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Purification and characterization of the Ner repressor of bacteriophage Mu

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The Ner protein of bacteriophage Mu acts as a λ cro-like negative regulator of the phage's early (transposase) operon. Using the band retardation assay to monitor *ner*-operator-specific DNA-binding activity, the 8 kDa Ner protein was purified to homogeneity. DNase I footprinting revealed that the purified protein bound and protected a specific DNA operator that contains two 12 bp sites with the consensus sequence 5'-ANPyTAPuCTAAGT-3', separated by a 6 bp spacer region. Moreover, regions corresponding to a turn of the DNA helix flanking these 12 bp repeats are also protected by Ner. Unlike the functionally similar λ cro protein, gel filtration experiments show the native molecular mass of Mu Ner to be approx. 8 kDa. These results, plus the pattern of DNase I protection, suggest that the protein may bind as a monomer to each of its specific DNA substrates.

Bacteriophage Mu; Protein, Ner; DNA-protein interaction

1. INTRODUCTION

Variations in gene expression are frequently mediated at the level of transcriptional initiation. This phenomenon has been studied using various bacteriophages as model systems. Mu is a temperate bacteriophage of *Escherichia coli* that propagates its DNA by undergoing up to 100 cycles of DNA transposition per hour during the lytic phase of its life cycle [1]. The choice between lytic and lysogenic development is directed, in part, by two phage-encoded repressor proteins, called repressor (c) and Ner, which act at the level of transcription [2]. The repressor protein acts to maintain the integrated proviral DNA in a repressed state, whereas the Ner protein is expressed during the phage's lytic mode of existence and acts to turn off the expression of repressor, thereby potentiating the early to late transition in the expression of viral genes [3–5]. The Ner protein begins to exert its regulatory effect approx. 4 min after the start of early gene expression [2,6] by binding to the intercistronic regions between the early genes (transcribed left to right [7]) and the repressor gene (transcribed right to left [8]). As a result of this binding [4], repressor is not transcribed and the early gene products (which include *ner* and products responsible for DNA transposition and lytic development) are expressed. However, since Ner can autoregulate its own synthesis, transcription from these early genes is turned down later on during the lytic cycle so that Mu phage can switch from early (DNA transposition) to late (virion morphogenesis) functions.

This report describes the purification of Mu Ner to homogeneity. It precisely defines (by DNase I footprinting) the Mu *ner* operator as a 12 bp consensus sequence present twice in inverted orienta-

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Abbreviations: bp, base pairs; kDa, kilodalton; Pu, purine; Py, pyrimidine; DEAE, diethylamino ethyl

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tion and separated by a 6 bp AT-rich sequence. Ner is also observed to protect an additional 7 bp, from DNase I cleavage, adjacent to both sides of this bipartite operator. Moreover, gel filtration experiments demonstrate Ner to chromatograph as a monomer in solution, suggesting that this repressor may be significantly different from other procaryotic repressors which bind as dimers to sites of dyad symmetry [9].

2. MATERIALS AND METHODS

E. coli strain LF 123, containing plasmid pUD88 [4], expresses the Mu Ner protein under the control of the *lac* UV5 promoter and served as the source for its purification. All purification steps, unless stated otherwise, were performed at 4°C using sterile buffers and baked glassware. The activity of the Mu Ner protein was monitored using the band retardation assay [10]. This assay is a modification of the technique of Strauss and Varshavsky [11] as described previously [12]. DNA was radioactively labelled with ^{32}P according to Tolias and DuBow [4]. Restriction fragments used for the band retardation assay include the 145 bp pUD88 *Eco*RI-*Hae*III or the 94 bp pUD88 *Dra*I-*Hin*dIII fragments containing the Mu Ner-binding site (Mu-specific DNA substrates), and the 147 bp pBR322 *Hpa*II restriction fragments (non-specific DNA substrates). Protein concentrations were determined as described by Lowry et al. [13]. Proteins were subjected to electrophoresis in 15% SDS-polyacrylamide gels [14] and stained with silver as described by Morrissey [15]. Crude extracts of strain LF123 were prepared as previously described [4]. From 10 l of LB broth grown cells, 50 ml of crude extract was obtained, containing 1.75 g of protein (fraction I).

2.1. *DEAE Sephadex chromatography*

Fraction I was added to a 280 ml slurry of DEAE-Sephadex previously equilibrated with 25 mM NaCl in buffer A [25 mM Tris-HCl (pH 7.5); 1 mM EDTA; 5% (v/v) glycerol; 10 mM 2-mercaptoethanol], gently mixed and filtered through a 4 cm diameter column. The column was washed with 25 mM NaCl in buffer A and the vast majority of the Mu Ner-specific DNA-binding activity was eluted in this buffer and pooled (fraction II; 500 ml, 800 mg protein).

2.2. *Phosphocellulose chromatography*

Fraction II was loaded onto a 50 ml phosphocellulose column (25 cm diameter) previously equilibrated with 25 mM NaCl in buffer A. The column was washed with 250 ml of the same buffer and then eluted with a 560 ml linear gradient of 25–1000 mM NaCl in buffer A. Active fractions, which eluted at approx. 330 mM NaCl, were pooled and dialysed against 25 mM NaCl in buffer A (fraction III; 136 ml, 90 mg protein).

2.3. *Gel filtration*

Fraction III was concentrated by adsorption onto a 5 ml phosphocellulose column (1 cm diameter), washed with 25 ml of 25 mM NaCl in buffer A, and eluted with 500 mM NaCl in buffer A. The Mu Ner-specific DNA-binding activity was pooled and loaded onto a 140 ml Sephadex G-75 superfine column (2 cm diameter) previously equilibrated with 10 mM sodium phosphate (pH 6.8) in buffer B (1 mM EDTA; 5% (v/v) glycerol; 10 mM 2-mercaptoethanol). The column was run with 200 ml of the same buffer and the Mu Ner-specific DNA-binding activity was then pooled (fraction IV; 15 ml, 15 mg).

2.4. *Hydroxyapatite chromatography*

Fraction IV was loaded onto a 5 ml hydroxyapatite column (1 cm diameter) previously equilibrated with 10 mM sodium phosphate (pH 6.8) in buffer B. The column was washed with 25 ml of the same buffer and then run with a 60 ml linear gradient of 10–100 mM sodium phosphate (pH 6.8) in buffer B. Active fractions, which eluted at approx. 65 mM sodium phosphate (pH 6.8), were examined for purity by SDS-polyacrylamide gel electrophoresis and fractions 43–50 were found to contain a single 8 kDa polypeptide. These fractions were then pooled and dialysed against buffer C (25 mM Tris-HCl, pH 7.5; 1 mM EDTA; 0.5 mM dithiothreitol; 25 mM NaCl; 25% (v/v) glycerol), frozen in a dry ice-ethanol bath and stored at −20°C (fraction V; 5 ml, 0.7 mg protein).

2.5. *DNase I footprinting*

This is a modification of the technique of Galas and Shmitz [16]. The DNA substrate used for the footprinting and sequencing reactions was the 94 bp pUD88 *Dra*I-*Hin*dIII restriction fragment containing the Mu Ner-binding sites (labelled by 'backfilling' the *Hin*dIII site). Ner protein was allowed to bind to the DNA at 37°C for 10 min in a 93 μl reaction mixture containing buffer A, 100 mM NaCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 25–50 ng ^{32}P-end labelled pUD88 94 bp *Dra*I-*Hin*dIII DNA restriction fragment and increasing amounts of pure Mu Ner protein. DNase I (Worthington) was then added for 30 s at 25°C to a final concentration of 350 ng/ml. The reaction was terminated by adding 25 μl of stop solution (3 M ammonium acetate; 250 mM EDTA; 150 μg/ml sonicated calf thymus DNA). The DNA was then quickly extracted with phenol, chloroform, iso-amyl alcohol (45:45:10 by vol.) followed by precipitation with ethanol. The reaction products were then subjected to electrophoresis with the DNA sequencing products [17] on 10% polyacrylamide gels containing 7 M urea. The gel was then exposed to Kodak XAR-5 film under Dupont Cronex intensifying screens at −70°C.

2.6. *Gel filtration experiments*

Gel filtration of the purified protein sample (300 μl) was performed on a 5 ml Sephadex G-75 superfine column (0.6 cm diameter), collecting 110 μl/fraction, at a flow rate of 500 μl/h with 25 mM NaCl in buffer A. The position of sample elution was monitored by: (a) protein gel electrophoresis [14] followed by silver staining of the gels [15]; (b) measurement of the A_{280} with an ISCO UA-5 absorbance monitor; and (c) the band competition assay [10].

3. RESULTS

The various fractions at intermediate stages of the Mu Ner purification are displayed in fig.1 on a silver stained [15] 15% SDS-polyacrylamide gel

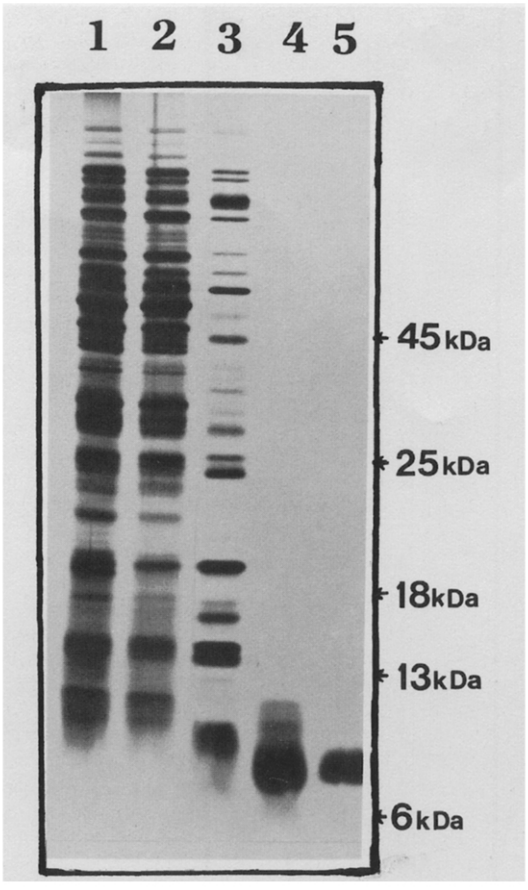

Fig.1. SDS-polyacrylamide gel electrophoresis of the fractions from the Mu Ner protein purification. Each lane was loaded with 2 μg of protein. Lanes: 1, fraction I (crude protein extract from strain LF123); 2, fraction II (after DEAE-Sephadex chromatography); 3, fraction III (after phosphocellulose chromatography); 4, fraction IV (after Sephadex G-75 gel filtration); 5, fraction V (after hydroxyapatite chromatography). The molecular mass markers shown are those of ovalbumin (45 kDa), α-chymotrypsinogen (25 kDa), β-lactoglobulin (18 kDa), cytochrome *c* (13 kDa) and bovine trypsin inhibitor (6 kDa) which were co-electrophoresed as standards.

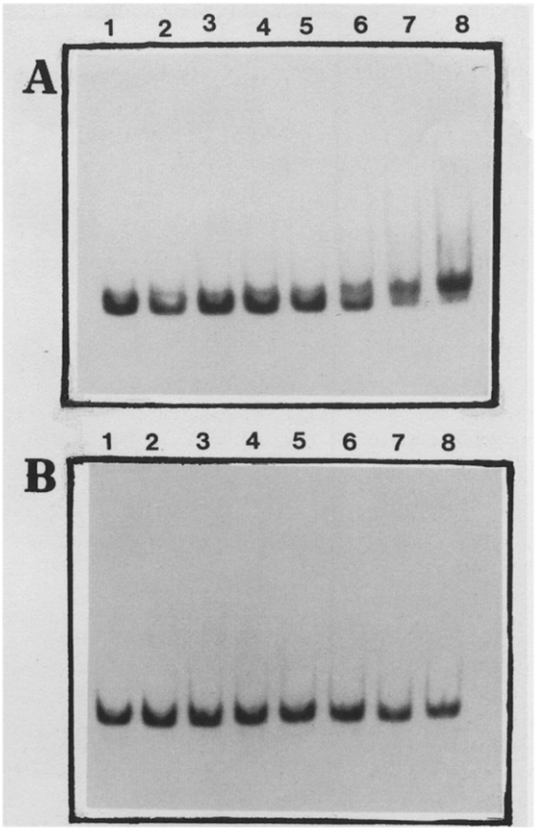

Fig.2. Detection of specific DNA-binding activity with purified Mu Ner protein. The band competition assay was performed as described in [10] except that the NaCl concentration was 100 mM and the binding reaction did not contain any sonicated calf thymus DNA. (A) Lane 1 displays 2 ng of the ^{32}P labelled Mu Ner-specific DNA substrate without addition of protein. Lanes 2–8 display the same but in the presence of 50, 100, 150, 200, 250, 300 and 350 ng of the purified Mu Ner protein, respectively. (B) Same as in A except that the ^{32}P-labelled DNA was the non-specific DNA substrate (see section 2).

[14]. Following hydroxyapatite chromatography (fraction V) the Mu Ner protein was found to be greater than 99% pure. Fig.2B shows that, at these concentrations, the Mu Ner protein fails to retard the electrophoretic migration of the non-specific DNA substrate (which lacks the Mu Ner-binding site). However, the specific DNA substrate (94 bp pUD88 *Dra*I-*Hin*dIII restriction fragment), which contains the Mu *ner* operator, is readily bound and retarded in its electrophoretic migration by the Ner protein under these same conditions (fig.2A).

Using a ^{32}P-end labelled 94 bp pUD88 *Dra*I-*Hin*dIII restriction fragment, we physically mapped the specific Ner-operator interaction by DNase I footprinting [16]. An enhancement of DNase I cleavage at nucleotide 1059 (fig.3A) with increasing amounts of Mu Ner is observable as the binding site becomes fully protected. The primary region protected from DNase I hydrolysis extends from base pairs 1027–1058 from the left end of Mu. This protected site also contains part of the previously hypothesized *ner* operators, OR1 and OR2 [18]. However, further examination reveals

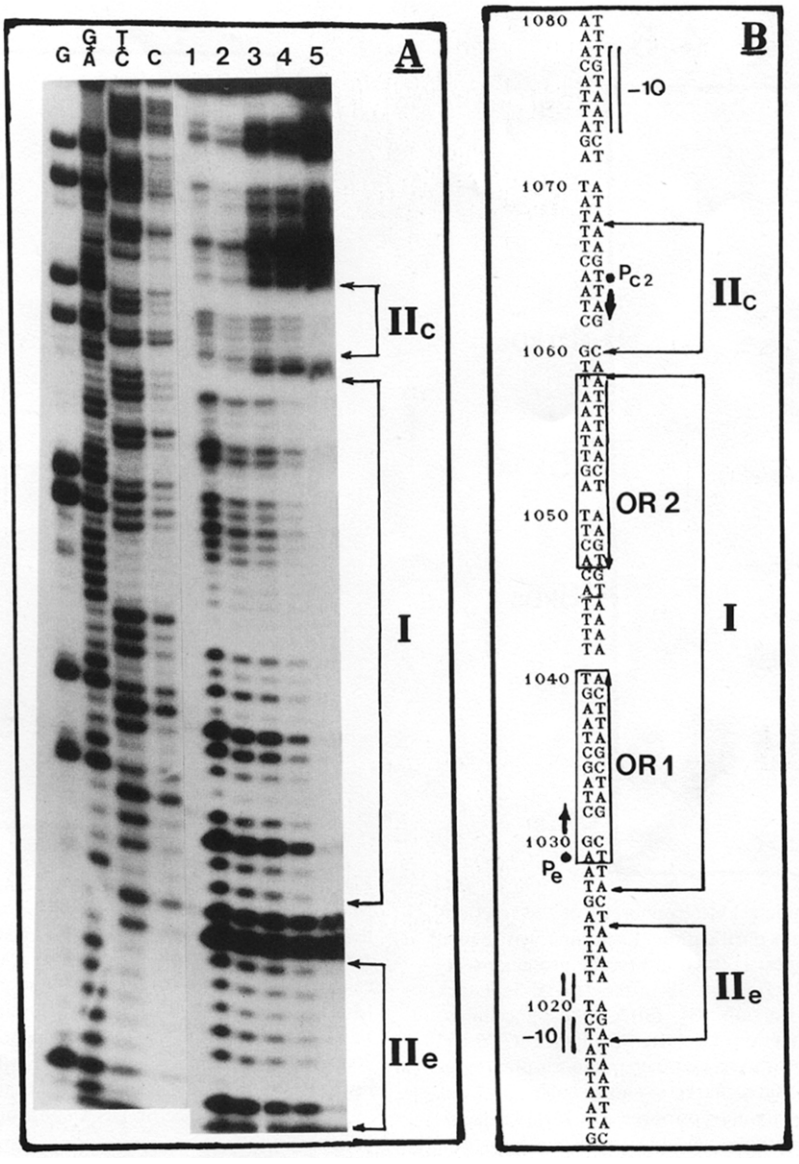

Fig.3. (A) DNase I footprinting with Mu Ner. Lanes 1–5 display the banding pattern obtained with purified Mu Ner at a concentration of 0, 2.5, 5, 7.5, and 10 μg/ml in each reaction, respectively. Lanes G, G + A, T + C, and C represent the respective Maxam and Gilbert DNA sequencing reactions. (B) DNA sequence of the *ner* operator region from bacteriophage Mu. Indicated are the regions protected from DNase I hydrolysis: I, includes the OR1 and OR2 inverted repeats; region II_e overlaps with the − 10 of Pe; and region II_c overlaps with the transcriptional initiation site for Pc_2.

two additional regions, denoted II_e and II_c, that are also protected from DNase I by Mu Ner binding to this substrate. Region II_e extends from bp 1024 leftwards to bp 1017, while region II_c spans rightwards from bp 1060 to bp 1067 along the Mu genome. These results suggest that in addition to Ner specifically binding to a primary region previously characterized [4,19], it also protects regions flanking both sides of this site from DNase I digestion.

To estimate the molecular mass of native Mu Ner, we subjected the purified protein to gel filtra-

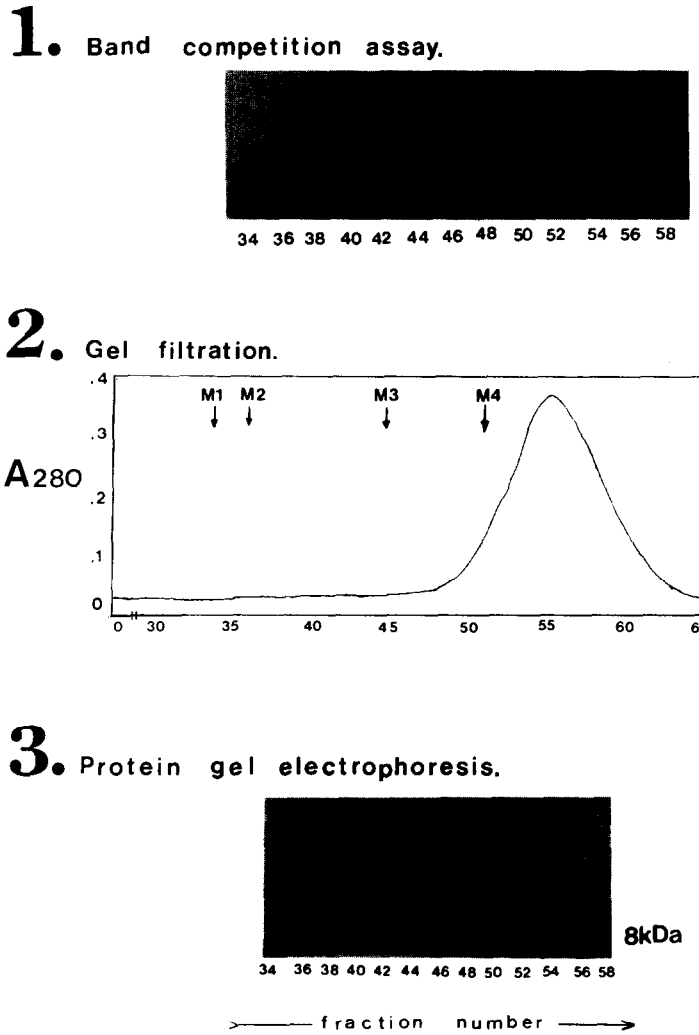

Fig.4. Gel filtration chromatography analysis of purified Mu Ner. Gel filtration: panel 1 displays measurement of specific DNA-binding activity in fractions 34–58 using the 145 bp, Mu Ner-specific DNA substrate in the band competition assay (as described in the legend to fig.2); panel 2 displays the A_{280} profile of each fraction; and panel 3 shows a silver stained 15% SDS-polyacrylamide gel of fractions 34–58. The vertical arrows in panel 2 denote the peak of the elution profile of the markers: M1, blue dextran 2000; M2, bovine serum albumin (68 kDa); M3, α-chymotrypsinogen (25 kDa); M4, cytochrome c (13 kDa).

tion as described in section 2 (fig.4). Mu Ner protein, at a concentration of 1.6×10^{-5} M (140 μg/ml), was loaded onto a 5 ml Sephadex G-75 superfine column and displayed an elution profile as seen in fig.4, panel 2. The position of this elution profile relative to that of the molecular mass markers suggests that the native molecular mass of Mu Ner is approx. 8 kDa. Moreover, this elution profile correlates with the specific DNA-binding activity of Mu Ner (fig.4, panel 1).

4. DISCUSSION

We report here the first purification of the DNA-binding, regulatory protein Ner from bacteriophage Mu. Fig.2 shows that purified Ner protein binds to its specific DNA substrate and not to a non-specific DNA substrate in vitro. The spatial organization of Ner, on its operator DNA, relative to the two overlapping and divergent promoters in this region was ascertained through

DNase I footprinting. We found that Mu Ner protects a region, spanning bp 1027–1058 from the Mu left end, from DNase I hydrolysis, as previously observed with crude protein extracts containing Mu Ner [4]. In addition, Goosen and Van de Putte [19] isolated five independent *ner*-insensitive mutants which also mapped to this region. This region is postulated to contain two 12 bp inverted operators, OR1 and OR2, each having the consensus sequence 5′-ANPyTAPuCTAAGT-3′ and which we found to be simultaneously occupied. However, the precise pattern of protection observed here with the purified protein extends this region on each side by approx. 3/4 of a turn of the DNA helix. Fig.3 denotes the adjacent regions from bp 1017–1024 and bp 1060–1067 from the Mu left-end as II_e and II_c, respectively. That a small protein like Ner protects such a large region is in sharp contrast to larger proteins such as λ and 434 repressors [20,21], whose interactions span smaller regions of DNA. Mu *ner* operator region II_c encompasses the transcriptional initiation site of the repressor promoter Pc_2 [22], whereas II_e spans a turn of the DNA that includes the '−10' promoter region of the early promoter Pe [7]. Upon binding, Ner may ultimately mediate its action by sterically hindering the initiation of transcription from both Pc_2 and Pe.

Integration host factor (IHF), comprised of the *him*A and *him*D gene products [23], is a heterodimer which positively regulates expression from both Pc_2 and Pe [22,24]. Expression from wild type Pe is markedly reduced in the absence of IHF [22]. *Pip* mutants, specifically localized to the −10 region of Pe, have been isolated that restore transcription from Pe in a *him*D$^-$ strain [19,25]. Since these changes occur within the II_e region protected by Ner from DNase I digestion, the *pip* mutants may actually influence Ner binding; this modification in the *ner* operator may translate to a decreased affinity for Ner and an increased affinity for RNA polymerase. That Ner-overproducing strains fail to maintain 'pseudo-immunity' against plaque forming Mu *pip* phage [19] may reinforce this hypothesis. The lack of sequence homology between regions II_e and II_c (as seen with OR1 and OR2) may suggest that binding of Ner to these extremities is not sequence specific; so how does a small protein like Ner cover a large region in such a manner? One possibility is that its operator DNA is bent around the protein such that the extremities contact Ner. Bending of DNA as a result of specific protein binding has been demonstrated with the CAP operator [26]. Alternatively, as has been recently found for the trp repressor [27], Ner may bind to specific conformations of its operator that are induced by sequence composition.

Gel filtration analysis of purified Mu Ner protein in conjunction with the detection of specific DNA-binding activity (fig.4) suggests the active Mu Ner protein to behave as a monomer in solution with a native molecular mass of approx. 8 kDa. This result is different from those obtained with most procaryotic DNA-binding proteins such as λ's cro and repressor proteins, which readily form dimers and even tetramers at much lower concentrations than those presented here [28–30]. These observations show that this relatively small (75 amino acids) monomeric protein forms a complex with its specific DNA substrate such that it protects an unusually large region from DNase I digestion. In corollary, Mu Ner does not display strong homology with the conserved helix-turn-helix regions of other bacterial and phage repressors [9,25] and may represent a new class of DNA-binding proteins.

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