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Purification and characterization of the DNA-binding protein Ner of bacteriophage Mu

(Recombinant DNA; phage λ p_L promoter; *c* repressor; clear-plaque phenotype; protease-deficient *Escherichia coli* B; thermoinducible Ner-mediated immunity; prophage; lysogeny)

Bernard Allet[a*], Mark Payton[a*], Robert J. Mattaliano[b], Angela M. Gronenborn[c], G. Marius Clore[c] and Paul T. Wingfield[a*]

[a] *Biogen S.A., Geneva (Switzerland);* [b] *Biogen Research Corporation, Cambridge MA 02143 (U.S.A.) Tel. (617)864-8900, and* [c] *Max-Planck Institut für Biochemie, 8033 Martinsried (F.R.G.) Tel. 49-89-8578*

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SUMMARY

The construction is described of a plasmid (pL-*ner*) which directs the high-level production of the bacteriophage Mu Ner protein in *Escherichia coli*. The protein, recovered in the soluble cellular fraction, was susceptible to in vivo proteolytic processing, in many host strains, but not in *E. coli* B, a natural *lon* $^-$ prototroph. A simple purification method is described which takes advantage of the basic nature of the protein. The purified protein was shown to be physically and chemically homogeneous and to have an amino acid sequence identical to that predicted for the authentic protein. The protein was also shown to have in vitro biological activity, as measured by specific binding to a DNA fragment containing the consensus Ner-binding sequence, and in vivo biological activity as the protein produced by the pL-*ner* plasmid allowed lysogenic-like maintenance of a Mu prophage *c* mutant unable to synthesise a functional Mu repressor.

INTRODUCTION

The Ner protein of bacteriophage Mu consists of the 74 aa encoded by nt 1102 to 1324 in the Mu DNA (Priess et al., 1982). The protein regulates the choice between the lysogenic and lytic development of the phage by modulating expression of both the repressor gene and the early functions which include *ner*

Correspondence to: Dr. P.T. Wingfield at his present address: Glaxo Institute for Molecular Biology, 46 route des Acacias, 1227 Geneva (Switzerland) Tel. (022)43-32-00.

* Present address: Glaxo Institute for Molecular Biology, 46 route des Acacias, 1227 Geneva (Switzerland) Tel. (022)-43-32-00.

Abbreviations: aa, amino acid(s); Ap, ampicillin; bp, base pair(s); buffers A–E, see MATERIALS AND METHODS, section **d**; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; Km, kanamycin; LB, see MATERIALS AND METHODS, section **d**; MPA, mega pascal; *ner*, Mu *ner* gene coding for Ner; Ner, a repressor-like protein which binds to the DNA sites 5′ANPyTAPuPyTAPuNT; nt, nucleotide(s); *p*, promoter; PAGE, polyacrylamide gel electrophoresis; pI, isoelectric point; p_L, phage λ leftward major promoter, PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; ^{R}, resistant; ^{S}, sensitive; SD, Shine–Dalgarno ribosome-binding sequence; SDS, sodium dodecyl sulfate; Tc, tetracycline; Tn, transposon; ts, temperature sensitive; v.v.m., volume of air per volume of liquid in fermentor per min; [ ], designates plasmid-carrier state; ::, novel joint.

itself (Van Leerdam et al., 1982). Regulation by Ner is negative and is exerted at the transcription level (Van de Putte et al., 1980). The target sequences on Mu DNA have been recently identified by genetic (Goosen, 1984; Goosen and Van de Putte, 1984) and by biochemical (Tolias and DuBow, 1986) techniques. Employing the latter approach footprinting analysis has shown that the Ner-binding sequences are located between nt 1028 and 1058 in the Mu DNA sequence and comprise two tandem repeats of the dodecanucleotide 5′–ANPyTAPuPyTAPuNT separated by a spacer of 6 bp (see Fig. 1B). As these binding sequences are flanked on one side by the mRNA initiation site for the early functions (*Pe*) and on the other by an mRNA initiation site for repressor synthesis (*Pc2*), it is likely that Ner inhibits RNA synthesis by preventing the binding of RNA polymerase.

Ner synthesis is autoregulated (Goosen, 1984), and so only very small amounts accumulate in *E. coli* infected with bacteriophage Mu. This led to the construction of multicopy plasmids in which the *ner* gene was placed under the control of promoters insensitive to the regulatory mechanism. With such plasmids, the *ner* gene product has been identified in mini-cells (Van Leerdam et al., 1982) and in an in vitro coupled transcription translation system (Tolias and DuBow, 1986). In these systems, however, Ner was still produced in small amounts.

Here, we describe the construction of a plasmid (pL-*ner*) which in *E. coli* directs high-level synthesis of Ner. The *ner* gene was placed under the control of the λ p_L promoter in a plasmid which includes a λ ts repressor gene (*c*Its). Ner was purified, characterised and shown to be biologically active using specific in vitro and in vivo assays.

MATERIALS AND METHODS

(a) Recombinant DNA technology

Restriction endonucleases, T4 DNA ligase and PolIk were purchased from New England Biolabs and used as recommended by the manufacturers. Strain storage, plasmid preparation, transformation, separation of DNA fragments in agarose and polyacrylamide gels were all performed as previously described (Maniatis et al., 1982).

(b) Construction of plasmid pL-*ner*

The plasmid expressing Ner was prepared as summarised in Fig. 1. Plasmid p236 is a pBR322 derivative containing the p_L promoter (Remaut et al., 1981). A high-copy variant was made by exchanging the large *Pst*I-*Eco*RI fragments of p236 and pAT153 (Twigg and Sherratt, 1980) to obtain plasmid p′236. The unique *Hae*III restriction site present in the p_L sequences upstream from the p_L was replaced by a *Pst*I site using a synthetic linker. The *Bgl*II-*Pst*I λ DNA fragment of 1102 bp containing the *c*Its repressor gene (Daniels et al., 1983) was purified from λ *c*I857*S*7 DNA and inserted between *Dra*I (pBR322 map position 3232; Sutcliffe, 1979) and the *Pst*I site in p′236 giving plasmid p′236*c*Its.

The exact Mu *ner* gene was prepared from plasmid pLP103-22 (Van Leerdam et al., 1982). A *Cla*I site was introduced between the ribosome binding sequence (SD) and the ATG start codon of *ner* by site-directed mutagenesis (Oostra et al., 1983). The sequence from nt position 1086 to 1104 in Mu DNA (Priess et al., 1982) was thereby replaced by the sequence 5′–GGAGGGATCGATAAATGTGT–3′. In addition, a synthetic *Xba*I linker was added to the *Hin*fI site at Mu DNA map nt position 1365. The 273-bp *Cla*I-*Xba*I Mu DNA fragment now included the *ner* gene followed by DNA encoding the first 13 aa of the Mu *A* (transposase) protein (Fig. 1B). This fragment was ligated at the *Cla*I side to a DNA fragment that contained a SD sequence (underlined) 5′–AATTCTTTATAGATTACAAAACTTAGGAGGGTAT–3′, and at the *Xba*I side, to a DNA fragment that included a Rho-independent transcription terminator, 5′–CTAGAGCTTGGGGAC-CCTAGAGGTCCCCTTTTTTATTTG–3′ (arrows mark the inverted repeat). The resulting 353-bp fragment was inserted within the unique *Eco*RI site of p′236 *c*Its in the orientation shown in Fig. 1C.

(c) Oligodeoxynucleotide synthesis

Oligodeoxynucleotides were synthesised on the Applied Biosystems 380A automated synthesizer. After deprotection by standard procedures (Beaucage and Caruthers, 1981), the crude oligodeoxynucleotides were desalted by HPLC using a C-18 SEPPAK column (Waters/Millipore Inc.) and purified by PAGE (Maniatis et al., 1975).

Double-stranded synthetic oligodeoxynucleotides were prepared by incubating at 95°C for 2 min equal amounts of the single-stranded oligodeoxynucleotides (50 μl each of A_{260nm} = 0.25) in 20 mM Tris · HCl, pH 8.0, containing 10 mM $MgCl_2$ and 50 mM NaCl. The temperature was then decreased over 4–5 h to 30°C. The reannealed fragments were stored at −20°C.

(d) Bacterial strains and culture media

Plasmids were propagated in *E. coli* C600 (Bachmann, 1972) lysogenic for wild-type λ. For plasmid preparations, transformants were grown at 37°C in LB broth [1% (w/v) Bactotryptone, 0.5% (w/v) Bacto yeast extract, 0.5% (w/v) NaCl, pH adjusted to 7.0 with NaOH] supplemented with 40 μg Ap/ml or 5 μg Tc/ml.

For Ner expression studies, the DNA of plasmid pL-*ner* was used to transform at 30°C the λ^- bacterial hosts, *E. coli* B (prototroph) and A89, a Tc-sensitive derivative of SG936 [F^- *lacZ*am *trp*am *pho*am *supC*(ts) *rpsL mal*am *htpR*am *tsx*::Tn*10 lonR*9] (Buell et al., 1985). Transformants were selected on Tc and purified.

E. coli hosts harbouring the plasmid pL-*ner* were grown in a 1.5-liter fermenter (MBR BioReactor A.G., Switzerland) in media comprising: 18 mM K_2HPO_4; 8 mM KH_2PO_4; 20 mM NH_4Cl; 0.10 mM $CaCl_2$; 15 mM K_2SO_4; 2 mM $MgCl_2$; 2.0% (w/v) Casamino acids; 0.03% (w/v) yeast extract; 4.0% (w/v) glycerol and trace elements. Growth was in the presence of 5 μg Tc/ml at 30°C. Fermentations were maintained at pH 7.0 by the addition of either phosphoric acid or ammonium hydroxide. Aeration was at 1 v.v.m. When cultures had reached an absorbance of about 30 at 650 nm, the temperature was raised to 42°C. After 1 h at 42°C, the cells were harvested to yield 100 mg (wet weight) cells per ml of culture. The cells could be frozen at −80°C without affecting the subsequent purification procedure.

The expression level of Ner following fermentation, and the assay of the protein in column fractions, were measured by 0.1% SDS–15% PAGE (Laemmli, 1970).

(e) Purification of Ner

The buffers used in protein purification were: buffer A, 25 mM Tris · HCl, pH 7.8, containing 1 mM benzamidine · HCl and 250 mM sucrose; buffer B, 50 mM Tris · HCl, pH 7.8, containing 5 mM benzamidine · HCl, 0.5 mM DTT and 2.0 mM EDTA; buffer C, 50 mM Tris · HCl, pH 7.8; buffer D, 50 mM sodium phosphate, pH 7.0, containing 0.5 mM DTT and 0.5 mM EDTA and buffer E, 100 mM Tris · HCl, pH 7.5, containing 2 mM sodium azide. The temperature of all operations was 0–8°C.

Cells (100 g wet weight) were washed by resuspending with 500 ml of buffer A and collected by centrifugation at 10000 × *g* for 30 min. The pelleted cells were resuspended with 300 ml of buffer B and passed through a French pressure cell at 124 MPa (18000 lbs/inch^2). The suspension was centrifuged at 10000 × *g* for 30 min and the supernatant re-centrifuged at 60000 × *g* for 60 min.

The supernatant (300 ml) was applied to a column (20 cm × 5 cm diameter) of DEAE-Sepharose (Pharmacia) equilibrated with buffer C. The column was eluted with buffer C; Ner was located in the flow-through fractions which were pooled (640 ml) and diluted two-fold with buffer D. The slightly cloudy solution was filtered using 0.8 μm pore size nitrocellulose membranes (Millipore filter type AAWP) and applied to a column (5 cm × 10 cm diameter) of CM-Sepharose (Pharmacia) equilibrated with buffer D. A linear gradient of 0–0.5 M NaCl in buffer D (2000 ml) was applied; Ner was eluted with about 160 mM NaCl. To the CM-Sepharose pooled fraction (480 ml) solid ammonium sulphate was added to 3.4 M at 0°C (87.2% saturation). After a 30-min incubation, the precipitate was collected by centrifugation at 10000 × *g* for 30 min and dissolved in 15 ml of buffer E, filtered with a Millex-GV 0.22-μm pore size filter unit (Millipore), and applied to a column (98 cm × 2.5 cm diameter) of Ultrogel AcA54 (LKB) equilibrated with buffer E. Ner-containing fractions were pooled (45 ml), concentrated five-fold by ultrafiltration using Diaflo YM2 membranes (Amicon) and stored at −80°C.

(f) Analytical ultracentrifugation

Prior to analysis, Ner was dialysed using Spectrapor No. 3 dialysis tubing against 50 mM

Tris · HCl, pH 7.0, containing 1% (w/v) KCl. Sedimentation equilibrium measurements were made using a Beckman LB-70 preparative ultracentrifuge equipped with a Prep UV Scanner. Ner was analysed at an initial protein concentration of 0.21 mg/ml (A_{280nm} = 0.48). The centrifuge was operated at 28000 rev./min at 15°C. Measurements were taken after 17 h and a corrected base line was established by centrifugation at 50000 rev./min for an additional 5 h. The M_r was calculated from the least-square fit of plots of the logarithm of absorbance, ln A_{280nm}, against r^2, the square of distance from axis of rotation.

(g) Amino-terminal amino acid sequence determination by Edman degradation

Automated Edman degradation was performed with a Model 470A gas-phase sequencer (Applied Biosystems). Identification and quantitation of phenylthiohydantoin derivatives were made on-line using an Applied Biosystems Model 120A PTH-amino acid analyser with external quantitation.

(h) Protein determination

The concentration of purified protein was determined by measuring the ultraviolet absorbance using a calculated value of $A^{1\%}_{1cm}$ = 23.2 at 280 nm.

(i) Protein binding to synthetic oligodeoxynucleotides

For protein binding studies, 20 μl (5 pmol) of the oligodeoxynucleotides 0.4 μM in 20 mM Tris · HCl, pH 8, containing 10 mM $MgCl_2$, and 0.1 mM DTT were mixed with 1 μl (8–200 pmol) of various proteins. The DNA-protein mixtures were incubated for 10 min at 37°C, applied to a 10% polyacrylamide gel with the Tris · borate buffer system previously described (Maniatis et al., 1982) and electrophoresed at room temperature at 25 V/cm. Bands were visualised by soaking the gel in a solution of ethidium bromide.

(j) Construction of W3110[pL-*ner*] lysogenic for repressor-less Mu *c*$^-$

Plasmid pLP103-22-7 (Van Leerdam et al., 1982) has a *ner* deletion and expresses the Mu *A* gene for transposase constitutively. The plasmid is Ap^R, Km^S. A Km^R, Ap^S derivative was prepared by inserting a 1500-bp *Hin*dIII-*Xho*I fragment containing the Km^R gene of Tn*5* (Berg et al., 1975) into the unique *Pvu*I site present in the β-lactamase gene of pLP103-22-7. The resulting plasmid, pLP103-22-7-Km, was used to transform a purified W3110 clone harbouring the plasmid pL-*ner*, and colonies resistant to both Tc and Km were purified. Cultures of the doubly transformed strain W3110[pL-*ner*; pLP103-22-7-Km] were then applied to agar plates containing Ap and Tc and incubated at 37°C with lysate from Muc3213pAp1 which contained an Ap^R gene. The phage mutant was isolated as a spontaneous clear derivative of Muc$^+$pAp1, a recombinant between Muc$^+$ (Taylor, 1963) and Mucts62pAp1 (Leach and Symonds, 1979). Antibiotic-resistant colonies were purified. Some colonies retained the Km^R plasmid which was removed (cured) by a few cycles of growth in the absence of Km.

RESULTS AND DISCUSSION

(a) Plasmid constructions

The individual components necessary for Ner expression were combined (MATERIAL AND METHODS, section **b**) to form the plasmid pL-*ner* shown in Fig. 1C. With this plasmid, Ner expression is regulated by a λ p_L promoter repressed by a temperature-sensitive repressor gene (*c*Its) expressed from the same plasmid. The natural SD translation initiation sequence of the *ner* gene was replaced by a synthetic but similar sequence.

Features of the plasmid construction we suggest responsible for the high-level protein production are: the omission of Ner-binding sequences (Fig. 1B) which thus prevents feed-back inhibition of protein synthesis by Ner binding and the inclusion of a synthetic Rho-independent transcription terminator (Ter) which stabilises *ner*-specific messenger RNA (B.A., unpublished work).

(b) Protein purification

With the host strains *E. coli* A89 and *E. coli* B harbouring the expression plasmid, Ner was pro-

duced to about 5 and 10%, respectively, of the total cell protein as estimated by SDS–PAGE (Fig. 2). The protein was located in the soluble cellular fraction as shown by differential centrifugation (MATERIALS AND METHODS, section e). The basic nature of Ner (calculated pI = 9.90) facilitated its isolation from cell extracts. The protein was partially purified by applying cell extract to a DEAE-Sepharose anion exchanger which did not bind Ner, whereas contaminating *E. coli* proteins and non-proteinaceous material (nucleic acid etc.) bound tightly. The partially purified protein bound tightly to the CM-Sepharose cation exchanger at neutral pH. Although the remaining *E. coli* contaminants also bound to the cation exchanger, they eluted from the column before Ner. The protein was >90% pure at this stage.

When the purification was carried out with extracts from a protease-deficient (*lon* mutant) *E. coli* strain, namely *E. coli* B (Donch and Greenberg, 1968; Donch et al., 1969), Ner eluted from CM-Sepharose in a single peak at about 160 mM NaCl. However, when the purification was carried out with extracts from the protease-deficient strain, *E. coli* A89 (for review see Goldberg and Goff, 1986), in addition to the Ner peak, another major peak was observed which eluted with about 125 mM NaCl. As shown below, this peak contained a proteolytically processed form of Ner.

About 1 mg of Ner was obtained per g wet weight of *E. coli* B cells using the purification method described.

(c) Physical and chemical characterization

SDS–PAGE of the purified protein indicated a single band of less than 14 kDa (Fig. 2, lane f). The M_r of the native protein was determined by sedimentation equilibrium analysis. The plot of the integral of absorbance, ln A_{280nm}, with respect to r^2, the distance from the axis of rotation, was linear as expected for a homogeneous and ideal protein solution. From the average value of the slope (0.433 ± 0.006, n = 4) and a partial specific volume ($\bar{v}$) of 0.725 g/ml, evaluated from the amino acid composition (Cohn and Edsall, 1943), an M_r of 9050 (± 150, n = 4) was estimated. This result is in good agreement with that predicted by the covalent structure (M_r 8500) and indicates that the native protein is monomeric over the concentration range studied (0.05–0.31 mg/ml).

The results of amino acid analysis showed excellent correlation with the amino acid composition predicted from the gene sequence. The complete amino acid sequence, except the N-terminal residue, was confirmed by direct sequencing of the protein (aa residues 2 to 56) and by sequencing of the tryptic peptides tn4 (aa residues 46 to 49), tn10 (aa residues 50 to 69) and tn2 (aa residues 70 to 74) (Fig. 3). The determined amino acid sequence (aa residues 2 to 74) was in exact accord with that predicted from the DNA sequence (Priess et al., 1982).

As mentioned above, partially purified protein from *E. coli* A89 was fractionated into two major peaks during CM-Sepharose chromatography. The first peak eluted was identified by gas-phase sequencing as an N-terminal peptide (aa residues 1 to 22) of Ner. This peptide was most likely produced by intracellular protease action rather than protease action after cell breakage. Fermentation studies with the *E. coli* A89 strain indicated that induction at 42°C for periods longer than 1 h resulted in a dramatic loss of Ner as judged by SDS–PAGE of cell extracts. On the other hand, similar studies with *E. coli* B indicated that the gene product was stable over much longer periods. This result was consistent with the fact that no proteolytically processed protein was detected during protein purification from the strain.

According to our results, the yield of small proteins produced in recombinant *E. coli* in a soluble state will be greatly influenced by the selection of the host strain and when p_L systems are used, the length of time used for temperature induction. Optimization of these factors may be crucial for the expression of proteins containing peptide bonds unusually sensitive to proteases as, for example, the bond between aa residues 22 and 23 of Ner.

(d) Specific binding of Ner to a synthetic oligodeoxynucleotide

Using extracts of bacteria containing Ner, Tolias and DuBow (1986) identified by footprinting analysis the Ner-specific binding sequence in Mu DNA. This sequence was found upstream from the translation start codon for *ner*, the first gene to be transcribed from the early promoter *P*e, and consists of a tandem duplication of the dodecanucleotide 5′–ANPy-

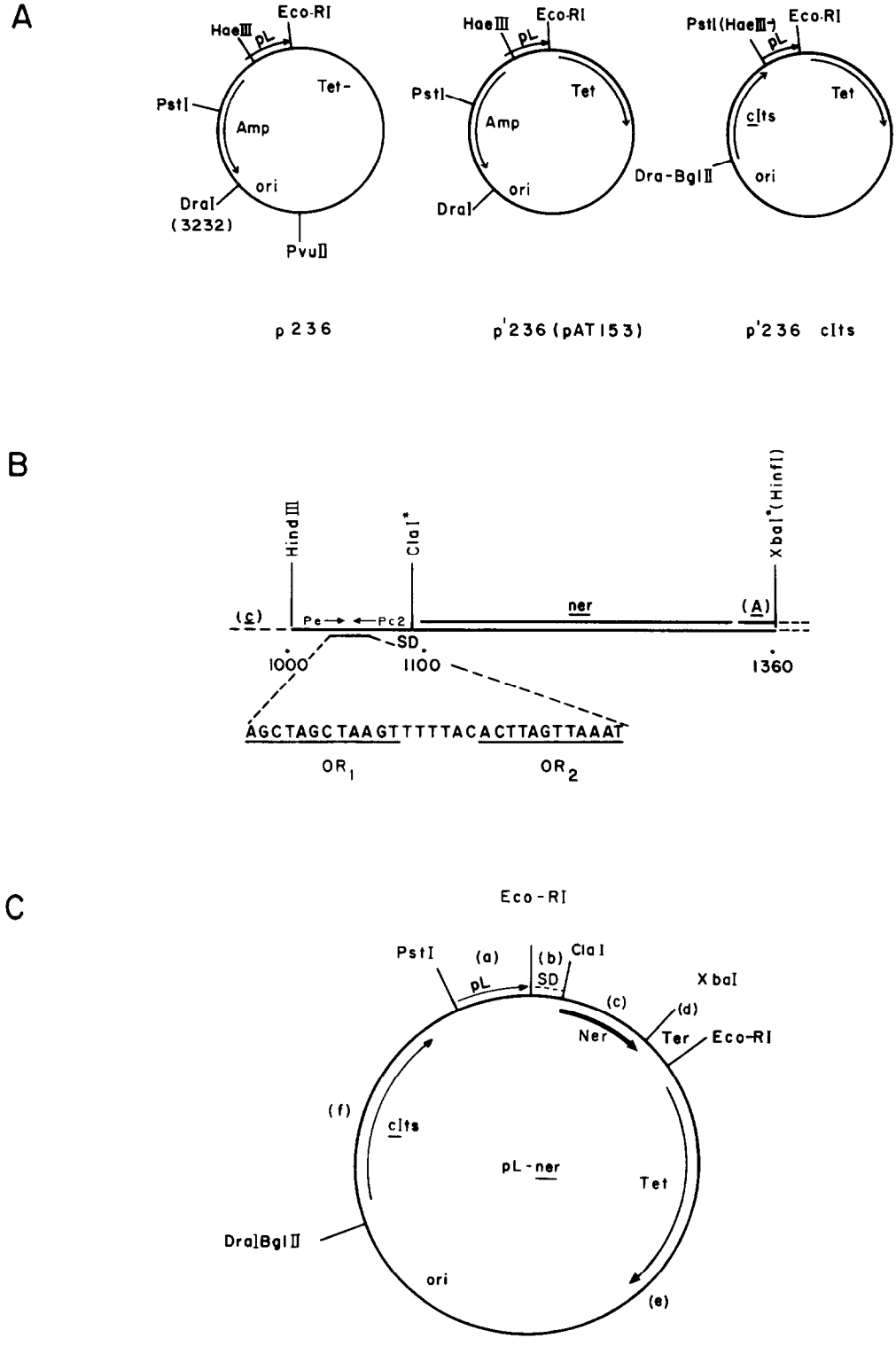

Fig. 1. Construction of plasmid pL-*ner*. (**A**) Structure of intermediate plasmids. The plasmids p236, p'236 and p'236*c*Its were constructed as described in MATERIALS AND METHODS, section **b**. The genes [Amp(Ap^R), Tet(Tc^R) and *c*Its] and the restriction sites are indicated inside and outside the circles, respectively. Symbol pL (and arrow) specifies phage λ p_L promoter. Tet$^-$ indicates absence of the Tc^R gene. (**B**) Schematic representation of a portion of Mu DNA. The horizontal solid line represents Mu DNA between the *Hin*dIII site at nt position 1001 (Priess et al., 1982), and the *Hin*fI site at nt position 1365. The restriction sites marked with an asterisk

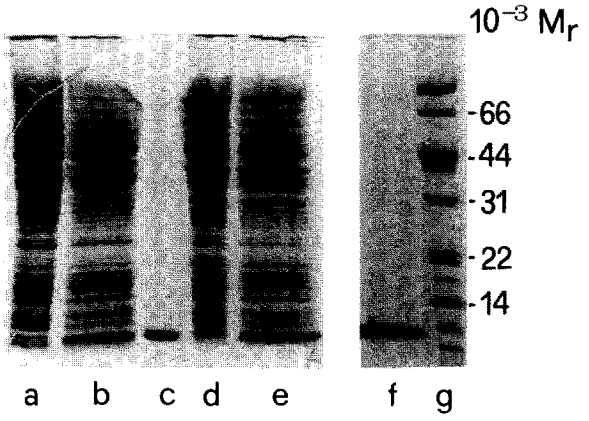

Fig. 2. Gel analysis of whole-cell extracts and of the purified Ner protein. The gel shows pre-induction (lane a) and post-induction (lane b) samples for *E. coli* A89[pL-*ner*] and pre-induction (lane d) and post-induction (lane e) samples for *E. coli* B[pL-*ner*]. Purified Ner (5 μg (lanes c and f)] and the M_r markers, phosphorylase b, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme (lane g; sizes in kDa on the right margin). PAGE was carried out on a 15% (w/v) polyacrylamide gel using a Mini-Protean II vertical electrophoresis system (BioRad) with the 0.1% (v/w) SDS buffer system described by Laemmli (1970). The polyacrylamide gel was stained for 60 min with 0.2% (w/v) Coomassie blue G250 in 45% (v/v) methanol and 10% (v/v) acetic acid and destained in 10% (v/v) methanol and 7.5% (v/v) acetic acid.

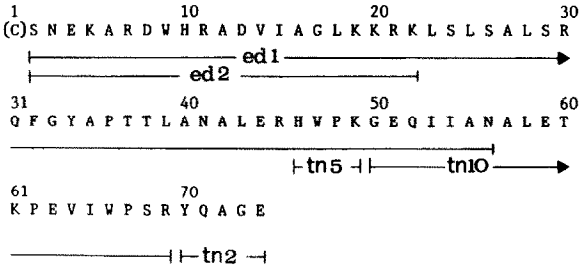

Fig. 3. Amino acid sequence determination of the purified Ner protein. Sequences determined by automated edman degradation of Ner and a peptide isolated by CM-Sepharose chromatography (see RESULTS AND DISCUSSION, sections **b** and **c**) are indicated by ed1 (residues 2 to 56) and ed2 (residues 2 to 22), respectively. The isolated tryptic peptides that were sequenced are indicated by tn2, tn5 and tn10. The numbers following the designation tn indicate the order in which the peptides were eluted during purification by reverse-phase HPLC. The N-terminal residue, predicted to be cysteine, is indicated in parentheses because it was not identified on the first cycle of edman degradation. The amino acid sequence is indicated by the one-letter code and is numbered from the N-terminal cysteine residue (residue 1).

TAPuPyTAPuNT separated by a spacer of 6 bp.

We obtained exactly the same result with the purified Ner by footprinting analysis using a *Hin*dIII-*Cla*I fragment containing the binding sequence shown in Fig. 1B.

In polyacrylamide gels, DNA fragments complexed with proteins migrate slower than the corresponding free DNA fragments (Strauss and Varshavsky, 1984). This property, incorporated into a band competition assay, was used by Tolias and DuBow (1986) to detect Ner in bacterial cell extracts. Using a simplified version of this assay, we studied the direct binding of purified Ner to a synthetic DNA fragment which included the consensus Ner-binding sequence (underlined):

5′-CGAACTAGTTAACTAGTACGAATTAGTTAAGTAAAAAGGGTATCGATTC
TTGATCAATTGATCATGCTTAATCAATTCATTTTTCCCATAGCTAAGTAC.
O_{R1} O_{R2}

(*Cla*I and *Xba*I) were introduced as described in MATERIALS AND METHODS, section **b**. The two convergent arrows above the map indicate the direction of transcription initiated at the *Pe* and *Pc*2 promoters. The genes *c* (Mu repressor), *ner*, and *A* are also shown above the map, whereas the position of the Ner-binding sequences are represented under the map as O_{R1} and O_{R2}. (C) Structure of the pL-ner plasmid. The plasmid was constructed as described in MATERIALS AND METHODS, section **b**. The genes [*c*Its, *ner* and Tc^R(Tet)] and restriction sites are indicated inside and outside the circle, respectively. The various components of the plasmid are represented by letters (a–f) in parentheses. (a) p_L sequences: 270 bp (nt 35737–35467; Daniels et al., 1983) including the entry site for the *E. coli* RNA polymerase, the Pribnow box and the transcription start point; (b) synthetic oligodeoxynucleotides of 40 bp including a ribosome-binding site; (c) the Mu DNA fragment of 273 bp, including *ner*, followed by the first 13 codons of the Mu *A* gene; (d) a synthetic DNA fragment of 40 bp that contains a Rho-independent transcription terminator; (e) plasmid pAT153 DNA (2525 bp) including the Tc^R gene (Tet) and the plasmid origin of replication (*ori*); (f) the fragment of 1102 bp (nt 38103–37005; Daniels et al., 1983) prepared from λ*c*I857*S*7 DNA and containing the temperature-sensitive λ repressor gene and its p_M promoter.

Mixing Ner and the DNA fragment before polyacrylamide gel electrophoresis resulted in retardation in the migration of the fragment indicating protein binding. The change in band position of the DNA was complete when the protein : DNA molar ratio was greater than 4 (Fig. 4A, lanes a and b).

Binding specificity with respect to protein was indicated by the fact that several proteins unrelated to Ner, including the basic protein murine IFN-γ (Fig. 4A, lane d), failed to bind to that specific DNA fragment, even in large molar excess. The basic N-terminal Ner peptide (aa residues 1 to 22), referred to above, also failed to bind to the DNA fragment (Fig. 4A, lane c). Binding specificity with respect to nucleic acid sequence was tested using a DNA fragment with a random sequence (see legend to Fig. 4B). Binding of Ner to the fragment was only observed at molar ratios of protein : DNA greater than 10 (Fig. 4B). This binding must therefore have been nonspecific and not unexpected in view of the basic nature of Ner.

The above results strongly suggest that the in vitro binding assay we have described is specific. In addition, the assay is sensitive, permitting the detection of pmol amounts of Ner. Many sequence-specific DNA-binding proteins such as, for example, Cro bind to DNA as dimers (for review see Pabo and Sauer, 1984). Although Ner appears monomeric in solution (see above), from a consideration of the stoichiometry of binding to DNA (protein : DNA molar ratio = 4) the protein may associate upon binding. Confirmation of this and other details of the protein–DNA interaction must await structure determinations.

(e) In vivo maintenance of a Muc$^-$ prophage by Ner-mediated immunity

It was previously shown that in vivo expression of a cloned *ner* gene from a plasmid confers protection against superinfection with Mu (Van Leerdam et al., 1982; Tolias and DuBow, 1986) termed 'pseudo-immunity', to distinguish it from conventional immunity conferred by the *c*-coded repressor, normally synthesised by a Mu prophage. Certain mutations in the *c* gene inactivate the repressor ensuring lytic development. Such phage mutants (clear mutants) produce clear rather than turbid plaques on lawns of permissive bacteria and cannot maintain

Fig. 4. Binding of Ner to a synthetic DNA fragment. (**A**) Specific binding. The 50-bp double-stranded synthetic DNA fragment (see RESULTS AND DISCUSSION, section **d**) containing the Ner-binding sequence (5 pmol) was mixed with various proteins (the amounts of which are indicated below, in parentheses) and analysed by PAGE as described in MATERIALS AND METHODS, section **i**. Lanes: (a) Ner (8 pmol); (b) Ner (20 pmol); (c) N-terminal fragment of Ner, aa residues 1 to 22 (40 pmol); (d) BSA (200 pmol); (e) murine IFN-γ (40 pmol); (f) no protein; (g) *Hin*fI digest of pBR322 DNA, the fragment of 75-bp is indicated as a size reference. Retardation (i.e., Ner binding) of the synthetic DNA fragment is seen only in lane b, which corresponds to a Ner protein : DNA molar ratio of 4. (**B**) Non-specific binding. A 52-bp double-stranded synthetic DNA fragment which does not contain the Ner-binding sequence, 5′–C-GATACTATGGCTGAAAATGGTGATAATGAAAAGGCT-GCCCTGCAGGCCAA–3′ (5 pmol), was mixed with Ner and analysed as described for panel A. Lanes: (d), no protein; (e), Ner (25 pmol); (f), Ner (125 pmol). Controls are shown in which 5 pmol of the DNA fragment containing the Ner-binding sequence are mixed with no protein (lane a); 25 pmol of Ner (lane b), and 125 pmol of Ner (lane c). Lane (g) is the same as lane (g) in panel A.

stable lysogeny. It was conceivable that over-expression of Ner could overcome the absence of active *c* repressor by establishing 'pseudoimmunity' on an incoming Mu *c* mutant, allowing such a phage to become a stable prophage.

Strain W3110 was transformed with the pL-*ner* plasmid (MATERIALS AND METHODS, section **a**). As expected, lysogenisation of that host with the Mu clear mutant (Muc3213pAp1) by selection of Ap-resistant derivatives after infection was not successful. Indeed Ner made from the plasmid should have inhibited synthesis by the incoming phage of the Mu transposase (pA) required for integration thus for lysogenisation (for review see Symonds et al., 1987). Therefore, a second plasmid which expressed the Mu transposase constitutively from a promoter insensitive to inhibition by Ner was transformed into W3110[pL-*ner*]. This new strain, W3110[pL-*ner*; pPL103-22-7-Km], was efficiently lysogenised by Muc3213pAp1. Moreover, the transposase was not necessary to maintain the prophage as the lysogen could be cured from the transposase-producing plasmid (MATERIALS AND METHODS, section **j**) without loss of cell viability. The resulting strain carried a Muc3213 prophage and the pL-*ner* plasmid.

The role of Ner in maintaining the prophage was indicated by the growth properties of the strain at various temperatures. Normal growth was observed at 37°C where Ner is expressed from p_L as a result of inactivation of the λ *c*Its repressor but the strain did not grow at 30°C where Ner expression is repressed. This suggested that at low temperature the absence of Ner resulted in the lytic development of the Muc3213 prophage. This was tested by growing the strain at 37°C to an absorbance of 1.0 at 575 nm, and then lowering the temperature to 30°C. Lysis was observed within a few hours and phage titers up to 10^9 per ml were measured in the supernatants of the cultures. Moreover, the strain did not require antibiotic for plasmid maintenance as loss of the plasmid would automatically result in lysis. Practical applications for the expression of heterologous proteins in *E. coli* will be described elsewhere.

(f) Conclusions

By using the techniques described in this paper it is possible to prepare and purify large amounts of the Ner protein of bacteriophage Mu. The availability of pure and well-characterised protein will enable its structure and interaction with DNA to be studied. NMR spectroscopy will be especially important for these studies in view of the small size (M_r 8500) and monomeric nature of the protein. The in vitro and in vivo DNA-binding properties of the plasmid-derived protein suggest that it has a similar conformation to the authentic Ner protein.

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