Mapping of binding sites for Mu repressor and *ner* product within the left-end *Eco*RI.C fragment of the Mu genome

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Bacterial cells containing the *ner* gene of phage Mu inserted into pBR322 express a binding activity with specificity for the left-end *Eco*RI.C fragment of Mu DNA. Crude extracts containing either Mu repressor or *ner* protein have been used to localize binding sites on *Taq*I subfragments of the *Eco*RI.C fragment. There are at least 3 distinct binding sites for the Mu repressor and 1 binding site for the *ner* protein on the *Eco*RI.C fragment. The possible biological function of these binding sites is discussed.

Phage Mu *Repressor* ner *Protein* *DNA-binding assay* *Binding site* *Regulation*

1. INTRODUCTION

The temperate *E. coli* phage Mu has been shown to code for a gene product, the Mu repressor, which is synthesized during the lysogenic state and which specifically binds to Mu DNA [1,2]. Furthermore, it has been demonstrated that there are at least two different binding sites for the Mu repressor on the left-end *Eco*RI.C fragment (5.1. kb), and none on the rest of the Mu genome [1]. From genetic and biochemical experiments, it was concluded that the product of another gene, the *ner* protein, could also bind to Mu DNA, thereby regulating the expression of the early functions [3]. Here, we show that the *ner* gene indeed codes for a protein which binds specifically to Mu DNA. In addition, 3 distinct binding sites for the repressor and one for the *ner* protein have been localized.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

The *E. coli* strain EB505 (*thy rpsL recA*) is a K12 derivative. The plasmids pBR322 [4], pMB9 [5], pKN001 [6], and pKN05 [7] have been described. The construction of the plasmids pKN140, pKN240, pKN241, pKN242, and pKN243 will be published elsewhere (in preparation). These latter pKN plasmids harbour different parts of the left end *Eco*RI.C fragment of Mu DNA (fig.1). Bacteria were routinely grown in LB broth (per liter: tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g; pH 7.4).

2.2. *Preparation of crude extracts*

Bacteria were grown in 500 ml LB broth to 4×10^8 cells/ml, chilled on ice and pelleted. The pellets were frozen in liquid nitrogen and stored at $-70°C$. Crude extracts were prepared from frozen pellets as in [8] and used without dialysis. The protein concentration was determined as in [9] using bovine serum albumin as a standard.

2.3. *DNA-binding assay*

The DNA-binding assay in [10] was modified slightly by using sonicated chicken-blood DNA and by incubating for 5 min at 0°C. The background binding of labeled DNA in the absence of any extract was below 1% of the input radioactivity for all substrate DNAs and was subtracted from all values. The preparation of [^{3}H]thymidine labeled plasmid DNA has been described [1].

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3. RESULTS AND DISCUSSION

3.1. *The recombinant plasmid pKN240 inheriting gene* ner *expresses a Mu DNA specific binding activity*

Bacteria which harbour the hybrid plasmid pKN240 encoding the *ner* gene (fig.1) do not allow propagation of infecting Mu phages (not shown). To correlate this superinfection immunity to the action of a DNA-binding protein expressed from pKN240, crude extracts were prepared from EB505/pKN240 and tested for the presence of a DNA-binding protein with specificity for Mu DNA. The binding of pKN001 harbouring the *Eco*RI.C fragment of Mu DNA and of pMB9 (control; vector plasmid of pKN001) is given in fig.2 and exhibits a more pronounced increase as compared to the control plasmid pMB9. The specific binding activities calculated from the linear part of the binding curves at 100 μg protein are given in table 1. In a control experiment, crude extracts from EB505/pMB9 produced specific binding activities of about 80 with both pMB9 and pKN001 as substrate DNAs (not shown).

To ascertain the specificity of the binding reaction for the left end Mu DNA, a competition experiment was carried out with increasing amounts of unlabeled DNA of pMB9 and pKN001; 5 μg unlabeled pKN001 DNA is sufficient to reduce the amount of labeled pKN001 DNA retained on filters by ~70% (fig.3). Addition of the same amount of unlabeled pMB9 DNA to the binding mixture resulted in a reduction of only 10%. From these results we conclude that pKN240 codes for a binding activity which specifically binds to the left end Mu DNA. As the *Taq*I-fragment of Mu DNA carried by pKN240 codes for only one complete Mu gene, the *ner* gene [11], we further conclude that this binding activity is caused by the *ner* protein in the crude extract.

3.2. *Mapping of Mu repressor binding sites*

The left-end *Hin*dIII fragment of Mu DNA (1.0 kb; contained within pKN05) expresses the repressor gene *c* [1,2]. We also found two different binding sites for the Mu repressor on the *Eco*RI.C fragment. To localize these binding sites more precisely, recombinant plasmids containing all but 1 of the 5 *Taq*I subfragments of the *Eco*RI.C fragment inserted into pBR322 were tested as substrates for the Mu repressor. Crude extracts from EB505/pKN05 which contains the repressor protein, were incubated with [^{3}H]DNA of pBR322 (control), pKN140, pKN240, pKN241, pKN242

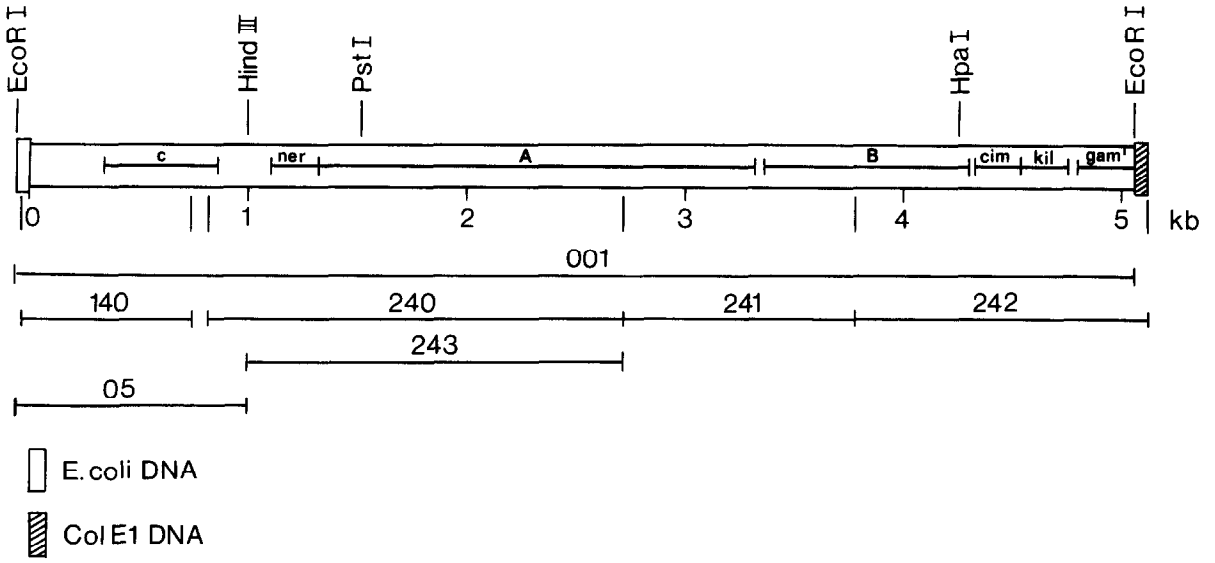

Fig.1. Physical and genetic map of the left-end *Eco*RI.C fragment of Mu indicating the DNA present in the pKN plasmids. The vertical lines below the Mu DNA represent *Taq*I restriction sites. The location of Mu genes is based on work of [11] and [12].

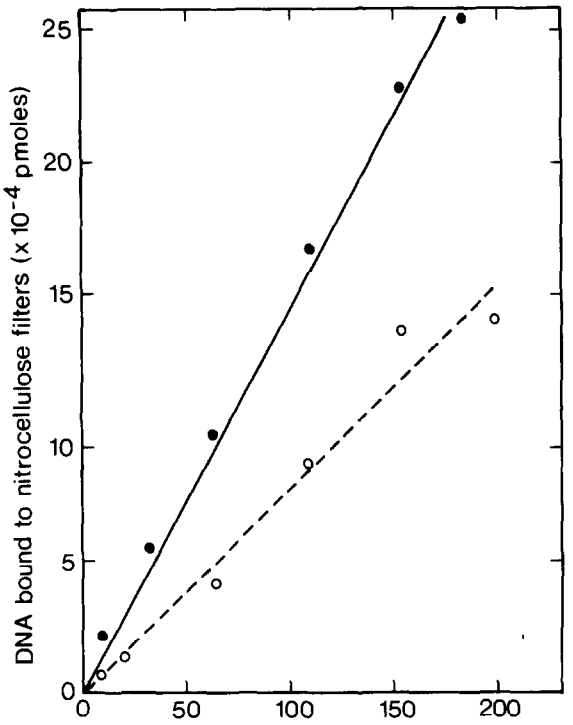

Fig.2. Binding of plasmid DNA to nitrocellulose filters by crude extract from EB505/pKN240. The indicated concentrations of crude extract were incubated with 40×10^{-3} pmol [^{3}H]DNA. Each point represents the average retention on 3 filters after subtraction of the background binding in the absence of any extract. Triplicate determinations differed by $\leqslant 10\%$ from each other. Radioactively labeled DNA of pKN001 (●), and pMB9 (○).

and pKN243. The binding curves are presented in fig.4; the specific binding activities are given in table 1. Whereas the binding curves with pKN140 and pKN242 as substrate are not different from those with the vector plasmid pBR322, much more DNA from plasmids pKN240, pKN241 and pKN243 was retained on nitrocellulose filters when increasing amounts of protein were used in the binding mixture.

We conclude that there are at least 3 distinct binding sites for Mu repressor on the *Eco*RI.C fragment: one to the left of the *Hin*dIII site [1]; a second to the right of the *Hin*dIII site (pKN243); and a third around genes *A* and *B* (pKN241). The possible biological significance of these binding sites will be discussed at the end of section 3.3.

Table 1

Specific binding of cloned fragments of Mu DNA by extracts containing repressor or *ner* protein

Plasmid	Extract containing			
	Repressor protein		*ner* Protein	
	DNA bound[a]	Binding efficiency[b]	DNA bound	Binding efficiency
pMB9	–	–	80	1
pKN001	–	–	150	1.9
pBR322	60	1	62	1
pKN140	50	0.8	62	1
pKN240	132	2.2	47	0.8
pKN241	110	1.8	150	2.4
pKN242	50	0.8	66	0.9
pKN243	142	2.4	–	–

[a] 10^{-4} pmol retained on filter/mg protein in binding mixture

[b] Binding relative to the vector plasmid

3.3. *Mapping of a* ner *binding site*

Crude extracts from EB505/pKN240 containing *ner* protein were incubated with the same set of plasmids described in fig.4, and from the specific binding activities listed in table 1, it is evident that plasmid pKN241 is more efficiently trapped on

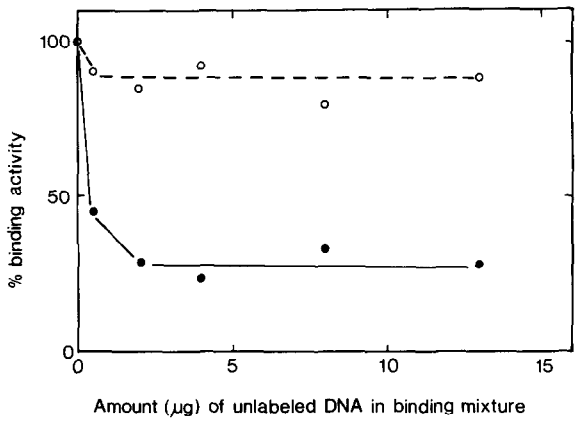

Fig.3. DNA competition for a fixed amount of crude extract. The reaction mixture contained 330 ng pKN001 [^{3}H]DNA and the indicated concentration of unlabeled pKN001 (●) or pMB9 (○) competing DNA plus a constant amount of crude extract (60 μg protein) of EB505/pKN240.

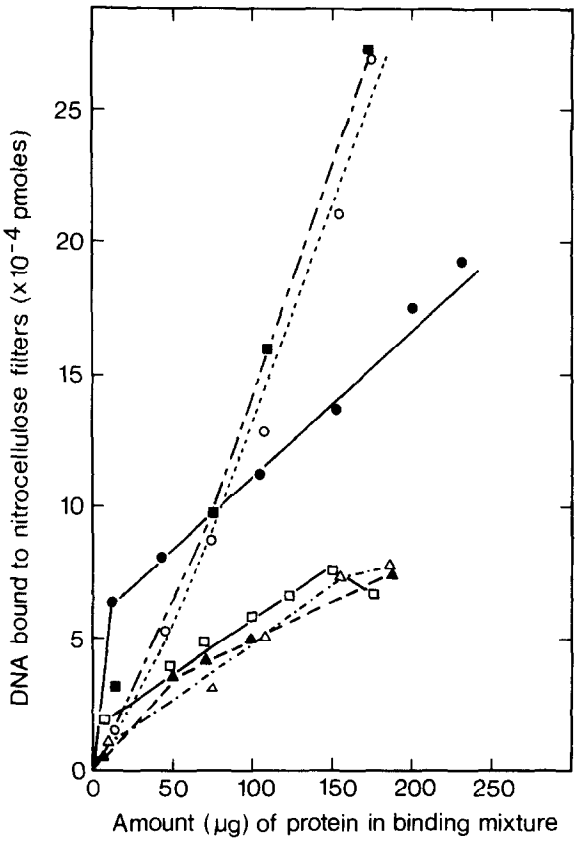

Fig.4. Binding of plasmid DNA to nitrocellulose filters by crude extracts from EB505/pKN05 containing repressor protein. The conditions were as in fig.1. Radioactively labeled DNA of pBR322 (□), pKN140 (△), pKN242 (▲), pKN240 (○), pKN241 (●), and pKN243 (■).

nitrocellulose filters than all the other plasmids tested (see also fig.5). This result suggests that there is only one *ner* protein binding site on the *Eco*RI.C fragment. Recent experiments have revealed that there is at least one additional binding site for the *ner* protein on the right-end *Eco*RI.B fragment of Mu DNA (unpublished).

What might be the biological significance of the repressor and *ner* binding sites on the left-end Mu DNA? By binding to the left of the *Hin*dIII site, the repressor most probably regulates its own synthesis. The binding site to the right of the *Hin*dIII restriction site might overlap with the early promoter of Mu DNA, and binding of repressor to this operator would then prevent expression of the early functions. The biological significance of the third binding site on the *Taq*I fragment within pKN241 is difficult to explain at the moment. This binding site might be located within the C-terminal part of gene *A*, between genes *A* and *B*, or within the N-terminal part of gene *B* (fig.1). It is tempting to speculate that the binding site might be located within the 41 bp between genes *A* and *B* [12] and represent a second operator within the early region. Repressor bound to the operator in front of gene *ner* does not completely prevent transcription of the early genes starting with gene *ner* and proceeding through genes *A* and *B* towards the *kil* gene (fig.1). As a result there is a low level expression of, e.g., the *kil* gene which is deleterious to the bacteria cell. Binding to the second operator may be necessary to fully abolish expression of the *kil* gene.

During the lytic cycle, expression of the early function is negatively regulated by the product of

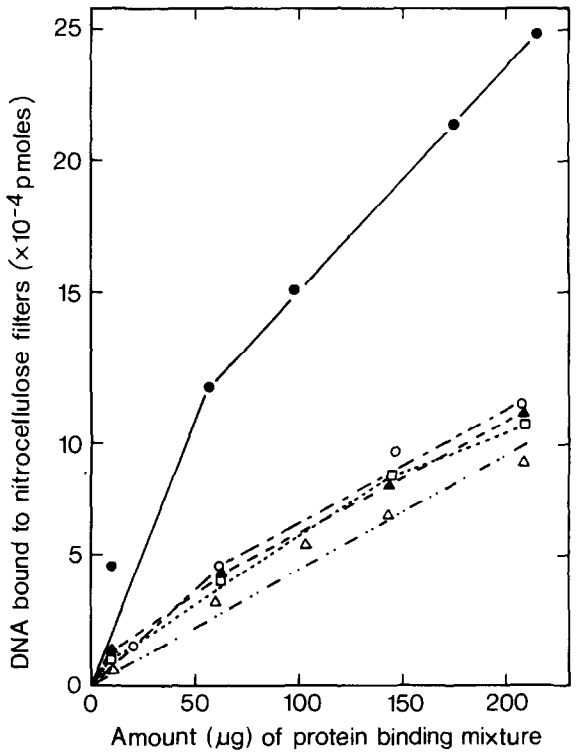

Fig.5. Binding of plasmid DNA to nitrocellulose filters by crude extract from EB505/pKN240 containing *ner* protein. The conditions were as in fig.1. Radioactively labeled DNA of: pBR322 (▲); pKN140 (□); pKN240 (△); pKN241 (●); and pKN242 (○).

the *ner* gene acting at the level of transcription [13]. The finding that a *ner* binding site is located near the boundary of genes *A* and *B* might be explained by assuming that continuous transcription of genes *ner* and *A* is required during the whole lytic cycle, while transcription of the other early genes will occur only during the first minutes of lytic development: The *ner* gene product may be responsible for shutting off their synthesis after this time. This hypothesis is consistent with the finding [14] that the activity of the Mu *A* protein is unstable and that therefore continued synthesis of this protein is required to maintain Mu DNA replication. Further experiments are required to elucidate the precise biological roles of the repressor and *ner* binding sites.

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