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Preparation and Specificity of Endonuclease IV Induced by Bacteriophage T4

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Bacteriophage-T4-induced endonuclease IV suitable for DNA sequence analysis has been prepared by a modified and easily reproducible method.

The specificity of T4-induced endonuclease IV has been investigated in order to verify whether this enzyme exhibits a single nucleotide recognition or a short sequence recognition.

The 5′-terminal dinucleotides and 3′-terminal nucleotides of oligonucleotides released by T4-induced endonuclease IV from three single-stranded DNAs (from bacteriophages ΦX174, fd, M 13) have been analysed. In different DNAs, 74–82% of the 5′-terminal dinucleotides end in 5′-deoxycytidylic acid; small but significant levels of several dinucleotides ending in 5′-deoxyadenylic acid, 5′-thymidylic acid and 5′-deoxyguanylic acid are also found. As far as 3′-terminal nucleotides are concerned all nucleotides are present with a large predominance of thymidylic acid.

It is concluded that T4-induced endonuclease IV recognizes short nucleotide sequences like all other DNases investigated so far. The spectrum of such sequences is, however, very narrow.

Recent investigations on the composition of terminal and penultimate nucleotides of DNA fragments released by five different deoxyribonucleases (DNase) have shown that these enzymes (hog spleen acid DNase, bovine pancreatic DNase, snail hepatopancreas DNase, *Escherichia coli* endonuclease I, and crab testis DNase) recognize and split specific sets of short oligonucleotide sequences [1,2]. It has been suggested [1] that such sequence recognition, also shared by restriction enzymes [3], may be a general feature of DNases, in sharp contrast with the single nucleotide recognition typical of ribonucleases (RNases), such as pancreatic and T1 RNases.

A study of the specificity of bacteriophage-T4-induced endonuclease IV [4] appeared to be of interest for the following reasons: (a) this enzyme was reported to cleave fd DNA and denatured λ DNA leaving deoxycytidylic acid at the 5′ end of all the product oligonucleotides [4], a finding which might indicate a single nucleotide recognition [5]; more recent work [6–8] has shown that endonuclease IV splits only dT-dC and dG-dC bonds in the fragments investigated; only the clearly separated major fragments were, however, the subject of these studies; (b) in contrast with restriction enzymes and the DNases mentioned above, which have a strict requirement and a strong preference, respectively, for double-stranded DNA, endonuclease IV much prefers single-stranded DNA. These features seem to suggest that this enzyme belongs in a different class of DNases [5].

We wish to report here results showing that endonuclease IV also exhibits the sequence recognition typical of DNases. The use of endonuclease IV in sequence studies having been hampered by difficulties encountered in its preparation, we will report here some alterations in the purification and assay procedure of Sadowski and Bakyta [10]. In the modified procedure, 8–9 working days are required to prepare a large supply of purified enzyme from only 40 g of infected cells of *E. coli*.

Enzyme. Phage T4-induced endonuclease IV (EC 3.1.4.–).

MATERIALS AND METHODS

Materials

Bacteriophage fd DNA was prepared with tritium in the thymine moiety by growing *E. coli* 993 or 2027 [10] in 200 ml tryptone broth to 3×10^8 cells/ml and infecting with phage fd at multiplicity 10 after adding 50 mg deoxyadenosine dissolved in a small volume of water, 2.0 ml 0.1 M $CaCl_2$ and 0.5 mCi of

[^{3}H]thymidine (specific activity ≈ 20 Ci/mmol). After shaking at 37 °C for 4 h the phage was titrated and purified according to Ling [11]. The CsCl band containing the phage was diluted with 3 ml of 50 mM Tris-Cl, pH 8.0, dialyzed briefly and extracted with water-saturated phenol; the aqueous phase was then exhaustively dialyzed against 50 mM Tris-Cl, pH 8.0 containing 0.5 mM EDTA and stored frozen. Phage fd DNA was also labelled with ^{32}P as described by Ling [11].

Bacteriophage M13 DNA was prepared essentially as fd DNA. Bacteriophage ΦX174 DNA was prepared by treating phage particles with water-saturated phenol.

Exonuclease I, the enzyme used to assay endonuclease IV, was prepared according to Lehman [12] from 20 g of *E. coli* B cells just before (or simultaneous with) the start of the endonuclease IV preparation. Fractions purified on DEAE-cellulose were stable for approximately two weeks when kept in ice.

T4 bacteriophage was an amber mutant defective in DNA synthesis, such as N82 (gene 44) or N122 (gene 42). Stocks were grown in small voumes (25 ml) for 4 h at 34 °C in broth containing per l: 8 g nutrient broth, 5 g bacto-peptone, 5 g NaCl and 1 g glucose. The titers on *E. coli* CR63 and *E. coli* B (permissive and non-permissive strains, respectively) were monitored for every stock prepared. When their ratio dropped below 10^4 the mutant was plaque-purified.

Phosphocellulose (Whatman P11) was prepared by the alkaline and acid treatment of Burgess [13] and titrated to pH 7.0 before exhaustive equilibration first with 0.2 M, then with 20 mM potassium phosphate at pH 6.1.

Protamine sulphate was salmine sulphate from British Drug Houses Chemicals Ltd (Poole, England).

Purification and Assay of T4 endonuclease IV

The method described by Sadowski and Bakyta [9] was used with the following modifications.

a) *E. coli* B cells were infected at 5×10^8 cells/ml with T4 am N82 at a multiplicity of 4, aerated for 40 min and poured on ice cubes at −20 °C before harvesting the cells, which were stored overnight or longer at −20 °C. Sonication of 40 g cells in 130 ml of buffer A was carried out in a 150-ml stainless steel beaker immersed in a mixture of crushed ice and ice water to dissipate the heat.

b) The enzyme assay was done at pH 8.4 with 50 µl of exonuclease I (DEAE-cellulose peak fraction) present from the start; no intermittent addition or adjustment of pH was made.

c) After the percolating step through DEAE-cellulose (the second column chromatographic step of Sadowski and Bakyta [9] the enzyme was rechromatographed on a 5 × 1-cm phosphocellulose column. The enzyme solution was carefully adjusted to pH 6.4 with 1% orthophosphoric acid and dialyzed against 2 l (one change) of 20 mM potassium phosphate, pH 6.1 containing 10 mM mercaptoethanol, 1 mM EDTA and 30% glycerol before application to this column. The gradient (volume 120 ml) was from 0−0.35 M $(NH_4)_2SO_4$ in dialysis buffer containing 10% glycerol.

d) All fractions from the second phosphocellulose column which contained endonuclease IV activity were inspected with regard to the gel electrophoretic banding pattern produced upon incubation with ^{32}P-labelled fd DNA. Under the conditions of Galibert *et al.* [6], 1 µg of freshly prepared ^{32}P-labelled fd DNA was incubated with 2−5 µl of enzyme at 37 °C for 30 min and subjected to electrophoresis on 12% or 15% polyacrylamide slab gels with bromophenolblue as a marker. The DNA fragments were visualized on X-ray film after a suitable exposure time (1−3 h).

DNA Degradation

DNAs from bacteriophage fd, M13 and ΦX174 were digested in 0.04 M Tris-Cl, pH 8.5, 0.02 M $MgCl_2$, 0.01 M 2-mercaptoethanol at 37 °C for different times (15−240 min). Enzyme and DNA concentrations were such that the longest time corresponded to a final digest.

Analysis of 5′-terminal dinucleotides was carried out according to Bernardi and Gaillard [14]. Analysis of 3′-terminal nucleotides was done according to Bernardi and Bertazzoni [15].

RESULTS AND DISCUSSION

The results of the modified purification procedure have been checked by the gel electrophoretic patterns obtained with limit digests of ^{32}P-labelled fd DNA prepared at 37 °C [16], as well as with partial digests prepared at cold-room temperature [4 °C) in high salt (0.2 M NaCl) [6]. These conditions were found by Galibert *et al.* [6] to yield large fragments of ΦX DNA.

Of great technical importance was the observation that the tail fractions of the activity peak obtained with the first phosphocellulose column contain an exonuclease contaminant. This undesirable activity can not effectively be removed by rechromatography. This circumstance makes it essential to select endonuclease IV fractions from the first column that are low in (or free of) the exonuclease. This selection is facilitated in the modified assay when the amount of exonuclease I is varied to make its concentration slightly limiting. In many cases the endogenous exonuclease contaminant gave itself away in the tail fractions because it tended to produce some extra

Table 1. *5′-Terminal nucleotides released by T4-induced endonuclease IV*
5′-Terminal dinucleotides are written with the 5′-terminal mononucleotide on the left

5′-Terminal dinucleotides	Released from DNA of phage ΦX 174	fd	M13
dA-dA	1.4 ± 0.70	3.3 ± 0.57	4.7 ± 0.42
dA-dC	1.5 ± 0.14	4.6 ± 0.32	6.8 ± 0.08
dA-dG	4.5 ± 0.35	2.7 ± 0.19	0.9 ± 0.08
dA-dT	1.4 ± 0.59	0.8 ± 0.03	3.3 ± 0.52
dC-dA	36.3 ± 0.70	30.1 ± 1.26	21.8 ± 1.15
dC-dC	15.8 ± 0.71	17.1 ± 0.63	15.5 ± 1.08
dC-dG	15.8 ± 1.76	12.1 ± 1.11	15.6 ± 0.83
dC-dT	14.4 ± 1.16	19.3 ± 0.76	21.4 ± 1.27
dG-dA	1.3 ± 0.28	0.4 ± 0.03	1.2 ± 0.27
dG-dC	0.5 ± 0.08	0.04 ± 0.02	0.5 ± 0.08
dG-dG	2.2 ± 0.48	1.2 ± 0.07	3.2 ± 0.58
dG-dT	1.1 ± 0.11	0.4 ± 0.04	1.4 ± 0.23
dT-dA	0.3 ± 0.07	1.7 ± 0.11	0.7 ± 0.09
dT-dC	0.3 ± 0.00	1.4 ± 0.23	0.5 ± 0.06
dT-dG	1.0 ± 0.17	1.3 ± 0.17	1.0 ± 0.14
dT-dT	2.2 ± 0.31	2.5 ± 0.26	1.9 ± 0.34
5′-Terminal nucleotides			
dA	8.8 ± 0.40	11.2 ± 0.52	15.6 ± 0.78
dC	82.4 ± 0.73	79.5 ± 0.59	74.2 ± 1.51
dG	5.0 ± 0.59	2.4 ± 0.12	6.1 ± 0.72
dT	3.8 ± 0.13	6.9 ± 0.55	4.1 ± 0.48
5′-Penultimate nucleotides			
dA	39.3 ± 0.66	35.4 ± 0.93	28.3 ± 1.28
dC	18.1 ± 0.80	23.6 ± 0.34	22.7 ± 0.95
dG	23.5 ± 2.09	17.7 ± 1.44	21.1 ± 0.91
dT	19.1 ± 1.39	23.7 ± 1.01	27.9 ± 1.47
Number of determinations	4	7	4

Table 2. *3′-Terminal nucleotides released by T4-induced endonuclease IV*

Nucleotide	Released from DNA of phage ΦX174	fd	M13
dA	26.2 ± 0.94	23.0 ± 1.67	30.7 ± 2.11
dC	13.4 ± 0.59	12.5 ± 1.58	8.8 ± 1.30
dG	19.8 ± 0.29	18.0 ± 0.70	18.7 ± 1.73
dT	40.6 ± 1.30	46.5 ± 1.24	41.8 ± 3.01
Number of determinations	4	3	3

acid-soluble nucleotides from ^{3}H-labelled fd DNA (the elution profile became indented). Based on these observations we have found it good practice never to include the last third of the activity peak in the fraction selected for further processing. A typical purification as described here yielded 24 ml of enzyme of very satisfactory quality of which 2 µl suffices for a standard fingerprint of highly labelled DNA material (J. Fiddes, personal communication).

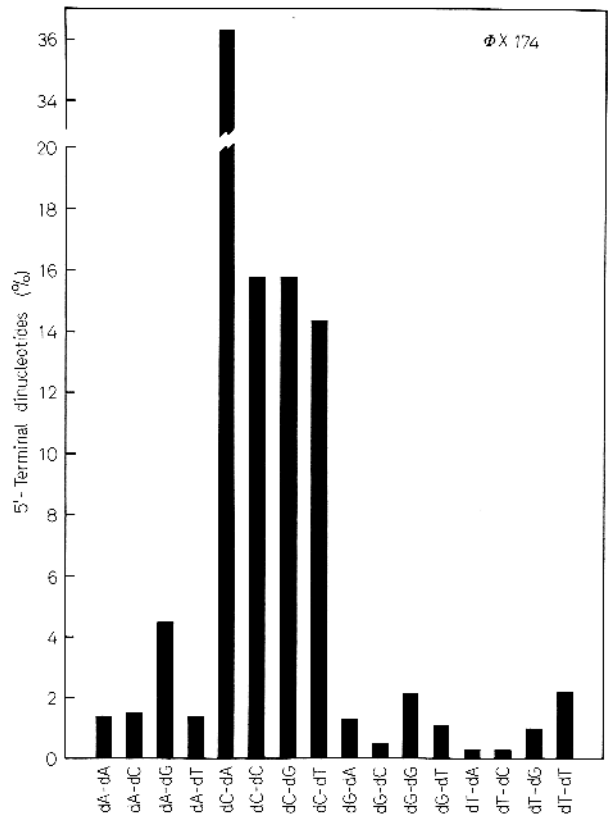

Fig. 1. *5′-Terminal dinucleotides of fragments released by T4-induced endonuclease IV from ΦX DNA*

Table 1 summarizes the results obtained in the analyses of 5′-terminal dinucleotides of fragments released from the different DNAs investigated. Table 2 presents the base composition of the 3′-terminal nucleotides. No significant trends were found in the composition of terminal dinucleotides and mononucleotides from digests obtained at different incubation times; therefore, all results for a given DNA could be averaged out.

The histograms of 5′-terminal dinucleotides in the oligonucleotides released from ΦX174, fd and M 13 DNAs (Fig. 1–3; Table 1) are generally similar in that the dinucleotides ending in 5′-deoxycytidylic acid are largely predominant (74–82%) and that a number of the other dinucleotides are below the 1% level. Likewise, the 3′-terminal nucleotides (Table 2; Fig. 4) obtained by digestion of these single-stranded DNAs exhibit a rather similar composition with predominance of thymidylic acid and lowest level of deoxycytidylic acid. Individual features for each one of these DNAs can be seen, particularly in the distribution of 5′-terminal dinucleotides.

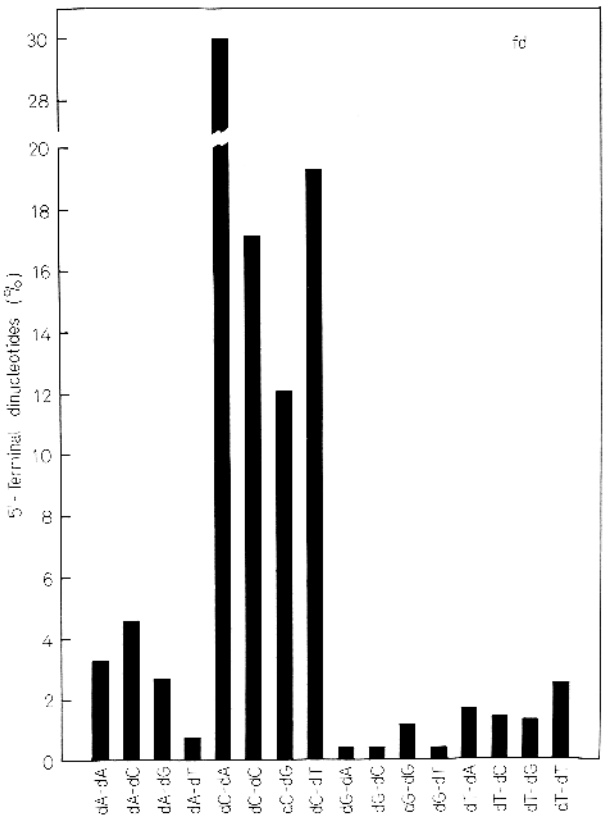

Fig. 2. *5'-Terminal dinucleotides of fragments released by T4-induced endonuclease IV from fd DNA*

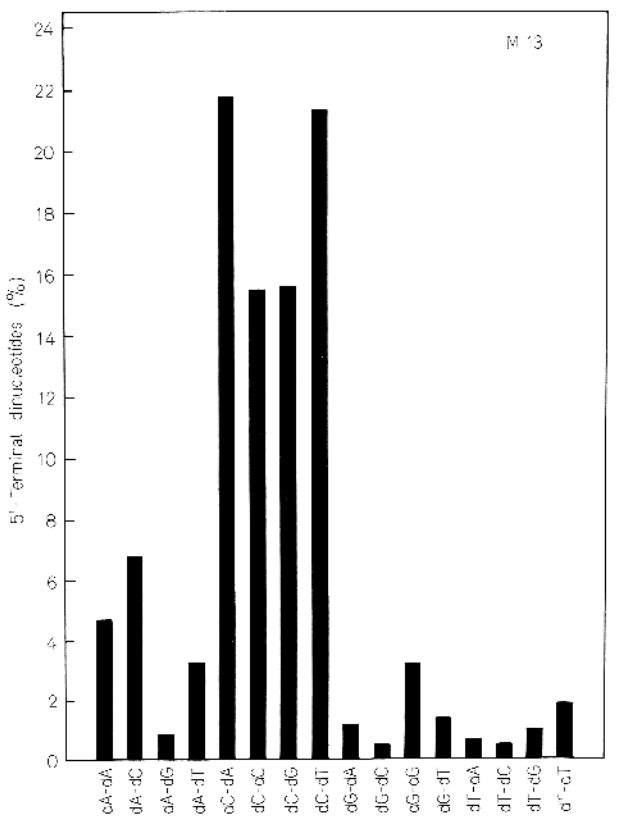

Fig. 3. *5'-Terminal dinucleotides of fragments released by T4-induced endonuclease IV from M13 DNA*

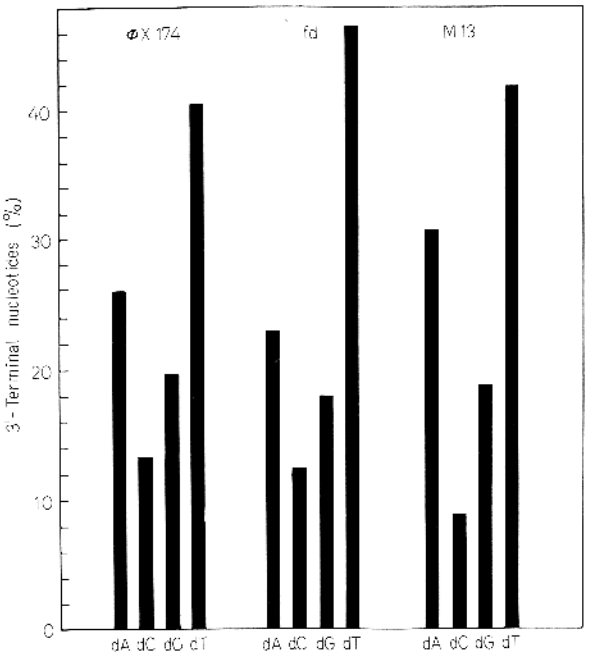

Fig. 4. *3'-Terminal nucleotides of fragments released by T4-induced endonuclease IV from the three different DNAs used in the present work*

A histogram of 'calculated' trinucleotides split by the enzyme from DNA of ΦX174 is shown in Fig. 5. This is shown to provide a comparison with the results obtained on *E. coli* DNA with five other DNAases [2]. It is very evident that the specificity of T4-induced endonuclease IV, as judged by this criterion, is by far the narrowest one exhibited by DNAases analyzed by us.

A major conclusion of the present work is that T4-induced endonuclease IV exhibits a short-sequence recognition, like all other DNAases investigated so far [1, 2], and not a single nucleotide recognition as previously claimed [4]. The present results are not contradictory with those reported by previous authors [6–8], since in this case what is examined is a small aliquot of a partial digest obtained under conditions where the secondary structure of the phage DNA is likely to limit the access of the enzyme.

Under these circumstances, T4-induced endonuclease IV can be used, like the other DNases, to assess the frequency of the sequences which it is able to recognize and split [1]. Preliminary experiments with heat-denatured DNAs from bacteriophage λ, *E. coli* and calf thymus showed terminal nucleotide patterns exhibiting an overall similarity with the results reported here. Interestingly, in the case of calf thymus DNA 5'-terminal dCpdG was almost absent, as expected from the shortage of this nucleotide doublet in vertebrate DNAs [17], and as found with all other DNAases [18] (and unpublished results).

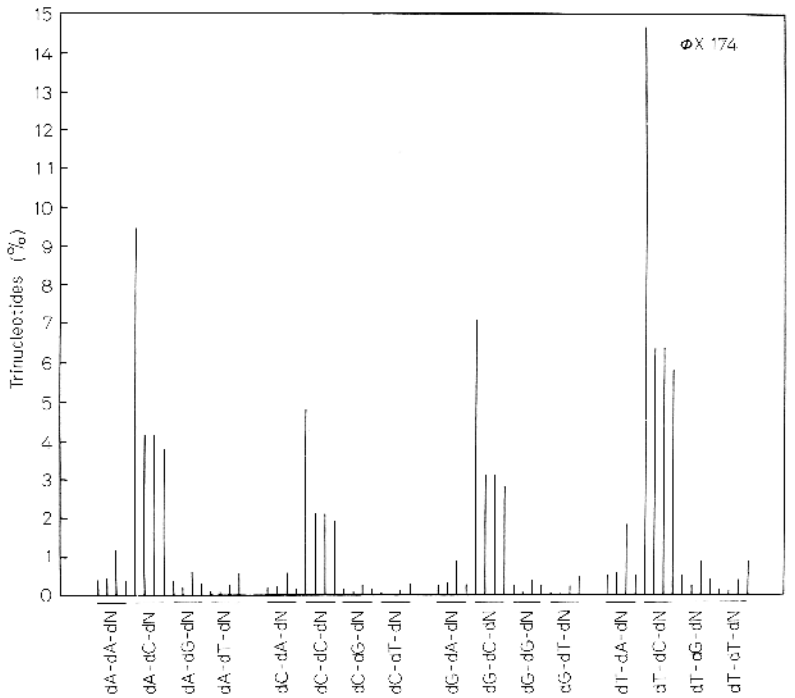

Fig. 5. *Histogram of 'calculated' trinucleotides split by T4-induced endonuclease IV in ΦX174 DNA.* Experimental values for 3′-terminal nucleotides were multiplied by experimental values for 5′-terminal dinucleotides. dN corresponds to dA, dC, dG, dT, in this order

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