JOURNAL OF BACTERIOLOGY, Aug. 1986, p. 503–507
0021-9193/86/080503-05$02.00/0

Vol. 167, No. 2

Role of Ner Protein in Bacteriophage Mu Transposition

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Received 6 December 1985/Accepted 29 April 1986

The Ner protein of bacteriophage Mu negatively regulates transcription initiated at the early promoter and at the major repressor promoter. The construction and isolation of a Ner⁻ mutant of Mu is described. Ner is an essential function for Mu, because the mutant phage only forms plaques when complemented for Ner. Mutations in the repressor protein did not abolish the need for Ner. However, when transcription of the repressor gene *c* was blocked by deleting the major repressor promoter, Ner was no longer essential for normal Mu development.

Functions involved in Mu transposition are situated on the leftmost 5,000 base pairs (bp) of the Mu genome. Apart from the transposition genes *A* and *B*, two regulatory genes, *c* and *ner*, have been recognized which control the expression of these transposition functions. The *c* gene, located in the leftmost part of the Mu genome, is transcribed on the *l*-strand from two promoters p_{c-1} and p_{c-2} (6). Expression of *c* enables the phage to enter the lysogenic state, by shutting off the expression from the early promoter p_e. Transcription from the major repressor promoter p_{c-2} and from p_e is overlapping (Fig. 1).

ner is the first gene that is transcribed from p_e. The function of the *ner* gene product is comparable to the function of Cro of bacteriophage λ. In λ, Cro has been shown to control the expression from two promoters, p_{re} and p_{rm}, by binding to three operator regions located between these promoters. At a low concentration of Cro protein, first p_{rm}, from which the *c*I repressor is expressed, will be turned off. At increasing concentrations Cro will shut off transcription from p_{re}, thereby negatively controlling its own synthesis and the expression of the replication functions O and P (for review, see reference 8).

The *ner* gene product of bacteriophage Mu has also been shown to be a negative regulator of both *c* transcription (9) and early transcription (12). When *ner* is constitutively expressed from a foreign promoter on a multicopy plasmid, the early transcription of an infecting Mu phage is shut off, resulting in an inhibition of Mu replication (5, 9). This phenotype is similar to immunity caused by the *c* repressor and is therefore called pseudoimmunity.

Plasmids that constitutively express the cloned *ner*, *A*, and *B* genes under control of the kanamycin resistance promoter have been found to induce a Mu prophage upon transformation of a Mu lysogen, whereas isogenic plasmids that express only the transposase functions A and B do not (E. van Leerdam, unpublished results). These results suggested that Ner might play an essential role in the Mu life cycle. To study this role in more detail we have isolated Ner⁻ mutants by using partly in vitro DNA manipulations. The properties of these mutants suggest that transcription of the repressor gene interferes with the replicative transposition of Mu. For normal transposition to occur, repressor transcription has to be shut off by Ner, which therefore is an essential function.

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MATERIALS AND METHODS

Bacteria and plasmids. The *Escherichia coli* strains employed were KA52 (*thi galK*), KMBL1646 (*thi met tonB-trp*::Mu Δ*c*), and PP258 (*argA galE rpsL recA tonB-trp*::Mu *c*ts62 Δβ).

pGP7 contains the left *Hin*dIII fragment of Mu in the vector pBR322 (10). pGP134 has a 65-bp DNA fragment located to the right of the *Hin*dIII site of Mu fused to the structural *galK* gene in the vector pKO1 (6). pLP103-22-7 expresses Ner from the kanamycin resistance promoter (pKm) of pACYC177 (9). pLP103-6 expresses the Ner, A, and B functions under control of pKm of pACYC177 (9). pLP103-6-3 is a derivative of pLP103-6 in which translation start site of the *ner* gene has been deleted by excision of a *Bal*I-*Sma*I fragment.

Media and determination of phage burst. Media, plating procedures, and preparation of phage stocks have been described (14). Transformation of bacterial strains with plasmid DNA or with ligation mixtures was carried out by the method of Cohen et al. (2). After transformation the cells were allowed to grow in L broth for 1.5 h at 32°C before plating on agar containing 20 μg of ampicillin per ml.

For the determination of the burst time of the Ner⁻ phages in the different genetic backgrounds, the strains were infected with the phage with a multiplicity of infection of about 0.5. At different times after infection, samples were shaken with chloroform, and the supernatant fluid was plated on a lawn of bacteria containing pLP103-6 (*ner A B*).

Isolation of plasmid DNA and enzymatic reactions. Isolation of plasmid DNA was done as described previously (1). Restriction nucleases *Eco*RI, *Sma*I, and *Hin*dIII were purchased from Amersham Corp., *Bal*I and T4 ligase were from New England Biolabs, Inc., and Klenow DNA polymerase was from Boehringer Mannheim Biochemicals. All enzymes were used according to the manufacturers' specifications. The *Eco*RI linker used (GGAATTCC) was from Bethesda Research Laboratories, Inc. For the measurement of Mu specific transcription, RNA was labeled with 1-min pulses of 75 μCi of [^{3}H]uridine and extracted as described previously (12). Hybridization of the RNA preparations was performed with Mu DNA on nitrocellulose filters by using the procedure for DNA-DNA hybridization (13).

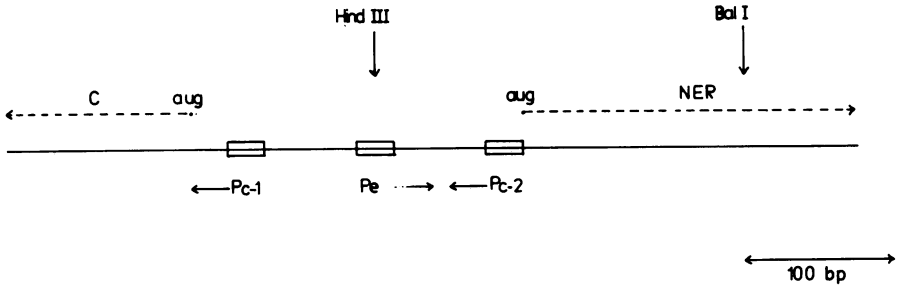

FIG. 1. Schematic representation of the early promoter region. p_{c-1} and p_{c-2} represent the minor and major repressor promoters respectively. Transcription from p_{c-2} and the early promoter (p_e) overlap (6). *Hin*dIII cleaves within p_e, and *Bal*I cleaves within the *ner* gene.

RESULTS

Construction of a Ner frameshift mutant. Because no Ner mutants of Mu have been reported among the numerous mutants isolated, we decided to construct a Ner$^-$ phage in vitro. In the first step of this construction a *ner* frameshift mutation was introduced in the plasmid pLP103-6 by inserting an *Eco*RI linker into the *Bal*I site present in the *ner* gene (Fig. 2). In minicells the Ner$^-$ plasmid (pGP90) produced a 5,500-dalton mutant protein instead of the 8,500-dalton Ner protein (results not shown). In the second step the *ner* mutant fragment was cloned adjacent to the Mu *c*ts gene in pGP7 (Fig. 2). The resulting plasmid (pGP94) conferred immunity to the transformed cells. As a result of the Ner$^-$ phenotype, immunity was also observed at 43°C, where normally the thermosensitive repressor is expected to be inactivated, indicating that the repressor was overproduced (9). In the last step of the construction, the plasmid pGP94 was recombined in vivo with a defective Mu prophage in which the part to the left of gene *A* was deleted (KMBL1646). This last recombination step was performed in the presence of pLP103-6 (on which the genes *ner*, *A*, and *B* are expressed under control of the Km promoter), to complement for the *ner* mutation. The phage that was released in this procedure was identified as Ner$^-$ by testing for the presence of the *Eco*RI linker in the *ner* gene (data not shown).

Properties of the Ner$^-$ frameshift mutant. The Ner$^-$ phage was not able to form plaques on a wild-type *E. coli* strain, indicating that the mutant phage was blocked in an essential function. The *ner* mutation was complemented in strains in which *ner*, *A*, and *B* were expressed either from a plasmid (pLP103-6) or from a defective prophage in the chromosome (PP258). However, when only A and B were provided without Ner (pLP103-6-3), the mutant phage was not able to form plaques. Since it has been shown that pLP103-6-3 provides enough A and B for the complementation of *Aam* and *Bam* mutants, we conclude from the experiments that the inability of the Ner$^-$ phage to plate on wild-type *E. coli* strains was the direct result of the absence of Ner and not due to a regulating effect of Ner on the expression of *A* and *B*.

Complementation of the *ner* mutation with the *ner* gene product alone, however, could only be achieved indirectly. When the Ner$^-$ phage was grown in strains in which *ner* was expressed from the Km promoter on a multicopy plasmid, Ner was overproduced, and early transcription of the phage to be complemented was shut off; therefore complementation could not be measured (9). Mu phages have been isolated which have become insensitive to Ner overproduction and which have a mutation in the Ner binding site (5). With the same frequency mutants of the Ner$^-$ phage can be isolated that plate on the Ner-overproducing strains. These

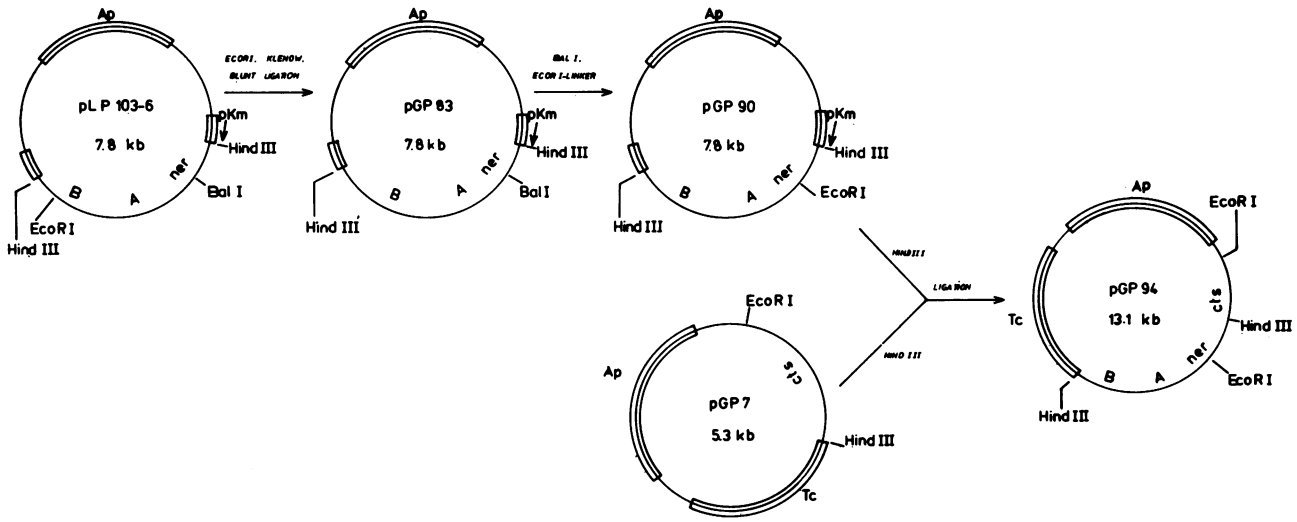

FIG. 2. Construction of pGP94. The *Eco*RI site of pLP103-6 was removed by filling in the sticky ends with Klenow DNA polymerase followed by blunt end ligation (pGP83). The 8-bp *Eco*RI linker was inserted in the *Bal*I site located in *ner* (pGP90). The *Hin*dIII fragment of pGP90 containing the Mu sequences was ligated in the *Hin*dIII site of pGP7, resulting in pGP94.

mutants are still Ner$^-$ because they do not form plaques in the absence of Ner. This suggests that these phages also have a mutation in the Ner binding site and that the defect of the Ner$^-$ phage can indeed be complemented by the *ner* gene product only.

Although the lytic development of Mu was strongly disturbed in the Ner$^-$ mutant, the mutation did not seem to affect the process of lysogenization. When an *E. coli* strain was infected with the Ner$^-$ phage with a multiplicity of infection of 10, about 26% of the cells survived. Among these survivors about 30% appeared to be immune, which is in the same order of magnitude as for a Mu Ner$^+$ phage.

A trivial reason why Ner is essential for Mu development could be that the repressor in the Ner$^-$ phage is overproduced, since Ner also acts as a negative regulator of repressor synthesis (8). To test this possibility, c^- Ner$^-$ mutants were isolated by plating the *c*ts Ner$^-$ phage on PP258 (which can be lysogenized by this phage) and screening for clear plaques. All of these c^- Ner$^-$ mutants, which were no longer able to lysogenize *E. coli* cells, also did not form plaques without complementation for Ner.

A Mu *c*ts Ner$^-$ lysogen was used to measure the effect of the *ner* mutation on Mu-specific transcription after induction (Table 1). Three conclusions could be drawn from these experiments. First, during the first 4 min after induction the amount of early transcription of the Ner$^-$ mutant was the same as that of the isogenic Ner$^+$ phage. Second, as was expected from a Ner$^-$ phage, no decrease in early transcription due to negative regulation of the early promoter was observed. Finally, the amount of RNA produced by the Ner$^-$ phage at 30 min was much lower than the amount of RNA produced by the Ner$^+$ phage and was of the same order of magnitude as transcription from only one phage copy.

***ner* mutation leads to a delayed burst of Mu.** During at least the first 30 min after induction of a Ner$^-$ phage, transcription seemed to occur from only one or a few copies of Mu DNA, suggesting that replication was severely impaired. After prolonged incubation, however, a few phage of the Ner$^-$ phenotype were produced (about 10^4 PFU/10^8 bacteria), indicating that some replication had occurred. Therefore we followed the production of Ner$^-$ phage particles over a longer period of time to investigate whether the inability of the mutant phage to form plaques on a wild-type strain was due to a delay in burst time. To exclude the possible effect of the Mu repressor these experiments were carried out with a c^- Ner$^-$ phage. *E. coli* strains with or without plasmids that provided different early Mu functions in *trans* were infected with the c^- Ner$^-$ mutant, and at different times after infection the number of phages produced was measured

TABLE 1. Transcription of Ner$^+$ and Ner$^-$ phage after induction

Time (min after induction)	% Mu-specific RNA[a]	
	Ner$^+$ phage	Ner$^-$ phage
0	0.05	0.04
2	0.17	0.18
4	0.30	0.34
8	0.21	0.54
30	3.19	0.63

[a] *E. coli* strains lysogenic for the Mu *c*ts Ner$^+$ and Mu *c*ts Ner$^-$ phages were labelled with 1-min pulses of [^{3}H]uridine at different times after thermal induction. The purified RNA was hybridized to filters containing Mu DNA, and the amount of hybridization was corrected for binding to filters containing calf thymus DNA (less than 0.05%). Hybridization was calculated as the percentage of the trichloroacetic acid-precipitable input radioactivity, which ranged from 2.8×10^5 to 4.8×10^5 cpm.

TABLE 2. Development of the c^- Ner$^-$ and Ner $\Delta p_{c\text{-}2}$ phages in strains where early Mu functions were provided in *trans* from multicopy plasmids

Phage	Time after infection (min)	No. of phages after infection (PFU/ml)			
		No plasmid	pLP103-6-3 (A B)	pLP103-22-7 (A)	pLP103-6 (Ner A B)
c^- Ner$^-$	50	$<10^2$	$<10^2$	$<10^2$	$<10^2$
	60	$<10^2$	$<10^2$	$<10^2$	10^8
	70	$<10^2$	$<10^2$	$<10^2$	10^9
	80	$<10^2$	9×10^6	$<10^2$	10^9
	90	$<10^2$	8×10^7	$<10^2$	
	100	5×10^6	2×10^8	3×10^7	
	110	7×10^7	10^9	10^8	
	120	1×10^8	10^9	2×10^8	
	130	5×10^8		5×10^8	
	150	5×10^8		5×10^8	
Ner $\Delta p_{c\text{-}2}$	60	$<10^2$	$<10^2$		$<10^2$
	70	$<10^2$	5×10^7		10^7
	80	$<10^2$	5×10^8		8×10^7
	90	$<10^2$	10^9		10^9
	100	$<10^2$	10^9		10^9
	110	$<10^2$			
	120	$<10^2$			
	130	$<10^2$			
	150	$<10^2$			

(Table 2). Infection of a wild-type *E. coli* strain with the c^- Ner$^-$ phage lead to a practically normal burst size, but at a very late stage, 130 min after infection. When extra A and B were provided in *trans*, the development of the phage was slightly improved, resulting in a burst at 110 min. Apparently the effect of the *ner* mutation could be partially overcome by the presence of additional A and B proteins, albeit at too low a level to allow plaque formation. The presence of extra A alone did not accelerate the burst of the mutant phage. In the presence of A, B, and Ner the infection of the c^- Ner$^-$ led to a burst at 70 min, which is about the same as found for a Ner$^+$ phage.

Isolation and properties of a Ner$^-$ mutant with a deleted repressor promoter. Since, as shown above, the inability of the Ner$^-$ mutant to form plaques on a wild-type *E. coli* strain was not due to a polar effect on the expression of A and B or to an overproduction of repressor protein, another possible reason for Ner being essential for normal Mu development was that transcription of the repressor gene has to be shut off before lytic transposition can occur. The *c* transcription which proceeds in the direction toward the left end of the Mu genome could possibly interfere with the transposase complex which acts on the Mu ends. To test this hypothesis, a Ner$^-$ phage was constructed in which the major repressor promoter ($p_{c\text{-}2}$; Fig. 1) was deleted.

To this purpose first the 240-bp *Hin*dIII-*Eco*RI fragment of pGP94 was replaced by the 65-bp *Hin*dIII-*Eco*RI fragment of pGP134, resulting in pGP160 (Fig. 3). Since pGP134 was isolated by deletion of 185 bp from the *Eco*RI linker inserted in the *ner* gene (6), both the $p_{c\text{-}2}$ promoter and the translation start site of the *ner* gene were no longer present in pGP160. Bacterial strains with pGP160 were no longer immune against superinfection with Mu, indicating that the expression of the repressor gene was severely reduced in this plasmid, due to deletion of $p_{c\text{-}2}$.

To obtain a complete phage, pGP160 was recombined in vivo with the defective Mu prophage in KMBL1646. The Ner$^-$ $\Delta p_{c\text{-}2}$ phage resulting from this recombination was

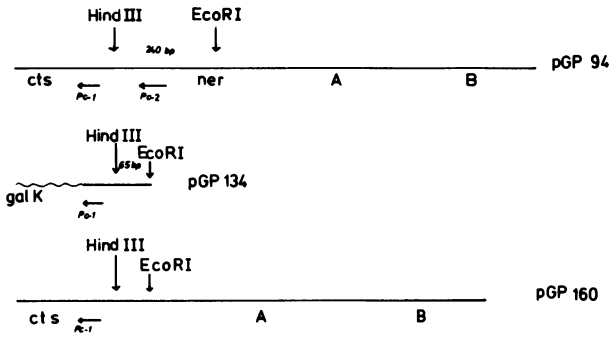

FIG. 3. Construction of pGP160. The 240-bp *Hin*dIII-*Eco*RI fragment (containing p_{c-2}) was replaced by the 65-bp *Hin*dIII-*Eco*RI fragment of pGP134.

tested for the deletion of the repressor promoter by restriction enzyme analysis (results not shown).

The Ner$^-$ Δp_{c-2} phage formed normal plaques on an *E. coli* strain that contained either pLP103-6 (expressing *ner*, *A*, and *B*) or pLP103-6-3 (expressing only *A* and *B*), indicating that for this mutant Ner was no longer essential.

However, the mutant phage did not produce viable progeny without A and B provided in *trans*, not even after prolonged incubation (Table 2). This was probably due to the fact that by deleting p_{c-2} the translation start site of the *ner* gene had also been removed, which might result in a premature termination of early transcription. In the presence of A and B the development of the Ner$^-$ Δp_{c-2} mutant was almost up to the level of a wild-type Mu phage (Table 2). The slight delay in development that was observed could be explained by the polar effect that the deletion might have on early transcription. As a result of this presumed polar effect, functions located byond gene *B*, which are believed to have a stimulating effect on Mu replication (3, 10), were probably expressed at a lower level.

In the presence of A, B, and Ner, the development of the Ner$^-$ Δp_{c-2} phage was not further increased.

Summarizing, we can conclude that repressor transcription interfered with Mu transposition and that Ner is essential to block this transcription.

DISCUSSION

The properties of the Ner$^-$ mutants described in this paper support the conclusions which were drawn from the properties of plasmids expressing the cloned *ner* gene in combination with the Mu transposition genes. By means of these plasmids, indirect evidence was obtained that Ner plays an essential role in Mu replicative transposition. To induce a Mu prophage to transpose, it is not sufficient to provide only the transposition functions A and B in *trans*; the *ner* gene product must also be supplied. Our results make it seem plausible that this essential function of Ner was connected with one of its regulatory functions, the negative regulation of repressor gene transcription. When the major repressor promoter was deleted, Ner was no longer essential for Mu transposition. Since Ner was still required when the Mu phage produced an inactive repressor protein, this strongly suggests that it was the repressor transcription and not the repressor protein itself which interfered with the transposition process. Since it has been shown that the *c* repressor also binds to the A binding sites (3), one could argue that the mutated repressor of the *c*$^-$ Ner$^-$ phage still has some affinity for these sites, thereby inhibiting A binding and consequently Mu transposition. However, we do not favor this hypothesis, because several *c*$^-$ Ner$^-$ phages have been isolated, among which repressor mutations are expected that also inhibit binding to these A binding sites. None of them was able to plate in the absence of a complementing Ner function. Moreover, no spontaneous mutants of the Ner$^-$ phage were found that could suppress the *ner* mutation. If the repressor protein itself were the cause of the defect of the Ner$^-$ phage, one would expect to find such mutants with a rather high frequency.

The interference with the process of transposition by repressor transcription can be explained by the assumption that polymerase molecules transcribing the repressor gene proceed to the A binding sites, thereby inhibiting the binding of the Mu transposase to these sites. This also explains the observation that, when the transposition functions A and B are provided in *trans* from a multicopy plasmid, the inhibitory effect can be partly overcome. Transcription coming from the outside of a transposon can interfere with the transposition of that transposon as has been shown for IS*1* (7).

In phage Mu, the inhibition of transposition by repressor transcription may be important for the lysogenization of the host. In contrast to phage λ, Mu utilizes the gene products A and B both for integration, which is essential to obtain lysogeny, and for replication. When, after the first integration event transcription of the *c* gene interferes with transposition, further lytic development could be physically blocked even in the presence of transposition functions.

ACKNOWLEDGMENTS

We thank A. Kuijpers for technical assistance, T. Goosen for the performance of the transcription experiments, and J. Brouwer for critical reading of the manuscript.

Part of this work was supported by Euratom contract BI0-E-408-81-NL(G).

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