Alteration of the specificity of PvuII restriction endonuclease

Moncef Nasri and Daniel Thomas

Laboratoire de Technologie Enzymatique, UA No.523 du CNRS, UTC BP.233, 60206 Compiègne cedex, France

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ABSTRACT

The restriction endonuclease PvuII which cleaves the sequence CAGCTG, at the position indicated by the arrow, was found to decrease its substrate specificity in the presence of organic solvents. Thirty-three sites, that we have named PvuII* sites, were identified on the nucleotide sequence of pBR322 DNA. The new recognition sequences cleaved in pBR322 DNA, at the positions indicated by the arrows, were shown to be AAGCTG, GAGCTG, CNGCTG, CANCTG, CAGNTG, CAGCNG, CAGCTC and CAGCTT. (TAGCTG and the complementary sequence CAGCTA are not present in pBR322 DNA).

From these recognition sequences, we deduced that PvuII* activity recognizes and cleaves degenerate sequences which differ from the standard PvuII sequence CAGCTG at only one of the recognition site. Any substitution can occur at any one of the six positions in the hexanucleotide sequence.

The optimum incubation medium for PvuII* activity was found to be: 10-50 mM Tris-HCl, pH 8.5, 12-15 mM MgCl₂, 50 mM NaCl, 10 % ethanol + 10 % dimethylsulfoxide (DMSO).

INTRODUCTION

Type II restriction endonucleases are widely used in the analysis and manipulation of DNA molecules (1, 2). Recently, many type II restriction endonucleases, which specifically cleave DNA at a limited number of sites, were found to decrease their substrate specificity under modified conditions : (at higher pH, lower ionic strength, substitution of the metallic cofactor and the addition of organic solvents), (3-10). In most cases the nucleotide sequences of the new sites correspond to degenerate sequences, derived from the standard sites. For BstI (7) and BsuI (4), relaxation occurs at the two external bases. The BamHI relaxation (11, 12) occurs at the internal nucleotide sequence of the hexanucleotide site. For HindIII (9) and EcoRI (3) the relaxation specificity may occur at the internal and the outer nucleotide sequence of the standard hexanucleotides.

We have previously reported that the cleavage specificity of PvuII endonuclease is altered in the presence of dimethylsulfoxide, and cleavages occur at additional sites (13). This secondary activity was referred to as PvuII*, as designated for EcoRI* (3).

Here, we examine this reduced specificity in more detail. The pBR322 DNA provides a

very convenient substrate for this study, since the entire nucleotide sequence of the pBR322 was already determined (14).

Here, we have present the effects of several factors on PvuII* activity such as organic solvents, etc... We have also attempted to optimise the PvuII* activity. In addition to the restriction sites determined previously (13), we have determined all the PvuII* recognition sites present in pBR322. An explanation of how DMSO may alter the specificity of PvuII endonuclease is also attempted.

MATERIALS AND METHODS

Enzymes and digestion conditions

PvuII restriction endonuclease was purchased from New England Biolabs Inc. Standard-Conditions of reaction for PvuII were : 6 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 50 mM NaCl and 6 mM 2-mercaptoethanol. Modified conditions of reaction were: standard conditions + organic solvents.

Substrate

pBR322 was purchased from Boehringer Mannheim

Gel electrophoresis

Electrophoresis of restriction fragments of DNA was carried out in either horizontal slab gels of 1 % (w/v) agarose, or in vertical slab gels of 7 % (w/v) polyacrylamide. The DNA bands were visualized under ultraviolet light after staining with ethidium bromide.

5'-terminal labelling of restriction fragments

pBR322 DNA was cleaved with PvuII endonuclease under relaxed conditions (10 mM Tris-HCl, pH 8.5, 12 mM MgCl₂, 50 mM NaCl, 10 % ethanol and 10 % DMSO) for 26-h at 37°C. The resulting mixture of PvuII* fragments was first treated with alkaline phosphatase, and then labelled with ³²P using polynucleotide kinase and (γ -³²P) ATP (15). Labelled fragments were redigested with a second restriction enzyme (HinfI endonuclease) and the resulting mixture was then fractionated by 8 % (w/v) polyacrylamide gel electrophoresis.

Sequence determination

The DNA fragments labelled at only one of the 5' ends were removed from the gel, and subjected to sequence analysis according to the procedure of Maxam and Gilbert (16).

RESULTS

Effect of organic solvents on the PvuII digestion

Under the standard conditions, PvuII endonuclease cleaves the DNA sequence CAGCTG, located at position 2067, as indicated by the arrow (17). Thus, it converts the circular pBR322 DNA into the linear form. However, in the presence of organic solvents, the specificity of the cleavage is modified and the enzyme cleaves pBR322 DNA at additional sites. In addition to

Fig. 1. Effect of ethanol on PvuII* activity under standard conditions.

Each reaction mixture contained 0.5 μ g pBR322 DNA, 40 units of PvuII endonuclease and the following concentrations (v / v) of ethanol :

(1) 0 % ; (2) 5 % ; (3) 10 % ; (4) 15 % ;
(5) 20 % ; (6) 25 % ; (7) 30 % ; (8) 40 % ;
and (9) 50 % . (A) pBR322 DNA standard.

All incubations were carried out for 4 hours at 37°C.

the effect of DMSO (13) on the PvuII digestion under the standard conditions, other organic solvents, which lower the polarity of the medium, were examined. Ethanol also alters the specificity of PvuII endonuclease. A maximal effect was obtained for ethanol concentrations ranging between 5 and 30 % (v/v). Higher concentrations (> 40 %) suppress both PvuII and PvuII* activities (Fig. 1).

Similar effects were produced by the addition of glycerol to the incubation mixture, when using supercoiled pBR322 DNA as substrate. Here the maximal effect is observed with a concentration of 26 % glycerol (result not shown). However, the PvuII* activity was not found in the presence of glycerol, when linear pBR322 DNA, generated either by PvuII or EcoRI, was used as possible substrate (Nevertheless, DMSO and ethanol show an activating effect with such substrate). The pBR322 DNA linearized by EcoRI was cleaved only at the standard site CAGCTG in the presence of glycerol. The PvuII* activity was also found in the presence of other organic solvents, such as: dimethyl-formamide, acetone ...

Other experiments were performed by incubating superhelical pBR322 DNA with PvuII endonuclease in the presence of a mixture of glycerol and DMSO, ethanol and DMSO. For this, different concentrations of DMSO were added to the reaction mixtures containing 26% glycerol or 10 % ethanol. The patterns of digestion obtained are shown in Figure 2. The analysis and comparison of DNA fragments produced by PvuII* activity under standard conditions + 15 % DMSO (Fig. 2A, track 2), or 26 % glycerol (Fig. 2A, track 3), or 10 % ethanol (Fig. 2B, track 3) show that DNA fragments are identical. These results indicate that the additional sites recognized and cleaved by PvuII* activity are very likely to be the same in these different modified conditions of reaction. On the other hand, the relaxation of PvuII specificity increases if two solvents are in

the reaction mixture. With mixtures of glycerol-DMSO and ethanol-DMSO, there is a maximum of pBR322 hydrolysis at 26 % glycerol-10 % DMSO, 10 % ethanol-5 % DMSO and 10 % ethanol-10 % DMSO respectively (Fig. 2).

Optimization of PvuII* activity

Under the conditions of maximum digestion (standard conditions +10 % ethanol and 10 % DMSO, or 26 % glycerol and 10 % DMSO), the complete cleavage of pBR322 DNA to final products was not obtained. In order to identify all the recognition sites for PvuII*, we have significantly modified the standard conditions of reaction (6 mM Tris-HCl, pH 7.4, 6 mM MgCl₂, 50 mM NaCl and 6 mM mercapto-ethanol) to obtain complete pBR322 DNA cleavage.

The optimum temperature, pH, Tris, NaCl and MgCl₂ concentrations, for the PvuII* activity were determined after addition of 10 % ethanol and 10 % DMSO. The activity was maximal at pH 8.5. The PvuII* activity was maximal between 37 and 45°C. The PvuII* is present even at a higher NaCl concentrations (200 mM). Both PvuII and PvuII* activities were lost when reaction mixture was depleted with MgCl₂.

Hsu and Berg (5) reported that the substitution of Mg²⁺ by Mn²⁺, under standard conditions, alters the specificity of the EcoRI endonuclease. For PvuII enzyme, Mn²⁺ may be able

Fig. 2. PvuII* digests of pBR322 DNA analysed by electrophoresis in a 1 % agarose gel.. Each reaction mixture contained 0.5 µg pBR322 DNA, 30 units of PvuII endonuclease, 26% glycerol (A) or 10 % ethanol (B) and the following concentrations (v/v) of DMSO: (3) 0 % ; (4) 5 % ; (5) 10 % ; (6) 15 % ; (7) 20 % ; and (8) 25 %.

(1) pBR322 DNA standard.

(2) 0.5 µg pBR322 DNA, 30 units of PvuII enzyme and 15 % DMSO.

Incubations were carried out for 6 h 30 mn at 37°C.

Fig. 3 : Comparison of AluI (1) and PvuII* (2) cleavage patterns.

to alter the sequence specificity of the enzyme only when organic solvents, such as DMSO, were added to the reaction mixture.

Neither PvuII nor PvuII* activity were obtained when MgCl_2 was replaced by Co^{2+} , Ba^{2+} or Zn^{2+} under the standard and relaxed conditions, while with Cu^{2+} , PvuII preparation converts superhelical pBR322 DNA into only open circular from (PvuII endonuclease cleaves the recognition sequence on only one of the two strands).

Finally, the optimum conditions for PvuII* activity was : 10-50 mM Tris-HCl, pH 8.5, 12-15 mM MgCl_2 , 50 mM NaCl, 10 % ethanol and 10 % DMSO.

Limit digest

Under optimum conditions of PvuII* activity (10-50 mM Tris-HCl pH 8.5, 12-15 mM MgCl_2 , 50 mM NaCl, 10% ethanol and 10% DMSO) and even after a 26-h incubation, a complete cleavage of the pBR322 DNA to the final products was not obtained. More than twenty fragments were produced under these conditions. The lengths of these fragments were estimated to be 400, 385, 360, 325, 315, 265, 260, 240, 220, 200, 175, 160, 155, 135, 130, 95, 80, 75, 74 and 70

base pairs, by comparing the electrophoretic mobility with pBR322 DNA fragments produced by Alu I digestion. The sum of the lengths of these fragments is less than 3900 bp, while the length of the whole pBR322 DNA is 4362 bp.

The central tetranucleotide of the PvuII recognition sequence, AGCT, is also recognized by AluI endonuclease (18). Under the standard conditions, PvuII generates the linear form, whereas AluI generates 16 fragments. Under the optimum conditions of PvuII* activity, PvuII* generates more than 21 fragments, whose mobilities are different from those of AluI enzyme (Fig. 3). This suggests that the alteration of PvuII endonuclease did not result in the reduction of the recognition site from a hexanucleotide to the central tetranucleotide as was suggested for BstI enzyme (7).

Determination of the cleavage sites of PvuII* activity using the technique of Maxam and Gilbert

After cleavage of pBR322 DNA by PvuII endonuclease under relaxed conditions, the mixture of fragments was dephosphorylated with alkaline phosphatase and the 5'-termini were then labelled with T₄ polynucleotide kinase and (γ -³²P) ATP. The end-labelled PvuII fragments were cleaved with endonuclease HinfI, in order to obtain fragments with only one labelled terminus and the digestion mixture was fractionated on a 7 % polyacrylamide gel. Fragments labelled only at the 5'-end of the PvuII* sites were selected for sequencing, as described in Materials and methods.

In addition to the restriction sites which we had determined previously (13) (CAGCTG, CCGCTG, CATCTG, CAGATG, CAGGTG and CAGCGG), we also discovered additional recognition sites for PvuII*:

- CAGCAG, substitution of T by A at the fifth position of the site. This sequence is found at positions 1405, 2884 and 3093.
- CAGCCG, substitution of T by C at the fifth position of the site. This sequence is found at positions 1408 and 3481.

In the previous paper (13), we have shown that PvuII* recognizes the sequence CAGCGG, where at the fifth position, T is substituted by G. Thus, under relaxed conditions, the specificity of PvuII endonuclease at the fifth position is completely lost, since all four possible hexanucleotides CAGCNG are found.

Since, PvuII* recognizes and cleaves double-stranded DNA, the sites CTGCTG and CGGCTG, (substitution of A by T or G at the second position, respectively) which are complementary to the sites CAGCAG and CAGCCG, respectively, are also recognized by PvuII* activity. In the same way, the specificity of PvuII enzyme at the second position is completely lost, since all four possible hexanucleotides CNGCTG are found.

Recognition sites determined by the double digestion of pBR322

The positions and nucleotide sequences of several of additional PvuII* sites were determined relative to other restriction sites on the pBR322 DNA. The restriction endonucleases used for the double digestion are: EcoRI, BamHI, HindIII, SalI, AvaI, PstI and HindII.

Fig(4) shows the fragments obtained from digestion of pBR322 DNA by PvuII* activity and their comparison to those generated in the double digestion.

For example, in the double digest with PvuII* and SalI (Fig. 4, track 2), the fragment B (about 340 bp) (Fig. 4, track 1 and 5) in the single PvuII* digest disappeared and two new fragments, a and b, appeared. The lengths of these fragments were about 230 and 115 bp, respectively. Fragment a has mobility similar to that of the D fragment, resulting of the single PvuII* digest. SalI cuts the pBR322 DNA at position 650. From these results, the two PvuII* cleavage sites were concluded to be at positions 422 and 765. For each of these two cleavage sites the sequence, CAGGTG, (determined according to the procedure of Maxam and Gilbert) was found. In the most prolonged digest, the PvuII* B fragment disappeared. It is obvious that this fragment is an intermediate product. The only recognition site, CAGCTC, located at position 686, within the PvuII* B fragment, appears to be the only plausible site since it was also found in the double digest with PvuII* and PstI.

The PvuII* nucleotide recognition sequences determined by the combinations of endonucleases are summarized in Table 1.

DISCUSSION

The data described in this paper, indicate that the specificity of PvuII restriction endonuclease is strongly relaxed when the reaction conditions are modified (by addition of organic solvents, such as DMSO, ethanol, glycerol...). The PvuII* endonucleolytic digestion of

Fig. 4 : Gel electrophoresis of pBR322 DNA digests.

Lane 1 and 5, PvuII* digest of pBR322 DNA ; Lane 2, PvuII* plus SalI ; Lane 3, PvuII*+ EcoRI; Lane 4, PvuII* + HindIII ; Lane 6, PvuII* + Pst I; Lane 7, Pvu II* + Ava I.

The samples were analysed on 8 % (w / v) polyacrylamide gel.

TABLE 1
Combinations of endonucleases used in the determination of the PvuII* nucleotide recognition sequence

First restriction endonuclease	Second restriction endonuclease	Sequences deduced	Location
PvuII*	EcoRI	CACCTG	4882
	HindIII	CAGGTG*	422
		CAGCTT	16
PvuII*	PstI	CATCTG*	3406
		CAGCCG	3481
		CAGCTC	3656
PvuII*	SalI	CAGGTG*	422
		CAGGTG*	765
		CAGCTC	686
PvuII*	AvaI	CAGCAG*	1405
		CAGCCG*	1408
		CTGCTG	1558
PvuII*	HindII	CAGCTC	3656
		CAACTG	4043
		CAGGTG*	422
		CAGGTG*	765

*, relaxed nucleotide sequences, determined also by the Maxam and Gilbert DNA sequence.

pBR322 DNA, which contain a single PvuII recognition site, results in complex fragment patterns. The optimum conditions for PvuII* activity was: 10-50 mM Tris-HCl, pH 8.5, 12-15 mM MgCl₂, 50 mM NaCl, 10% ethanol and 10% DMSO.

Comparison of the patterns of pBR322 digested by PvuII* under the optimum PvuII* medium and by AluI enzyme under the standard conditions of the reaction (which recognizes the central tetranucleotide of the PvuII hexanucleotide sequence) has shown that the alteration of the PvuII specificity does not involve the loss of the selectivity to both terminal nucleotides of the hexanucleotide site.

These data in addition to those reported in the previous paper (13), indicate that the PvuII relaxation occurs at the hexanucleotide sequence of the CAGCTG standard site. Only one of the six nucleotides of the standard site may be substituted under relaxed conditions. Any base can be substituted at the internal nucleotide sequence of the CAGCTG: CNGCTG, CANCTG, CAGNTG, CAGCNG.

TABLE 2
PvuII relaxed sites

Standard	NAGCTG	CNGCTG	CANCTG	CAGNTG	CAGCNG	CAGCTN
CAGCTG ↓	GAGCTG	CTGCTG	CACCTG	CAGATG ↓	CAGCAG ↓	CAGCTC
	(b)	(a) (b)	(a)			(a)
	AAGCTG	CCGCTG ↓	CAACTG	CAGGTG ↓	CAGCCG ↓	CAGCTT
	(b)		(a)			(a)
	TAGCTG	CGGCTG	CATCTG ↓	CAGTTG	CAGCGG ↓	CAGCTA
	?	(b)		(b)		?

↓, Relaxed sites as determined by the Maxam and Gilbert DNA sequence.

(a), Relaxed sites determined by the double digestion.

(b), Relaxed sites determined from complementary sites (The sites GAGCTG, AAGCTG, CCGCTG, CAGTTG and CTGCTG are complementary to the sites: CAGCTC, CAGCTT CAGCCG, CAACTG and CAGCAG, respectively).

?, Potential PvuII* sites, which are not present in the pBR322 DNA.

The relaxation of PvuII specificity occurs also at the outer nucleotide sequence of the standard site. PvuII* activity is found to cleave the sequences CAGCTT and CAGCTC (substitution of G by T or C at the sixth position, respectively). Because cleavage of DNA by PvuII enzyme requires double-stranded DNA, the complementary sequences AAGCTG and GAGCTG (substitution at the first position of C by A or G, respectively) have also to be recognized by PvuII* activity.

From the present data, it seems likely that the sites, TAGCTG (substitution of C by T at the first position) and the complementary sequence CAGCTA (substitution of G by A at the sixth position), which are not present in pBR322 DNA, are potential sites for PvuII* activity.

The nucleotide sequences recognized by PvuII* are presented in Table.2 . The nucleotide sequences of the region of pBR322 DNA containing cleavage sites for PvuII* and their positions are presented in Table.3 .

Two hypotheses may be put forward to explain the relaxation process: (a) the PvuII endonuclease is contaminated by an additional enzyme, masked under standard conditions; or (b) PvuII endonuclease is responsible for the relaxation specificity. Arguments supporting the second hypothesis were obtained: (a) PvuII* activity is not found, under standard conditions, when large amounts of endonuclease are used; (b) Both PvuII and PvuII* produce fragments with flush ends; (c) Moreover, the recognition sequence of PvuII* activity is derived from the standard one, with only one base changed (substitution can occur at any single position within

TABLE. 3
Nucleotide sequences in pBR322 DNA containing a cleavage site for PvuII* activity.
Recognition sites are underlined.

Site	Sequence determined	Location	Site	Sequences determined	Location
1	TGA <u>CAGCTT</u> ATC	16	18	TCACAGCTT <u>GTC</u>	2135
2	CCACAGGTGCGG	422	19	CGTCAGCGG <u>GTG</u>	2186
3	AGTCAGCTCCTT	686	20	GCACAGATGCGT	2321
4	GGACAGGTGCCG	765	21	GTT <u>CGGCTG</u> CGG	2397
5	GGACAGCTTCAA	1089	22	TATCAGCTCACT	2416
6	GGACCGCTGATC	1140	23	TCCAAGCTGGGC	2778
7	CTCCAGCAGCCG	1405	24	CGACCGCTGCGC	2816
8	CAGCAGCCGCAC	1408	25	TGGCAGCAGCCA	2884
9	CGACTGCTGCTG	1558	26	GCTCTGCTGAAG	3000
10	CTGCTGCTGCAA	1561	27	CCACCGCTGGTA	3061
11	ATGCTGCTGGCT	1686	28	AAGCAGCAGATT	3093
12	CTACATCTGTAT	1713	29	TACCATCTGGCC	3406
13	CGCCAGTTGTTT	1782	30	AGCCAGCCGGAA	3481
14	AACGAGCTGGAC	1999	31	ATTCAGCTCCGG	3656
15	AGACATCTGTGA	2025	32	ACCCA <u>ACTG</u> ATC	4043
16	CCGCAGCTGCCT	2067	33	TGCCACCTGACG	4282
17	ATGCAGCTCCCG	2116			

the hexanucleotide); (d) Both PvuII and PvuII* are inhibited by glutaraldehyde which reacts with amino groups and by carbodiimide which reacts specifically with carboxyl groups.

From these observations, and results showing that the relaxation in specificity was related to the loss of hydrogen bonds between the endonuclease and each base pair to be recognized (manuscript in preparation), we conclude that PvuII endonuclease alone is responsible for the relaxed specificity observed. Since, the same enzyme recognizes different relaxed sites, the loss of specific contacts between functional groups of the enzyme and the DNA, could result from a changes in conformation of the quaternary structure of the protein, caused by organic solvents.

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