*Nde*I: a restriction endonuclease from *Neisseria denitrificans* which cleaves DNA at 5′-CATATG-3′ sequences

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Received 4 October 1982

Palindrome Isoschizomer Cleavage site Restriction mapping DNA sequencing

1. INTRODUCTION

Type II restriction endonucleases recognize and cleave near specific DNA sequences, usually 4–6 basepair palindromes. They are of fundamental importance as tools for the recombinant DNA technology as they provide the selective cleavages required for the analysis and restructuring of DNA in vitro. The flexibility of these techniques is proportional to the number of DNA sequences which can be cleaved by available enzymes. Here, we describe the isolation and characterization of a restriction enzyme from *Neisseria denitrificans* with a new recognition sequence, 5′-CATATG-3′.

2. MATERIALS AND METHODS

2.1. *Strain growth*

Neisseria denitrificans NRCC strain #31009 was grown in either BBL trypticase soy broth or BBL brain-heart infusion at 37°C with aeration. Late logarithmic-phase cells were collected by centrifugation, washed in 0.85% saline and either used immediately or mixed with an equal volume of 0.85% saline in 40% glycerol and frozen at −80°C until use.

2.2. *Purification of* Nde*I and* Nde*II*

Cells (5 g) were suspended in 10 ml buffer A (20 mM Tris–HCl (pH 8.0), 0.5 mM EDTA, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 100 μg BSA/ml) and broken by five 30 s treatments with the small probe of a Branson sonifier cell disruptor 185. The lysate was clarified by centrifugation at 250000 × *g* for 17 h and the crude extract applied to a 1.5 × 20 cm DEAE-cellulose column equilibrated with buffer A. Three void volumes of buffer A were passed through the column followed by successive 100 ml additions of buffer A containing 0.05, 0.10, 0.25, 0.50 and 1.0 M NaCl. Restriction endonuclease activity corresponding to *Nde*I was eluted by 0.1 M NaCl. A second enzyme activity, that of *Nde*II, was eluted at 0.25 M NaCl. Fractions with *Nde*I activity were pooled and concentrated by dialysis against buffer A containing 40% glycerol and 0.10 M NaCl. *Nde*I was further purified by heparin–agarose chromatography as in [1,2] using stepwise additions of 0.10, 0.25, 0.50 and 1.0 M NaCl in buffer A to develop the column. *Nde*I activity eluted in the 0.50 M NaCl fraction. Active fractions were concentrated by dialysis against buffer A containing 0.10 M NaCl and 40% glycerol and stored at −20°C.

2.3. *Enzyme assay and characterization*

DNA digestions with *Nde*I were done in 150 mM NaCl, 10 mM Tris–HCl (pH 7.8), 7 mM $MgCl_2$, 6 mM 2-mercaptoethanol and 100 μg BSA/ml at 37°C. The substrates used were pBR322, SV40, φX174, M13mp9 and λ DNAs. These DNAs were purified or purchased as in [1]. Except for λ DNA their sequences are known [3–8]. Searches for

Issued as NRCC no. 20688, CBRI no. 1353

Published by Elsevier Biomedical Press
001457593/82/0000—0000/$2.75 © Federation of European Biochemical Societies

restriction sites within these sequences and prediction of sizes of their restriction endonuclease digest fragments were aided by computer as in [1]. Techniques for single and double restriction enzyme digests and gel electrophoretic techniques have also been described [1,9].

DNA sequencing to determine the cleaveage site for *Nde*I was done by the dideoxy chain termination technique [10] using the single-stranded form of M13mp9 [7,8] as a template. A 53 basepair double-stranded primer for the reaction was generated from M13mp7 RF DNA by digestion with *Cla*I and *Bgl*II.

3. RESULTS AND DISCUSSION

Crude *N. denitrificans* extracts were found to have two restriction endonuclease activities separable by DEAE-cellulose chromatography (section 2.2). *Nde*II, which elutes from this column in 0.25 M NaCl was found to cleave DNA at 5′-GATC-3′ sequences based on the DNA fragment sizes derived by treatment of SV40 DNA with this enzyme. Thus, this enzyme is an isoschizomer of *Mbo*I [11].

*Nde*I, which elutes from the column in 0.1 M NaCl, was found to cleave pBR322 once, SV40

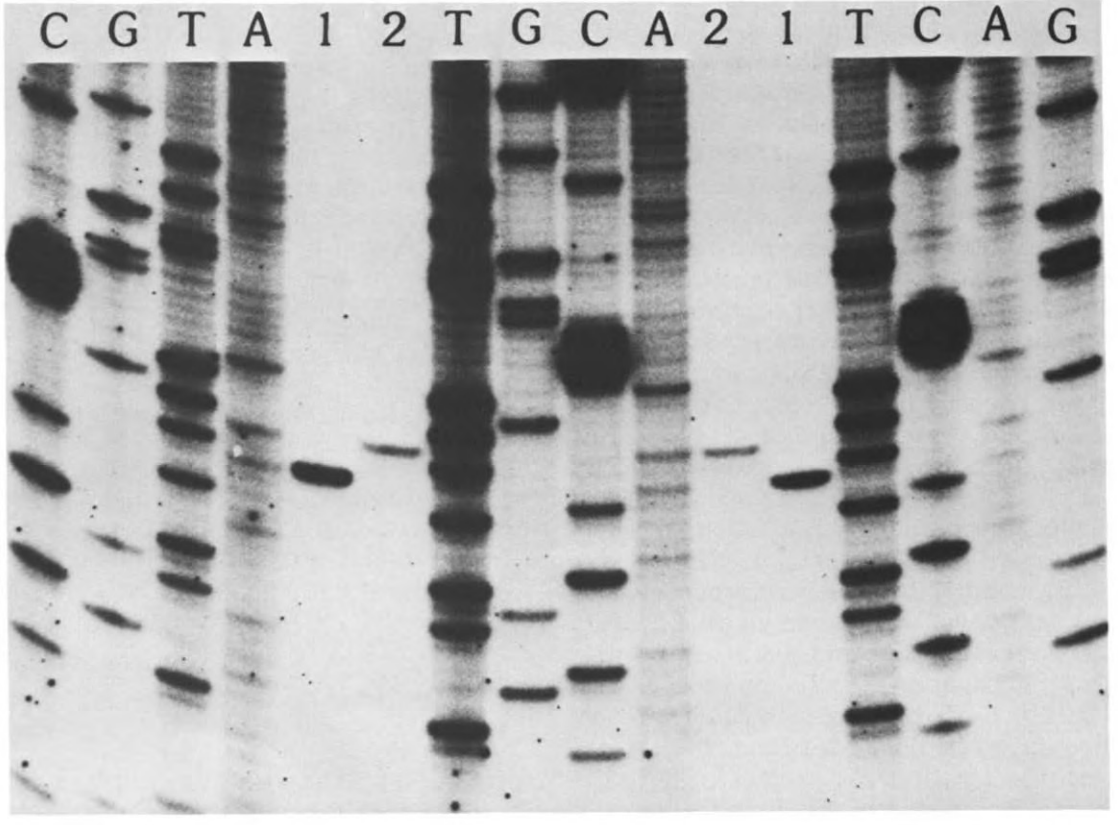

Fig. 1. Recognition and cleavage site of *Nde*I. The single-stranded form of M13mp9, which contains an *Nde*I site at 6825 was used as a template, and a double-stranded primer (53 basepair) was generated by digesting M13mp7RF DNA with *Cla*I and *Bgl*II. The sequence channels were obtained by the chain termination procedure [10]. Channels 1 and 2 were obtained from a parallel reaction in which the primer was extended in the absence of chain terminators. The reaction was treated with *Nde*I following the elongation. After *Nde*I treatment one half of this mixture was loaded in channel 1, the other half was further treated with DNA-polymerase I and loaded in channel 2.

DNA twice, M13mp9 DNA 3 times, lambda DNA ~7 times, and was inactive against ϕX174 DNA. To identify the recognition sequence of *Nde*I double digests of pBR322 with this enzyme together with *Bam*HI, *Pst*I, *Ava*I, *Nci*I, *Hin*fI and *Hae*III were analyzed to finely localize the site. By this means it was found to map at basepair 2300 ± 10 basepair. This corresponds to the position of the palindrome 5′-CATATG-3′, which occurs uniquely in pBR322 at basepair 2296–2301 [3]. This sequence also appears at basepair 3745 and 4763 in SV40 DNA, in agreement with the 4.2 and 1.0 kilobase sizes of the fragments found after treatment of this DNA with *Nde*I, and is absent from ϕX174 DNA. No sequence other than 5′-CATATG-3′ occurring near basepair 3200 in pBR322, including non-palindromic sequences, was found to occur in pBR322, SV40 and ϕX174 DNAs with the frequencies noted above for *Nde*I cleavage. Also, no other candidate sequences were found by testing various sequences related to CATATG (e.g., PyATATPu and CAPuPyTG). From these data we conclude that 5′-CATATG-3′ is the recognition sequence of *Nde*I.

To determine the cleavage site of *Nde*I, the following experiment was performed. The single-stranded form of M13mp9 which contains an *Nde*I site at basepair 6825 was used as a template, and a double-stranded primer (53 basepairs) was generated by disgesting M13mp7 RF DNA with *Cla*I and *Bgl*II. The template and primer were incubated with the Klenow fragment of *E. coli* DNA polymerase I such that the primer was extended through and beyond the *Nde*I site. Following elongation the polymerase was inactivated by heat treatment and the extended chains cleaved with *Nde*I. To one half of the reaction a further amount of DNA polymerase was added plus all 4 deoxynucleoside triphosphates, and the reaction continued. To the other half no addition was made. These 2 samples were then electrophoresed on a DNA sequencing gel alongside a standard set of sequencing reactions [10]. The results are shown in fig. 1.

From this experiment it can be seen that cleavage takes place within the recognition sequence 5′-CATATG-3′. In the case of the newly synthesized strand, it can be seen that the band obtained in the absence of added polymerase comigrates with the band in the A channel, indicating the A is the 3′-terminal nucleotide, whereas in the reaction in which polymerase has been added a band two nucleotides longer is produced due to the addition to the 3′-terminal nucleotide by polymerase I. This longer fragment co-migrates with the other A in the site, indicating cleavage by *Nde*I is symmetrical and occurs at the same location on both strands producing a 2 base 5′ extension. The data establish that the cleavage site for *Nde*I is 5′-CA↓TATG-3′.

ACKNOWLEDGEMENTS

We thank J. Bardwell, G. Masson and W. Rowsome for technical assistance.

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