THE MAPPING AND SEQUENCE DETERMINATION OF THE SINGLE SITE IN ϕX174*am*3 REPLICATIVE FORM DNA CLEAVED BY RESTRICTION ENDONUCLEASE *Pst* I

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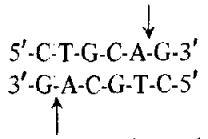
1. Introduction

A large number of restriction endonucleases have now been isolated from prokaryotes. We have tested some of these enzymes on the covalently-closed replicative form (RFI) of ϕX174*am*3 DNA. The restriction endonuclease from *Providencia stuartii* 164 (*Pst* I) [1] was found to convert RFI DNA to a linear form of apparent unit length.

In this paper we describe the mapping of the single *Pst* I site in ϕX174*am*3 RFI DNA and the determination of the sequence cleaved. This is the symmetrical hexanucleotide sequence:



The methods used to determine the sequence recognised by *Pst* I and the site of cleavage within that sequence are novel, and should be of general use.

2. Experimental and results

2.1. *Preparation of* Pst *I*

Cells were grown in Nutrient Broth. The enzyme was isolated as described for *Hae* II by Roberts et al. [2] except in that the ammonium sulphate fractionation was omitted. There were no contaminating endo- or exonuclease activities.

2.2. *Mapping of the* Pst *I site in* ϕ*X174*am*3 RFI DNA*

The cleavage pattern of *Pst* I on ϕX174*am*3 RFI DNA was determined by comparing the DNA fragment pattern obtained when RFI DNA was digested with both *Pst* I and a second restriction endonuclease (a double-digest) with the fragment pattern obtained by using the second enzyme alone.

ϕX174*am*3 RFI DNA (a gift from Dr C. A. Hutchison III) was radioactively labelled with [α-^{32}P] dATP (New England Nuclear; 100 Ci/mmol) by 'nick-translation' [3] using *E. coli* DNA polymerase I (Boehringer Corporation, Ltd.). *Hha* I was a gift from Dr R. J. Roberts. Other restriction endonucleases were prepared by methods similar to those described in [2]. Restriction enzyme digests were performed in 50 mM NaCl, 6.6 mM $MgCl_2$, 6.6 mM β-mercaptoethanol, 6.6 mM Tris-HCl, pH 7.4. In double-digests both enzymes were present simultaneously. The products of restriction endonuclease digestion were analysed on 5% acrylamide slab-gels in TBE buffer (90 mM Tris-borate, 2.5 mM Na_2EDTA, pH 8.3). The sizes of the restriction fragments cleaved by *Pst* I, and the sizes of the products were calculated from their mobilities relative to ϕX174*am*3 fragments of known size ([4] and unpublished data).

In each double-digest only one of the fragments of ϕX174*am*3 DNA produced by the second enzyme was cleaved by *Pst* I. Comparison of the sizes of the restriction fragments cleaved by *Pst* I and the sizes of the products (table 1) shows that *Pst* I cut ϕX174*am*3 DNA at one site. This *Pst* I site was located within the known restriction fragment map of ϕX174*am*3 [4] and allowed a more detailed map of this region to be drawn (fig.1).

2.3. *Sequence of the cleavage site*

Mapping of the *Pst* I site showed that the site was

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Table 1
Double-digestion of ϕX174*am*3 with *Pst* I and other restriction endonucleases

Restriction fragment cleaved[a]	Size of fragment (nucleotide pairs)	Size of fragment products in double-digest (nucleotide pairs)	
Alu I 5	340	170	162
Hae III 3	895	455	440
Hha I 8b	193	161	37
*Hin*d II 5	400	365	35
*Hin*f I 6	303	250	59

[a]Description of these enzymes and their fragment maps is given in [4]

amenable to sequence determination by primed synthesis with DNA polymerase [5] using *Hin*d II fragment 7b (R7b) as the primer on the viral DNA strand as the template. The sequence in the non-viral strand primed by fragment R7b was determined by the 'plus and minus' method of Sanger and Coulson [5]. The location of the *Pst* I cleavage site within this sequence was determined by a modification of the plus and minus method. This involved the construction of the plus and minus pattern of electrophoretic bands for the region of DNA containing the *Pst* I site in the normal way. The site of cleavage was located in the DNA sequence by parallel electrophoresis of extension products [5] which had been cleaved with *Pst* I. The position of this site relative to the cleavage site in the template DNA was determined by treatment of the *Pst* I-cleaved elongation products with T4 DNA polymerase and unlabelled deoxyribonucleoside triphosphates followed by parallel electrophoresis. The change in position of the band at the *Pst* I site defines the relationship of the two points of cleavage.

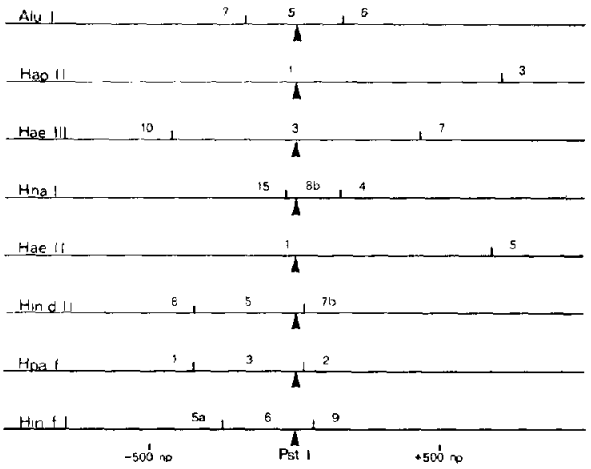

Fig. 1. Partial map of the cleavage sites of some restriction endonucleases in relation to the *Pst* I cleavage site. The orientation of the fragments cleaved by *Pst* I was determined from data given in [4]. The map is drawn with the 5′–3′ direction of the viral strand from left to right. np = nucleotide pairs.

After extension of the R7b primer with [α-^{32}P] dATP and unlabelled dGTP, dCTP and dTTP, the DNA polymerase and excess triphosphates were removed. Aliquots of the extension product were incubated with the plus and minus mixtures, then cleaved with *Hin*d II [5]. Another aliquot (O_{RP}) was not incubated with plus or minus mixtures, but was cleaved directly with *Hin*d II and *Pst* I. Control aliquots (O_R and O_P) were cleaved directly with *Hin*d II and *Pst* I. Control aliquots (O_R and O_P) were cleaved directly with *Hin*d II or *Pst* I alone. A further aliquot (C_{RP}) was incubated for 30 min at 37°C with T4 polymerase (approx. 0.02 unit) in the presence of all four unlabelled deoxyribonucleoside triphosphates (0.4 mM), then cleaved with *Hin*d II and *Pst* I. The products were denatured and analysed by electrophoresis on a 12% acrylamide-7 M urea slab gel in TBE buffer. An autoradiograph of the gel is shown in fig.2.

In sample O_{RP} all molecules in which the extension reaction proceeded past the *Pst* I site gave rise to the fragment lying between the *Hin*d II site and the *Pst* I site. The intense band in channel O_{RP} in fig.2 was not present in either of channels O_R or O_P and was probably due to this fragment. This band had the same mobility as bands in the + A channel and the – G channel. The *Pst* I cut in the non-viral strand is therefore as shown in the sequence.

A-A-C-T-C-T-G-C-A↓G-G-T-T-G-G

In channel C_{RP} the intense band had the same mobility as bands in the + C and – T channels, and the fragment was four nucleotides shorter than that in channel O_{RP}.

We propose that, in ϕX174*am*3 DNA, *Pst* I

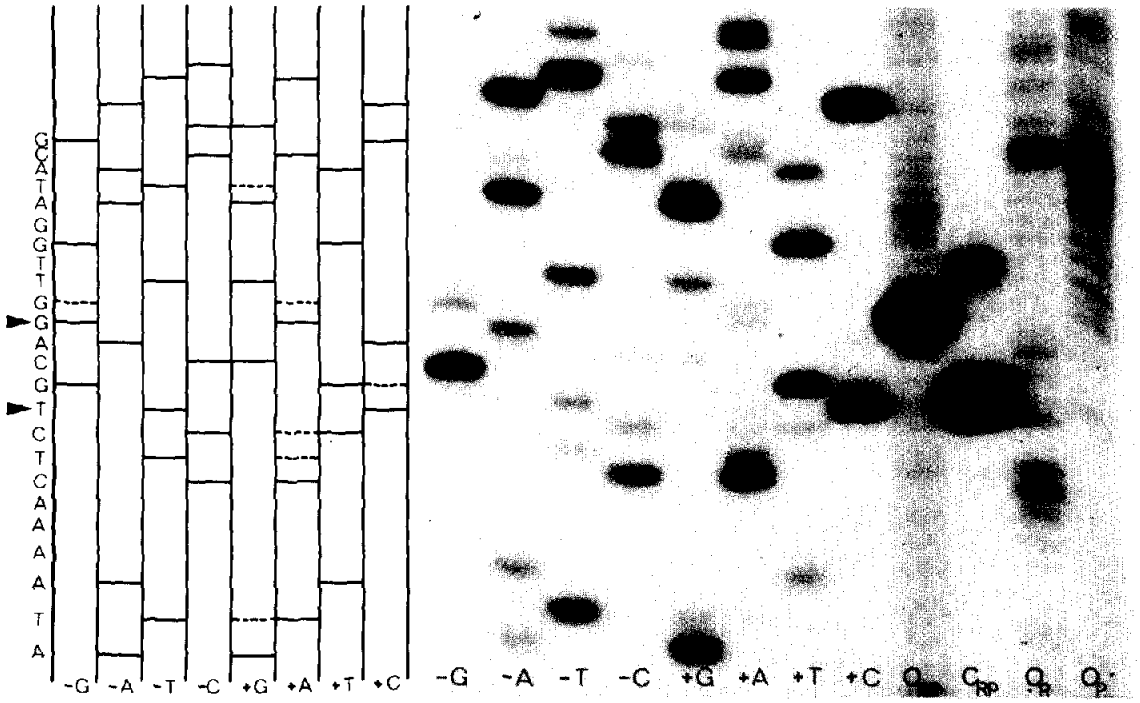

Fig. 2. Autoradiograph of part of a sequencing gel [5] showing the sequence primed by fragment R7b on viral DNA as template, and the determination of the *Pst* I cleavage site. A diagrammatic interpretation of the sequence is also shown. Artefact bands are represented by dashed lines.

recognises and cleaves the symmetrical hexanucleotide sequence:

5′-C-T-G-C-A-G-3′
3′-G-A-C-G-T-C-5′

giving a duplex, unit-length fragment with tetranucleotide 3′-cohesive ends.

In the presence of T4 polymerase and all four deoxyribonucleoside triphosphates (sample C_{RP}) the 3′-exonuclease activity of T4 polymerase [6] probably removes the 3′-cohesive ends. The 3′-terminal nucleotide of the duplex region would then be removed and replaced due to the concerted action of the 3′-exonuclease and polymerase activities [7].

3. Discussion

Pst I produces more than 18 cleavages in λ DNA [1], and one cleavage in ϕX174*am*3. An enzyme with a hexanucleotide recognition site would be expected to give approximately this number of cuts. Although the hexanucleotide C-T-G-C-A-G in ϕX174*am*3 shows perfect two-fold symmetry, our data do not eliminate the possibility of the recognition site being of the types:

(i) C-T-Pu-Py-A-G
or (ii) Py-Py-T-G-C-A-Pu-Pu

(where Py and Pu are any pyrimidine and any purine respectively). However, the sequences C-T-G-T-A-G (type (i)) [8] and T-T-T-G-C-A-A-Pu (type (ii)) (P. M. Slocombe, personal communication) are found in ϕX174*am*3 DNA and are not cleaved by *Pst* I. Consequently we believe that the hexanucleotide C-T-G-C-A-G defines the *Pst* I site.

The method described for the determination of the sequence cleaved by *Pst* I should be applicable to other restriction endonucleases. We have described the

results obtained for an enzyme which gives 3′-cohesive ends. For an enzyme which gives flush-ended fragments, the fragment produced by digestion with two restriction endonucleases would be the same length in the sample containing T4 polymerase and all four deoxyribonuclease triphosphates as in the sample where these were absent. If the enzyme gives 5′-cohesive ends the T4 polymerase activity would 'repair' the end using the 5′-terminus as a template. The length of the fragment obtained in the sample containing T4 polymerase and triphosphates would be greater than that obtained in the sample cleaved directly, and the difference in electrophoretic mobility of the fragments would give the length of the 5′-cohesive ends.

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