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DNA-directed oligomerization of the monomeric Ner repressor from the Mu-like bacteriophage D108

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We have purified the 8.6 kd *ner* gene product (a λ Cro-like protein which negatively regulates transcription from two divergent and overlapping promoters) from the Mu-like transposable bacteriophage D108. Chemical and enzymatic protection experiments show the D108 *ner*-operator to contain two perfect 11 bp (5′-CCG-TGAGCTAC-3′) inverted repeats separated by an 8 bp AT-rich region. Ner makes base-specific contacts in the major groove spanning the 11 bp repeats and also interacts with regions flanking these sites such that its operator comprises five turns of the DNA helix. Furthermore, gel filtration chromatography and dimethyl suberimidate crosslinking experiments indicate that D108 Ner (at concentrations exceeding 5 μM) is a monomer in solution, yet crosslinks as a dimer when bound to its operator site. As a small (73 amino acids) monomeric protein, Ner does not display strong homology with any known DNA-binding proteins. By virtue of the interactions with its operator it appears to bind DNA in a markedly different manner from other known prokaryotic repressors thus adding to the growing catalog of protein motifs used for specific binding to DNA.

Key words: DNA protection/DNA−protein interaction/ D108 Ner purification/protein crosslinking/transposable bacteriophage

Introduction

The specific interactions of proteins with nucleic acids is critical to gene regulation and development of virtually all biological systems. D108 is a temperate bacteriophage of *Escherichia coli* that propagates its 37 000 bp linear double-stranded DNA genome via transposition during its life cycle (Hull *et al.*, 1978). Heteroduplex analysis has shown D108 to be ~90% homologous at the DNA level to phage Mu (Gill *et al.*, 1981). However, differences such as the heteroimmunity between the two phages reflect the non-homology in their left-end regulatory genes *c* (repressor) and *ner* (Toussaint *et al.*, 1983; Mizuuchi *et al.*, 1986) which act at the level of transcription (van de Putte *et al.*, 1980) and mediate the choice between lytic and lysogenic development in addition to expression of the crucial early (transposase) operon. The two phages' regulatory regions are located between the repressor and early genes (Schumann *et al.*, 1979; Krause and Higgins, 1986; Levin and DuBow, 1987). Transcription directed by the early promoter (Pe) proceeds from left to right and overlaps with transcription initiated from one of the repressor promoters (Pc_2), which proceeds from right to left (Krause *et al.*, 1983; Goosen *et al.*, 1984; Levin and DuBow, 1989). *Ner* is the first gene encoded by the early operon and expressed during lytic development (Wijffelman *et al.*, 1974). The binding of Ner to the intercistronic region between Pe and Pc_2 (Tolias and DuBow, 1985, 1986) appears to negatively regulate transcription from both of these promoters (van de Putte *et al.*, 1980; van Meeteren and van de Putte, 1980; van Leerdam *et al.*, 1982; Goosen and van de Putte, 1986) in a functionally analogous manner to the Cro protein of phage λ (Johnson *et al.*, 1981).

D108 *ner* has previously been cloned and overexpressed under the control of the *lac* UV5 transcriptional promoter. Its 73 amino acids are ~50% homologous to the 75 amino acids comprising the Ner protein from bacteriophage Mu, which has recently been suggested to be monomeric in solution (Allet *et al.*, 1988; Kukolj *et al.*, 1989). In spite of this similarity, the *ner* operators to which these proteins bind and regulate are quite different in the two phages, and the Ner proteins confer pseudo-immunity *in vivo* only to their respective phages (Tolias and DuBow, 1985, 1986).

Many DNA-binding regulatory proteins exhibit stringent sequence specificity for certain sites along their DNA substrates, and this selectivity has been well characterized among prokaryotic proteins such as the cyclic AMP receptor protein (CRP) (McKay and Steitz, 1981), Trp R repressor (Otwinowski *et al.*, 1988), and bacteriophages λ and 434 Cro and cI repressors (W.F.Anderson *et al.*, 1981; J.E.Anderson *et al.*, 1987; Aggarwal *et al.*, 1988; Jordan and Pabo, 1988; Wolberger *et al.*, 1988). The structure of these proteins, as determined from crystallographic analysis, reveals a common α-helix−turn−α-helix motif which makes physical contact with key nucleotides within the major groove of the specific DNA substrate. Furthermore, each of these proteins mediate their high binding affinity as dimers, which associate to appropriate sites of dyad symmetry. In this paper, we describe the purification of the D108 Ner regulatory protein. We demonstrate that it makes base-specific contacts over two identical 11 bp sequences in its operator. However, the Ner-bound operator extends to include a full turn of the helix on each side of this bipartite region, as determined by DNase I and hydroxyl radical protection, even though these flanking regions share no common DNA sequence motifs. D108 Ner is demonstrated to be predominantly monomeric in solution and oligomerizes only upon association with its binding site. This atypical interaction with its operator, and the lack of homology with conserved helix−turn−helix regions (Pabo and Sauer, 1984; Dodd and Egan, 1987), suggest that Ner is a member of a different class of DNA-binding proteins.

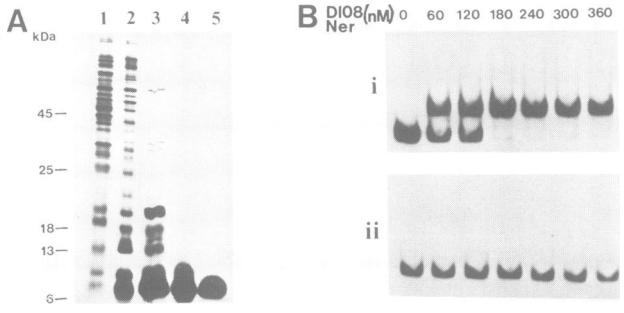

Fig. 1. Purification of D108 Ner monitored by the operator binding assay. **(A)** SDS−20% PAGE of samples containing 2 μg of total protein from successive steps during the purification. **Lane 1**, fraction I (crude protein extract from strain LF4331); **2**, fraction II (after phosphocellulose chromatography); **3**, fraction III (after DEAE−Sephadex chromatography); **4**, fraction IV (after Sephadex G-75 gel filtration); **5**, fraction V (after heparin−Sepharose chromatography). The mol. wt markers, shown in decreasing mol. wt, are ovalbumin, α-chymotrypsinogen, β-lactoglobulin, cytochrome *c* and bovine trypsin inhibitor. **(B)** Operator binding assay was performed as described (Tolias and DuBow, 1985; Kukolj *et al.*, 1989) except that the binding reactions did not contain any sonicated calf thymus DNA. **(i)** Displays the effect of increasing amounts (as indicated above each slot) of D108 Ner on the mobility of the 186 bp pUD78 *DraI*−*BglII* restriction fragment containing the *ner*-operator. **(ii)** Effect of increasing amounts (same as in Bi) of D108 Ner on the mobility of the non-specific 147 bp pBR322 *Hpa*II restriction fragment.

Results

D108 Ner purification

D108 Ner was purified as described in Materials and methods, and following heparin−Sepharose chromatography (fraction V), was found to be >99% pure (Figure 1A). The N-terminal amino acid sequence (H_2N-Met-His-Met-Asn- . . .) was determined with an Applied Biosystem Model 470A gas-phase sequencer using a Model 120A PTH-amino acid analyzer, and found to be colinear with that predicted from the DNA sequence (Tolias and DuBow, 1985). This purified protein binds to and retards the migration of a specific DNA substrate which contains the D108 *ner*-operator (Figure 1Bi). However, at the same concentrations, Ner fails to retard the electrophoretic migration of a non-specific DNA substrate lacking the D108 Ner-binding site (Figure 1Bii).

D108 Ner protects regions that include and flank the inverted repeats of the D108 ner-operator from DNase I cleavage

To determine the precise binding site(s) of D108 Ner, DNase I footprinting experiments were performed on both strands of a fragment containing the proposed operator. As the concentration of D108 Ner is increased, a region extending from nucleotides 997 to 1027 from the left end of D108 is protected from DNase I hydrolysis (Figure 2A). Similarly, on the complementary strand (Figure 2B), the association of D108 Ner to the DNA protects nucleotides 994−1024. This middle region encompasses the previously proposed (Tolias and DuBow, 1985) D108 *ner*-operator (nucleotides 996−1026) composed of two perfectly inverted 11 bp repeats (5′-CCGTGAGCTAC-3′) separated by an 8 bp spacer. However, the footprint extends on both sides of this central site. On the top strand (Figure 2A), these flanking sites respectively extend from nucleotides 989−995 and 1028−1036, while the additional sites protected on the complementary strand respectively encompass nucleotides 985−992 and 1026−1032, with two enhanced DNase I cleavages observed between positions 1024 and 1026 (Figure 2B). Unlike the perfectly conserved and inverted sequences that characterize the centrally protected domain, the two adjacent sites display no apparent sequence homology with each other.

Purines contacted by D108 Ner are restricted to the inverted repeat domain

To determine the sequence-specific DNA−Ner interactions, the ability of the protein to alter the susceptibility of purines to methylation by dimethylsulfate was examined (Craig and Nash, 1984). The methylation protection and enhancement patterns are indicated on both strands of the operator (Figure 3) at positions respectively marked '−' and '+'. It is interesting to note that the purines, which show altered methylation due to D108 Ner binding, are clustered within the central domain possessing the 11 bp inverted repeats. However, only certain purines within the 11 bp repeats appear to be altered; of these, the adenines are hyper-methylated, whereas the guanines are blocked from methylation in the presence of D108 Ner. A summary of the methylation protection results is presented in Figure 7. We also observed that the modified purines on one strand within one 11 bp site correspond to the identical purines on the complementary strand of the other 11 bp repeat. This pattern suggests that D108 Ner contacts the inverted repeats in a symmetrical manner.

D108 Ner discontinuously covers five helical turns of the DNA-sugar backbone

To obtain a higher-resolution interpretation of D108 Ner's interaction with its binding site, we used the hydroxyl radical footprinting technique described by Tullius and Dombroski (1986). The footprint on the top strand (Figure 4A) shows five discontinuous blocks of protected sequences, each covering 3−4 nucleotides. These blocks (labeled a−e) appear between nucleotides 985 and 1038 and are centered at 12- to 13-nucleotide intervals (approximately one helical turn) from each other. Nucleotides encompassed by brackets 'b' and 'd' each belong to one of the 11 bp inverted repeats, whereas those bracketed by 'c' fall within the spacer region. The outer boundaries of the D108 Ner-binding site on this strand are delineated by brackets 'a' and 'e' (nucleotides 985−987 and 1035−1038 respectively). Hydroxyl radical footprinting on the bottom strand (Figure 4B) also results in five discontinuous regions of protection (a′−e′) spanning bases 983−1035. This protection pattern is virtually identical to the one observed with the top strand; however, the protected nucleotides are not the complement of those observed on the top strand. This protection pattern (as summarized in Figure 7A) is staggered between the two strands; hence regions a and a′ are offset by 2 bp, as are regions b, c, d and e from b′, c′, d′ and e′ respectively.

Native D108 Ner monomers crosslink as dimers only when bound to their operator

The apparent mol. wt of native Ner repressor was determined by analytical Sephadex G-75 gel filtration chromatography. At an initial concentration of 16 μM, the elution profile of Ner (Figure 5B,C), relative to that of the mol. wt markers,

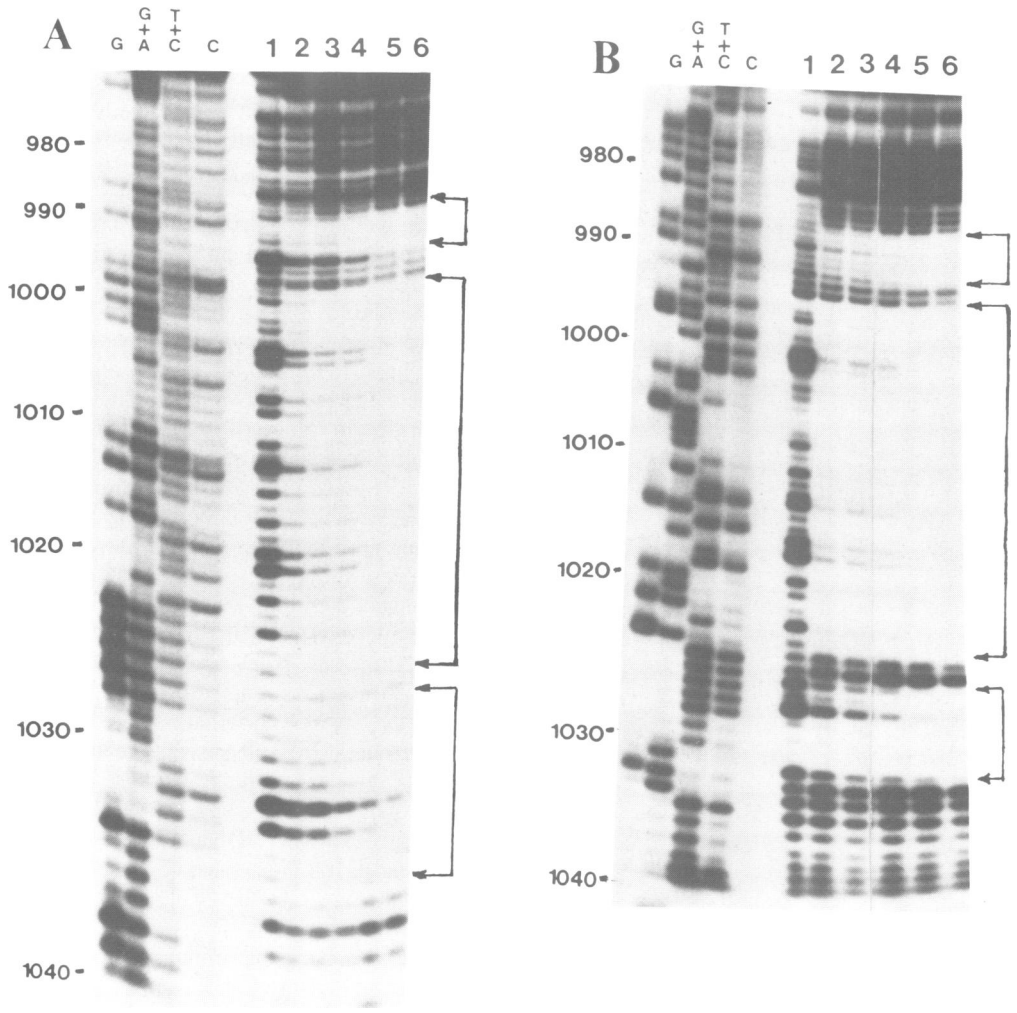

Fig. 2. DNase I footprints in the D108 early region. (**A**) Footprints on the 93 bp pUD78 *DraI*–*NdeI* restriction fragment labeled at the 3′ end of the *NdeI* site; this corresponds to the top strand in Figure 7A. G, G + A, T + C and C are the Maxam and Gilbert (1980) sequencing reactions; **lanes 1–6** represent fragments partially digested with DNase I in the presence of D108 Ner at 0, 60, 120, 480, 840 and 1200 nM. (**B**) Same as in (A) except that the sequencing and footprints are on the 93 bp pUD78 *DraI*–*NdeI* restriction fragment labeled at the 5′ end of the *NdeI* site; this corresponds to the bottom strand in Figure 7A. Vertically aligned numbers adjacent to the sequence indicate the positions of the bases from the conventionally denoted left end of the D108 genome as described by Mizuuchi *et al.* (1986). Brackets mark the regions protected by D108 Ner.

suggests that the native mol. wt of D108 Ner is ~8.6 kd. Moreover, this elution of Ner monomer also corresponds to the profile of specific DNA-binding activity (Figure 5A). Similar results were obtained when D108 Ner was subjected to gel filtration at concentrations as high as 200 μM (data not shown).

Support for the fact that D108 Ner displays monomeric behavior comes from crosslinking studies using the bi-functional agent dimethyl suberimidate (DMSI), which acts by forming covalent amide linkages between lysine residues (Davies and Stark, 1970). Its ability to delineate the interactions between subunits of a protein is demonstrated by DMSI-promoted inter-subunit crosslinking of *E.coli* SSB protein (Figure 6A), a homotetramer of 18.9-kd subunits (Weiner *et al.*, 1975). The bands visualized at 19, 38, 57 and 65 kd respectively represent the monomer, dimer, trimer and tetramer forms of the protein. Crosslinking of D108 Ner at a concentration of 6–10 μM followed by denaturing gel electrophoresis (Figure 6B) demonstrates that the vast majority (>98%) of the protein migrates as a single band of 8.6 kd, however, a trace amount of dimer (18 kd) species is detectable. The slight decrease in migration of Ner monomer on SDS gels following reaction with DMSI presumably reflects the intramolecular addition of up to six molecules of the reagent to the polypeptide. Crosslinking in the presence of non-specific DNA (at a 4:1 Ner monomer to DNA fragment molar ratio) yields only a slight increase (~3-fold) in the dimer species and a preponderance of Ner monomer. The low levels of Ner dimer observed at these high protein concentrations in the presence of a non-specific DNA fragment may be due to high localized concentrations of the basically charged Ner monomer associated with the nucleic acid (Carpenter and Harrington, 1972). However, a marked increase in Ner dimer (30-fold more than that observed in the absence of DNA) is seen when a *ner*-operator-containing fragment (at a 4:1 Ner monomer to DNA molar ratio) is present in the crosslinking reaction and may reflect the close proximity of Ner monomers and/or a conformational change in crosslinkable residues when Ner is bound to its operator.

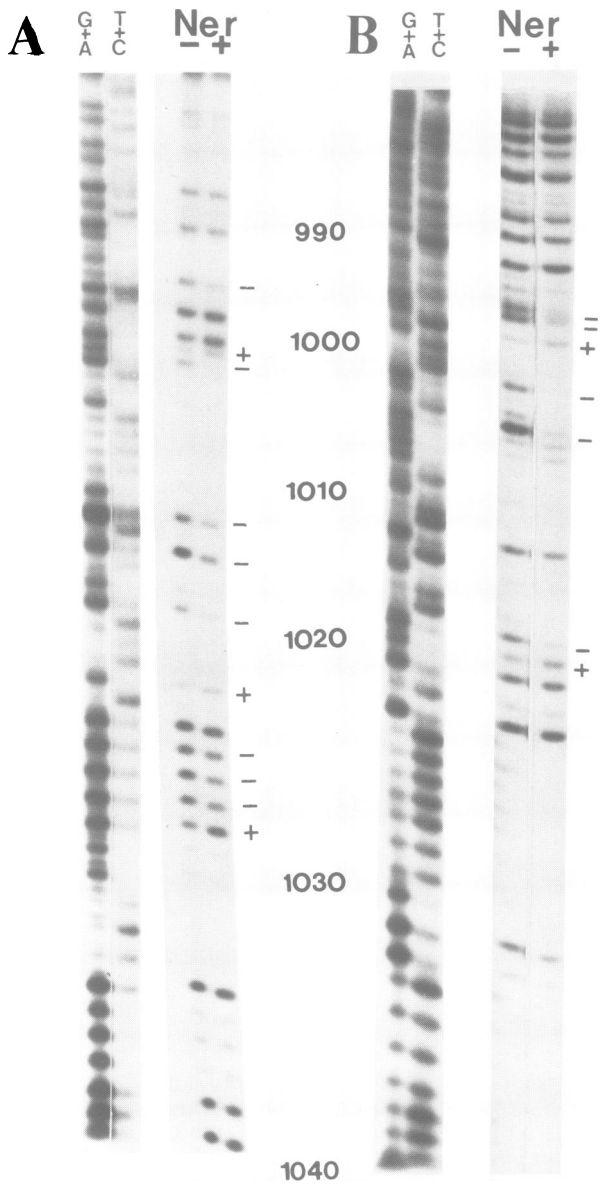

Fig. 3. Methylation protection of the operator's purine residues by D108 Ner. Each panel represents results with strands of the operator labeled as described in Figure 2. The array of '−' and '+' marks adjacent to the sequence denotes purines whose methylation is respectively diminished or enhanced in the presence of D108 Ner. (**A**) The top strand of the D108 *ner*-operator's G + A and T + C sequencing reactions, followed by its methylation pattern in the absence (−Ner) or presence (+Ner) of 500 nM D108 Ner. (**B**) Same as in (A) except the bottom strand of the D108 *ner*-operator is visualized.

Discussion

The Ner proteins from bacteriophages D108 and Mu act to regulate expression from the two overlapping and divergent promoters, Pe and Pc_2 (van Meeteren and van de Putte, 1980; van Leerdam *et al.*, 1982; Tolias and DuBow, 1985; Goosen and van de Putte, 1986). It has been demonstrated that the Pe and Pc_2 promoters in D108 are situated on either side of the *ner*-operator in a manner similar to Mu (Goosen *et al.*, 1984; Tolias and DuBow, 1986; Kukolj *et al.*, 1989; Levin and DuBow, 1989). The results presented here show that purified D108 Ner binds to an operator containing two perfectly inverted 11 bp (5′-CCGTGAGCTAC-3′) repeats which we found to be simultaneously occupied. These repeats have been shown to encompass the site of transcription initiation for Pe (position 1000) and Pc_2 (position 1020) (Levin and DuBow, 1989).

We have also presented evidence to show that the D108 *ner*-operator spans five turns of the DNA helix (Figure 7). Purine-specific contacting of the operator is limited to the area defined by the 11 bp repeats. Guanines were protected from methylation and adenines hypermethylated in a manner which indicates that the protein is positioned within the major groove at these locations in the operator. Stoichiometrically, our evidence suggests that only one D108 Ner monomer contacts each of the two 11 bp sites, since crosslinking of Ner in the presence of its operator was found to produce a significant quantity of a covalently dimerized form of the protein (Figure 6B). D108 Ner monomers must therefore associate with each of these repeats in an identical, and perhaps co-operative, manner allowing the protein to be positioned in major grooves on opposite sides of the helix.

Contacts with the DNA operator, as revealed by DNase I and hydroxyl radical protection, are not limited to the inverted repeats. Ner's association with the backbone at the outer extremities (denoted by a, a′ and e, e′) may be weak since the sugar−phosphate bond linking some of these deoxyriboses are still susceptible to DNase I hydrolysis. Within this large operator, there are only two specific (nucleotides 992−996 and 1024−1028) locations along the minor groove where DNase I accessibility is not impaired by bound D108 Ner. Each of these locations border the 5′ end of the 5′-CCGTGAGCTAC-3′ repeats, reiterating the possibility that D108 Ner is situated in the major groove, leaving the backside minor groove, at these locations, accessible to DNase I. The imprint of all deoxyribose sugars protected by Ner on a model of B DNA (Figure 7B) shows that pairs of localized sites (a−a′, b−b′, c−c′, d−d′ and e−e′) are separated by slightly more than one helical turn from a neighboring pair, such that these five regions lie on different faces of the DNA helix. Protection of the central minor groove in the spacer region (c−c′) may result from protein−protein interactions involved in dimerization over this area, or merely reflect their close proximity.

The detailed biochemical characterization of D108 Ner's interaction with its operator differs markedly from other prokaryotic repressors. It reveals the binding of two molecules of the relatively small D108 Ner protein (8.6 kd) to an operator spanning five turns of the DNA helix. The functionally similar cI repressor and Cro proteins in phages λ and 434 bind as dimers to sites of imperfect dyad symmetry which respectively encompass 17 and 14 bp (W.F.Anderson *et al.*, 1981; J.E.Anderson *et al.*, 1987; Aggarwal *et al.*, 1988; Jordan and Pabo, 1988; Wolberger *et al.*, 1988). A general feature determined from the structural studies with the lambdoid phage regulatory proteins is that these repressors commonly possess two α-helices separated by a β-turn. This motif has also been shown to exist in the *E.coli* cyclic AMP receptor and Trp R regulatory proteins (McKay and Steitz, 1981; Otwinowski *et al.*, 1988). The three-dimensional structure of D108 Ner is not known. However, its amino acid sequence does not display homology with the key amino acids that occur in the designated helix 2 and helix 3 binding domains, nor in the turn between them (Pabo and Sauer, 1984; Dodd and Egan, 1987).

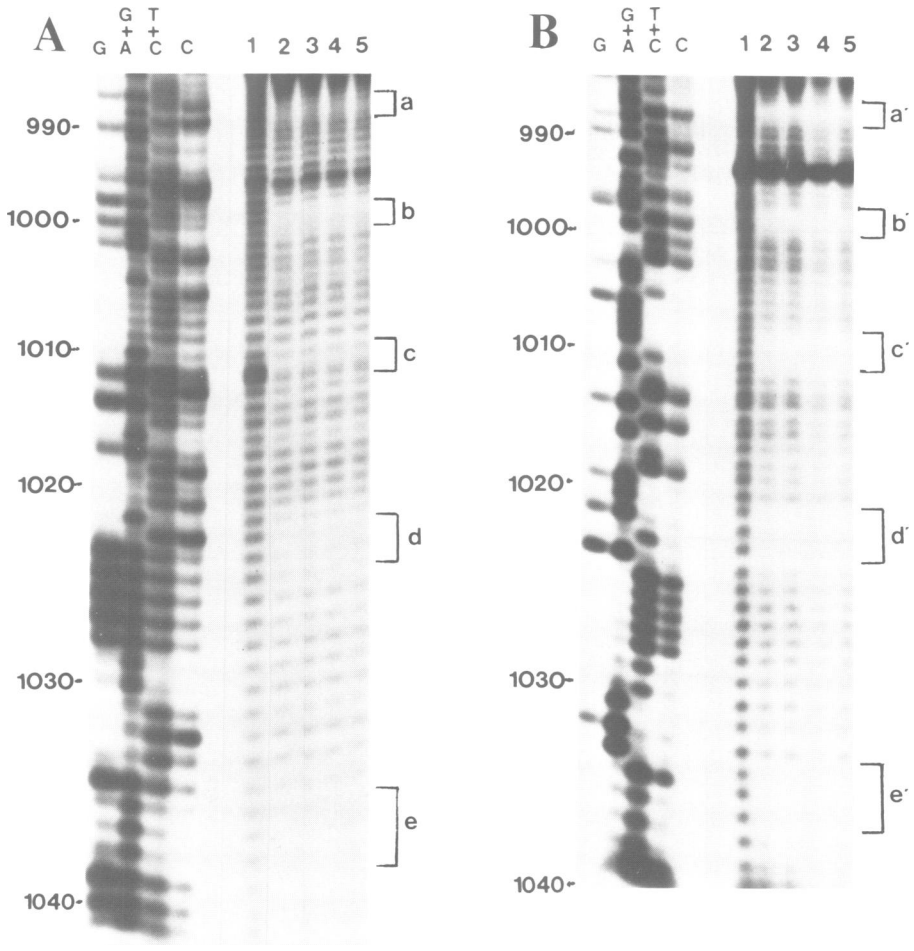

Fig. 4. Operator protection from hydroxyl radical cleavage by D108 Ner. Each panel represents results with the strands of the operator uniquely labeled as described in Figure 2. G, G + A, T + C and C are the sequencing reactions. **Lanes 1–5** represent the products of hydroxyl radical cleavage in the presence of D108 Ner at 0, 150, 500, 900, 1000 and 1500 nM. (**A**) On the top strand five regions of protection are denoted by the brackets a–e. (**B**) Same as in (A) except that the bottom strand's footprinted regions are marked by brackets a′–e′.

The symmetry displayed by an operator allows multiple subunits of the same protein to bind to this DNA region and form a thermodynamically stable complex. For most of the DNA-binding proteins mentioned above, their ΔGs of multimerization are such that they appropriately oligomerize without being bound to DNA. Thus, the energy of binding a multimer to a DNA site is much less than would be necessary if individual monomers associated independently (Schleiff, 1988). D108 Ner, at concentrations as high as 10^{-5}M (which is many orders of magnitude higher than the concentrations required for the oligomerization of other repressors), displays minor intermolecular interactions with itself and is predominantly monomeric in solution. This essentially monomeric behavior of Ner in solution has recently been observed with the eukaryotic Sp1 transcription factor (R.Tjian, personal communication). The bound D108 Ner–DNA complex may therefore possess minimal protein–protein interaction energy, and interactions with the operator must compensate for this discrepancy. Hence, it is not surprising that Ner displays extensive contacts with its operator.

How the 73 amino acid D108 Ner protein contacts this large region at numerous locations is not yet known. One possibility is that the DNA does not assume the B conformation but bends around the bound proteins to accommodate the necessary interactions such as are postulated to occur with the small histone-like proteins Hu and IHF (Drlica and Rouviere-Yaniv, 1987). Phage λ *att*P DNA has also been postulated to wrap around the Xis protein, which binds to a specific 40 bp region containing two 13 bp direct repeats separated by a 7 bp spacer (Yin *et al.*, 1985). Similar to D108 Ner, the 72 amino acid λ Xis is a highly basic (25% lysine and arginine content) protein that is monomeric in solution (Abremski and Gottesman, 1982); however, no homologous alignment with respect to specific amino acid positions is observed between any segments of the two proteins. Additionally, the Mnt repressor of bacteriophage P22 also contacts both sides of the DNA through the major groove by an unknown mechanism (Vershon *et al.*, 1987). However, Mnt binds DNA as a tetramer (Vershon *et al.*, 1985), and also displays no significant homology to D108 Ner at the amino acid level.

We have shown that D108 Ner interacts with its operator in a unique manner; that it displays no homology with previously characterized DNA-binding proteins suggests that it may belong to a new class of such proteins whose structure and unique mode of interaction with DNA remains to be elucidated. Our results add to the growing evidence that a variety of structural motifs can be used by proteins to bind specifically to DNA.

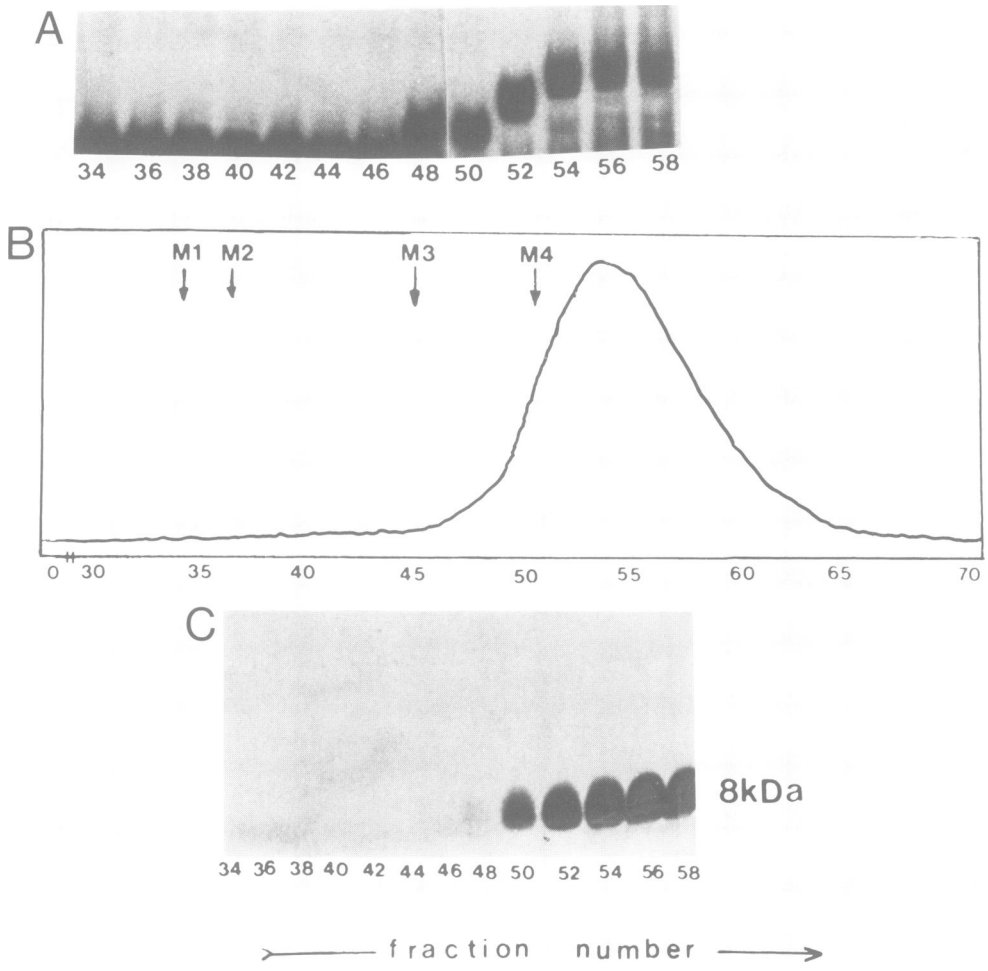

Fig. 5. Sephadex G-75 filtration of D108 Ner. (**A**) Measurement of specific DNA-binding in fractions 34–58 eluted from the column using the D108 Ner-specific DNA substrate in the operator binding assay (as described in the legend to Figure 1B). (**B**) The A_{280} elution profile of each fraction with the vertical arrows denoting the elution profile of the markers; M1, blue dextran 2000; M2, bovine serum albumin (68 kd); M3, α-chymotrypsinogen (25 kd); M4, cytochrome *c* (13 kd). (**C**) Silver-stained SDS–polyacrylamide gel of samples from fractions 34–58.

Materials and methods

Chromatography material, enzymes and reagents

Phosphocellulose (P-11, Whatman), DEAE–Sephadex A-50, Sephadex G-75 Superfine and heparin–Sepharose CL-6B (Pharmacia) were prepared according to the manufacturers' instructions. Restriction endonucleases were obtained from Pharmacia, *E.coli* polymerase I Klenow fragment and bacterial alkaline phosphatase were from BRL. Phage T4 polynucleotide kinase was from New England Biolabs and DNase I from Worthington. [α-^{32}P]dNTPs (3700 Ci/mmol) were purchased from ICN. [γ-^{32}P]ATP (5000 Ci/mmol) was obtained from Amersham and dimethylsulfate from Aldrich. Pierce was the supplier of DMSI.

DNA fragments and end labeling

DNA fragments containing the D108 *ner*-operator were isolated from the plasmid pUD78, which carries 1414 bp of the left end of the D108 genome (Tolias and DuBow, 1985). The D108 Ner-specific 186 bp pUD78 *Dra*I–*Bgl*II restriction fragment was labeled at the recessed 3′-*Bgl*II end with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I (Maniatis *et al.*, 1982) and the 147-bp *Hpa*II restriction fragments from pBR322 (labeled at the 3′ ends with [α-^{32}P]dCTP) was used as a non-specific substrate lacking the *ner*-operator.

The source of *ner*-operator DNA used in the protection experiments was the 93 bp *Dra*I–*Nde*I restriction fragment from pUD78. End-labeling the 3′ end of the fragment was achieved by incorporating [α–^{32}P]TTP at the *Nde*I site with DNA polymerase I Klenow fragment. The 5′-end-labeled fragment was obtained by hydrolyzing pUD78 with *Rsa*I and *Nde*I, removing the 5′ phosphates with bacterial alkaline phosphatase, labeling with [γ-^{32}P]ATP and T4 polynucleotide kinase followed by secondary digestion with *Dra*I. The labeled DNA fragments were subjected to electrophoresis on 5% polyacrylamide gels, located by autoradiography, and extracted by the 'crush and soak' procedure as previously described (Maxam and Gilbert, 1980).

Operator binding assay

Binding of Ner to ^{32}P-labeled fragments was assayed using a modification of the band competition assay as described previously (Tolias and DuBow, 1985; Kukolj *et al.*, 1989).

D108 Ner purification

Escherichia coli strain LF4331, containing plasmid pPT011 (Tolias and DuBow, 1985), expresses the D108 Ner protein under the control of the *lac*UV5 promoter and served as the source for its purification. All columns were equilibrated with 25 mM NaCl in buffer A (25 mM Tris–HCl, pH 7.5, 1 mM EDTA, 10 mM 2-mercaptoethanol, 5% v/v glycerol) prior to loading protein fractions. The activity of the D108 Ner protein was monitored using the operator binding assay (Kukolj *et al.*, 1989). Proteins were visualized by electrophoresis in 20% polyacrylamide/0.1% SDS gels (Laemmli, 1970) and stained with silver as described by Morrisey (1981). Protein concentrations were determined as described by Lowry *et al.* (1951). Eighteen liters of LB broth grown cells (supplemented with ampicillin, 40 μg/ml) were used to prepare crude extracts as described (Tolias and DuBow, 1985). A 30 ml volume of crude extract containing 1.11 g of protein was obtained (fraction I).

Fraction I was loaded onto a 2.5 cm × 10 cm phosphocellulose column, washed with 250 ml of 25 mM NaCl in buffer A, and then eluted with a 560 ml linear gradient of 25–1000 mM NaCl in the same buffer. Active fractions, which eluted over the range of 130–550 mM NaCl, were pooled and dialyzed extensively against 25 mM NaCl in buffer A (fraction II: 230 ml, 113 mg protein).

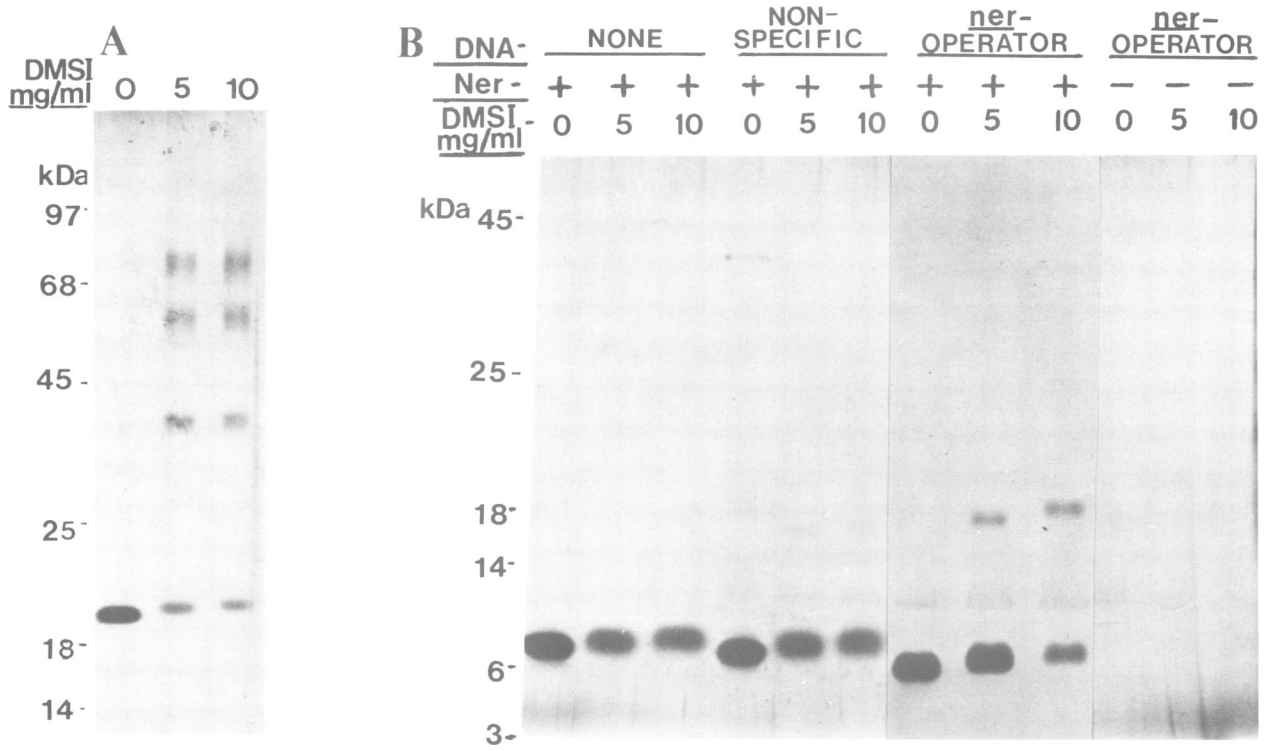

Fig. 6. SDS−PAGE of Ner crosslinked with dimethyl suberimidate (DMSI). (**A**) *Escherichia coli* single-stranded DNA binding protein (SSB, 18.9 kd) at a concentration of 5 μM was crosslinked in the presence of 0, 5 and 10 mg/ml DMSI. Markers in descending order are phosphorylase B, bovine serum albumin, ovalbumin, α-chymotrypsinogen, β-lactoglobulin and lysozyme. (**B**) D108 Ner (8.6 kd) at a concentration of 6−10 μM was crosslinked in the presence of 0, 5 and 10 mg/ml of DMSI in reactions containing either no DNA, a non-specific (217 bp pBR322 *Hpa*II) DNA restriction fragment or a *ner*-operator containing (186 bp pUD78 *Dra*I−*Bgl*II) restriction fragment. The migration of *ner*-operator DNA alone was shown to be unaffected by exposure to DMSI. Markers in descending order are ovalbumin, α-chymotrypsinogen, β-lactoglobulin, lysozyme, bovine trypsin inhibitor and insulin B chain.

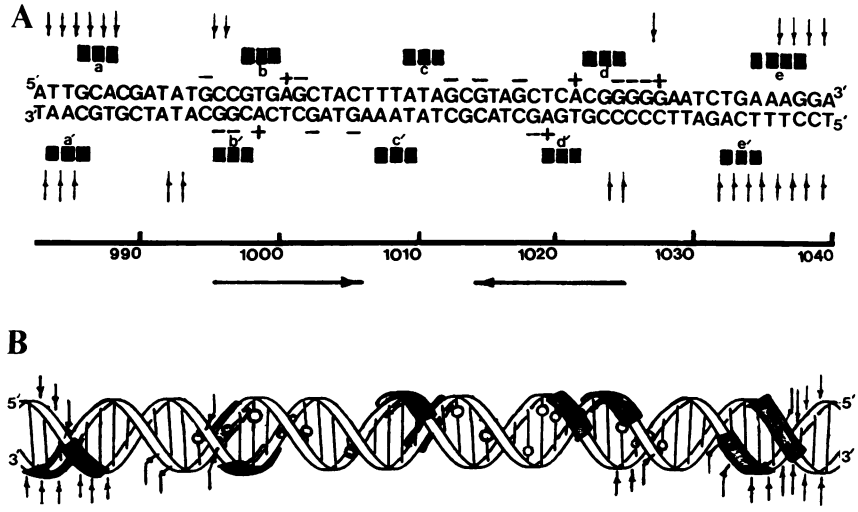

Fig. 7. DNA sequence and topology of D108 Ner interaction with its operator. (**A**) The intercistronic region of D108 spanning bp 982−1040 from the left end of the genome. The position of bonds that are accessible to DNase I cleavage in the presence of Ner are indicated by ↑. '−' or '+' overlining and underlining the sequence respectively represent the purines protected or enhanced in methylation upon D108 Ner binding. The closed squares, overlining and underlining the sequence, indicate positions of nucleotides whose deoxyribose backbones are protected from hydroxyl radical cleavage. The five blocks of protection on each strand (a−e and a′−e′) are as described in Figure 4. Horizontal arrows highlight the position of the 11-bp inverted repeats. (**B**) The results from the protection experiments are diagrammed on a B DNA model. ↑, positions of bonds cleaved by DNase I; ○, purines showing an altered susceptibility to methylation; shaded regions along the model represent nucleotides along the DNA backbone protected from hydroxyl radical cleavage by Ner.

Fraction II was loaded onto a DEAE−Sephadex column (2.5 cm × 10 cm). The column was washed with 25 mM NaCl in buffer A and the vast majority of the D108 Ner-specific DNA-binding activity was eluted in this buffer (fraction III: 480 ml, 25 mg protein).

Fraction III was concentrated by adsorption onto a phosphocellulose column (1 cm × 5 cm), washed with 25 ml of 25 mM NaCl in buffer A, and eluted with 500 mM NaCl in the same buffer. The D108 Ner-specific DNA-binding activity was pooled and loaded onto a Sephadex G-75 Superfine column (2 cm × 60 cm). The column was run with 200 ml of 25 mM NaCl in buffer A and the D108 Ner-specific DNA-binding activity was then pooled (fraction IV: 37.5 ml, 12 mg protein).

Fraction IV was loaded onto a heparin−Sepharose column (1 cm ×

5 cm). The column was washed with 25 ml of 25 mM NaCl in buffer A and then run with a 60 ml linear gradient of 25–1000 mM NaCl in the same buffer. Active fractions, which eluted at ~235 mM NaCl, were examined for purity by SDS–PAGE and fractions 19–25 contained a single 8.6 kd polypeptide. These fractions were then pooled and dialyzed against buffer B (25 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.5 mM dithiothreitol, 25 mM NaCl, 25% v/v glycerol; fraction V: 3.5 ml, 6.4 mg protein) and stored at −20°C. The *ner* operator-specific binding activity was stable for several months.

Protection experiments

DNase I protection (Galas and Schmitz, 1978) and dimethylsulfate protection experiments were performed as previously described (Craig and Nash, 1984; Kukolj *et al.*, 1989). Protection from hydroxyl radical cleavage was performed as described (Tullius and Dombroski, 1986), except that various amounts of D108 Ner (which was extensively dialyzed versus 25 mM Tris–HCl, pH 7.5, and 1 mM EDTA) were added to 1 pmol of end-labeled fragment in a binding buffer consisting of 25 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 50 mM NaCl, 3 μg/ml of sonicated calf thymus DNA and 100 μg/ml bovine serum albumin. The reaction products were subjected to electrophoresis with the DNA sequencing reactions as previously described (Maxam and Gilbert, 1980).

Gel filtration experiment

Gel filtration of the protein sample containing purified D108 Ner (1.6×10^{-5} M in 300 μl) was performed on a Sephadex G-75 Superfine column as described previously (Kukolj *et al.*, 1989).

Dimethyl suberimidate (DMSI) crosslinking with D108 Ner

This is a modification of the technique by Davies and Stark (1970). *Escherichia coli* single-stranded DNA-binding protein (SSB), used as a control, was purchased from USB. Between 60 and 100 pmol of D108 Ner and/or 15 pmol of specific (186 bp pUD78 *DraI–BglII ner*-operator containing restriction fragment) or non-specific (217 bp pBR322 *HpaII* restriction fragment) DNA were incubated in crosslinking buffer (80 mM triethanolamine–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1 mM dithioerythritol) for 10 min at 37°C in a 6.67 μl reaction volume to permit binding to DNA (not shown). The volume was brought up to 10 μl with varying amounts of DMSI in crosslinking buffer (pH 8.0) and incubated for 1 h at room temperature. The reaction was terminated by the addition of 10 μl of sample buffer (1% SDS, 4% 2-mercaptoethanol, 10% glycerol, 10 mM $NaPO_4$, pH 7.0, 0.02 mg/ml bromophenol blue) followed by heating at 90°C for 5 min. The products were subjected to electrophoresis on 20% polyacrylamide/0.1% SDS gels (Laemmli, 1970) and stained with silver (Morrissey, 1981).

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