‘Star’ activity and complete loss of specificity of *CeqI* endonuclease

Zsuzsa IZSVÁK and E. DUDA*

Institute of Biochemistry, MTA Biological Research Center, Hungarian Academy of Sciences, P.O.B. 521, H-6701 Szeged, Hungary

Restriction endonuclease *CeqI*, an isoschizomer of *EcoRV*, exhibits ‘star’ activity, a relaxation of specificity in the presence of Mn^{2+} , dimethyl sulphoxide or glycerol. The enzyme cleaves a set of sequences that differ from the canonical GATATC by only one nucleotide in positions 2, 3, 4 or 5. Two of these sequences are not cleaved if modified by *dam* methylase. A further loss of specificity can be observed in circumstances less favourable for the enzyme, namely low-ionic-strength buffers of pH values below 6.0 or above 9.4. This activity seems to cleave DNA at any sequence, producing a smear instead of well-defined bands. Partial renaturation of the denatured enzyme gives rise to a similar non-specific nuclease activity.

INTRODUCTION

Restriction endonucleases are known for their phenomenal precision, recognizing and cleaving DNA molecules at well-defined sequences of four, five, six or eight nucleotides. Replacement of Mg^{2+} by Mn^{2+} and the presence of organic solvents, e.g. dimethyl sulphoxide (DMSO) or glycerol, have been shown to disturb the recognition process, causing partial or total loss of specificity (Polisky *et al.*, 1975; Hsu & Berg, 1978; Tikchonenko *et al.*, 1978; Malyguine *et al.*, 1980; Wells *et al.*, 1981; Kuzmin *et al.*, 1984; Halford *et al.*, 1986; Nasri & Thomas, 1987).

Restriction endonuclease *CeqI* isolated from *Corynebacterium equi* (Duda *et al.*, 1987) is an isoschizomer of *EcoRV*, recognizing the sequence GATATC. *EcoRV* was purified to homogeneity from *Escherichia coli* (Schildkraut *et al.*, 1984; D’Arcy *et al.*, 1985). It was shown to exhibit ‘star’ activity in the presence of DMSO (Kuzmin *et al.*, 1984; Halford *et al.*, 1986). Of the 18 possible sequences that differ from GATATC by one base, all were cleaved readily, except the following four: TATATC, GATATA, CATATC and GATATG.

In the present paper we report a similar relaxation of specificity of *CeqI* when Mg^{2+} is replaced by Mn^{2+} . We have recently purified *CeqI* to homogeneity (Z. Iszvá & E. Duda, unpublished work). *CeqI* and *EcoRV* are produced by unrelated organisms, and the physical-biochemical properties of the two enzymes are rather different. Nevertheless, they recognize the same sequence, cleave at the same site, producing identical blunt-ended fragments, and their specificity can be relaxed to show very similar star activities. Comparison of the action of these two enzymes at the molecular level could reveal important features of high-precision DNA–protein interaction.

MATERIALS AND METHODS

Bacteriophage λ and plasmid pBR322 DNA were purified in our laboratory; *EcoRV*, *CeqI* and all other restriction enzymes used were purified in our Institute.

Cloned, sequenced DNA fragments were generously given by our colleagues (B. Arya, J. Gyuris, T. Lukácsovich and I. Takács). DNA sequencing was performed by using standard procedures (Maniatis *et al.*, 1982), with minor modifications.

DNA fragments were run in, and isolated from, agarose and polyacrylamide gels according to standard procedures (Maniatis *et al.*, 1982). Plasmids pBR322, π an7 and JE1880 were purified either from *E. coli* strain HB101 (*dam*-methylated form) or from strain GM119 (in the non-methylated form).

RESULTS

CeqI cleaves pBR322 at a unique site at position 185 (Duda *et al.*, 1987), linearizing the circular form of the plasmid. Replacing Mg^{2+} in the reaction buffer with Mn^{2+} (1–10 mM) results in a different cleavage pattern (Fig. 1). The enzyme cleaves pBR322 at several additional sequences, generating a rather complex pattern. Mg^{2+} alters the specificity of the enzyme, even in the presence of Mg^{2+} : in buffers containing 6 mM- Mg^{2+} the presence of 1–6 mM- Mn^{2+} results in a typical *CeqI* star-cleavage pattern (Fig. 1). Salt and pH optima for the Mg^{2+} and the Mn^{2+} enzymes are identical (50 mM-Tris/HCl, pH 8–8.5; 100–150 mM-NaCl).

Systematically surveying a whole collection of sequenced DNA fragments we have identified a set of restriction sites cleaved by *CeqI** (Table 1). These sequences can be described as 5/6 homologues of the authentic *CeqI* recognition sequence, GATATC. The Mn^{2+} enzyme shows a clear preference for sequences that differ from the canonical sequence in positions 3 and 4 over the ones that are altered in positions 2 and 5. A G substitution in position 2 or a C in position 5 are also preferred over pyrimidines in position 2 or purines in position 5. We could not detect cleavage of sequences substituted in positions 1 or 6.

Digestion of sequences GAGATC and GATCTC depends on *dam* methylation (Hattman *et al.*, 1978) of these sequences: only non-methylated sequences are cleaved. (*EcoRV* star activity cannot cleave methylated

Abbreviations used: DMSO, dimethyl sulphoxide; *dam* methylase, methylase activity specific for deoxyadenine.

* To whom correspondence and reprint requests should be addressed.

Fig. 1. Relaxed specificity of *CeqI* endonuclease in the presence of Mn^{2+}

Cleavage pattern of pBR322 DNA fragments after digestion with *CeqI* enzyme (20 units of enzyme/ μ g of DNA, 12 h digestion at 37 °C) in the presence of buffer H-[100 mM-Tris/HCl (pH 8.0)/100 mM-NaCl/1 mM-dithiothreitol]. Lane 1, 6 mM- $MnCl_2$; lane 2, 6 mM- $MnCl_2$ + 6 mM- $MgCl_2$; lane 3, 2 mM- $MnCl_2$ + 6 mM- $MgCl_2$; lane 4, 1 mM- $MnCl_2$ + 6 mM- $MgCl_2$; and lane 5, 6 mM- $MgCl_2$.

sequences either; Halford *et al.*, 1986). Sequences GGTATC and GATACC in pBR322 are cleaved in certain cases, whereas in others they remain uncut. Similar site preferences are known for several restriction enzymes (e.g. *EcoRI*; Thomas & David, 1975).

We have also tried DMSO and glycerol to decrease the specificity of *CeqI*. Both agents can be used in the presence of Mg^{2+} or Mn^{2+} at pH 8.0 [8 % (v/v) DMSO or 20 % (v/v) glycerol]. The Mg^{2+} enzyme is quite prone to denaturation in the presence of organic solvents during incubation; the Mn^{2+} enzyme, however, tolerates organic solvents better. There is a significant difference in the specific activity of *CeqI* enzymes: organic-solvent-induced star-like activity is extremely inefficient and unstable compared with the activity of the Mn^{2+} enzyme (results not shown).

Intercalating dyes and Methylene Blue inhibit activity of restriction enzymes (Polyanovsky *et al.*, 1979; Goppelt *et al.*, 1981) and interfere with their star activity (Hsu & Berg, 1978). We have investigated the effect of ethidium bromide, Acridine Orange, Methylene Blue and Methyl Green on *CeqI* and *CeqI*^{*} activity. Ethidium bromide showed the most discriminating effect: at concentrations between 5 and 50 μ g/ml it completely inhibited the star activity of the enzyme without interfering with its action on GATATC sequences. Acridine Orange, Methyl

Table 1. Recognition sites of *CeqI*^{*} enzyme

A collection of *CeqI*^{*} cleavage sites, identified on the basis of digestion patterns of several sequenced DNA fragments, is shown. These sites can be described as 5/6 homologues of the authentic *CeqI* recognition sequence, GATATC. We could not find digestion in cases where base substitutions occurred in positions 1 or 6. Short (0.5–2 kb) DNA fragments were digested in high-pH buffer in the presence of 6 mM with at least 20 units of *CeqI* enzyme for 24–32 h. (Digestions were carried out in sealed glass capillaries to prevent precipitation of manganese salts). Digested samples were deproteinized by phenol/chloroform treatment, diethyl ether-extracted, analysed on 5–8 %-(w/v)-polyacrylamide or 2–3 %-(w/v)-agarose gels, and stained with ethidium bromide (or silver). Each sample was run together with *BspRI*-digested pBR322 as marker, in at least five separate experiments. The estimated size of the DNA fragments found were compared with the sizes calculated by computer (partial and limit digests and both 5/6 and 4/6 homologues were calculated. (No 4/6 homologues were found.)

Recognition site (itali-cized)	Cleav-age	Recognition site (itali-cized)	Cleav-age	Recognition site (itali-cized)	Cleav-age
GAAATC	+	GTTATC	+	AATATC	—*
GATTTTC	+	GATAAC	+	GATATT	—*
GACATC	+	GGTATC	+/-	CATATG	—*
GATGTC	+	GATACC	+/-	GAGATG	—*
GAGATC	+/-†	GCTATC	+	TATATC	—*
GATCTC	+/-†	GATAGC	+	GATATA	—*

* Substitution in position 1 or 6.

† Depending on *dam*-methylation.

Table 2. Interference of intercalating dyes with *CeqI*^{*} activity

Dye	Concn. (μ g/ml) ...	Effect (—, no cleavage; +, no interference)			
		1	5	10	50
Methyl Green		+	+	+	—
Methylene Blue		+	+	+	—
Acridine Orange		+	+	—	—
Ethidium bromide		+	—	—	—

Green and Methyl Blue displayed less selectivity (Table 2).

Our studies indicated that an enzyme activity with further loss of specificity (producing a smear instead of well-defined bands) can be detected in circumstances very unfavourable for the stability of the enzyme: low-salt buffers (below 50 mM) of pH values below 6 or over 9, or high concentration of glycerol in the presence of Mg^{2+} . A similar loss of specificity of other enzymes caused by organic solvents and non-specific activity of *XbaI* in the presence of DMSO and/or glycerol has been described (Malyguine *et al.*, 1980).

DISCUSSION

Restriction endonuclease *CeqI* purified from *Corynebacterium equi* shows star activity: a lower degree of specificity in the presence of Mn^{2+} ions, DMSO or glycerol. The enzyme cleaves several additional sequences that differ from the original GATATC by only one nucleotide in positions 2, 3, 4 or 5. Base substitutions in position 3 and 4 are more readily tolerated and cleaved than in positions 2 and 5, where cleavage cannot be detected in all cases. Two of these sequences, GAGATC and GATCTC, are subjects of *dam*-methylation in most *E. coli* strains. The methylated form of these sequences is resistant to cleavage by *CeqI*^{*} activity.

The enzyme can be converted into a non-specific endonuclease form by partial denaturation or destabilization of the active *CeqI* enzyme. Experiments suggest two forms of the enzyme: (1) a binding form, recognizing the canonical sequence (and closely related ones), but unable to cleave it, and (2) a cleaving form. A conformational change of the nuclease domain is needed; the refolding requires a full match of the recognition sequence and Mg^{2+} (in a suitable buffer). As soon as the DNA has been cleaved by the 'cleaving' form of the enzyme, the enzyme itself refolds into the 'binding' form. Mn^{2+} would act by making the second form more favourable, facilitating structural change even if one of the inner nucleotides mismatches. The fact that the Mn^{2+} enzyme is more stable in the presence of organic solvents and works more efficiently at low temperatures than does the Mg^{2+} enzyme (Z. Izsvák & E. Duda, unpublished work), supports this idea. Stabilization of the enzyme in the second form as a result of either a damaging milieu or improper refolding after denaturation will result in an apparent loss of specificity of the nuclease.

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