a limited number of fragments migrating near the origin of the polyacrylamide gel.

Fig. 2(c) is a depurination fingerprint of band 23, a fragment migrating near the bromphenol blue dye on the polyacrylamide gel (see fig. 1). It is clear that the fingerprint of this band is different from that of whole phage DNA as well as band 14. Since band 23 should be considerably smaller than band 14, a simpler fingerprint would be expected, yet the fingerprint was still fairly complicated. This suggested that band 23 contained a number of fragments not resolved from each other. When band 23 was further purified by the two-dimensional thin-layer system employed here, except that the chromatography step utilized a 5% solution of unhydrolyzed yeast RNA, a number of components were observed. One of the components yielded a depurination fingerprint shown in fig. 2(d). This is a very simple fingerprint with only seven nucleotide spots and suggests that this fragment of band 23 was isolated as a single pure fragment.

The facts that $T_4$ endonuclease IV yielded DNA fragments that migrated as discrete bands on polyacrylamide gel and that these bands gave unique fingerprints, indicate that the enzyme has considerable specificity. While the specificity of endonuclease IV has not yet been rigorously examined, Sadowski and Hurwitz [8] have suggested that it may have preference for regions on the DNA containing clusters of cytosine. This enzyme is likely to be generally useful since we have observed that it produces discrete DNA fragments from a number of single-stranded phage DNAs in addition to fd, namely f1, M13 and ØX174. Thus it seems possible to obtain specific fragments of DNA on which DNA sequencing techniques may be worked out. It also seems possible that specific DNA fragments of biological interest could be generated by the use of this enzyme.

## Acknowledgements

I thank Dr. F. Sanger in whose laboratory this work was carried out for stimulating discussions and encouragement. I also thank Dr. P.D. Sadowski for the hospitality of his laboratory and Mrs. I. Bakyta for assistance during the preparation of endonuclease IV. I am supported in this research by a fellowship from the Medical Research Council of Canada.

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