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# The cloning and characterization of the bacteriophage D108 regulatory DNA-binding protein ner

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**From the transposable Mu-like bacteriophage D108 we have cloned the *ner* gene under the control of the *lac* UV5 promoter in the expression vector pOP95-15. The recombinant plasmid, pPT011, overproduced the 8-kd D108 ner protein (visualized by *in vitro*-coupled transcription-translation) and served as a substrate for DNA sequencing of the D108 *ner* gene. The ner protein of D108 was found to be 48% homologous to the Mu ner protein, though the DNA sequences that encode these proteins are quite divergent. We used the retardation of migration of $^{32}$P-labelled DNA restriction fragments by ner-containing crude protein extracts in polyacrylamide gels (band competition assay) to determine which DNA restriction fragment(s) contained the ner-binding sites. DNA footprinting using crude extracts physically identified the 47-bp DNA sequence that the ner protein was interacting with in the D108 early gene regulatory region. This sequence is located 10 bp downstream from the presumed D108 early gene transcription initiation site. Therefore, by binding strongly to this 47-bp DNA sequence, the D108 ner protein can regulate D108 early gene transcription.**

*Key words:* bacteriophage D108/band competition assay/DNA-binding regulatory protein/DNA-footprinting/*ner* gene sequence

## Introduction

D108 is a temperate bacteriophage of *Escherichia coli* that was isolated by Mise (1971) as a generalized transducing phage. It was later discovered that D108 is closely related to the temperate plaque-forming transposable phage Mu (Hull *et al.*, 1978). Heteroduplex analysis has shown the two phages to be ~90% homologous at the DNA level (Gill *et al.*, 1981). The most important aspect of the D108:Mu relationship is the non-homology which spans the left end early genes *c*, *ner* and into gene *A* (Toussaint *et al.*, 1983). The Mu-encoded repressor c and the product of the *ner* gene, both of which function at the level of transcription (van de Putte *et al.*, 1980), act to regulate the choice between lytic and lysogenic development and the expression of the transposase (early) operon. The Mu *A* gene product (transposase) mediates DNA transposition by binding to specific DNA sequences at the ends of the Mu and D108 genomes (Craigie *et al.*, 1984). Biological differences such as the heteroimmunity seen between the two phages (Gill *et al.*, 1981; Toussaint *et al.*, 1981, 1983) can all be attributed to differences at the DNA level in the left-end early genes.

We have previously cloned and characterized the Mu ner protein and proposed a possible model of how ner is involved in regulating lytic versus lysogenic development by virtue of its rapid association and dissociation to its operator (Tolias and DuBow, 1985). Here we describe the cloning, DNA sequencing and biochemical characterization of the specific regulatory DNA-binding properties inherent in the gene product of the D108 *ner* gene. The similarities and differences of the *ner* genes of the two phages at the DNA, protein and functional regulatory level are discussed.

## Results

### *Cloning and DNA sequencing of the D108 ner gene and visualization of its gene product*

The D108 *ner* gene was cloned under the control of the *lac* UV5 promoter using the multicopy pBR322 derivative expression vector, pOP95-15 (Fuller, 1982) as indicated in Figure 1 and described in Materials and methods. The resulting plasmid, pPT011, provided us with a good source of the ner protein whose expression could be induced by isopropyl-β-D-thio-galactopyranoside (IPTG) in *E. coli* strain JM103. This strain will be referred to as LF4331. Strain LF121 contains the vector plasmid pOP95-15 in *E. coli* strain JM103. Plasmid pUD78 (Figure 1), containing 1.7 kb of the D108 left-end genome, served as a source of the DNA sequences that the ner protein specifically binds to and regulates.

The DNA sequence of the D108 *ner* gene was determined (as indicated in Materials and methods) and is presented in Figure 2A along with the DNA sequence of the Mu *ner* gene (Priess *et al.*, 1982). The *ner* genes of these two phages differ significantly at the DNA sequence level. However, these two genes are 48% homologous at the encoded amino acid sequence level. Furthermore, the relative positions of 63% of the amino acids are conserved with respect to charge and are concentrated in several blocks of homology. DNA sequence analysis of D108 DNA cloned in plasmid pPT011 along with examination of the DNA sequences present between the *Eco*RI site and the β-lactamase gene of pBR322 (Sutcliffe, 1979), reveals two possible open reading frames which have been introduced into the recombinant plasmid pPT011. One encodes a 73-amino acid polypeptide (ner). The other encodes a 63-amino acid fusion protein composed of the first 47 amino acids of the D108 *A* gene product (Toussaint *et al.*, 1983) fused to 16 amino acids encoded in-frame by pBR322 DNA (Sutcliffe, 1979).

To visualize these pPT011-encoded gene products, we used a prokaryotic DNA-directed *in vitro* translation system. Figure 2B shows that in addition to polypeptides encoded by pOP95-15 (lane 4), pPT011 (lane 3) directs the synthesis of one or more polypeptides with an apparent mol. wt. of 8 kd on 20% SDS-polyacrylamide gels. When the 1.6-kb *Hin*dIII-*Pst*I pPT011 restriction fragment is subjected to *in vitro*-coupled transcription-translation, only one major band is visible (lane 5) which again corresponds to the size of the D108 ner protein and the amino terminus of the gene *A* fusion protein.

### *Characterization of specific DNA-binding activity using the band competition assay*

To demonstrate and characterize the specific DNA-binding activity of the D108 ner protein to its binding site, we modified

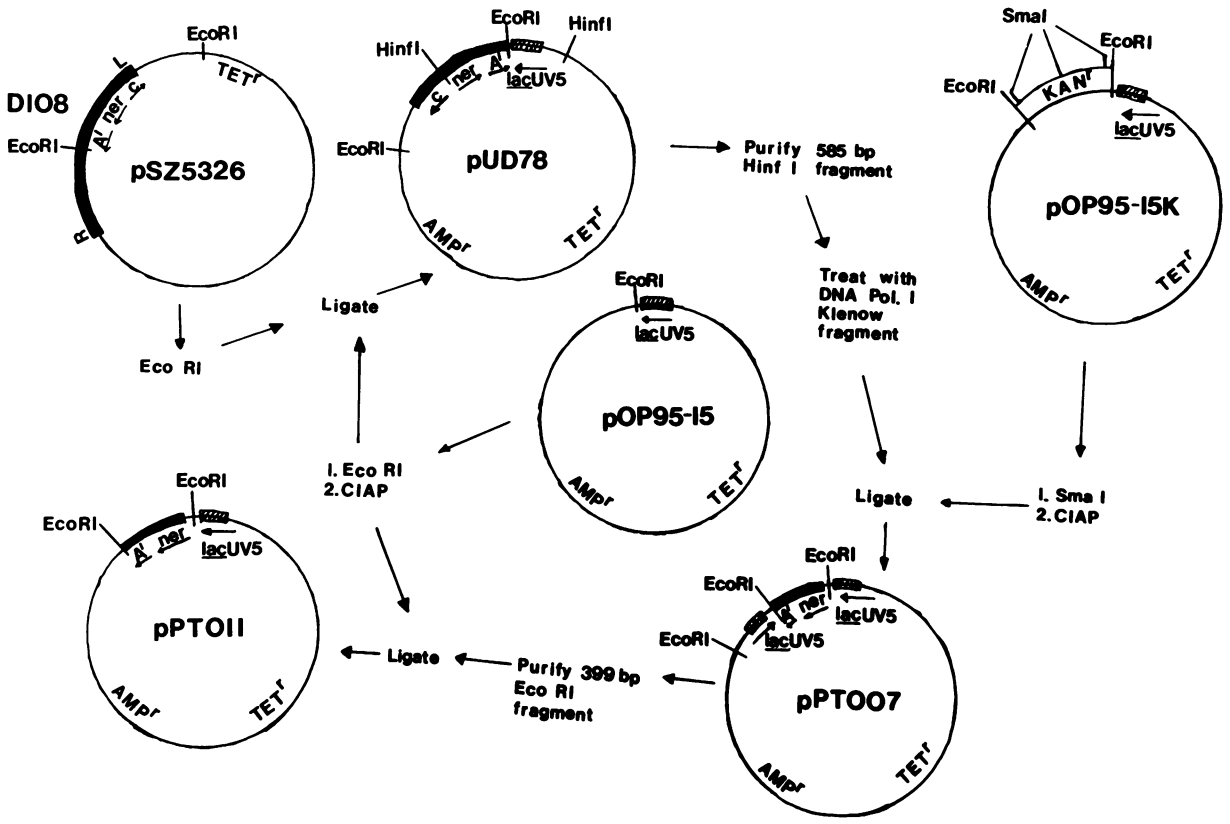


**Fig. 1.** Construction of recombinant plasmids containing the D108 *ner* gene and its regulatory sequences. All plasmids were constructed under the conditions described in Materials and methods. Mini-D108 plasmid pSZ5326 was constructed by Szatmari *et al.* (in preparation). Construction of plasmid pOP95-15 has been previously described (Fuller, 1982). Plasmid pOP95-15K was constructed by J.Harel by inserting the polylinker-flanked kanamycin-resistance gene of Tn903 from pUC71K (Vieira and Messing, 1982) into the *Eco*RI site of pOP95-15 (Fuller, 1982). Plasmid pUD78 was constructed as shown by inserting the D108 *ner* gene-containing *Eco*RI fragment of pSZ5326 into the *Eco*RI site of pOP95-15. Plasmid pPT007 was constructed by 'backfilling' the 585-bp *Hinf*I fragment of pUD78 using the Klenow fragment of DNA polymerase I and subsequent ligation of the blunt end DNA fragment into the outer *Sma*I sites of pOP95-15K. Plasmid pPT011 was constructed by inserting the *ner* gene-containing 399-bp *Eco*RI fragment of pPT007 into the *Eco*RI site of pOP95-15.

the band competition assay (Strauss and Varshavsky, 1984) as described in Materials and methods. This assay permits identification of DNA restriction fragments that are recognized by specific DNA-binding proteins by virtue of the reduced electrophoretic mobility of the DNA on low ionic strength polyacrylamide gels. Conditions were previously determined where a non-specific DNA restriction fragment was not retarded in its electrophoretic mobility (unpublished data).

Figure 3A, lane 1 displays the migration pattern of pUD78 *Sau*96(1)-*Bgl*II-restricted DNA labelled with $^{32}P$ as described in Materials and methods). When this DNA was incubated with LF121 crude extracts prior to electrophoresis (see band competition assay in Materials and methods), the migration pattern remained unaltered (lane 2). However, when the band competition assay was performed using LF4331 crude extracts, a 490-bp fragment was completely retarded in its electrophoretic migration (lane 3), presumably by the interaction of the D108 ner protein (either alone or as a complex with other proteins in the crude extract) with specific DNA sequences present within this DNA restriction fragment. Using this approach, we localized the ner-binding site to a $^{32}P$-labelled 182-bp pUD78 *Dra*I-*Bgl*II restriction fragment which will be referred to as the specific DNA substrate in the experiments that follow. The non-specific $^{32}P$-labelled DNA substrate used was the pBR322 217-bp *Hpa*II restriction fragment and will be referred to as the non-specific DNA substrate.

To demonstrate the specificity and strength of the specific binding of LF4331 crude extracts to the $^{32}P$-labelled specific DNA substrate, a titration experiment was performed where increasing quantities of sonicated calf thymus competitor DNA were added to each reaction of the standard band competition assay. Figure 3B, lane 1 shows that in the absence of LF4331 crude extract, the specific DNA substrate migrated in the gel as a distinct band, while in the presence of LF4331 crude extract (lane 2), the migration of the DNA is retarded. When a sufficiently large excess of unlabelled calf thymus competitor DNA (110 $\mu$g, or a calf thymus DNA/specific DNA substrate weight ratio of 5500) was added, the specific DNA substrate migrated in the gel as a distinct band (lane 10). This experiment was repeated using the $^{32}P$-labelled non-specific DNA substrate. Figure 3C, lane 2 shows that $< 10$ $\mu$g of calf thymus DNA (or a calf thymus DNA/non-specific DNA substrate weight ratio of $< 500$) is required to make the latter migrate as a distinct band. These results show that the specific DNA substrate was bound by LF4331 crude extracts with a much greater affinity compared with the non-specific DNA substrate.

The experiment described above was repeated using LF121 crude extracts. Strain LF121 does not produce ner and we failed to detect any preferential binding specificity for the specific DNA substrate over the non-specific DNA substrate (Figure 3D and E).

To determine the sensitivity of the standard assay, we varied the amount of the LF4331 crude extract added to each standard reaction. Figure 4A and B, lanes 7 – 13 show that the range of

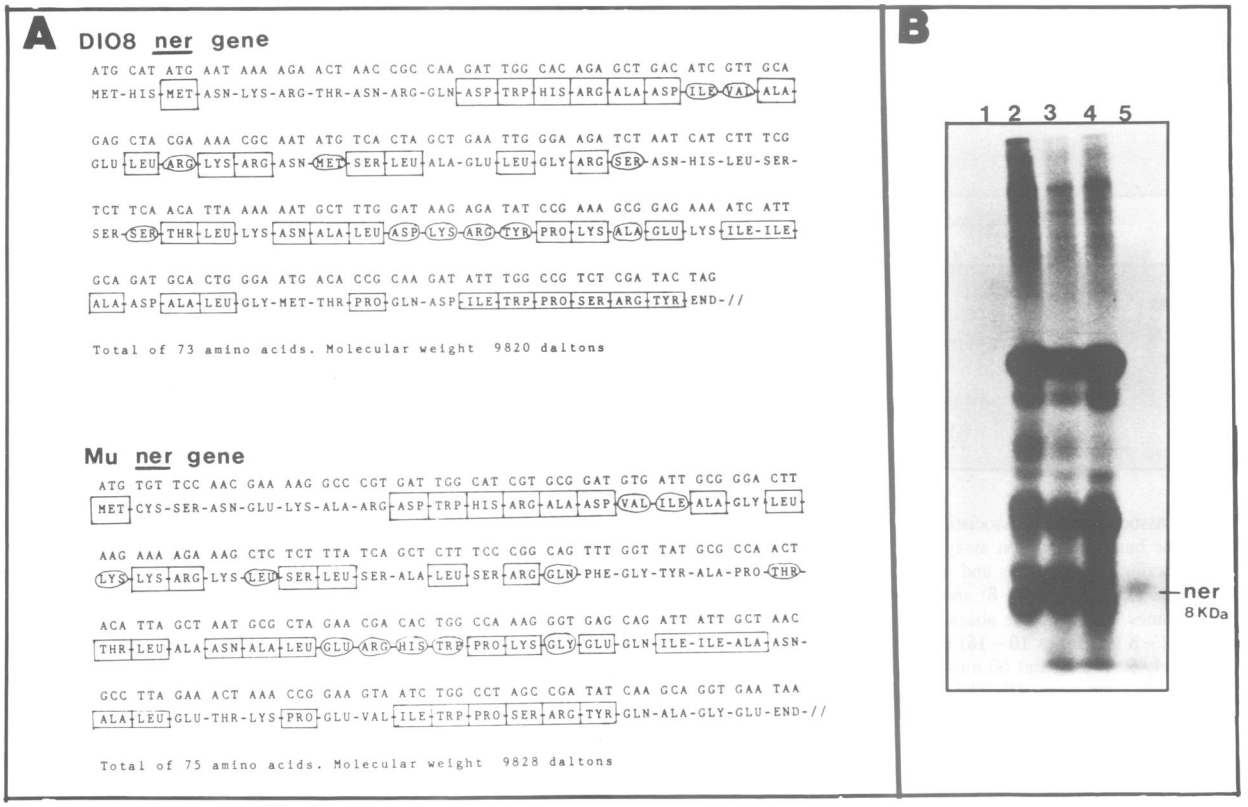


**Fig. 2.** Nucleotide sequence of the *ner* gene of phages Mu and D108 and visualization of the D108 *ner* gene product. (**A**) The nucleotide and amino acid-encoding sequence of the phage D108 *ner* gene (as determined in Materials and methods) is displayed and compared with the *ner* gene of phage Mu (Priess *et al.*, 1982). Boxed amino acids are completely conserved in the two proteins. Circled amino acids are conservative changes yielding no net difference in charge. (**B**) SDS-polyacrylamide gel (20%) electrophoretic analysis of the gene products produced by a DNA-directed *in vitro*-coupled transcription-translation reaction (see Materials and methods). **Lanes 1–5**, show the gene products encoded by no DNA (**1**), pAT153 (Twigg and Sherratt, 1980) (**2**), pPT011 (**3**), pOP95-15 (**4**) and a 1.6-kb pPT011 *Hin*dIII-*Pst*I restriction fragment (**5**).

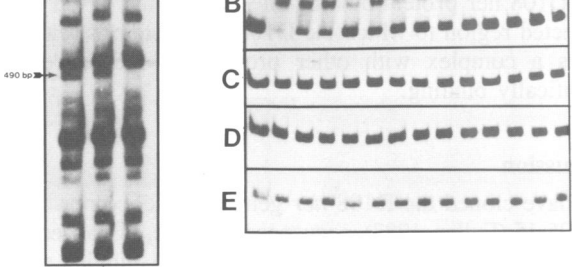


**Fig. 3.** Dectection of specific DNA-binding activity in LF4331 extracts by the band competition assay. (**A**) *Sau*96(1)-*Bgl*II hydrolysed pUD78 DNA was labelled with $^{32}P$ (as described in Materials and methods) and used as a probe in the band competition assay. **Lane 1**, displays the migration pattern of 400 ng of this $^{32}P$-labelled DNA without addition of crude protein extracts under the remaining conditions of the standard band competition assay (as described in Materials and methods); **lanes 2** and **3**, display the same, but in the presence of LF121 and LF4331 crude extracts, respectively. (**B**) The $^{32}P$-labelled 182-bp *Dra*I-*Bgl*II specific DNA substrate was mixed with 1 μg of LF4331 crude extract (as described in the band competition assay of Materials and methods) and increasing amounts of unlabelled sonicated calf thymus DNA. **Lane 1**, displays the migration pattern of the $^{32}P$-labelled specific DNA substrate without addition of LF4331 crude extracts; **lanes 2–14**, display the same but in the presence of LF4331 crude extract with 10, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140 and 150 μg of sonicated calf thymus DNA per assay, respectively. (**C**) Same as in **B**, except that the $^{32}P$-labelled DNA used was the 217-bp *Hpa*II pBR322 restriction fragment. (**D**) Same as in **B**, except that the crude extract used was from strain LF121. (**E**) Same as in **C**, except that the crude extract used was from strain LF121.

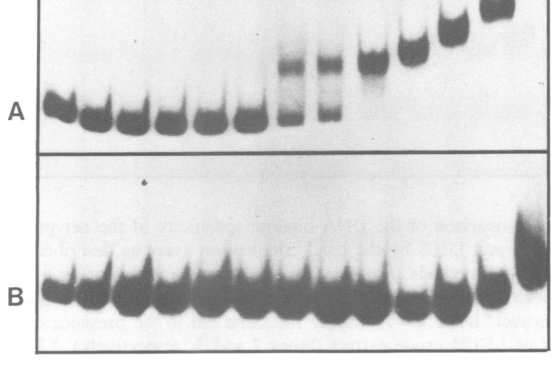


**Fig. 4.** The sensitivity of the band competition assay in detecting specific DNA-binding activity. (**A**) The 182-bp $^{32}P$-labelled pUD78 *Dra*I-*Bgl*II-specific DNA substrate was mixed under the conditions of the standard band competition assay (see Materials and methods) with increasing amounts of LF4331 crude extract. **Lane 1**, displays the migration pattern of the $^{32}P$-labelled DNA without addition of LF4331 crude extracts. **Lanes 2–13**, display the same but in the presence of $2 \times 10^{-3}$, $6 \times 10^{-3}$, $2 \times 10^{-2}$, $6 \times 10^{-2}$, 0.15, 0.35, 0.85, 2, 5, 12.7, 30 and 70 μg of crude extract, respectively. (**B**) Same as in **A**, except that the $^{32}P$-labelled DNA used was the 217-bp *Hpa*II pBR322 restriction fragment.

protein added to the reaction, which still shows specific DNA-binding activity, extends from 0.35 to 70 μg of LF4331 crude extract per standard reaction.

Using the standard assay conditions, we also examined the time

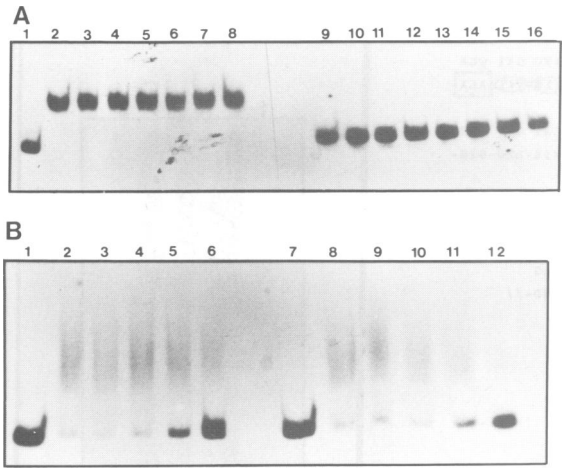


**Fig. 5.** Time of association and dissociation of ner to its binding sites as measured with the band competition assay. (**A**) The standard assay was performed as described in Materials and methods using $^{32}$P-labelled 182-bp *DraI-BglII* pUD78 DNA (**lanes 1–8**) and $^{32}$P-labelled 217-bp *HpaII* pBR322 DNA (**lanes 9–16**) in the absence (**lanes 1** and **9**) and in the presence (**lanes 2–8** and **lanes 10–16**) of LF4331 crude extract and allowing 0.5, 2, 4, 8, 15, 30 and 60 min of binding (**lanes 2–8** and **lanes 10–16**, respectively) per assay. (**B**) The standard assay was performed as described in Materials and methods using a $^{32}$P-labelled 182-bp *DraI-BglII* pUD78 restriction fragment and LF4331 crude extracts. **Lane 1**, displays the migration pattern of the $^{32}$P-labelled DNA without the addition of crude extract. **Lanes 2–6**, show the effect of adding a 20-fold molar excess of unlabelled 182-bp *DraI-BglII* pUD78 restriction fragment after pre-incubating for 10 min (for specific binding to occur) and incubating for an additional time period of 0.5, 2, 5, 15 and 45 min per assay, respectively. **Lanes 7–12**, show the same as **lanes 1–6** except that the 20-fold molar excess of unlabelled competitor used was the 217-bp pBR322 *HpaII* restriction fragment.

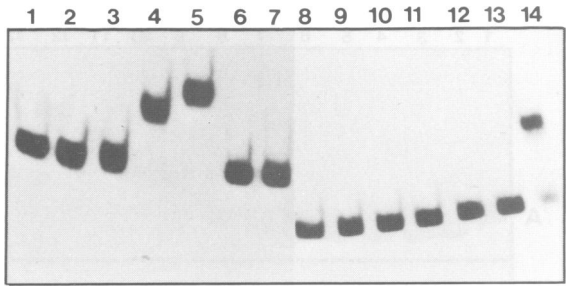


**Fig. 6.** Comparison of the DNA-binding specificity of the ner proteins of phages Mu and D108 by the band competition assay as described in Materials and methods. **Lane 1**, displays the migration pattern of the $^{32}$P-labelled 182-bp *DraI-BglII* pUD78 restriction fragment without addition of crude extract; **lanes 2–7**, display the same but in the presence of 1 and 12.5 μg of LF121 crude extract (**lanes 2** and **3**, respectively), LF4331 crude extract (**lanes 4** and **5**, respectively) and LF123 crude extract (**lanes 6** and **7**, respectively). LF123 crude extracts were from Tolias and DuBow (1985). **Lanes 8–14**, display the same as **lanes 1–7** except that the $^{32}$P-labelled DNA used was the 145-bp *Eco*RI-*Hae*III pUD88 restriction fragment (from Tolias and DuBow, 1985) containing the Mu ner-binding sites.

required for the specific protein-DNA association to be established. Figure 5A, lane 2 shows that by 30 s after the addition of the LF4331 crude extract to the specific DNA substrate, specific DNA-binding is established, whereas no binding specificity is observed with the non-specific substrate even after 60 min of incubation at 37°C (lane 16).

We also determined the length of time required to dissociate the specific binding observed in the standard assay after addition of a 20-fold molar excess of unlabelled specific DNA substrate. Figure 5B, lanes 5 and 6 show that most of the dissociation of the ner-containing LF4331 crude extract from the $^{32}$P-labelled specific DNA substrate was slow and occurred between 15 and 45 min. The unlabelled non-specific DNA substrate was only able to dissociate approximately half of the binding established between the $^{32}$P-labelled specific DNA substrate and LF4331 crude extracts by 45 min of incubation at 37°C (lane 12).

The Mu *ner* gene has previously been cloned and the ner-binding site has been localized (Goosen and van de Putte, 1984; Tolias and DuBow, 1985). This enabled us to determine whether the ner proteins of Mu and D108 are capable of binding to each other's DNA-binding site. Figure 6, lanes 4 and 5 show that the D108 specific DNA substrate is retarded in its electrophoretic mobility by the D108 ner-containing crude extract LF4331, but not by the Mu ner-containing crude extract LF123 (lanes 6 and 7). Furthermore, the Mu-specific DNA substrate containing the Mu ner-binding site [$^{32}$P-labelled 145-bp pUD88 *Eco*RI-*Hae*III restriction fragment from Tolias and DuBow (1985)], is only retarded by the Mu ner-containing extract LF123 in the band competition assay (lane 14) but not the D108 ner-containing crude extract LF4331 (lanes 11 and 12).

*Physical localization of the ner-binding site*

The $^{32}$P end-labelled specific DNA substrate used in the standard band competition assay was used as a substrate for mapping the ner-binding site. We modified a method to map physically the ner-binding region by DNase I footprinting using crude extracts of LF4331 and LF121. Figures 7A and B, lanes 7–16, show 10% polyacrylamide-urea gels with increasing LF4331 versus LF121 crude protein extracts in the DNase I footprinting reaction (see Materials and methods), respectively. The region protected from DNase I digestion by LF4331 (but not by LF121) crude extracts is 47 bp in length and is displayed in Figure 7C. Further examination of the structure of this region reveals two perfect inverted repeats of 11 bp composed of the consensus sequence 5′-CCGTGAGCTAC-3′ that are separated by a spacer region of 7 bp. This may suggest the existence of two D108 ner protein-binding sites within the 47-bp DNase I-protected region to which the D108 ner protein is (either alone or as a complex with other proteins in the crude extract) specifically binding.

## Discussion

We have cloned the D108 *ner* gene into the expression vector pOP95-15 (Fuller, 1982), sequenced the gene, visualized the gene product, and characterized its specific DNA-binding activity *in vitro*. Similar experiments have been performed with the *ner* gene of phage Mu (Tolias and DuBow, 1985). These studies enabled us to compare, physically and functionally, the *ner* genes of the two phages and their encoded gene products.

The *ner* gene of phage D108 encodes a 73-amino acid polypeptide as deduced from our sequencing of the gene. This polypeptide has an apparent mol. wt. of 8 kd on SDS-polyacrylamide gels. Similarly, the *ner* gene of phage Mu encodes a polypeptide composed of 75 amino acids (Priess *et al.*, 1982) with an apparent mol. wt. on SDS-polyacrylamide gels of 8 kd (Magazin *et al.*, 1978; Giphart-Gassler *et al.*, 1981). However, the *ner* gene of both phages seems to share little homology at the DNA level but displays a 48% homology at the deduced amino acid sequence level. Furthermore, conserved amino acids are concentrated in several distinct blocks of homology and the relative positions of 63% of the amino acids are conserved with respect to charge. Note also that the D108 ner protein is two amino acids

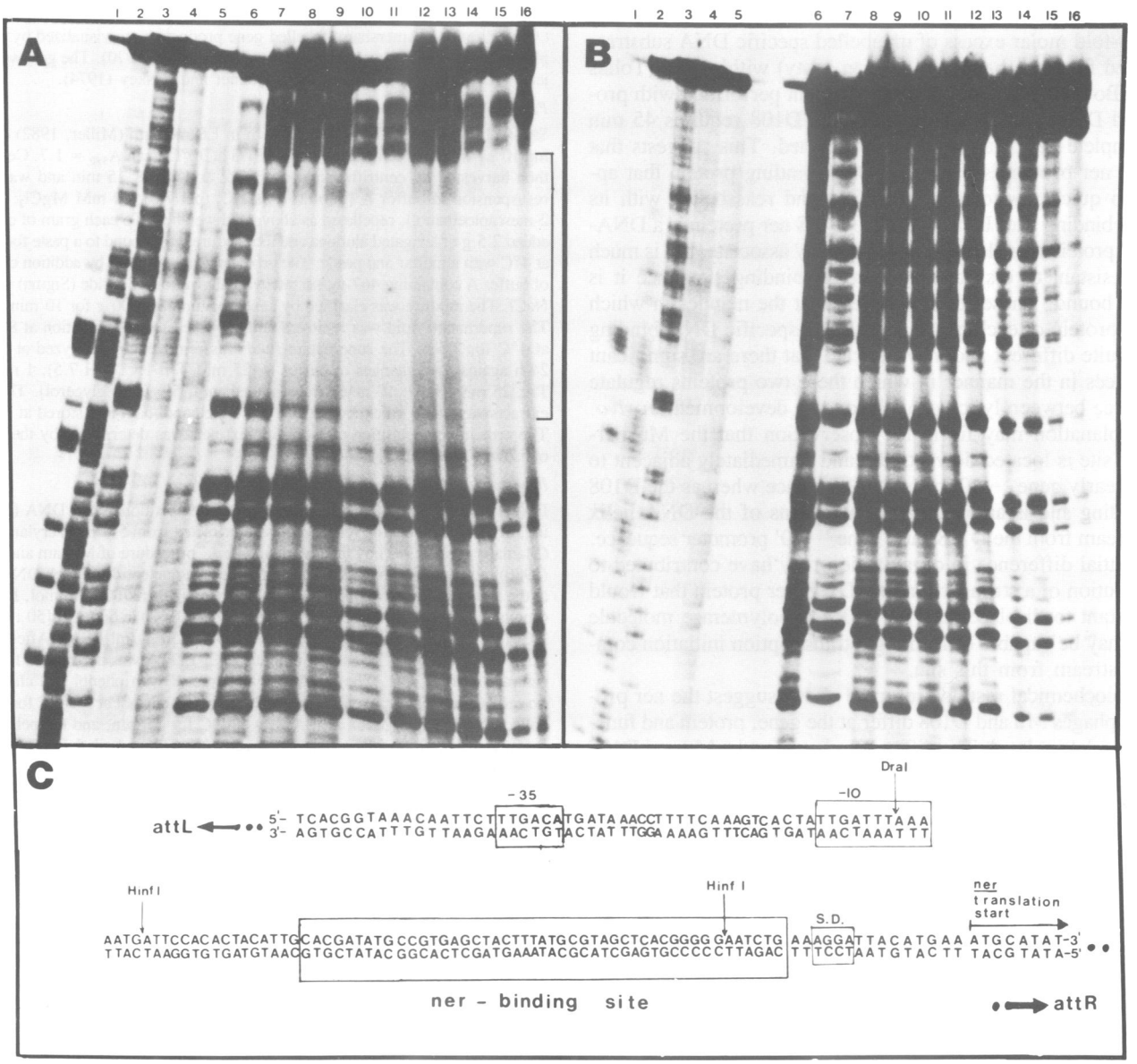


**Fig. 7.** DNase I-footprinting from crude extracts. (**A**) DNase I-footprinting and Maxam and Gilbert DNA sequencing were performed using the 182-bp *Dra*I-*Bgl*II pUD78 restriction fragment labelled with $^{32}P$ at the *Bgl*II site as described in Materials and methods. **Lanes 1–5**, display the DNA banding pattern obtained from the Maxam and Gilbert DNA sequencing reactions G, G+A, T+C, C and A>C, respectively. **Lanes 6–16**, display the banding pattern obtained when using LF4331 crude extracts at a concentration of 0, 10, 20, 30, 40, 50, 60, 75, 100, 150 and 200 μg/ml per DNA-footprinting reaction, respectively. (**B**) Same as in **A**, except that the crude extract used in the DNA-footprinting reactions was from strain LF121. (**C**) DNA sequence from the 5′ side of the D108 *ner* gene translation start codon with the relative orientation on the phage genome displayed by *att*L near the left-end of the sequence and *att*R near the right-end of the sequence. Indicated is the ner-binding site and sequences with a possible role in the initiation of translation ['.S.D.' from Shine and Dalgarno (1974)] and transcription ['−35' and '−10' from Rosenberg and Court (1979)].

longer at the amino terminus and four amino acids shorter at the carboxyl terminus compared with the Mu ner protein. These observations suggest that the ner proteins of phages Mu and D108 have diverged physically, though they have maintained a significant degree of structural homology.

Many DNA-binding proteins display considerable homologies at key amino acid positions which usually occur in the designated helix 2, helix 3 DNA-binding region and the link between them. Some of the proteins that show considerable homologies in this region include the phages λ, 434 and P22 repressor and cro proteins as well as the *lac* repressor protein of *E. coli* (Takeda *et al.*, 1983). However, the Mu and D108 ner proteins show no significant homologies to any of the above-mentioned proteins in the helix 2, helix 3 region nor in the link between them.

The results presented in Figure 6 suggest that the Mu and D108 ner proteins are incapable of binding to each other's DNA-binding site (as deduced by the band competition assay). Physical mapping of the DNA-binding site by DNA footprinting shows that the D108 ner-binding site (Figure 7A) is 47 bp in length and shows no sequence homology with the 33-bp Mu ner-binding site (Goosen and van de Putte, 1984; Tolias and DuBow, 1985). In contrast to the Mu ner-binding site which consists of the directly repeated consensus sequence 5′-ANPyTAGPyTAAPuT-3′ separated by a 6-bp spacer (Tolias and DuBow, 1985), the D108 ner-binding site is organized as two 11-bp perfect inverted repeats composed of the consensus sequence 5′-CCGTGAGCTAC-3′ separated by a 7-bp spacer. Furthermore, we have previously shown that the binding established between the Mu ner protein

and its $^{32}$P-labelled specific DNA substrate can be dissociated by a 20-fold molar excess of unlabelled specific DNA substrate (deduced from the band competition assay) within 30 s (Tolias and DuBow, 1985). This same experiment performed with protein and DNA components from phage D108 requires 45 min for complete dissociation to be established. This suggests that the Mu ner protein is a specific DNA-binding protein that appears to quickly associate, dissociate and reassociate with its specific binding site. In contrast, the D108 ner protein is a DNA-binding protein that also appears to quickly associate, but is much more resistant to dissociation from its binding site once it is already bound. These results suggest that the manner in which the ner protein of each phage binds to its specific DNA-binding site is quite different and may indicate that there are significant differences in the manner in which these two proteins regulate the choice between lytic versus lysogenic development *in vivo*. The explanation may lie in the observation that the Mu ner-binding site is located downstream and immediately adjacent to the Mu early gene '−10' promoter sequence whereas the D108 ner-binding site is approximately two turns of the DNA helix downstream from the D108 early gene '−10' promoter sequence. This spatial difference in organization may have contributed to the evolution of a stronger binding D108 ner protein that would be resistant to displacement by an RNA polymerase molecule which may be capable of forming a transcription initiation complex upstream from this site.

The biochemical results presented above suggest the ner proteins of phages Mu and D108 differ at the gene, protein and functional regulatory level. The differences between the Mu and D108 *ner* genes and their gene products that we and others have reported *in vitro* confirm differences observed *in vivo* (Gill *et al.*, 1981; Hull *et al.*, 1978; Toussaint *et al.*, 1981, 1983).

## Materials and methods

*Cloning of the D108 ner gene*

All enzymes were purchased from Bethesda Research Laboratories, Inc. (BRL), New England Nuclear (NEN), or Boehringer Mannheim Canada Ltd. (BMC). Buffers used for cloning included restriction endonuclease digestion buffer [75 mM NaCl; 125 μg/ml bovine serum albumin (BSA: Pentex, Fraction V, Miles Laboratories, Elkart, Indiana); 6 mM 2-mercaptoethanol; 6 mM Tris-HCl (pH 7.5); 6 mM $MgCl_2$] and T4 DNA ligase buffer [60 mM Tris-HCl (pH 7.4); 10 mM $MgCl_2$; 15 mM dithiothreitol (DTT); 1 mM spermidine; 0.75 mM ATP; 50 μg/ml autoclaved gelatin]. The D108 *ner* gene was subcloned as indicated in Figure 1 under *lac* UV5 control into the expression vector pOP95-15 (Fuller, 1982). Restriction enzyme-hydrolysed vector plasmid was treated with calf intestinal alkaline phosphatase (CIAP) (NEN) to prevent self-ligation (Ullrich *et al.*, 1977). Ligation of insert to vector at a molar ratio of 3:1, was carried out for 18 h at 15°C with T4 DNA ligase (BRL), *E. coli* strain JM103 [Δ (*lac pro*), *thi*, *rsp*L, *sup*E, *end*E *sbc*B, *hsd*R$^-$, F′ *tra*D36, *pro*AB, *lac*I$^q$, ZΔM15] was transformed (Mandel and Higa, 1970) with the ligated DNA mixture and recombinant plasmids were selected on LB plates (Miller, 1972) containing tetracycline (10 μg/ml). These colonies were then screened for ampicillin sensitivity and pseudo-immunity to D108 *c*ts10 superinfection in the presence of 1 mM IPTG at 37°C (Chaconas *et al.*, 1981). D108 *c*ts10 phage from strain LF4028 [Δ (*lac pro*), *trp*, *rps*E, *rps*L, D108$^R$, F′ *pro lac*I::D108 *c*ts10] were purified ($5.5 \times 10^{10}$ p.f.u./ml) according to Ljungquist and Bukhari (1977). Strain LF4331 contained plasmid pPT011 (Figure 1) and served as a source of the D108 *ner* gene product in the experiments that followed. Strain LF121 was constructed by transforming JM103 with pOP95-15 and served as an isogenic negative control strain in the experiments that followed. Strain LF123 is JM103 containing the plasmid pUD88 which encodes the Mu ner protein (Tolias and DuBow, 1985) and served as the source of the Mu ner protein.

*Visualization of pPT011-encoded gene products*

Gene products were visualized by *in vitro*-coupled transcription-translation of DNA templates. Plasmids purified by cesium chloride-ethidium bromide equilibrium centrifugation (Maniatis *et al.*, 1982) or a 1.6-kb *Hind*III-*Pst*I pPT011 restricton fragment purified by the 'crush and soak' procedure of Maxam and Gilbert (1980), were used as templates for prokaryotic DNA-directed *in vitro* translation (Amersham Kit N.380) in the presence of 0.6 mM IPTG. [$^{35}$S]methionine (1120 Ci/mmol, Amersham) labelled gene products were visualized by electrophoresis on 20% polyacrylamide SDS gels (Laemmli, 1970). The gels were subjected to fluorography according to Bonner and Laskey (1974).

*Preparation of crude extracts*

Strains LF4331 and LF121 were grown in LB medium (Miller, 1982) containing 10 μg/ml tetracycline and 1 mM IPTG at 32°C to an $A_{550} = 1.7$. Cells were then harvested by centrifugation at 4000 *g* at 4°C for 15 min and washed by resuspension in buffer A [10 mM Tris-HCl (pH 7.5); 10 mM $MgCl_2$; 10 mM 2-mercaptoethanol], repelleted as above and weighed. To each gram of cells was added 2.5 g of levigated alumina and the mixture was ground to a paste for 10 min at 4°C with a mortar and pestle. The proteins were extracted by addition of 1.5 ml of buffer A containing 467 μg/ml phenylmethylsulfonylfluoride (Sigma) and 1 M NaCl. The mixture was clarified by centrifugation at 4000 *g* for 10 min at 4°C. The supernatant fluid was removed and subjected to centrifugation at 80 000 *g* at 4°C for 2.5 h. The supernatant fluid was removed and dialyzed at 4°C for 24 h against two changes of buffer B [25 mM Tris-HCl (pH 7.5); 1 mM EDTA; 25 mM NaCl; 10 mM 2-mercaptoethanol; 5% (v/v) glycerol]. The crude extracts were then quick frozen in a dry ice ethanol bath and stored at −70°C. The protein concentration of the crude extracts was determined by the method of Lowry *et al.* (1951).

*In vitro labelling and sequencing of DNA*

DNA restriction fragments used for the band competition assay, DNA footprinting, or DNA sequencing reactions, were extracted from 5% polyacrylamide gels (Maniatis *et al.*, 1982) by the 'crush and soak' procedure of Maxam and Gilbert (1980), and end-labelled by 'backfilling' the first nucleotide of each DNA (2 μg) at the recessed 3′ end with 500 μCi/ml [α-$^{32}$P]dNTPs (3700 Ci/mmol, ICN) and 6 units of DNA polymerase I Klenow fragment (BMC) in buffer C [50 mM Tris-HCl (pH 7.2); 10 mM $MgSO_4$; 0.1 mM DTT; 50 μg/ml BSA]. After incubation for 90 min at 15°C and 30 min at 25°C, EDTA was added to a final concentration of 10 mM. The DNAs were extracted with phenol and chloroform (to remove proteins), precipitated with 2.5 volumes ethanol at −70°C for 10 min, collected by centrifugation at 15 000 *g* at 4°C for 15 min, and the pellets were resuspended in 100 μl of sterile deionized $H_2O$. The labelled DNAs were sequenced according to the procedure of Maxam and Gilbert (1980).

The *ner* gene was sequenced by 'backfilling' at the internal *Bgl*II site and by extracting the pPT011 399-bp *Eco*RI restriction fragment as above and removing the 5′ phosphates at 65°C for 1 h with 20 units/μg DNA of bacterial alkaline phosphatase (BRL) in 20 mM Tris-HCl, (pH 8). Following three extractions with phenol, two with chloroform, and one with ether, the DNA was precipitated with ethanol as above. The DNA pellet was resuspended in 50 μl of buffer D [50 mM Tris-HCl, (pH 7.6); 10 mM $MgCl_2$; 5 mM DTT; 0.1 mM EDTA; 0.1 mM spermidine] containing 150 μCi [γ-$^{32}$P]ATP (3000 Ci/mmol, Amersham) and 15 units of T4 polynucleotide kinase (BRL) at 37°C for 30 min. Each strand of the pPT011 399-bp *Eco*RI restriction fragment was separated on a strand separating gel (Maniatis *et al.*, 1982) and sequenced according to Maxam and Gilbert (1980).

*Preparation of sonicated calf thymus DNA*

Calf thymus DNA (Sigma) was allowed to dissolve into solution at a concentration of 5 mg/ml in TE buffer [10 mM Tris-HCl (pH 7.5); 1 mM EDTA] at 4°C for 5 days. RNase A (BMC) was added to a concentration of 5 μg/ml for 2 h at 37°C followed by hydrolysis with self-digested Pronase (BMC) at a final concentration of 1 mg/ml for 3 h at 37°C. The DNA was extracted three times with phenol and chloroform, twice with ether and then extensively dialyzed against TE buffer. The DNA was then sonicated to an average chain length of 500 bp with a Branson model 185 sonifier.

*Band competition assay*

This is a modification of the technique of Strauss and Varshavsky (1984). We determined the following conditions (which will be referred to as the standard assay) to display specific DNA-binding activity. 20 ng of $^{32}$P-labelled DNA restriction fragment, 10 μg sonicated calf thymus DNA and 1.0 μg of crude protein extract were incubated in a total reaction volume of 36 μl in buffer B. The crude extract was added last and the reaction was incubated at 37°C for 10 min, and subjected to electrophoresis on a 5% polyacrylamide gel (Maniatis *et al.*, 1982) in TBE buffer (89 mM Tris-borate; 89 mM boric acid; 2 mM EDTA). The gel (0.1 × 12 cm) was pre-electrophoresed for 1 h at 16 V/cm. Electrophoresis was performed at room temperature at 16 V/cm until the bromophenol blue dye reached the bottom of the gel. The gel was soaked in 5% (v/v) glycerol for 30 min, dried and autoradiographed on Dupont Cronex intensifying screens at −70°C using Agfa Curix RPI film.

*DNA footprinting with DNase I and crude extracts*

Proteins were allowed to bind to the DNA at 25°C for 15 min in a 100 μl reaction mixture containing buffer B, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 104 μg/ml sonicated calf thymus DNA, 50 − 100 ng $^{32}$P end-labelled (by 'backfilling' at the *Bgl*II site) pUD78 182-bp *Dra*I-*Bgl*II DNA restriction fragment and increasing

amounts of LF4331 or LF121 crude extracts. DNase I (Worthington) was then added for 30 s at 25°C to a final concentration of 400 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 according to Galas and Schmitz (1978)]. The DNA was quickly extracted with phenol and chloroform followed by precipitation with ethanol as above. The reaction products were then electrophoresed on 10% polyacrylamide-7 M urea gels as described by Maxam and Gilbert (1980). The 182-bp *DraI-BglII* pUD78 restriction fragment was sequenced according to Maxam and Gilbert (1980) and subjected to electrophoresis on the same gel as the footprinting reactions. The gels were autoradiographed on Dupont Cronex intensifying screens at −70°C using Agfa Curix RPI film.

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## References

Bonner,W.M. and Laskey,R.A. (1974) *Eur. J. Biochem.*, **46**, 83-88.
Chaconas,G., de Bruijn,F.J., Casadaban,M.J., Lupski,J.R., Kwoh,T.J., Harshey, R.M., DuBow,M.S. and Bukhari,A.I. (1981) *Gene*, **13**, 37-46.
Craigie,R., Mizuuchi,M. and Mizuuchi,K. (1984) *Cell*, **39**, 387-394.
Fuller,F. (1982) *Gene*, **19**, 43-54.
Galas,D.J. and Schmitz,A. (1978) *Nucleic Acids Res.*, **5**, 3157-3170.
Gill,G.S., Hull,R.C. and Curtis,R.,III (1981) *J. Virol.*, **37**, 420-430.
Giphart-Gassler,M., Reeve,J. and van de Putte,P. (1981) *J. Mol. Biol.*, **145**, 165-191.
Goosen,N. and van de Putte,P. (1984) *Gene*, **30**, 41-46.
Hull,R.C., Gill,G.S. and Curtis,R.,III (1978) *J. Virol.*, **27**, 513-518.
Laemmli,U.K. (1970) *Nature*, **227**, 680-685.
Ljungquist,E. and Bukhari,A.I. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 3143-3147.
Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J. (1951) *J. Biol. Chem.*, **193**, 265-275.
Magazin,M., Reeve,J.N., Maynard-Smith,S. and Symonds,N. (1978) *FEMS Microbiol. Lett.*, **4**, 5-9.
Mandel,M. and Higa,A. (1970) *J. Mol. Biol.*, **53**, 159-162.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*, published by Cold Spring Harbor Laboratory Press, NY.
Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499-560.
Miller,J.H. (1972) *Experiments in Molecular Genetics*, published by Cold Spring Harbor Laboratory Press, NY.
Mise,K. (1971) *J. Virol.*, **7**, 168-175.
Priess,H., Kamp,D., Kahmann,R., Brauer,B. and Delius,H. (1982) *Mol. Gen. Genet.*, **186**, 315-321.
Rosenberg,M. and Court,D. (1979) *Annu. Rev. Genet.*, **13**, 319-353.
Shine,J. and Dalgarno,L. (1974) *Proc. Natl. Acad. Sci. USA*, **71**, 1342-1346.
Strauss,F. and Varshavsky,A. (1984) *Cell*, **37**, 889-901.
Sutcliffe,J.G. (1979) *Cold Spring Harbor Symp. Quant. Biol.*, **43**, 77-90.
Takeda,Y., Ohlendorf,D.H., Anderson,W.F. and Matthews,B.W. (1983) *Science (Wash.)*, **221**, 1020-1026.
Tolias,P.P. and DuBow,M.S. (1985) *Virology*, in press.
Toussaint,A., Faelen,M. and Resibois,A. (1981) *Gene*, **14**, 115-119.
Toussaint,A., Faelen,M., Desmet,L. and Allet,B. (1983) *Mol. Gen. Genet.*, **190**, 70-79.
Twigg,A.J. and Sherratt,D. (1980) *Nature*, **283**, 216-218.
Ullrich,A., Shine,J., Chirgwin,J., Pictet,R., Tischer,R., Rutter,W.J. and Goodwin,H.M. (1977) *Science (Wash.)*, **196**, 1313-1319.
van de Putte,P., Giphart-Gassler,M., Goosen,N., Goosen,T. and van Leerdam,E. (1980) *Cold Spring Harbor Symp. Quant. Biol.*, **45**, 347-353.
Vieira,J. and Messing,J. (1982) *Gene*, **19**, 259-268.

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