Functional Domains of the Penicillinase Repressor of Bacillus licheniformis

VAUGHAN WITTMAN,1 H. C. LIN,2 AND H. C. WONG1*

Biology Skills Center, Dade Diagnostics Division, Baxter Healthcare Inc., 10555 West Flagler Street, Miami, Florida 33174,1 and National Defense Medical Center, Institute of Preventive Medicine, Taipei, Taiwan2

Received 2 July 1993/Accepted 21 September 1993

The penicillinase repressor (PENI) negatively regulates expression of the penicillinase gene (penP) in Bacillus licheniformis by binding to its operators located within the promoter region of penP. penl codes for a protein with 128 amino acids. Filter-binding analyses suggest that the active form of the repressor is a dimer. Genetic analyses of PENI derivatives showed that the repressor carrying either a 6-amino-acid deletion near the N terminus or a 14-amino-acid deletion at the C terminus was functionally inactive in vivo. A repressor derivative carrying a 6-amino-acid deletion within its N-terminal region was extensively purified and used in DNA footprinting and subunit cross-linking analyses. The results of these studies showed that the repressor derivative had lost its ability to bind operator specifically even though it could dimerize effectively. In similar studies, we demonstrated that an N-terminal portion of PENI with a molecular mass of 10 kDa derived by digestion with papain was able to bind operator specifically but with reduced affinity and had completely lost its ability to dimerize. These data suggest that the repressor has two functional and separable domains. The amino-terminal domain of the repressor is responsible for operator recognition, and the carboxyl-terminal domain is involved in subunit dimerization.

The inducible enzyme penicillinase produced by Bacillus licheniformis is encoded by the chromosomal gene penP (3). In the absence of penicillin, the expression of the penP gene is negatively regulated at the level of transcription by a repressor. Genetic analysis (3, 19) indicates that this penicillin repressor (PENI) is encoded by the closely linked gene penl.

The mechanism involved in the induction of the penP gene by exogenously penicillin is unknown, but two additional regulatory genes, penR1 and penR2, are believed to play a role in the signal transfer process. penR1 codes for the penicillin receptor protein, which is located in the bacterial membrane (7, 11). penP and penl are transcribed divergently from within a 364-nucleotide region separating the coding regions. The translation initiation codon of penl is located 5 bases downstream of the coding region of penl. Therefore, penl and penR1 are likely transcribed as a polycistronic mRNA from the penl promoter. penl was expressed in Escherichia coli, and its gene product was purified. The purified PENI was used in vitro for gel retardation and DNase I footprinting experiments to characterize its interactions with the regulatory regions of the pen genes, and three penl operators, positioned in the region between the penP and penl coding sequences, were identified (25). Since these operator sequences overlapped with the promoter sequence of penP and penl, we propose that the PENI repressed the expression of penP by physically blocking the RNA polymerase-binding site and that penl was autoregulated.

PENI is a protein with 128 amino acids. It binds to a nucleotide sequence 29 bp long that contains a region of dyad symmetry. The nucleotide sequences in the three operators are highly conserved and show a consensus half-site of 5'ANNTTACA2-3'. The nucleotides shown in uppercase appear exclusively at the positions indicated, with the exception of the first T in TTACA which is present in five of six half-sites. The nucleotides shown in lowercase share these positions, and each is found half the time. Although amino acid sequence comparison of PENI with other DNA-binding proteins suggests that it contains a helix-turn-helix motif within its amino terminus, there is nothing known about the structure-function relationship of the various regions of the protein. In this paper, we present evidence that PENI contains separate domains for DNA binding and protein dimerization.

MATERIALS AND METHODS

Bacterial strains and plasmids. E. coli DG101 (thi-1 hsdR17 endA1 supE44 lacF- lacZΔM15 F−) and DG116 (thi-1 hsdR17 endA1 supE44 [lacI857 ΔH1 bio70]) were obtained from D. Gelfand, Cetus Corporation. E. coli MC1000 (2) was provided by Malcolm J. Casadaban, University of Chicago. Plasmids pUC18 and pFC54.t (24) have been described previously. The restriction map of plasmid pVW37 is shown in Fig. 1. This plasmid is a derivative of pDH5413 (25). It carries penl under the control of the trp promoter (15), followed by a polylinker region and the crystal protein transcriptional terminator (26). Plasmid pVW31 was constructed by inserting a HindIII-BamHI restriction fragment containing penl into the HindIII-BamHI sites of pUC18.

Expression and purification of repressor protein. Expression and isolation of native PENI has been described previously (25). The repressor was further purified by using a MonoS column (Pharmacia) as described by Grossman and Lampen (5), except that KCl was substituted for NaCl. PENI eluted from the column as a discrete peak at 400 and 450 mM KCl. This method consistently yielded repressor preparations in which at least 75% of the PENI was active, as estimated by gel shift analysis (25). The truncated version of the repressor lacking six amino acids near the N terminus was expressed and purified by the same procedure. This mutant protein eluted from the MonoS column as a discrete peak at the same KCl concentration as the native protein. The papain-treated version of the repressor eluted from the MonoS column just after the void volume peak at 200 mM KCl.
PCR-based oligonucleotide-directed mutagenesis. Plasmid pVW31 contains the penI coding sequence inserted in pUC18 between the HindIII and HincII restriction sites with the 3' end of the coding sequence proximal to the HincII site. EcoRI-digested plasmid pVW31 was used as the template for the polymerase chain reactions (PCR). The primers for PCR are listed in Table 1. PCR contained 1 ng of template, 100 pmol (each) of the appropriate primers, 100 μmol of each deoxynucleotide triphosphate, 1 × PCR buffer (Perkin Elmer-Cetus), and 2.5 U of Taq DNA polymerase (Perkin Elmer-Cetus). Extension and amplification were carried out in a DNA Thermal Cycler (Perkin Elmer-Cetus) for 10 cycles with a denaturing temperature of 96°C, an annealing temperature of 37°C, and an extension temperature of 72°C. The amplification products were digested with HindIII and BamHI and separated on an 0.8% LMT agarose gel (Bethesda Research Laboratories). Restriction fragments with the appropriate sizes were excised from the gel and cloned directly into pVW37, which had been digested with HindIII and BamHI. The entire sequence of each deleted penI was confirmed by dyeoxy sequence analysis (18).

**S1 and exonuclease III digestion.** Plasmid pVW37 was digested with XbaI and KpnI to generate a linearized plasmid with a 3' protruding end adjacent to the remainder of the polylinker sequence and a 5' protruding end adjacent to penI. This linearized plasmid was then digested with exonuclease III and S1 nuclease as described previously (20) to create deletions at the 3' end of penI. Oligonucleotides with the sequences 5'-TGACTGACTGAGGATCCC-3' and 5'-GGGATCCTCAGTCA-3' were annealed, digested with BamHI, and added to the exonuclease III-S1-treated pVW37, which had been digested with BamHI. This mixture was ligated and used to transform E. coli MC1000 competent cells. The transformants were plated on L-agar containing 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml and 100 μg of ampicillin per ml. After overnight incubation at 37°C, the transformants with a blue appearance were picked, and their plasmid DNAs were isolated. The nucleotide sequence of the penI up to the deletion endpoints at the 3' end of the gene was determined by DNA sequencing by using the chain termination method.

**Filter-binding reactions.** The 32P-labeled plasmid DNA was dissolved at a concentration of approximately 10–11 M in the assay buffer containing 0.01 M Tris-HCl (pH 7.5), 0.05 M KCl, 0.01 M MgCl2, 10–4 M EDTA, 5% dimethyl sulfoxide, and 50 μg of bovine serum albumin per ml. Different amounts of repressor protein in 10-μl aliquots of the reaction buffer were added to 200 μl of this assay mix. After 20 min at 25°C, the samples were slowly filtered through 25-mm BA85 nitrocellulose filters (Schleicher & Schuell) that had been soaked in reaction buffer. The filters were washed twice with 500-μl aliquots of the same buffer and dried, and bound DNA was detected by scintillation counting in toluene-liquid.

**Cross-linking of the penI repressor and derivatives.** Four cross-linking agents, DSP (diethylbis(succinimidylpropionate)), DMS (dimethyl suberimidate), EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide], and EDC + sulfoNHS (N-hydroxysulfosuccinimide) obtained from Pierce, were reacted with native PENI at a concentration of 5 × 10–5 M. Initial screening was carried out in a solution containing 20 mM NaPO4 (pH 8.0), and 50 mM KCl for DMS and DSP or 20 mM NaPO4 (pH 7.0) and 50 mM KCl for EDC and EDC + sulfoNHS, with concentrations of the cross-linking reagents at 2 mg/ml (0.73 mM), 3 mg/ml (0.75 mM), 10 mM, and 100 mM + 5 mM, respectively. Cross-linking reactions were performed as previously described (6, 16, 22, 23). In the initial screening, we identified EDC as the most efficient cross-linking agent. Its optimal concentration for cross-linking the repressor was 50

![FIG. 1. A physical map of the reporter plasmid pVW37. Transcription of penI is under the control of the E. coli trp operon promoter (Trp), lacZ from E. coli is fused at its ninth codon with the penP promoter, penP translation initiation signal, and its first 13 codons (PenP). Ap, cry, and ori represent the structural gene of the ampicillin resistance gene from Tn3, the transcriptional terminator sequence of the Bacillus thuringenesis cry gene, and the replication origin of ColEI derivative pMB1, respectively. The sequence between the penP and cry (i.e., counterclockwise) is derived from the plasmid pBR322. Relevant restriction sites are shown.](http://jb.asm.org/)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-TGA CTG ACT CGA GTG ATC-3'</td>
<td>Stop codons and BamHI restriction site for ecoIII-S1 penI deletions</td>
</tr>
<tr>
<td>5'-GGGATCC TCA GTC ACG TCT-3'</td>
<td>Stop codons and BamHI restriction site for ecoIII-S1 penI deletions</td>
</tr>
<tr>
<td>5'-GTCGAC AGCT TTA TGA AAG ATG CCA GTG AG-3'</td>
<td>PCR deletion of codons for aa 3 to 8</td>
</tr>
<tr>
<td>5'-GTCGAC AGCT TTA TGA AAG ATG CCA GTG AG-3'</td>
<td>PCR deletion of codons for aa 3 to 13</td>
</tr>
<tr>
<td>5'-GTCGAC AGCT TTA TGA AAG ATG CCA GTG AG-3'</td>
<td>PCR deletion of codons for aa 3 to 23</td>
</tr>
<tr>
<td>5'-GTCGAC AGCT TTA TGA AAG ATG CCA GTG AG-3'</td>
<td>Back primer for PCR deletions</td>
</tr>
</tbody>
</table>

* Start codons, stop codons, and restriction sites are underlined.

aa, amino acids.
mM. In a typical reaction, an aliquot of the peak fraction from a MonoS column containing either the native repressor, its derivative lacking six amino acids at the N terminus, or the papain-treated form was diluted to a final concentration of 5 μM in a solution containing 20 mM NaPO₄ (pH 7.0) and 50 mM EDC. The reaction mixtures were incubated at room temperature for 4 to 5 h, and cross-linking was terminated by the addition of trichloroacetic acid to a final concentration of 8%. After precipitation, the samples were washed with acetone, dried, resuspended in loading dye containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol, electrophoresed on an SDS–12.5 or 15% polyacrylamide gel, and stained with Coomassie blue.

DNA footprinting. A 216-bp HindIII–EcoRI fragment carrying the penI operator from plasmid pVW/46 was labeled at the 5' end with T4 polynucleotide kinase and [γ-32P]ATP for DNA footprinting. DNase I footprinting was performed as described by Galas and Schmitz (4) in a solution containing 10 mM Tris–HCl (pH 7.4), 2 mM MgSO₄, 50 μg of bovine serum albumin per ml, and 50 mM KCl at an operator concentration of less than 1 nM.

Enzymes and radiolabeling procedure. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs, Inc.; calf intestinal alkaline phosphatase and T4 polynucleotide kinase were obtained from Boehringer Mannheim Biochemicals. All reaction conditions for the enzymes were used as recommended by the suppliers. Previously described methods (14) were used for 5'-end labeling of DNA fragments with [γ-32P]ATP (3,000 Ci/mmol; New England Nuclear Corp.).

Miscellaneous techniques. The methods for transformation and preparation of competent cells of E. coli (13), protein determination (21), and SDS-polyacrylamide gel electrophoresis (PAGE) of protein samples (12) are described elsewhere. β-Galactosidase activity was determined by the method of Miller (15). The specific activity of β-galactosidase is expressed as millimoles of o-nitrophenol formed per minute per milligram of protein at 28°C.

RESULTS

The active form of PENI is a dimer. The equilibrium parameters of PENI–operator interaction were measured by a filter-binding assay. A 29-bp oligonucleotide fragment containing the penI operator (5'-GATCTCATAATCTTACAAATG TAGTCTTTG-3') was synthesized and cloned into the BamHI site of pUC19. The resulting plasmid was digested with EcoRI, dephosphorylated with calf intestinal alkaline phosphatase, and end labeled with [γ-32P]ATP. Various amounts of purified penI repressor were then added to the binding solution containing 10⁻¹¹ M labeled penP2 operator. After equilibration, the mixtures were filtered through nitrocellulose filters. The filters were then washed, and the amount of bound operator was determined by scintillation counting. Figure 2 shows the binding of PENI to labeled plasmid bearing a single penI2 operator at various repressor concentrations. The curve is sigmoid, suggesting that the repressor monomers are in concentration-dependent equilibrium with an oligomeric form of the repressor and that the oligomer is the active form for binding the penP2 operator DNA. Thus, at the low repressor concentrations (at the beginning of the curve) most of the repressor present are monomers, and monomers do not bind operator DNA strongly. As the repressor concentration rises, the proportion of the oligomers increases, giving a sigmoidal increase in binding. Thus, the shape of the binding curve reflects two equilibria. One is the association of repressor monomers to form oligomers; the other is the binding of the repressor oligomers to DNA. These equilibria can be represented as follows:

\[ R_n \rightleftharpoons nR \quad K_1 = \frac{[R]^n}{[R]^0} \]  

\[ R_n O \rightleftharpoons R_n + O \quad K_2 = \frac{[R_n O]}{[R_n][O]} \]

Therefore, the overall equilibria for the binding of the repressor monomers to operator can be represented as:

\[ K = K_1 \times K_2 = \frac{[R]^n[O]}{[R_n O]} \]

where [R] is the concentration of repressor monomer, [Rₙ] is the concentration of the active oligomers, [Rₙ O] is the concentration of the bound operator, and [O] is the concentration of the unbound operator. Equation 3 predicts that a plot of ln[R] versus ln[Rₙ O]/[O] would yield a straight line with its slope...
In vivo activity of amino-terminal-truncated repressors. The PCR loop out approach was used to substitute the wild-type penI in pVW37 with different 5'-truncated penI genes. The resulting plasmids were used to transform E. coli MC1000. The transformants were then grown in M9 minimal medium without tryptophan, and their β-galactosidase activities in the crude extracts were assayed. Bars indicate the expected size of each PENI derivative based on the deletion endpoints of the constructs. Shaded areas represent deleted amino acids. Negative numbers indicate the extent of the deletion starting with amino acid 3. The specific activity (spec. act.) of β-galactosidase is defined as millimoles of o-nitrophenol formed per minute per milligram of protein at 28°C.

In our filter-binding experiments, the concentration of the penP2 operator is low (10^{-11} M) in comparison with the repressor concentration at which half-maximal DNA binding occurs. Therefore, the overall equilibrium dissociation constant, K, is approximately equal to the square of repressor concentration at the half-maximal binding point (9). Thus, the overall dissociation constant, K, is calculated to be 2.5 \times 10^{-15} \text{mol/liter} on the basis of the data presented in Fig. 2. This represents the value for binding of the PENI dimer to the single penP2 operator site.

In vivo genetic analysis of PENI. Deletions starting from both the 5' and 3' ends of the penI gene were constructed in vitro, and the repressor functions of their derivatives were examined in vivo. The plasmid pVW37 (Fig. 1) was designed to evaluate the repressor functions in vivo. This plasmid carries penI under the control of the E. coli trp promoter and a penP-lacZ fusion gene. Transcription of the penP-lacZ fusion gene is regulated by the penP promoter-operator. Therefore, in the absence of L-tryptophan, transcription from the trp promoter results in the production of PENI, which represses the expression of the penP-lacZ fusion gene.

Repressor genes coding for proteins lacking 6, 11, or 21 amino acids at the N terminus [PENIA(3-8), PENIA(3-13), or PENIA(3-23), respectively] were constructed by PCR-directed mutagenesis (see Materials and Methods for details). Sequencing of the whole penI insert for each construct confirmed that each one contained the first codons of the wild-type penI and a deletion from the third codon through codon 8, 13, or 23. The first two codons were retained in each construct to ensure translation of the deleted gene's transcript. To examine whether these deleted genes coded for functional repressors, we subcloned them into the HindIII-BamHI sites of the reporter plasmid pVW37, substituting them for the coding region of the wild-type penI. Evaluation of each of the deleted gene product's ability to repress expression of the penP-lacZ fusion was accomplished by monitoring β-galactosidase activity. As is shown in Fig. 3, cells harboring anyone of the three constructs yielded at least sevenfold higher levels of β-galactosidase activity than cells carrying the parental plasmid pVW37 under conditions in which the repressor is induced. Immunoblot analysis of the soluble cell extracts using an anti-PENI polyclonal antibody confirmed the presence of truncated repressor protein in cells containing each of the three deletion constructs (Fig. 4). Analysis of each deletant demonstrated that whether the penI was induced or uninduced, the total units of β-galactosidase activity produced were essentially the same (the maximum variation in activity between

---

**FIG. 3.** In vivo activity of amino-terminal-truncated repressors. The PCR loop out approach was used to substitute the wild-type penI in pVW37 with different 5'-truncated penI genes. The resulting plasmids were used to transform E. coli MC1000. The transformants were then grown in M9 minimal medium without tryptophan, and their β-galactosidase activities in the crude extracts were assayed. Bars indicate the expected size of each PENI derivative based on the deletion endpoints of the constructs. Shaded areas represent deleted amino acids. Negative numbers indicate the extent of the deletion starting with amino acid 3. The specific activity (spec. act.) of β-galactosidase is defined as millimoles of o-nitrophenol formed per minute per milligram of protein at 28°C.

<table>
<thead>
<tr>
<th>repressor derivative</th>
<th>Spec. act. β - galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>PENI Δ(3-8)</td>
<td>-6 aa 292</td>
</tr>
<tr>
<td>PENI Δ(3-13)</td>
<td>-11 aa 269</td>
</tr>
<tr>
<td>PENI Δ(3-23)</td>
<td>-21 aa 355</td>
</tr>
<tr>
<td>PENI</td>
<td>128 aa 37</td>
</tr>
</tbody>
</table>

---

**FIG. 4.** Expression of amino-truncated repressors. E. coli MC1000 was transformed with plasmids constructed by substituting the wild-type penI in pVW37 with penI derivatives encoding PENIA(3-8), PENIA(3-13), and PENIA(3-23). These strains were designated VW40, VW41, and VW42, respectively. Strain VW43 carried pVW37 and expressed the wild-type PENI. VW40 (lanes 1 and 2), VW41 (lanes 3 and 4), VW42 (lanes 5 and 6), and VW43 (lanes 7 and 8) were grown in M9 medium containing 0.5% Casamino Acids (15) either in the presence (i.e., the penI genes were repressed [lanes 2, 4, 6, and 8]) or in the absence (i.e., the penI genes were derepressed [lanes 1, 3, 5, and 7]) of 80 μg of L-tryptophan per ml. Lane 9 contains 5 ng of purified PENI. Cellular extracts prepared from these four transformants under induced or uninduced conditions were fractionated by SDS-PAGE, transferred to an Immobilon-P membrane (Millipore), probed with an affinity-purified anti-PENI polyclonal antibody derivatized with horseradish peroxidase, and then visualized by the ECL method (Amersham).
induced and uninduced was less than 2%, two isolates for each mutant were tested; data not shown) while the induction of the wild-type penI resulted in a threefold decrease in the β-galactosidase level, from 0.1 to 1.7 U. The results indicate that PENI lacking 6, 11, or 21 amino acids at its N terminus has lost its ability to repress expression from the penP promoter. Thus, the N terminus of the repressor is essential to the in vivo function of PENI.

To make deletions at the 3' end of penI, we utilized unique restriction sites that were engineered into pWW37 (Fig. 1). Since these sites were engineered in the 3' noncoding region of penI, we were able to linearize the plasmid and use the exonuclease III-S1 nuclease protocol (see Materials and Methods for details) to create deletions at the 3' end of penI. It should be noted that we also inserted a linker carrying the termination codon TGA in all three reading frames downstream of the deletion endpoints in penI (Table 1). As a result, all the deleted PENI derivatives contain no more than two additional amino acids beyond their remaining native PENI sequences. pWW37 derivatives carrying these deleted penI genes were introduced into E. coli MC1000, and the transformants were plated on agar media containing ampicillin and X-Gal but lacking tryptophan. Blue colonies result from cells which produce nonfunctional repressors because of deletions in penI. Large numbers of blue colonies were picked, and their plasmids were isolated. The endpoints of these deletions were then determined by DNA sequencing. Such analysis indicated that PENI was not able to maintain its in vivo activity after the loss of as few as 14 amino acids from its C terminus. This conclusion was confirmed by examining the lacZ activities of E. coli transformants carrying pWW37 derivatives with deletions of 14, 25, 53, or 65 codons at the 3' end of the penI gene. As shown in Fig. 5, none of these deleted PENI derivatives was able to repress the expression of the penP-lacZ fusion gene in E. coli. This demonstrates that the C terminus of the repressor is also essential for the in vivo function of PENI.

To investigate whether the derepression of the penP-lacZ fusion gene was due to the instability of the carboxyl truncated repressors, we carried out immunoblot analyses to demonstrate the accumulation of these truncated PENIs in the E. coli host containing the pWW37 derivatives (data not shown). The anti-PENI polyclonal antibody was able to detect the PENI derivatives which had 14 or 25 amino acid residues deleted from their C termini [PENIΔ(115–128) or PENIΔ(104–128), respectively] in E. coli extracts prepared from the cells carrying plasmid pHCW26 or pHCW15, respectively. The failure of the anti-PENI antibody to detect the repressors which lacked their last 53 or 65 amino acid residues in the cell extracts was likely due to the reduced affinity of our polyclonal antibody for these truncated repressors. When the carboxyl truncated repressors were overexpressed in a P1 vector, the expression level of each was approximately equal as estimated on a Coomassie blue-stained SDS-polyacrylamide gel. However, when the same lysates were appropriately diluted and immunoblotted with the anti-PENI polyclonal antibody, the signal generated by the repressors decreased significantly as the size of the carboxyl terminus decreased (data not shown). On the basis of these observations, we believe that the repressors lacking 53 or 65 amino acids were undetected by the polyclonal antibody even though their expression levels were equivalent to those of PENIΔ(115–128) or PENIΔ(104–128).

The C-terminal region of the PENI is responsible for the dimerization of the protein. We performed cross-linking studies and DNase I footprinting analysis with the wild-type and truncated derivatives of PENI to examine whether the DNA binding and the dimerization properties are carried by separable domains of the repressor. Cross-linking studies were carried out with three forms of the repressor; the wild-type protein, a truncated repressor lacking six amino acids near the amino terminus, PENIΔ(3–8), and the papain-digested form of the repressor, PENIΔ(87–128). When PENI was digested with papain, a derivative lacking 42 amino acids at the carboxyl terminus was obtained (i.e., Fig. 6b, lane 5). All forms of the repressor were purified by using a protocol developed previously (25), with an additional step of elution from a MonoS column (Pharmacia) (5). Four cross-linking reagents, DSP, DMS, EDC, and EDC + sulfoNHS obtained from Pierce, were tested for efficacy with the wild-type repressor. These reagents cross-link protein via two different mechanisms. DMS and DSP are homobifunc-

<table>
<thead>
<tr>
<th>repressor derivative</th>
<th>Spec. act. β-galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>PENI</td>
<td>128 aa</td>
</tr>
<tr>
<td>PENIΔ(115–128)</td>
<td>114 aa</td>
</tr>
<tr>
<td>PENIΔ(104–128)</td>
<td>103 aa</td>
</tr>
<tr>
<td>PENIΔ(76–128)</td>
<td>75 aa</td>
</tr>
<tr>
<td>PENIΔ(64–128)</td>
<td>63 aa</td>
</tr>
<tr>
<td>PENIΔ(87–128)</td>
<td>86 aa</td>
</tr>
</tbody>
</table>

FIG. 5. In vivo activity of carboxyl-terminal-truncated repressors. Restriction cassettes carrying different 3'-truncated penI constructs were cloned into the HindIII-BamHI sites of the plasmid pWW37 to substitute the intact penI repressor gene. The resultant plasmids were used to transform E. coli MC1000. The transformants were then grown in M9 minimal medium without tryptophan, and their β-galactosidase activities in the crude extracts were assayed. Bars indicate the expected size of each penI repressor protein based on the deletion endpoints of the constructs. PENIA indicates the repressor derivative, with the amino acids (aa) that are deleted given in parenthesis. PENIΔ(87–128) is generated by digestion of the native repressor with papain and is shown for comparison. The specific activity (spec. act.) of β-galactosidase is defined as millimoles of o-nitrophenol formed per minute per milligram of protein at 28°C.

The C-terminal region of the PENI is responsible for the dimerization of the protein. We performed cross-linking studies and DNase I footprinting analysis with the wild-type and truncated derivatives of PENI to examine whether the DNA binding and the dimerization properties are carried by separable domains of the repressor. Cross-linking studies were carried out with three forms of the repressor; the wild-type protein, a truncated repressor lacking six amino acids near the amino terminus, PENIΔ(3–8), and the papain-digested form of the repressor, PENIΔ(87–128). When PENI was digested with papain, a derivative lacking 42 amino acids at the carboxyl terminus was obtained (i.e., Fig. 6b, lane 5). All forms of the repressor were purified by using a protocol developed previously (25), with an additional step of elution from a MonoS column (Pharmacia) (5). Four cross-linking reagents, DSP, DMS, EDC, and EDC + sulfoNHS obtained from Pierce, were tested for efficacy with the wild-type repressor. These reagents cross-link protein via two different mechanisms. DMS and DSP are homobifunc-
tional cross-linking reagents which cross-link through primary amino groups. EDC and EDC + sulfoNHS are heterobifunctional cross-linking reagents which cross-link primary amines to carboxyl groups. We found that a solution containing PENI, at a concentration of $5 \times 10^{-6}$ M, treated with these reagents in the appropriate buffers demonstrated that EDC was the most efficient cross-linker (data not shown). As a result, we chose EDC as the reagent for our further studies.

The monomers of PENI and PENIΔ(3-8) migrate as proteins with molecular sizes of approximately 15 and 14 kDa, respectively, in SDS-12.5% PAGE (Fig. 6a, lanes 5 and 2, respectively). When they were cross-linked with EDC and resolved by SDS-PAGE, approximately 28- and 30-kDa proteins appeared in the preparations of amino terminally truncated the wild-type repressors (Fig. 6a, lanes 3 and 4, respectively). Since the sizes of these cross-linked products were approximately twice those of their corresponding monomers, we believe that the proteins which migrate at 30 and 28 kDa represent dimer forms of the PENI and PENIΔ(3-8) monomers, respectively. In contrast, when the papain-digested form of the repressor was cross-linked with EDC, we did not detect significant amounts of dimer-sized material in the protein preparation on SDS-15% PAGE (Fig. 6b, lane 4). The cross-linked form of the papain-treated PENI appears to be smaller than the untreated form. This could be due to cross-linking within the amino-terminal domain, making it more compact and thus causing it to migrate faster on SDS-PAGE. These results suggested that at a concentration of $5 \times 10^{-6}$ M, the wild-type and the amino terminally truncated repressors exist primarily in the dimer form and the deletion of the C terminus of the repressor by papain treatment eliminated the protein’s ability to form dimers.

The N terminus of PENI is responsible for operator recognition. To examine whether the N-terminal portion of the repressor is responsible for operator recognition, we carried out DNase I footprinting analysis on the penI operator using wild-type and deletion derivatives of the repressor. A 216-bp restriction fragment that carried the penI operator was used as the target DNA in this footprinting analysis. As shown in Fig. 7, when PENI was present at a concentration of 10 nM, the

FIG. 7. DNaseI protection footprinting analysis with truncated repressors. A HindIII-EcoRI fragment containing the penI operator sequence was 5' end labeled at the HindIII site, incubated with wild-type or truncated repressor, and digested with DNaseI. (a) Operator plus PENI. Lanes 1, 2, and 3 represent 0, 2, 10, and 50 µM repressor, respectively. (b) Operator plus PENIΔ. Lanes 1, 2, and 3 represent 0, 10, and 50 nM wild-type repressor, respectively. (c) Operator plus PENIΔ(7-128). Lanes 1 to 4 represent 0, 2, 10, and 50 µM repressor, respectively.

FIG. 6. Cross-linking of amino- and carboxyl-terminal-truncated repressors with EDC. (a) Intact and amino-terminal-truncated repressors. Lanes: 1, molecular size (in kilodaltons [kD]) markers; 5 and 2, the purified intact repressor and its amino-terminal-truncated peptide, respectively; 4 and 3, material from the cross-linking reactions with intact repressor or amino-terminal-truncated peptide, respectively. (b) Intact and carboxyl-terminal-truncated repressors. Lanes: 1, molecular size (in kilodaltons [kD]) markers; 2 and 5, purified intact and carboxyl-terminal truncated repressors, respectively; 3 and 4, material resulting from the cross-linking reactions with intact repressor or carboxyl-terminal-truncated peptide, respectively.
27-bp-long penI operator was completely protected from DNase I digestion (Fig. 7b, lane 2). In contrast, neither PENIΔ(3–8) or PENIΔ(87–128) was able to protect the penI operator when they were used in nanomolar quantities (data not shown). At a concentration of 2 μM or higher, PENIΔ(87–128) preferentially protected a region corresponding to the center of the penI operator sequence from DNase I digestion (Fig. 7c, lanes 2 and 3). This indicated that the papain-digested repressor still specifically recognized the penI operator but with much reduced affinity. However, at a concentration of 2 μM, the repressor lacking the six amino acids near its N terminus did not preferentially protect the penI operator region from DNase I digestion (Fig. 7a, lane 2). This suggested that the six-amino-acid deletion at the N terminus of the repressor eliminated the operator recognition function of PENI. When the N terminally truncated form or the papain-digested form of the repressor was used at a concentration of 10 μM or higher in DNase I footprinting, regions surrounding the operator sequence were indiscriminately protected (Fig. 7a and c, lanes 3 and 4). We believe this to be the result of nonspecific binding of repressor derivatives to the DNA substrate.

**DISCUSSION**

We employed the S1 nuclease-exonuclease III (20) and PCR loop out approaches to create deletions both at the 5' and 3' ends of penI. The constructs expressing these mutant repressors were tested for their ability to suppress transcription from the penP promoter linked to the reporter gene lacZ. Results of these analyses indicated that amino acid residues positioned at both the amino and carboxy termini of PENI were essential for in vivo activity. Since immunoblot analysis demonstrated that the mutant repressors accumulated to significant levels in cells, we further concluded that the mutant repressors' failure to control the expression from the penP promoter was not due to their instability in vivo.

In vitro characterization of the purified repressor proteins, either carrying a six-amino-acid deletion at its N terminus, PENIΔ(3–8), or containing the first 86 amino acid residues, PENIΔ(87–128), provided us with some clues about the functionality of the amino acid residues positioned at the repressor termini. PENIΔ(87–128) was obtained by digesting the repressor with papain. Using DNase I analysis, we demonstrated that this peptide was able to maintain its operator-binding specificity. However, it lost its capacity to form dimers in chemical cross-linking reactions with EDC as the cross-linking agent. In contrast, PENIΔ(3–8), lost its operator-binding activity in our DNase I footprinting analysis but cross-linked efficiently with EDC. Therefore, we suggest that the repressor contains two functional domains. The domain located at the N terminus of the repressor functions as the operator recognition unit, and the domain positioned at the C terminus of the repressor is responsible for dimerization.

PENIΔ(87–128) binds the operator specifically but with significantly lower affinity than the wild-type repressor. The results of DNase I footprinting-titration experiments indicated that a 500- to 1,000-fold higher concentration of this form of the repressor is required to protect the operator against DNase I digestion compared with the native repressor. These results are consistent with those reported by Johnson et al. (8) for the N-terminal fragment of the lacI repressor, with which a significantly higher concentration of truncated repressor was necessary for protection of the operator. We propose that this decreased affinity results from the truncated repressor's inability to dimerize. The PENIΔ(87–128) monomer binds only one operator half-site and cannot be stabilized by interaction with its neighboring monomer occupying the other half-site. The suggestion for such a subunit cooperativity in operator binding is consistent with the results from our filter-binding experiments which show sigmoid kinetics.

The weak interaction of the PENIΔ(87–128) monomer with its operator is possibly due to the inability of the monomer to distort the operator DNA enough to obtain the nonspecific interactions which usually occur between the repressor dimer and the operator sequence in the protein-DNA complex. The crystal structure derived from the phage 434 repressor-operator complex (1) suggests that stabilization of the repressor-operator complex results from this repressor's ability to distort the DNA helix. This repressor "overwinds" the helix at the center of the operator, compressing the minor groove and "unwinds" the helix at the end of the operator sequence, widening the minor groove. The result is an increase in the contact surface between operator and repressor. The formation of the PENI dimer may be necessary to increase the residence time of the repressor on the operator or to trigger an organizational change in the domains of the monomer to initiate the nonspecific protein-DNA interaction.

Genetic analysis indicates that PENI is inactivated upon penicillin induction (3, 19). Immunoblot analysis indicated that the repressor remained intact during induction and that its intracellular concentration increased roughly fivefold (data not shown). Therefore, it appears that the inactivation of PENI does not take place at the level of transcription or by proteolytic cleavage, as is the case with the lacI repressor. It is possible that the repressor is modified in a way which disallows dimer formation upon penicillin induction. Isolation of the penR2 gene and identification of its gene product may shed some light on the inactivation mechanism. However, we can not eliminate the possibility that the failure of the repressor to function results from direct modification of its DNA-binding domain.

Examination of the protein sequences of PENI and the ARC repressor of bacteriophage P22 indicates that there is a region of similarity from amino acids 4 through 9 of ARC with positions 1 through 6 of PENI. This is particularly intriguing because the first nine amino acids of ARC have been shown to determine operator-binding specificity (10).

\[
\begin{array}{cccccccccccc}
1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 \\
\text{ARC: Met Lys Gly Met Ser Lys Met Pro Gln} \\
\text{PENI: Met Lys Lys Ile Pro Gln} \\
1 & 2 & 3 & 4 & 5 & 6
\end{array}
\]

Our results demonstrate the importance of the amino-terminal residues of PENI for its operator recognition. In addition, it is interesting to note that the nucleotide sequence of the consensus half-site of both operators is quite similar (10, 25). The important bases are underlined in the half-sites presented below.

\[
O_{\text{ARC}}: \text{AT}^g \text{AG}^g \text{TAC}^g \text{A}^g \text{C}^g \\
O_{\text{PENI}}: \text{ANN}^g \text{TAC}^g \text{A}^g
\]

We suggest that the similarities in the half-site sequences of the operators and the amino acid sequence of the repressors reflect the close relationship of the DNA-binding motifs of PENI and ARC. We are in the process of examining this possibility.

**ACKNOWLEDGMENTS**

We gratefully acknowledge Mervin Bibb for helpful criticism of the manuscript, Clayton Casipit and Pedro Romero for invaluable assis-
REFERENCES


