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*Nae*I Endonuclease Binding to pBR322 DNA Induces Looping†

Michael D. Topal,* Randy J. Thresher, Michael Conrad, and Jack Griffith

Lineberger Comprehensive Cancer Center, Departments of Pathology, Microbiology, and Biochemistry and Biophysics, and Curriculum in Genetics, University of North Carolina Medical School, Chapel Hill, North Carolina 27599-7295

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ABSTRACT: Previous work has demonstrated the existence of both resistant and cleavable *Nae*I sites. Cleavable sites introduced on exogenous DNA can act in trans to increase the catalysis of *Nae*I endonuclease cleavage at resistant sites without affecting the apparent binding affinity of the enzyme for the resistant site [Conrad, M., & Topal, M. D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9707-9711]. This activation suggests allosteric regulation of *Nae*I cleavage by distant cis- and trans-acting sites in DNAs containing both resistant and cleavable sites. Plasmid pBR322 contains four *Nae*I sites, at least one of which is resistant to cleavage. Electron microscopy is used here to demonstrate that *Nae*I endonuclease simultaneously binds to multiple recognition sites in pBR322 DNA to form loops with *Nae*I protein bound at the loop's base. The maximum number of loops formed with a common base suggests four binding sites per enzyme molecule. Looping was inhibited by addition of enzyme-saturating amounts of double-stranded oligonucleotide containing an *Nae*I site, whereas another double-strand oligonucleotide without the *Nae*I site had no effect. The number of loops seen was not above background when double-stranded M13 DNA, which contains only a single *Nae*I recognition site, was used as substrate.

Examples of one DNA site affecting an event at another distant site are known in transcription, replication, and recombination systems in both prokaryotes and eukaryotes (Majumdar & Adhya, 1984; Dynan & Tjian, 1985; Ptashne, 1986; Gellert & Nash, 1987; Moitoso de Vargas et al., 1988; Schleif, 1988). A number of protein-driven mechanisms have been proposed to account for communication between cis(intramolecular)-acting sites on DNA. These mechanisms include protein sliding, cooperative binding of protein to span the distance between sites, and looping-out of intervening DNA to bring the sites in contact with a common protein structure [see Ptashne (1986) for a review]. It is probable that looping-out of intervening DNA may be a general mechanism used for communication between distant cis-acting sites (Griffith et al., 1986; Kramer et al., 1987; Theveny et al., 1987; Amouyal et al., 1988; Chattoraj, 1988; Mukherjee et al., 1988).

In the *lac* repressor system, *lac* repressor monomers bind to sites on the same face of the helix; dimerization then causes the DNA between the sites to loop-out (Ptashne, 1986; Griffith et al., 1986). In the *Nae*I restriction endonuclease system, *Nae*I endonuclease cleavage of resistant sites has been shown to be activated in trans (intermolecular) by the addition of a separate cleavable site either on supercoiled plasmid DNAs or on small DNA fragments (Conrad & Topal, 1989). Trans-activation strongly suggests that *Nae*I endonuclease might be subject to cis-activation by DNA looping when cleavable and resistant sites reside on the same DNA molecule. This looping should be visible with the aid of an electron microscope.

The activation of *Nae*I endonuclease catalysis of DNA cleavage, according to the kinetic models of Monod et al. (1965) and Koshland et al. (1966), suggests an allosteric change in the protein upon activator binding to perhaps correctly position the catalytic site. Thus, *Nae*I endonuclease regulation by DNA provides a paradigm for regulation of enzyme activity by allosteric modification of the enzyme by trans- and cis-acting sites. In this study, electron microscopy was used to demonstrate that in a DNA containing both cleavable and resistant *Nae*I recognition sites, *Nae*I protein induces the formation of a high frequency of protein–DNA structures in which DNA is held stably in loops by *Nae*I protein bound at the loop's base. In contrast, DNAs containing a single recognition site exhibited very low levels of looping, and looping within the multiple-site DNA was reduced to background by competition for enzyme binding sites by short duplexes containing the cognate site.

Materials and Methods

Materials. *Nae*I, *Nde*I, and *Pvu*II endonucleases and bacteriophage T4 DNA polymerase were purchased from New England Biolabs (Beverly, MA). Biotinylated dCTP was

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* Address correspondence to this author at the Lineberger Comprehensive Cancer Center, University of North Carolina Medical School, Chapel Hill, NC 27599-7295.

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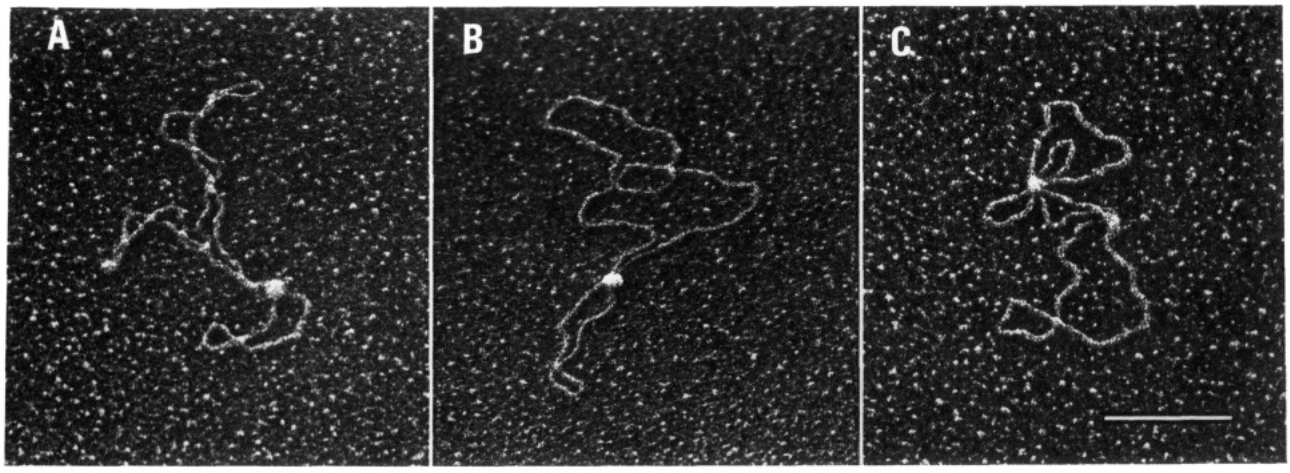

FIGURE 1: Visualization of *Nae*I endonuclease bound to pBR322 DNA. *Nae*I (New England Biolabs) is shown bound to supercoiled (A) and relaxed (B and C) DNA, as visualized by electron microscopy. Bar equals 0.1 μm.

obtained from ENZO Biochemicals (New York, NY). Unlabeled dCTPs, streptavidin, diethylaminoethyl (DEAE)-Sephadex, and other chemicals were purchased from Sigma Chemical Corp. (St. Louis, MO).

Protein–DNA Interactions. One microgram of pBR322 DNA was incubated with 4 units (where 1 unit is the amount of enzyme required to cleave 1 μg of Adeno-2 DNA in 1 h at 37 °C) of either commercial *Nae*I (~50% pure) or *Nae*I further purified by DEAE-Sephadex chromatography to >95% purity as judged by SDS–polyacrylamide gel electrophoresis and silver staining (M. Conrad and M. D. Topal, unpublished results). The 25-μL reactions were prepared to contain 10 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 20 mM NaCl, and 5 mM β-mercaptoethanol; reactions were incubated for 10 min at 37 °C. To limit the amount of cleavage, yet retain protein binding (Fox, 1988), $MgCl_2$ was replaced with 2.5 mM $CaCl_2$ in some reactions.

No *Nae*I methylase activity could be detected in the purified *Nae*I endonuclease preparation as judged by the ability of *Nae*I endonuclease to cleave M13 dsDNA in the presence of activator. The DNA had first been incubated with purified *Nae*I endonuclease plus *S*-adenosylmethionine in the absence of activator; initial incubation of the DNA with *Hpa*II methylase replacing *Nae*I endonuclease completely inhibited activated cleavage.

Avidin-Labeled DNA. DNA labeled at a unique end was generated by incubating *Nde*I-linearized pBR322 DNA with bacteriophage T4 DNA polymerase (1.4 units/μg of DNA) in 33 mM Tris–acetate (pH 7.9), potassium acetate (66 mM), magnesium acetate (10 mM), DTT (0.5 mM), and 100 μg of bovine serum albumin per milliliter at 37 °C for 30 s to briefly digest the 3′ ends. To this were added dNTPs (including biotinylated dCTP) to a final concentration of 100 μM each, and incubation was continued for 2 min. After the reaction was stopped by adding EDTA to 50 mM, the biotinylated DNA was precipitated in ethanol, cut with *Pvu*II endonuclease, phenol-extracted, and ethanol-precipitated again. Streptavidin (1 μg/mL) was added directly to the reactions for 30 s at 37 °C just prior to fixation.

Electron Microscopy. Following the 10-min reactions, DNA–protein complexes were fixed by the addition of glutaraldehyde to 0.6% for 5 min at 20 °C. The fixed samples were diluted 50–100-fold in 10 mM HEPES (pH 7.6) and directly adsorbed to thin carbon films, washed, dehydrated in ethanol, air-dried, and rotary-shadowcast as previously described (Griffith & Christiansen, 1978). Unfixed samples were rapidly frozen and freeze-dried by briefly adsorbing the samples to thin carbon films. Excess liquid was blotted away, and the grids were immediately plunged into liquid ethane chilled with liquid nitrogen. The frozen samples were transferred into a Wiltek-modified Balzers 300 freeze–etch machine and freeze-dried for 2 h at −85 °C. These were rotary-shadowcast with tantalum in a vacuum of 10^{-7} torr. Micrographs were taken with a Phillips EM400 TLG electron microscope operated at 40 kV. Protein binding sites were determined by measurements taken directly from micrographs using a Summagraphics digitizer coupled to an IBM-AT computer.

Results and Discussion

The DNA of pBR322 contains four *Nae*I cognate restriction sites located at nucleotide positions 401, 769, 929, and 1283 (Sutcliffe, 1979; Peden, 1983). All of these sites are cleaved in pBR322 DNA, albeit with different efficiencies, by *Nae*I restriction endonuclease under appropriate conditions (A. Oller and M. D. Topal, unpublished results). One site (at nucleotide 401), however, is resistant to cleavage in the absence of the other three (Conrad & Topal, 1989).

Samples containing *Nae*I protein and DNA substrates were prepared for EM by using Mg^{2+} either with incubation times too short to observe significant cleavage or with Ca^{2+} in place of Mg^{2+} to allow protein binding but eliminate cleavage (Fox, 1988; Frediani and Topal, unpublished results). Either we used *Nae*I endonuclease directly from the supplier (~50% pure) or we additionally purified it by DEAE-Sephadex chromatography. Enzyme purity was greater than 95% as judged by silver staining of column fractions analyzed by SDS–polyacrylamide gel electrophoresis (M. Conrad and M. D. Topal, unpublished results). DNA–protein loops were seen in the presence of either cation. However, a significant amount of cleavage occurred in the presence of Mg^{2+}, so samples containing Ca^{2+} were used to obtain statistics about the occurrence of loops induced by *Nae*I. The relative amount of looping was similar between templates using either cation.

About 45 ± 1% of the pBR322 DNA molecules (n = 554) contained loop(s) with protein bound at the base after 10 min of incubation using purified *Nae*I protein (Table I). Commercial-grade *Nae*I protein was found to act similarly by inducing loops in supercoiled plasmid molecules (Figure 1A), in nicked molecules (Figure 1B,C), and in cleaved molecules (not shown). The looping and cleaving activities of *Nae*I endonuclease were found to copurify (results not shown).

To demonstrate that *Nae*I recognition sites were involved in loop formation, pBR322 DNA was linearized and specifically tagged at one end (Figure 2). In this DNA, *Nae*I

Table I: Elimination of DNA Loops By Use of either DNA with Only One *Nae*I Site, Competitor DNA, or Enzyme That Lacks an Activator Binding Site

DNA	enzyme	competitor DNA[a]	% loops
M13	*Nae*I		18 (32/178)
pBR322	*Nae*I		45 ± 1 (159/352, 90/202)[b]
pBR322	*Nae*I	no *Nae*I site	46 (107/234)
pBR322	*Nae*I	*Nae*I site	12 (35/282)
pBR322	*Fsp*I		3 (1/38)

[a] Either a 21 base pair oligonucleotide duplex (6.2 μM) without an *Nae*I site (no *Nae*I site) or a 35 base pair oligonucleotide duplex (3 μM) that contains a single cleavable *Nae*I site (*Nae*I site) was added to the binding reaction so that the molar ratio of oligonucleotide duplex to pBR322 DNA (5.2 nM) was 300:1 and 144:1, respectively, in terms of *Nae*I sites. [b] The results from two independent experiments.

protein was found to induce the formation of single, double, and triple loops of DNA (Figure 3A–C), which suggests allosteric protein–DNA interactions that may be responsible for the cis- or trans-activation of *Nae*I endonuclease activity (Conrad & Topal, 1989).

The size of *Nae*I protein bound to any common base of loops was similar to that of the bound streptavidin (67 kDa). This similarity is consistent with the size of the active enzyme measured by gel permeation chromatography (~70 kDa; M. D. Topal and M. Conrad, unpublished results). While it is difficult to estimate protein masses in the multiloop structures, the complexes do not appear to be much larger than when *Nae*I protein was bound to single sites (Figure 3A) as determined by position mapping. The maximum number of loops with a common base per circular molecule was four, and the maximum number per linear molecule was three. These results suggest four binding sites per enzyme molecule.

Intermolecular DNA trans interactions were found at about 1% the frequency of intramolecular cis interactions (Figure 3E). The number of loops indicated four sites bound by the enzyme. The rarity of trans interactions between pBR322 DNAs, as seen by EM, probably reflects the relative effective concentrations of intra- and intermolecular sites under the conditions employed here.

The DNA sites involved in the protein-induced loops were mapped on streptavidin-labeled templates from digitized tracings of electron micrographs. A plot of the distribution of purified *Nae*I protein binding at different sites on pBR322 DNA (four sites) revealed a background of binding throughout the DNA molecule. Therefore, a more stringent selection criterion required mapping the binding on molecules with multiple loops having a common base. Such selection reduced the inclusion of loops that results from a chance cross-over rather than protein binding. This selection method consistently identified *Nae*I cognate sites in protein-mediated loop formation between experiments (Figure 4). The *Nae*I cognate site at nucleotide 401 of pBR322 DNA always gave the strongest binding signal. Since the 401 site is resistant to cleavage in the absence of an activating sequence (Conrad & Topal, 1989), it is reasonable that this site should be commonly detected in loop formations. This is especially true when cleavage, but not protein binding, is impaired by the substitution of Ca^{2+} for Mg^{2+}. Other protein binding sites were detected outside of the *Nae*I binding sites. We do not know the significance of this binding; it could represent enzyme–DNA binding intermediates trapped in the process of scanning through the DNA for correct binding sites.

The induction of DNA loops by *Nae*I endonuclease appears to depend upon specific interactions between a DNA molecule with multiple binding sequences and a restriction enzyme with multiple binding sites. To test the requirement for multiple

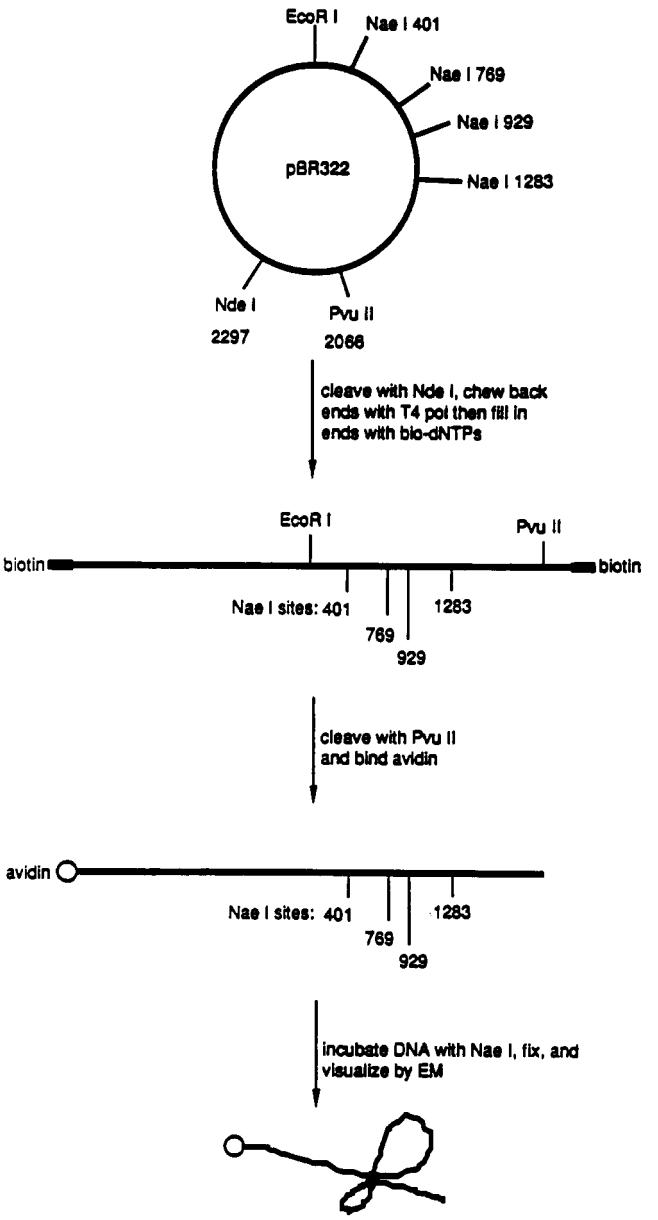

FIGURE 2: Production of linear pBR322 DNA molecules end-tagged with avidin for EM mapping of protein binding sites. Uniquely end-labeled DNAs were generated by incubating pBR322 DNA (cleaved with *Nde*I endonuclease) with bacteriophage T4 DNA pol in the absence of dNTPs for 30 s. Precursors (including biotin–dCTP) were then added to resynthesize the 3′ ends. Subcutting with *Pvu*II yielded long pBR322 DNA fragments with one end labeled for the orientation of molecules as visualized by EM after incubation with avidin.

binding sequences, the interaction of *Nae*I endonuclease with M13 double-strand DNA, which contains only a single *Nae*I site, was studied by EM. Only 18% of M13 DNA molecules incubated with purified *Nae*I endonuclease had protein-induced loops as compared with 45 ± 1% of pBR322 DNA, which contains four *Nae*I sites (Table I). In addition, competition for enzyme sites by a synthetic oligonucleotide duplex containing a single *Nae*I site reduced *Nae*I-induced looping of pBR322 DNA to just 12%. In contrast, competition using a synthetic oligonucleotide duplex without the *Nae*I site had no effect. Protein-based DNA loops were also seen when pBR322 DNA was used and samples were prepared for EM without fixation by rapid freezing and freeze-drying (Figure 3D), indicating that the loops were not an artifact of chemical fixation. These results demonstrate the specific involvement

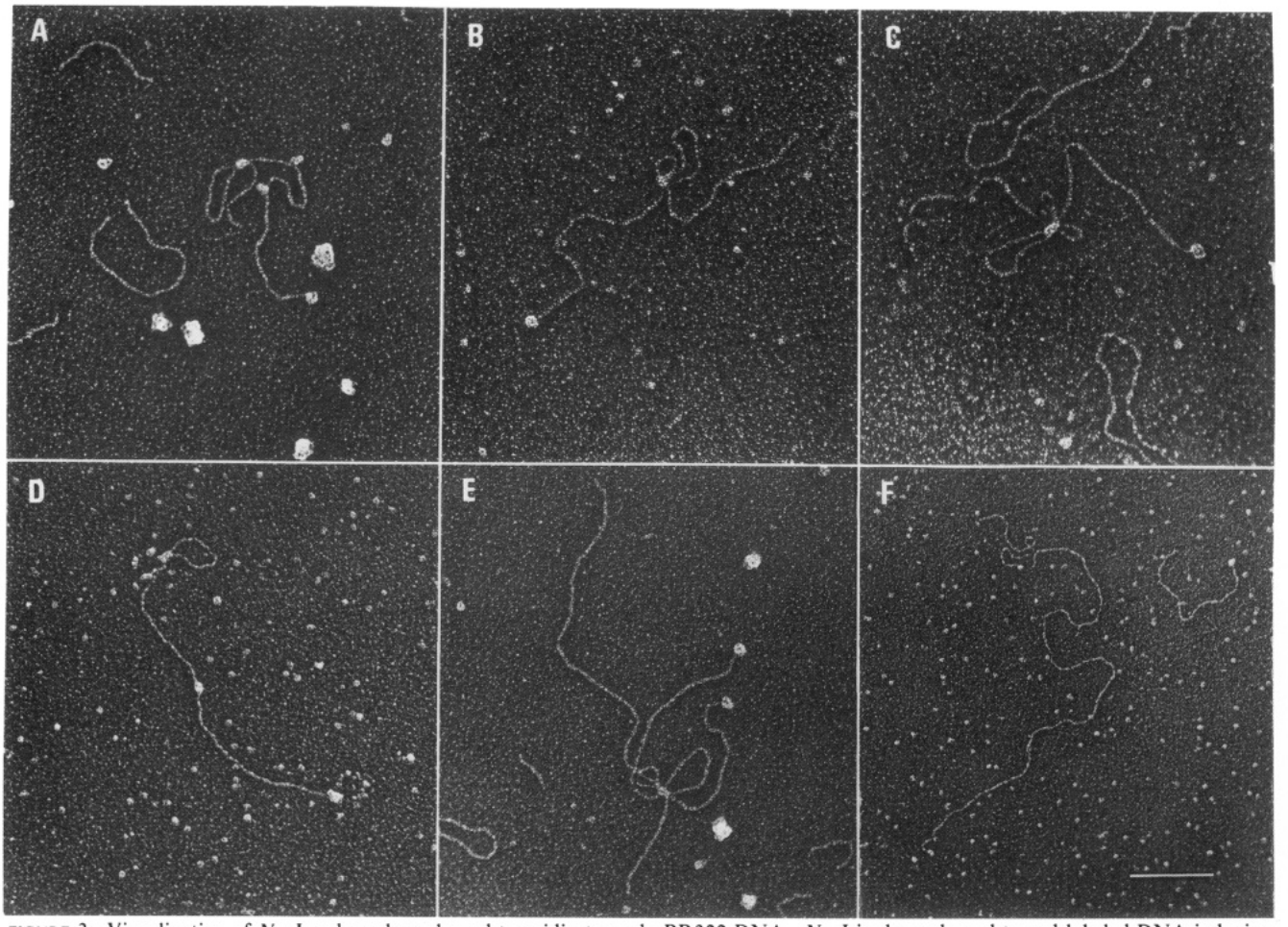

FIGURE 3: Visualization of *Nae*I endonuclease bound to avidin-tagged pBR322 DNA. *Nae*I is shown bound to end-labeled DNA inducing the formation of single- (A), double- (B), and triple-loop (C) structures. *Nae*I is also shown bound to a cleaved DNA molecule where the cleavage site participates in loop formation (D). Occasionally, trans interactions between two DNA molecules could be seen (E). *Fsp*I is shown bound to the same DNA (F). Reaction conditions and EM preparation were as described under Materials and Methods. Bar equals 0.1 μM except for (F) where it equals 0.14 μM.

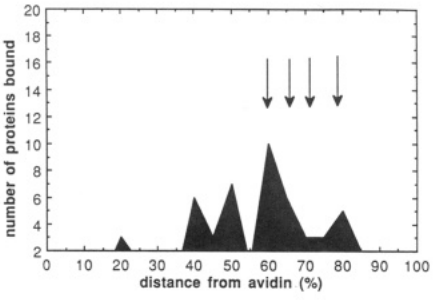

FIGURE 4: Frequency distribution of protein binding locations involved in DNA looping. The locations were determined by digitized measurements from the avidin-labeled end of *Nde*I-linearized pBR322 DNA (see Materials and Methods). The four *Nae*I recognition sites are shown (arrows) as determined from the pBR322 DNA sequence (Sutcliffe, 1979; Peden, 1983). The outlying peaks varied from experiment to experiment and probably represent trapped nonspecific interactions (see text).

of *Nae*I sites in the majority of loops induced by *Nae*I endonuclease binding.

Evidence that DNA loop induction by *Nae*I endonuclease is related to the novel regulatory properties of this enzyme comes from another type II restriction enzyme, *Fsp*I. Like *Nae*I protein, *Fsp*I has four recognition sequences in pBR322 DNA. *Fsp*I, however, apparently does not exhibit the regulatory properties observed with *Nae*I protein (Oller et al., 1991). Incubation of *Fsp*I protein with avidin-labeled pBR322 DNA under similar conditions as *Nae*I endonuclease (suitable for *Fsp*I activity) showed mainly linear molecules with proteins bound (Figure 3F and Table I).

In this study, we have directly demonstrated for the first time the induction of loops by the binding of a type II restriction endonuclease, *Nae*I, to DNA that contains distant cis-acting recognition sites. Type I endonucleases *Eco*B and *Eco*K also induce loops in DNA (Bickle et al., 1978; Rosamond et al., 1979). Looping by these enzymes, however, is related to the requirement that they bind a recognition site and then track along the DNA to cleave randomly within certain limits of distance from the recognition site.

DNA loops were found bringing together cis-acting *Nae*I sites. This, coupled with the regulatory properties of *Nae*I seen in vitro (Conrad & Topal, 1989), provides precedent for DNA looping as a means for DNA to act as an allosteric effector of a DNA binding protein. It is also interesting to note the similarity between the ability of *Nae*I to juxtapose and cleave distant recognition sequences and some aspects of site-specific recombination and transposition.

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CORRECTION

Purification, Characterization, and Structure of Pseudobactin 589 A, a Siderophore from a Plant Growth Promoting *Pseudomonas*, by Magnus Persmark, Torbjörn Frejd, and Bo Mattiasson*, Volume 29, Number 31, August 7, 1990, pages 7348–7356.

Page 7350. In Figure 1A, the ordinate label should read $\epsilon \times 10^{-3}$ (M^{-1} cm^{-1}).