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Location of Putative Binding and Catalytic Sites of *Nae*I by Random Mutagenesis*

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Endonuclease *Nae*I is a prototype for an unusual group of type II restriction endonucleases that must bind two DNA recognition sequences to cleave DNA. The *naeI*R gene, expressed from a Ptac promotor construct, was toxic to *Escherichia coli* in the absence of *Nae*I-sequence specific methylases. The *naeI*R gene was mutagenized with *N*-methyl-*N'*-nitrosoguanidine; four classes of *Nae*I variants were isolated in the absence of protecting methylase activity. Class I variants (T60I, 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, G245E) displayed significantly attenuated cleavage and binding activities. Class IV variants (G197D, G214R/A219T, G236S, L241P, G245E, 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 *Nae*I: 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 residues of *Eco*RI, *Eco*RV, and *Bam*HI.

The *Nae*I restriction endonuclease, isolated from *Nocardia aerocolonigenes*, is a member of the unique type IIe class of endonucleases. This family includes *Bsp*MI, *Hpa*II, *Nae*I, *Nar*I, and *Sac*II (Conrad and Topal, 1989; Oller *et al.*, 1991) as well as *Eco*RII (Krüger *et al.*, 1988; Gabbara and Bhagwat, 1992). *Nae*I 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).

*Nae*I recognizes and cleaves the sequence GCC/GGC. Binding of the second DNA recognition sequence assembles an active form of *Nae*I homodimer. Dimer formation in the absence of two-site binding gives an inactive conformation of *Nae*I (Baxter and Topal, 1993). Although a significant amount of work has been done to understand the *Nae*I 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 *Eco*RI, *Eco*RV, and *Bam*HI, identification of cleavage-deficient variants that bind DNA have defined a Mg^{2+} binding motif. Such variants also provided critical information to help assign the amino acids involved in DNA recognition by *Eco*RI (reviewed by Heitman, 1992 and 1993).

Here we employ random mutagenesis with MNNG[1] to isolate cleavage-deficient variants of *Nae*I endonuclease. Bacteria were selected by their ability to survive in the presence of mutagenized *Nae*I but lacking protecting methylases. Analysis of cleavage and DNA binding ability of the resulting *Nae*I variants enabled the identification of four classes of *Nae*I variants with reduced cleavage activity.

EXPERIMENTAL PROCEDURES

Materials

Bacterial Strains—*Escherichia coli* strains CAA1 (*F⁻ e14⁻ (mcrA⁻) lacY1 or Δ(lac)⁶ SupE44 galK2 galT22 mcrA rfbD1 mcrBa hsd($r_k^-m_k^+$) M·MspI⁺*) was obtained from Ellen Guthrie (New England Biolabs); ER1992 (*F⁻ λ⁻ endA1 thi-1 supE44 mcr-67 Δ(lac)U169 Δ(mcr-hsd-mrr)114::1510 dinDA::MudI1734*) was obtained from Elisabeth Raleigh (New England Biolabs); WA802 (*lacY1 Δlac-6 glnV44(AS) galK2 galT22 λ⁻ rfbD1 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 *naeI*R 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 *Nae*I gene. This region contained several out-of-frame start sites. Plasmid pBR322 DNA was prepared by banding in CsCl (Sambrook *et al.*, 1989).

Oligodeoxyribonucleotides for Gel Mobility Shifts—Fragments were:

Specific fragment:

5'GGGTGCCGGCAGGG3'

3'CCCACGGCCGTCCC5'

Nonspecific fragment:

5'GCTGGTGGTGGGTGAATTCAGGGTGGCAGCT3'

3'CGACCACCACCCACTTAAGTCCCACCCGTCGA5',

Sequencing primers—Primers were CGATTACGAGATTGCAGGAGT, GAAATCCAGCCGCGGTAATC, GGAATTGTGAGCGGATAACA, AGATGACCAGACAGATGTGCC, and GGACACCCTTCATCATCTCGA.

All oligodeoxyribonucleotides were synthesized by machine using an Applied Biosystems 380A synthesizer.

Nucleotides, Chemicals, and Restriction Enzymes—[γ-^{32}P]dATP was purchased from DuPont-NEN. T4 polynucleotide kinase and *Bam*HI

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[1] 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; bp, base pair(s).

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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_{18} cartridges were from Waters.

Methods

MNNG Mutagenesis—Random mutations were generated *in vivo* with MNNG according to Miller (1972). 200 μl of MNNG (1 mg/ml) were added to 5 ml of log-phase growth CAA1 (Me^+) cells containing the *naeI*R-containing plasmid pCY786. After treatment, the cells were grown overnight at 37 °C in 5 ml of LB/ampicillin medium. Minipreparations of *naeI*R-plasmid DNA were transformed into either WA802 or ER1992 competent cells, which lack specific methylases to protect against *NaeI* cleavage. Cleavage defective *naeI*R-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 *naeI*R 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 minipreparations, putative *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 1 min, and the cell debris was pelleted by spinning for 15 min in a microcentrifuge. DNA was precipitated from the resulting supernatant by adding isopropyl alcohol to 50% and centrifuging an additional 15 min. The DNA pellet was resuspended in 100 μl of H_2O.

For larger-scale DNA preparations, *naeI*R mutants were grown overnight in 100 ml of LB/ampicillin medium. The DNA was isolated using Qiagen-tip 100 columns according to the manufacturer, except that the DNA was resuspended in 100 μl of H_2O 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 μg/ml), and grown to saturation. Cells from 2.5 ml of growth were pelleted and then resuspended in 1 ml of buffer (20 mM KPO_4, 0.1 mM EDTA, 1 mM β-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 Assays—A volume of cell-extract containing 3 μg (or serial dilutions) of total protein from mutant and wild type *NaeI*-containing cells was incubated with 200 ng to 1 μg of pBR322 at 37 °C for 1 h in a 20-μl reaction volume containing 50 mM β-mercaptoethanol, 200 mM NaCl, 100 mM $MgCl_2$, 100 mM Tris (pH 8.0), and bovine serum albumin (50 μg/ml). Reactions were stopped by incubating 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 nM of oligodeoxyribonucleotide was radioactively labeled by incubation with 0.02 mCi of [γP^{32}]dATP and 16 units of T4 Kinase, and 1 × kinase buffer in a 50-μl reaction volume for 30 min at 37 °C. The reaction was stopped by incubating at 65 °C for 10 min. Unincorporated label was removed with G-50 Sephadex spin columns. The recovered radiolabeled oligoribodeoxnucleotide was annealed to its complement by heating at 70 °C for 5 min in buffer and slowly cooling to 25 °C. Cell-free extracts (3 μg of total protein) from mutant and wild type *NaeI*-containing cells were incubated with 10 nM [γ-^{32}P]dATP labeled double-stranded probe for 10 min at 37 °C in a 10-μl reaction volume containing 100 mM Tris (pH 8.0), 50 mM β-mercaptoethanol, 200 mM NaCl, 20 mM $CaCl_2$, 100 ng/ml bovine serum albumin, 10% glycerol, 300 ng of poly(dI-dC). Samples were immediately loaded onto a 6% (37.5:1) polyacrylamide gel and run for 2 h at 200 V.

The *NaeI* mutants that bound to DNA were tested for specific *versus* nonspecific binding. Assay conditions were the same as the general binding assays except either 1 μM unlabeled sequence-specific competitor or 1 μM unlabeled nonspecific competitor were added to the reactions.

DNA Sequencing—Oligonucleotide primers for sequencing were designed based upon the *naeI*R gene sequence (Taron *et al.*, 1994). The primers were desalted using the Sep-Pak plus C_{18} cartridges. 40 nM primer was resuspended in 500 μl of H_2O, and NaCl was added to 0.7 M. The cartridges were rinsed with 5 ml of MeOH followed by 5 ml of H_2O. The primer was applied to cartridge, then washed with 2 ml of H_2O. The primer was eluted four times with 1 ml of 60% MeOH/H_2O. The fractions containing primer were pooled together, dried in a speed vac, and resuspended in 100 μl of H_2O.

DNA was sequenced at the UNC-CH Automated DNA sequencing facility on a model 373A DNA Sequencer (Applied Biosystems) using the *Taq* DyeDeoxy™ terminator cycle sequencing kit (Applied Biosystems).

RESULTS

Mutagenesis of the NaeI Restriction Endonuclease Gene—To analyze the domains of *NaeI* involved in endonuclease function, we isolated *NaeI* variants defective in DNA cleavage, DNA binding or both. *NaeI* is lethal to the host cell in the absence of protecting methylase activity. Random mutagenesis of *naeI*R by MNNG was used to select for defective variants.

To optimize conditions we determined an MNNG survival curve and the amount of *NaeI* mutagenesis at each MNNG dose used. We isolated plasmid DNA from cells treated with 10 and 50% survival doses of MNNG, transfected the DNA into cells lacking methylase, and randomly picked surviving colonies. CAA1 (*MspI*·me^+) was used as the host cell strain for mutagenesis of *naeI*R. *MspI* activity (mCCGG) is highly protective against cleavage by *NaeI* (McClelland and Nelson, 1985).

To avoid DNA rearrangements and deletions, minipreparations of *naeI*R-plasmid were prepared from each of the mutants, cleaved with restriction enzyme, and checked for appropriate fragment size by gel electrophoresis. Only those mutants showing expected fragment patterns were selected.

DNA Cleavage Assays—We compared the sequence-specific DNA cleavage activity of mutant and wild type *NaeI* protein. Serial dilutions of wild type and mutant cell-free extracts were incubated with pBR322, a plasmid DNA substrate containing four *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, *NaeI* variants were also analyzed for DNA binding using the gel mobility-shift assay (Fig. 2). This assay measures protein-DNA binding because *NaeI*·DNA complexes migrate slower than the free DNA during electrophoresis. Ca^{2+}, instead of Mg^{2+}, was used as the divalent cation in the gel mobility shifts because *NaeI* binds but does not cleave DNA in the presence of Ca^{2+} (Baxter and Topal, 1993). The DNA probe used in these assays was a 14-bp "specific" fragment containing the 6-bp *NaeI* cognate recognition site (GCCGGC). 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 *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 *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 *NaeI* recognition sequence did not. Therefore, the gel mobility shift depended on the presence of an *NaeI* recognition sequence, which implies that the

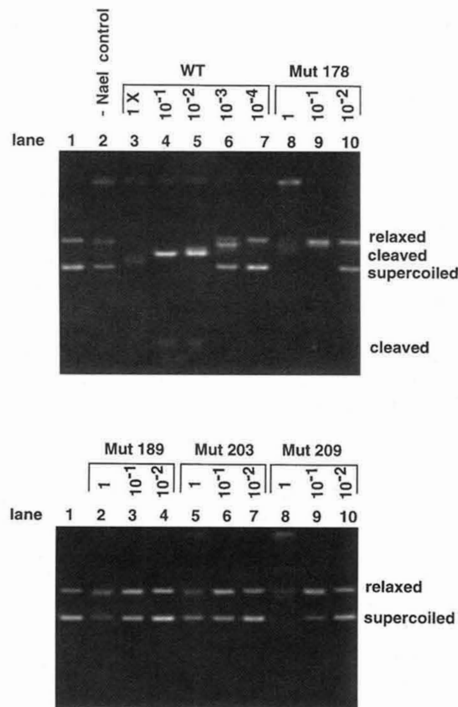

FIG. 1. **Cleavage activity of wild-type and mutant *NaeI* extracts.** Serially diluted (*1X* = 3 µg of total cell free protein) wild type (*lanes 3–7, upper panel*) and mutant *NaeI* cell-free extracts were assayed as described under "Experimental Procedures" using pBR322 DNA as the substrate. Untreated DNA is shown in *lane 1* of both panels. Separation of the reaction products by gel electrophoresis on 1% agarose for several example mutants and wild type is shown. Production of linear product is used as the indicator of *NaeI* activity. Note, for example, that dilution 10^{-2} for mutant 178 is approximately equivalent to dilution 10^{-4} of wild type. This indicates that this variant has approximately 1% the activity of wild type.

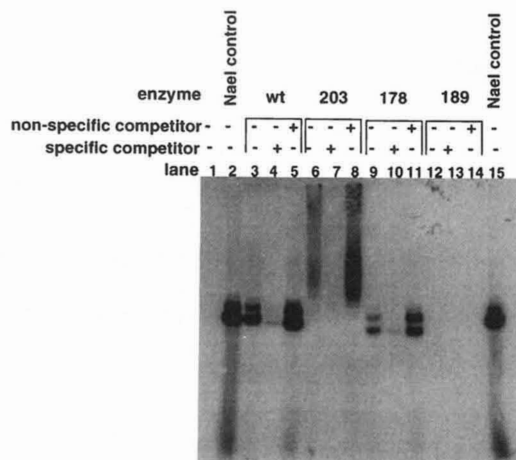

FIG. 2. **Gel mobility shift assays of wild-type and variant *NaeI*.** Wild type and variant *NaeI* cell free extracts were assayed for DNA binding as described under "Experimental Methods." Specificity of binding was tested by competition with specific and nonspecific competitor DNAs as indicated. Variants *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.

TABLE I
Characteristics of NaeI variants

Class	Variant	Amino acid change	Relative cleavage	Relative binding
			%	
	Wild type		100	100
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
IV	58, 59	G245R	<0.1	<1
	109	G214R/ A219T	<0.1	<1
	171	L241P	<0.1	<1
	172, 184	G197D	<0.1	<1
	173, 185	G236S	<0.1	<1
	189	G250E	<0.1	<1
	192	G270E	<0.1	<1
Temperature sensitive	TS8	A188T/ A222T	0.2	<1
	TS42	R200C	0.4	<1

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 survive 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

```
                               c*          c*
 41  DQLYDGQRTG RWNFDQLHKT EKTHMGTLVE INLHREFQFG
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  1  MTELPLQFAE PDDDLERVRA TLYSLDPDGD RTAGVLRDTL

                               c*          c*
 41  DQLYDGQRTG RWNFDQLHKT EKTHMGTLVE INLHREFQFG

                           c
 81  DGFETDYEIA GVQVDCKFSM SQGAWMLPPE SIGHICLVIW

                b               c              b
121  ASDQQCAWTA GLVKVIPQFL GTANRDLKRR LTPEGRAQVV

                                        t      b* t
161  KLWPDHGKLQ ENLLLHIPGD VRDQIFSAKS SRGNQHGQAR

                   b*    b*  t             b*
201  VNELFRRVHG RLIGRAVIAT VAQQDDFMKR VRGSGGARSI

         b    b*                      b*
241  LRPEGIIILG HQDNDPKVAN DLGLPVPRKG QVVAARVVPA

281  DEGDQRQTAE IQGRRWAVAV PGDPIVEAPV VPRKSAE
```

FIG. 3. **DNA sequence of *NaeI* showing the MNNG induced amino acid changes and possible structural elements.** Amino acids essential for catalysis are indicated by **c***. Amino acids that reduce binding are indicated by a **c**. Amino acids essential for binding are indicated by a **b***. Amino acids that effect binding and therefore also cleavage are indicated by a **b**. Amino acids involved in temperature sensitivity are indicated by **t**: amino acids Ala-188 and Ala-222 are part of temperature sensitive double mutant TS8. The two regions proposed to mediate DNA binding and cleavage are *underlined*. The DNA sequence was provided by Ellen Guthrie of New England Biolabs before publication and was confirmed by sequencing the gene for each of the variants as described under "Experimental Procedures."

determined. The amino acid changes fell close to or within the second putative DNA binding region (Fig. 3). We could detect low levels of cleavage by these variants *in vitro*. The cleavage *in vitro*, however, was not temperature sensitive. Binding of the temperature-sensitive variants to DNA could not be detected by gel mobility shift assay at either temperature. Thus, as one would expect for amino acid changes in this region of the protein, cleavage apparently was reduced by reduction in the affinity of the protein for DNA.

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 complexes than those produced by wild type protein. The increased binding phenotype has been seen with variants of other restriction enzymes (Wright *et al.*, 1989; Xu and Schildkraut, 1991) and is usually associated with creation of additional positive charges in the protein.

NaeI mutations D95N, G141D, G155D, and G245E bind DNA and display weak cleavage activity. *NaeI* mutations G131R and G131E bind DNA so weakly that the lack of detectable cleavage can be attributed to the low binding activity.

The *NaeI* amino acids apparently essential for catalysis have similarity to the developing signature for the endonuclease catalytic site (Fig. 4). The roles these residues play in *Eco*RI, *Eco*RV, and *Bam*HI have been deduced from their crystal structure (McClarin *et al.*, 1986; Kim *et al.*, 1990; Winkler *et al.*, 1993; Newman *et al.*, 1994) and from each protein's apparent inability to tolerate substitutions at these positions (King *et al.*, 1989; Xu and Schildkraut, 1991; Thielking *et al.*, 1992). The *Eco*RV catalytic center shares a similar structure with *Eco*RI (Selent *et al.*, 1992). Residues Asp-74 and Asp-90 in *Eco*RV, and Glu-111 and possibly Asp-91 in *Eco*RI are involved in chelating Mg^{2+} (Heitman, 1992). When Asp-90 chelates Mg^{2+} in *Eco*RV, it distorts the DNA and activates the catalytic center of the endonuclease (Thielking *et al.*, 1992). Lys-92 contacts the scissile phosphate in *Eco*RV; the Lys-113 residue in *Eco*RI may have a similar role (Selent *et al.*, 1992). *Bam*HI has a slight variation on this model. The Asp-94, Glu-111, and Glu-113 residues of *Bam*HI are conserved with those in *Eco*RI and *Eco*RV. However, it also requires Glu-77 for catalysis.

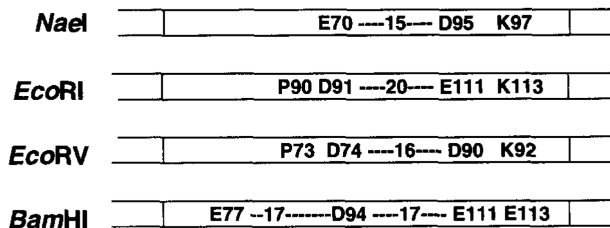

FIG. 4. **The catalytic-site signature for *Bam*HI, *Eco*RI, and *Eco*RV.** The emerging catalytic site signature is based on the crystal structure of *Eco*RI, *Eco*RV, and *Bam*HI (McClarin *et al.*, 1986; Kim *et al.*, 1990; Winkler *et al.*, 1993; Newman, 1994). *NaeI* may contain some elements of this signature: Glu-70 (*E70*) appears essential for catalysis and substitution of Asp-95 (*D95*) gives reduced cleavage but normal binding (see Table I); Lys-97 (*K97*) is indicated because it occurs at a position predicted to be important by the signature of the other enzymes.

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 *Bam*H1, *Eco*RI, and *Eco*RV 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.[2]

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 *Bam*HI, *Eco*RI, and *Eco*RV. 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

[2] K. J. and M. D. Topal, unpublished results.

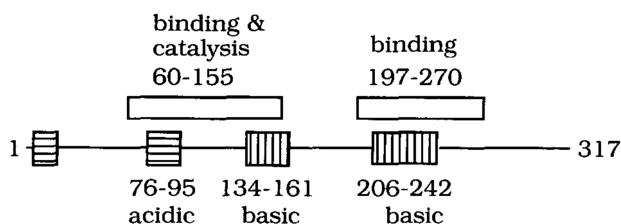

FIG. 5. **The proposed DNA substrate and effector binding sites shown relative to the acidic and basic regions of *Nae*I.** The acidic and basic regions were deduced from the DNA sequence shown in Fig. 3.

associated with a basic region located within the domain. These results imply that this domain is the substrate-binding site of *Nae*I. 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 *Nae*I. This result suggests that this domain may be the effector-binding site of *Nae*I (Yang and Topal, 1992; Baxter and Topal, 1993).

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