Endonuclease NaeI is a prototype for an unusual group of type II restriction endonucleases that must bind two DNA recognition sequences to cleave DNA. The naeIR gene, expressed from a Ptac promoter construct, was toxic to Escherichia coli in the absence of NaeI sequence specific methylases. The naeIR gene was mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine; four classes of NaeI variants were isolated in the absence of protecting methylase activity. Class I variants (T601, E70K) lacked detectable cleavage activity, but displayed good sequence-specific DNA binding. Class II variants (D95N, G141D) displayed 1-5% of the wild-type cleavage activity and normal DNA binding. Class III variants (G131E, G131R, G155D, G245R, G250E, G270E) displayed significantly attenuated cleavage and binding activities. Class IV variants (G197D, G214R, G219T, G236S, L241P, G245R, G250E, G270E) lacked both cleavage and binding activities. These results imply two amino acids (Thr-60, Glu-70) essential for catalysis. In addition, two domains are indicated in NaeI: one (Thr-60 to Gly-155) mediates substrate binding and catalysis, the other (Gly-197 to Gly-270) may mediate binding of the activating DNA sequence. Our results are compared with the active site sequences of EcoRI, EcoRV, and BamHI.

The NaeI restriction endonuclease, isolated from Nocardia aerocolonigenes, is a member of the unique type IIe class of endonucleases. This family includes BspMI, HpaII, NaeI, NarI, and SacII (Conrad and Topal, 1989; Oller et al., 1991) as well as EcoRII (Krüger et al., 1988; Gabbara and Bhagwat, 1992). NaeI is characterized by the strong sigmoidal dependence of its cleavage velocity on substrate concentration. This sigmoidal dependence is indicative of the need to bind two DNA recognition sequences for cleavage. The two DNA-binding sites manifest different preferences for sequences flanking the recognition sequence (Yang and Topal, 1992). This gives the interesting situation that DNAs containing sequences without sufficient affinity to occupy one or the other sites are resistant to cleavage (Conrad and Topal, 1989; Oller et al., 1991; Yang and Topal, 1992).

NaeI recognizes and cleaves the sequence GCC/GGC. Binding of the second DNA recognition sequence assembles an active form of NaeI homodimer: Dimer formation in the absence of two-site binding gives an inactive conformation of NaeI (Baxter and Topal, 1993). Although a significant amount of work has been done to understand the NaeI reaction scheme (Yang and Topal, 1992; Baxter and Topal, 1993), little is known about the structure of the protein and the amino acid residues that mediate its functions.

Together with the crystal structures for EcoRI, EcoRV, and BamHI, identification of cleavage-deficient variants that bind DNA have defined a Mg2+ binding motif. Such variants also provide critical information to help assign the amino acids involved in DNA recognition by EcoRI (reviewed by Heitman, 1992 and 1993).

Here we employ random mutagenesis with MNNG to isolate cleavage-deficient variants of NaeI endonuclease. Bacteria were selected by their ability to survive in the presence of mutagenized NaeI but lacking protecting methylases. Analysis of cleavage and DNA binding ability of the resulting NaeI variants enabled the identification of four classes of NaeI variants with reduced cleavage activity.

EXPERIMENTAL PROCEDURES

Materials

Bacterial Strains—Escherichia coli strains CAAl (F' e14 merAl lacY1 or lacY6 SupE44 galK2 galT22 merA rfiD1 merB haedir; m0) M-Map1 was obtained from Ellen Guthrie (New England Biolabs); ER1992 (F' λ endA1 thi-1 supE44 mer-67 ΔlacU169 Δmer-hadr 14Δ::1510 dinA::Mud11734) was obtained from Elisabeth Raleigh (New England Biolabs); WA803 (lacY1 Δlac-6 glnV444A5) galK2 galT22 rfiD1 metB1 hsdR2) was obtained from Barbara Bachmann (E. coli Genetic Stock Center). All cells were grown in Luria-Bertani (LB) medium (Sambrook et al., 1989) and supplemented with ampicillin to 125 µg/ml, when necessary, to maintain plasmid selection.

Plasmids—Plasmid pCY786 contains the naeIR gene in the pAGR3 tac expression vector. pCY786 is a derivative of pNEB786 (from Ellen Guthrie, New England Biolabs) obtained by deleting the 130-bp region between the tac promoter and the translational start site of the NaeI gene. This region contained several out-of-frame start sites. Plasmid pBR322 DNA was prepared by banding in CaCl2 (Sambrook et al., 1989).

Oligodeoxyribonucleotides for Gel Mobility Shifts—Oligodeoxyribonucleotides were synthesized by machine using an Applied Biosystems 380A synthesizer. The abbreviations used are: MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; IPTG, isopropyl-1-thio-β-D-galactopyranoside; X-gal, 5-bromo-4-chloro-3-indoyl β-D-galactoside; hp, base pair(s).

Specific fragment:

5'GGTCCGGGCAAGGG3'
5'CCACGCGGCCTCC3'

Non-specific fragment:

5'GCTGGTTGGGATATCGGCTGGCGACCTC'5'CGAAGCCAGCAGCTTCGAA',

Sequencing primers—Primers were CGATTACGAGATTGCAAGGGCTTCAAAACAGAAGAGTtgCGCAGCTC.

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restriction endonuclease were from Promega. Ampicillin, MNNG, X-gal, and IPTG were from Sigma. Protein dye reagent was from Stratagene. Sep-Pak plus C\textsubscript{18} cartridges were from Waters.

**Methods**

**MNNG Mutagenesis**—Random mutations were generated in vitro with MNNG according to Miller (1972); 0.01 \( \mu \)g of MNNG (1 mg/ml) were added to 5 ml of log-phase growth CAA1 (\( \text{Me}^+ \)) cells containing the \( \text{naeR} \)-containing plasmid pCY786. After treatment, the cells were grown overnight at 37 °C in 5 ml of LB/ampicillin medium. Mini-preparations of \( \text{naeR} \)-plasmid DNA were transformed into either WA802 or ER1992 competent cells, which lack specific methylases to protect against \( \text{nauR} \) cleavage. Cleavage defective \( \text{naeR} \)-containing variants were selected based on their ability to form colonies on ampicillin plates. Mutants were selected at random and numbered in chronological order. Control cells containing wild type \( \text{naeR} \) gave no colonies under these conditions when plated at a density of 10\(^6\) colony-forming units/plate.

**Temperature-sensitive Mutants**—MNNG-treated pCY786 was transformed into WA802 cells and grown overnight at 42 °C. Colonies that survived were replica-plated (Miller, 1972); replicas were grown at 25 °C overnight. Temperature-sensitive variants were selected that grew at 42 °C but not at 25 °C.

**DNA Isolation**—For mini-preparations, putative \( \text{NaeI} \) mutant colonies were grown overnight in LB/ampicillin medium. 3 ml of the culture were pelleted and resuspended in STET buffer (8% sucrose, 5% Triton X-100, 50 mM EDTA, pH 8.0, 10 mM Tris, pH 8.0). The cells were boiled for 10 min, and the debris was pelleted by microcentrifuge. DNA was precipitated from the resulting supernatant by adding isopropl alcohol to 60% and centrifuging an additional 15 min. The DNA pellet was resuspended in 100 \( \mu \)l of H\textsubscript{2}O.

**For larger-scale DNA preparations, \( \text{naeR} \) mutants were grown overnight in 100 ml of LB/ampicillin medium. DNA was isolated using Qiagen-tip 100 columns according to the manufacturer, except that the DNA was resuspended in 100 \( \mu \)l of H\textsubscript{2}O instead of Tris-EDTA for DNA sequencing purposes.

**Cell free Extracts**—Overnight cell cultures were subcultured, grown approximately 2 h to mid-log phase, induced with IPTG (220 \( \mu \)g/ml), and grown to saturation. Cells from 2.5 ml of growth were pelleted and then resuspended in 1 ml of buffer (20 \( \mu \)M KPO\textsubscript{4}, 0.1 mM EDTA, 1 mM \( \beta \)-mercaptoethanol, 5% glycerol). Cells were lysed by sonicating three times for 15 s, and cell debris was pelleted. The total protein concentration of the cell-free extracts was determined by the Bradford (1976) method.

**Cleavage Activity Assay**—A volume of cell-extract containing 3 \( \mu \)g (or serial dilutions) of total protein from mutant and wild type \( \text{NaeI} \)-containing cells was incubated with 200 ng to 1 \( \mu \)g of pBR322 at 37 °C for 1 h in a 20-pl reaction volume containing 50 mM \( \beta \)-mercaptoethanol, 200 mM NaCl, 100 mM MgCl\textsubscript{2}, 100 mM Tris (pH 8.0), and bovine serum albumin (50 \( \mu \)g/ml). Reactions were stopped by incubation for 10 min at 65 °C. SDS was added to 0.2% to each sample, and samples were incubated for 10 min at 65 °C and then electrophoresed on 1% agarose gels. Assay conditions for the temperature-sensitive mutants were the same as above except the temperature-sensitive mutants were incubated with 400 ng of pBR322 at both 25 °C and 42 °C.

**Gel Mobility Shift Assays**—100 \( \mu \)g of oligodeoxyribonucleotide was radiolabeled by incubation with 0.02 \( \mu \)c of \( \text{[\text{\beta}}\text{P} \text{dATP]}\) and 16 units of T4 Kinase in \( 20 \mu \)l of T4 kinase buffer in a 50-\( \mu \)l reaction volume for approximately 2 h to mid-log phase, induced with IPTG (220 \( \mu \)g/ml), and grown to saturation. Cells from 2.5 ml of growth were pelleted and then resuspended in 1 ml of buffer (20 \( \mu \)M KPO\textsubscript{4}, 0.1 mM EDTA, 1 \( \mu \)M \( \beta \)-mercaptoethanol, 5% glycerol). Cells were lysed by sonicating three times for 15 s, and cell debris was pelleted. The total protein concentration of the cell-free extracts was determined by the Bradford (1976) method.

**DNA Cleavage Assays**—We compared the sequence-specific DNA cleavage activity of mutant and wild type \( \text{NaeI} \) protein. Serial dilutions of wild type and mutant cell-free extracts were incubated with pBR322, a plasmid DNA substrate containing four \( \text{NaeI} \) cognate sites. Activity was measured from the intensity and banding pattern of the pBR322 cleavage products (Fig. 1). Of the 81 mutants compared to the wild type cell-free extract, 76 had no detectable activity and 5 (174, 175, 178, 188, 209) had partial activity.

**The temperature-sensitive mutants TS8 and TS42 were tested for in vitro cleavage activities at the permissive (25 °C) and non-permissive (42 °C) temperatures. Both of the temperature-sensitive mutants showed small but detectable amounts of cleavage at both temperatures (not shown). Only very small differences in cleavage were detectable at the two temperatures.

**Gel Mobility Shift Assays**—To determine whether the variants with reduced cleavage activity resulted from changes in catalysis or changes in DNA binding ability, \( \text{NaeI} \) variants were also analyzed for DNA binding using the gel mobility-shift assay (Fig. 2). This assay measures protein-DNA binding because \( \text{NaeI} \)-DNA complexes migrate slower than the free DNA during electrophoresis. Ca\textsuperscript{2+}, instead of Mg\textsuperscript{2+}, was used as the divalent cation in the gel mobility shifts because \( \text{NaeI} \) binds but does not cleave DNA in the presence of Ca\textsuperscript{2+} (Baxter and Topal, 1993). The DNA probe used in these assays was a 14-\( \mu \)l "specific" fragment containing the 6-bp \( \text{NaeI} \) cognate recognition site (GCGGGG). Of 51 variants tested, 41 did not shift the mobility of the DNA probe, four (variants 60, 174, 175, 209) bound similar to the wild type \( \text{NaeI} \) cell-free extract, two (203, 212) bound tighter and shifted the mobility to that of higher apparent molecular weight complexes, and four (61, 178, 188, 196) showed reduced but measurable binding.

Addition of large concentrations of cold \( \text{NaeI} \)-specific probe (100-fold excess over labeled probe) to the binding reaction competed away the mobility shift. Addition of similar concentrations of a probe lacking the \( \text{NaeI} \) recognition sequence did not. Therefore, the gel mobility shift depended on the presence of an \( \text{NaeI} \) recognition sequence, which implies that the
DNA as the substrate. Untreated DNA is shown in lane tracts. Serially diluted All binding reactions were reproducible based on at least two determinations using extracts prepared from a different bacterial colony for each determination.

The second region contains only substitutions that attenuate binding; most of these reduced binding to below detectable levels. All, but one, of the variants (178: G245E) in the second region lack detectable DNA cleavage and binding activities compared to the wild type (Table I). The first region contains amino acids Gly-197 and Gly-250 in the NaeI enzyme that give sequence ambiguity. Three primers, spaced approximately 300 bp apart, sequence the template (+) strand. Two primers sequence the first and last thirds of the complementary (−) strand of the NaeI gene.

Although MNNG tends to give a high frequency of double mutations (Foster, 1991), only 8 out of the 31 mutants sequenced contained double mutations and 2 contained triple mutations. The triple mutations were not considered because of ambiguity in assigning structure-function relationships. Only one of the double mutants is considered for the same reason. Results of both the DNA sequencing data and the biochemical analysis of the remaining 22 variants (including the two temperature-sensitive variants) are compiled in Table I.

**DISCUSSION**

We randomly mutated the NaeI gene using MNNG and selected for cells that survived in the presence of mutated naeIR. The NaeI variants isolated display deficiencies in DNA cleavage, DNA binding, or both. By this method, we have started to characterize substrate and effector DNA binding domains of NaeI and amino acids essential for catalysis.

**Mutations Involved in DNA Binding**—Two clusters of mutations between amino acids Thr-60 and Gly-155 and between amino acids Gly-197 and Gly-250 in the NaeI amino acid sequence indicate potential DNA binding sites (Fig. 3). The first region contains an intermingling of amino acids that attenuate catalysis with substitutions that attenuate binding (Fig. 3). The second region contains only substitutions that attenuate binding; most of these reduced binding to below detectable levels. All, but one, of the variants (178: G245E) in the second region lack detectable DNA cleavage and binding activities (Table I). Temperature-sensitive Mutations—The amino acid changes responsible for the two temperature sensitive variants were sequenced the entire naeIR gene for each variant characterized for cleavage and DNA binding activity shown in Table I. This included 31 mutants: all 10 of the variants that showed binding but reduced cleavage, 19 randomly chosen non-binding mutants, and 2 temperature-sensitive mutants. Five oligonucleotide primers were designed to completely sequence the naeIR gene and to sequence both strands of regions that gave sequence ambiguity. Three primers, spaced approximately 300 bp apart, sequence the template (+) strand. Two primers sequence the first and last thirds of the complementary (−) strand of the NaeI gene.

| Class | Variant | Amino acid change | Relative cleavage | Relative binding |
|-------|---------|------------------|------------------|-----------------|
| I     | 60      | T60I             | <0.1             | 50–100          |
|       | 203, 212| E70K             | <0.1             | 200             |
| II    | 174, 175| G141D            | 1–5              | 100             |
|       | 209     | D95N             | 1–5              | 100             |
| III   | 61      | G131R            | <0.1             | 5–10            |
|       | 178     | G245E            | 1–5              | 50              |
|       | 188     | G155D            | 0.1–1            | 10–50           |
|       | 196     | G131E            | <0.1             | 1–5             |

**FIG. 1. Cleavage activity of wild-type and mutant NaeI extracts.** Serially diluted (1X = 3 pg of total cell free protein) wild type (lanes 3–7, upper panel) and mutant NaeI cell-free extracts were assayed as described under “Experimental Procedures” using pBR322 DNA as the substrate. Untreated DNA is shown in lane 1 of both panels. Separation of the reaction products by gel electrophoresis on 1% agarose gel indicated. Variants 203 (E70K), 178 (G245E), and 189 (G197D) are caused by the larger amino acid changes in the NaeI control (lane 1) versus the wild type and variant NaeI cell free extracts (lanes 3–7).

**FIG. 2. Gel mobility shift assays of wild-type and variant NaeI.** Wild type and variant NaeI cell free extracts were assayed for DNA binding as described under “Experimental Methods.” Specificity of binding was tested by competition with specific and nonspecific competitor DNAs as indicated. Variants 203 (E70K), 178 (G245E), and 189 (G250E) are shown as examples of tight binding, reduced binding, and no binding, respectively. Identical amounts of total protein, as determined by Bradford assay, were used in each lane; the higher amounts of binding in the NaeI controls (compare lanes 2 and 3) are caused by the larger amounts of pure NaeI in the controls relative to that in the cell extracts. All binding reactions were reproducible based on at least two determinations using extracts prepared from a different bacterial colony for each determination.

protein-DNA interactions were sequence-specific.

DNA Sequence Analysis of the NaeI Mutants—To link the biochemical phenotypes to specific amino acid changes, we sequenced the entire naeIR gene for each variant characterized for cleavage and DNA binding activity shown in Table I. This included 31 mutants: all 10 of the variants that showed binding but reduced cleavage, 19 randomly chosen non-binding mutants, and the 2 temperature-sensitive mutants. Five oligonucleotide primers were designed to completely sequence the naeIR gene and to sequence both strands of regions that gave sequence ambiguity. Three primers, spaced approximately 300 bp apart, sequence the template (+) strand. Two primers sequence the first and last thirds of the complementary (−) strand of the NaeI gene.

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**Temperature-sensitive Mutations**—The amino acid changes responsible for the two temperature sensitive variants were
EcoRV, and BamHI have been deduced from their crystal structure (Xu and Schildkraut, 1991) and is usually associated with catalytic site (Fig. 4). The roles these residues play in EcoRI, DNA and display weak cleavage activity. NaeI mutations binding are indicated by a.

Amino acids that effect binding and therefore also cleavage are indicated by a b. Amino acids in common with BamHI, EcoRI, and EcoRV. Two amino acids in this domain, T60 and E70, appear to be essential for catalysis. These amino acids are defined two domains in the protein separated by a similar basic region in NaeI. This basic region supports the notion that this is the second DNA binding site.

Mutations Involved in Catalysis—Protein variants that bind DNA, but have no detectable cleavage activity are useful to identify mutations involved in catalysis (Fig. 3). NaeI mutations T60I and E70K may be essential for catalysis: they bind DNA with differing capabilities, but have no detectable cleavage activity. The E70K mutation increases binding and produces higher mobility shift complexes than those produced by wild type protein. The increased binding phenotype has been seen with variants of other restriction enzymes (Wright et al., 1991; Newman et al., 1994). The EcoRV catalytic center shares a similar structure with EcoRI (Selent et al., 1992). Residues Asp-74 and Asp-90 in EcoRV, and Glu-111 and possibly Asp-91 in EcoRI are involved in chelating Mg2+ (Heitman, 1992). When Asp-90 chelates Mg2+ in EcoRV, it distorts the DNA and activates the catalytic center of the endonuclease (Thielking et al., 1992). Lys-92 contacts the scissile phosphate in EcoRV; the Lys-113 residue in EcoRI may have a similar role (Selent et al., 1992). BamHI has a slight variation on this model. The Asp-94, Glu-111, and Glu-113 residues of BamHI are conserved with those in EcoRI and EcoRV. However, it also requires Glu-77 for catalysis.

NaeI amino acids Glu-70, Asp-95, and Lys-97 appear similar to the catalytic site signature discussed above (Fig. 4). Glu-70 appears to be essential for catalysis by NaeI, Asp-95 also appears to be involved in catalysis, but is not essential since reduced cleavage was observed in the D95N variant. In addition, Thr-60 is apparently essential for catalytic function of NaeI, but has no apparent counterpart in the other restriction enzymes mentioned.

The NaeI catalytic domain, defined by the cleavage-deficient variants Thr-60, Glu-70, Asp-95, and Gly-141, is intermingled with substitutions that significantly affect binding to DNA (Fig. 3). The substrate binding domains for BamHI, EcoRI, and EcoRV also contain the amino acids essential for catalysis. Thus, this region in NaeI appears be the substrate binding domain, which also contains the catalytic site. The second domain is independent of the first and only affects DNA binding. This domain appears to be the activation domain of NaeI, which kinetic studies imply is not directly involved in catalysis.

It is informative to lay the two putative NaeI protein domains over a map of the basic and acidic regions of NaeI (Fig. 5). The substrate-binding domain coincides with an acidic and a basic region. These two different regions correlate with the catalytic and binding variants defining the first domain, respectively. The activation domain coincides with another significant basic region in NaeI. This basic region supports the notion that this is the second DNA binding site.

In summary, we have used random mutagenesis to locate amino acids involved in catalysis and DNA binding. These amino acids define two domains in the protein separated by significant linear distance in the protein sequence, but not necessarily in three-dimensional space. The domain closest to the amino terminus shares relative position and acidic amino acids in common with BamHI, EcoRI, and EcoRV. Two amino acids in this domain, T60 and E70, appear to be essential for catalysis. This domain also contains amino acids that when substituted attenuate DNA binding. These amino acids are

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**Table 1:** (Note: The table is not fully visible in the transcription.)

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2 K. J. and M. D. Topal, unpublished results.
binding &
catalysis
binding
60-155
197-270
1
76-95
134-161
206-242
acetic
basic
basic
317

Fig. 5. The proposed DNA substrate and effector binding sites shown relative to the acidic and basic regions of NaeI. The acidic and basic regions were deduced from the DNA sequence shown in Fig. 3.

associated with a basic region located within the domain. These results imply that this domain is the substrate-binding site of NaeI. The second domain contains almost exclusively amino acids that are required for DNA binding. These amino acids coincide with a larger, second basic region in NaeI. This result suggests that this domain may be the effector-binding site of NaeI (Yang and Topal, 1992; Baxter and Topal, 1993).

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