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Translation of Two Nested Genes in Bacteriophage P4 Controls Immunity-Specific Transcription Termination

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In phage P4, transcription of the left operon may occur from both the constitutive P_{LE} promoter and the regulated P_{LL} promoter, about 400 nucleotides upstream of P_{LE}. A strong Rho-dependent termination site, t_{imm}, is located downstream of both promoters. When P4 immunity is expressed, transcription starting at P_{LE} is efficiently terminated at t_{imm}, whereas transcription from P_{LL} is immunity insensitive and reads through t_{imm}. We report the identification of two nested genes, *kil* and *eta*, located in the P4 left operon. The P4 *kil* gene, which encodes a 65-amino-acid polypeptide, is the first translated gene downstream of the P_{LE} promoter, and its expression is controlled by P4 immunity. Overexpression of *kil* causes cell killing. This gene is the terminal part of a longer open reading frame, *eta*, which begins upstream of P_{LE}. The *eta* gene is expressed when transcription starts from the P_{LL} promoter. Three likely start codons predict a size between 197 and 199 amino acids for the Eta gene product. Both *kil* and *eta* overlap the t_{imm} site. By cloning *kil* upstream of a tRNA reporter gene, we demonstrated that translation of the *kil* region prevents premature transcription termination at t_{imm}. This suggests that P4 immunity might negatively control *kil* translation, thus enabling transcription termination at t_{imm}. Transcription starting from P_{LL} proceeds through t_{imm}. Mutations that create nonsense codons in *eta* caused premature termination of transcription starting from P_{LL}. Suppression of the nonsense mutation restored transcription readthrough at t_{imm}. Thus, termination of transcription from P_{LL} is prevented by translation of *eta*.

Phage-plasmid P4 enjoys multiple ways of propagation in its host, *Escherichia coli*. If the bacterial cell harbors the genome of a helper phage, such as P2, P4 can perform the lytic cycle, relying on the morphogenetic functions of the helper for the construction of its capsid and tail and for cell lysis. In the absence of the helper, P4 can propagate as a multicopy plasmid. Both in the presence and in the absence of the helper phage, P4 can establish lysogenic conditions, integrating its genome in the bacterial chromosome and establishing the immune state (for a review, see reference 29).

Under lysogenic conditions, P4 prevents the expression of the lytic genes by a peculiar mechanism based on premature transcription termination (14, 19). The P4 left operon encodes both the immunity and the replication functions (Fig. 1). Early during infection, this operon is transcribed from the constitutive P_{LE} promoter; within 15 min, the P4 immunity control is established and transcription from P_{LE} is subject to strong premature termination at a Rho-dependent termination site, t_{imm}, located about 450 nucleotides (nt) downstream of the promoter (7, 38). Moreover, the transcripts are readily processed to ≤0.3-kb RNAs (immunity transcripts) (7, 17, 38). Thus, only the leader region of the operon is transcribed, and expression of the replication functions, located in the distal part of the operon, is prevented.

The P4 immunity determinants are located in the leader region of the left operon (Fig. 1 and 2). The immunity factor, encoded by the *cI* gene, is a small RNA, the CI RNA, produced by processing of longer transcripts (17). A sequence internal to CI, *seqB*, shows complementarity to two sequences, *seqA* and *seqC*, located upstream and downstream of *cI*, respectively (the *seqC* sequence is split into *seqC′* and *seqC″*) (38) (Fig. 2). The *seqA* and *seqC* sequences represent the target sites of the CI RNA. P4 immunity is controlled by RNA-RNA interactions between the CI RNA and the *seqA* and *seqC* sequences on the nascent transcript, causing premature transcription termination at t_{imm} (7, 38). How the CI RNA elicits transcription termination is still unexplained.

Mutations either in the *cI* gene or in the *seqA* and *seqC* target sequences may impair the immunity control. In these mutants, transcription from P_{LE} is not subject to efficient termination at t_{imm}, thus leading to protracted expression of the replication genes and impairment of the ability of P4 to lysogenize the bacterial cell (17, 38).

In the plasmid state and late in the lytic cycle, expression of the replication functions encoded by the left operon is achieved by activating the late P_{LL} promoter, located about 400 bp upstream of P_{LE} (Fig. 1 and 2) (11, 13, 28, 39). This promoter is under the control of both positive (δ gene product [11]) and negative (*vis* gene product [33]) P4-encoded regulators. Although transcription from P_{LL} covers the t_{imm} region, it is not subject to premature termination. In particular, when P4 establishes the plasmid state, both the P_{LE} and P_{LL} promoters are active but only transcription from P_{LL} can read through t_{imm}. Thus, the immunity control acts only on transcription starting at P_{LE} (7, 14, 38). The P4 virulent mutant, P4 *vir1*, carries a promoter-up mutation in P_{LL} (28) (Fig. 2) that enables it to bypass the immunity control by allowing early expression of the left operon from the mutated promoter (13).

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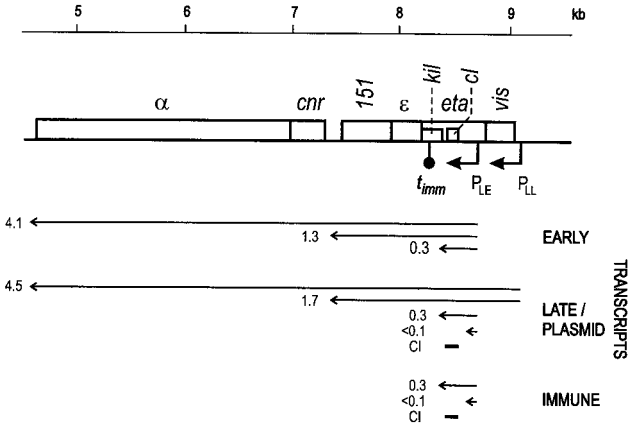

FIG. 1. Genetic map and transcription profile of the P4 essential left operon. The map of the nt 4500 to 9500 P4 genome is shown. The promoters and the t_{imm} transcription termination site are indicated. The transcripts synthesized early after infection, late in the lytic cycle, or under the plasmid conditions and in the immune state are indicated (11, 13, 14). CI indicates the small CI RNA, produced by processing (17).

P4 *c*I mutants form clear plaques (6, 8). They can also be isolated by selecting for the Ash phenotype, i.e., the ability to grow on a host lysogenic for P3, a P2-like helper phage (6, 28). Several P4 Ash$^-$ mutants have been sequenced and found to carry a base substitution in *cI* (6, 28) (Fig. 2). Conversely, the *cI405* mutation is also found to exhibit the Ash$^-$ phenotype (28). The mutant phages are affected in lysogenization ability, and premature transcription termination at t_{imm} is not efficient; hence, the expression of the downstream genes of the operon is protracted (14, 28). This suggests that the Ash$^-$ phenotype might be correlated to overexpression of one or more genes of the left operon. A peculiar kind of P4 Ash$^-$ mutant is represented by the *ash10* mutation, a base insertion in *cI* (28). This mutation suppresses the virulence conferred by the *vir1* mutation, as shown by the inability of P4 *vir1 ash10* to plate on a P4 P2 double lysogen (6, 28). A possible explanation for this phenotype is reported below.

Several P4 *cI* mutants (*cI405*, *ash3*, and *ash7*) kill the host after infection (8, 26, 27, 38). Cell death does not depend on P4 lytic growth, since it occurs in the absence of the P2 helper phage. Moreover, it is not observed when the infected cells are lysogenic for P4 or carry P4 in the plasmid condition (1). These observations led us to hypothesize the presence of a lethal function, which is normally under the control of P4 immunity and which is overexpressed in P4 *cI* mutants. The isolation of a P4 *cI405* derivative, P4 *cI405 kil1*, which is unable to establish the immune lysogenic state but does not cause cell death after infection, supported this hypothesis (1). The *kil1* mutation is recessive and linked to *cI405*.

In this paper, we describe two nested genes, *kil* and *eta*, whose coding sequences cover the t_{imm} region and demonstrate that their translation prevents transcription termination at t_{imm}. Moreover, the *cI* gene is also nested within the *eta* gene. Thus, the *cI* DNA segment encodes both the CI RNA and the amino acid residues in the middle of the Eta polypeptide.

MATERIALS AND METHODS

Bacteria and phages. The bacterial strains used were the *Escherichia coli* C strains C-1a (prototrophic) (42), C-8 (polyauxotropic; *str-1*) (4), C-236 (C-8 lysogenic for P2 and P4) (44), C-283 (C-8 lysogenic for P3) (from the Six collection), C-295 (C-1a lysogenic for P2) (42), C-520 (*supD*) (48), C-5205 (polyauxotrophic; *str-1 supD*) (12), and C-5580 (C-520 lysogenic for P2 and P4) (this work) and the *E. coli* K-12 strain JM101 (50). The phages used were P2 (3); P3 (5); P4 (44); P4 *ash7* (from the Six collection); P4 *ash23* (27); P4 *cI405* (8); P4 *cI405 kil1* (1); P4 *vir1* (30); P4 *vir1 ash10* (28); P4 *vir1 ash2* and P4 *vir1 ash4* (from the Six collection); P4 *vir1 ash28*, P4 *vir1 ash29*, P4 *vir1 ash31*, P4 *vir1 ash32*, and P4 *vir1 ash33* (reference 26 and this work), and P4 *vis2* (from the Milan collection). The P4 genome coordinates are from the updated P4 DNA sequence (GenBank accession no. X51522 [20, 51]).

Plasmids. The plasmid vectors used were pUC8, pUC18, and pUC19 (49, 50) and pGM331, a pGZ119EH derivative carrying the tRNA^{Gly} reporter gene downstream of the *ptac* promoter (7). pGM216 was constructed by insertion of the nt 8130 to 8626 DNA fragment of P4 *cI405 kil1* in the *Acc*I-*Sma*I sites of the pUC19 vector. pGM236 carries the nt 6447 to 10657 P4 region cloned in the *Bam*HI-*Nde*I sites of pUC18. pGM260 carries the P4 nt 9023 to 8657 region inserted into the *Sma*I site of the pUC8 vector. The resulting plasmid contains the *plac* promoter, the Shine-Dalgarno sequence and the first 11 codons of *lacZ* fused in frame with the third codon of *vis*, followed by *eta* fused with the terminal part of *lacZ* (see Fig. 5). pGM262 was derived from pGM260 by *Eco*RI digestion, filling in, and religation, thus creating a nonsense mutation in codon 6 of *lacZ*. pGM672, pGM673, and pGM674 are pGM331 derivatives in which the nt 8401 to 8130 region from P4$^+$, P4 *cI405 kil1*, and P4 *kil343*, respectively, obtained by PCR amplification, has been cloned between *ptac* and the tRNA^{Gly} reporter gene.

Construction of *kil-lacZ* fusions. The *lacZ* gene of pUC19, lacking the first amino-terminal codons and the upstream ribosome binding site, was obtained by PCR amplification with three pairs of oligonucleotides (263 [TGCAGGATCC CTATGCGGCATCAGAGCAG] plus 264 [GTACGGATCCACTGGCCGTC GTTTTACAAC]; 263 plus 265 [GTACGGATCCCACTGGCCGTCGTTTTAC AAC], and 263 plus 266 [TGCAGGATCCGCACTGGCCGTCGTTTTACAAC]). The amplified fragments differ from each other for 1 or 2 bp at the 5′ end of the *lacZ* gene. The fragments were digested with *Bam*HI and cloned in the *Bam*HI site of pGM331. A set of three plasmids in which the *lacZ* reading frame is shifted by 1 or 2 bp was obtained. The P4 nt 8401 to 8342 DNA fragment was cloned in the above plasmids, and expression of β-galactosidase activity was monitored by observing the colony color in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG) (40 μg/ml).

Generation of the *kil343* mutation. The *kil343* mutation (a GC-to-CG mutation at nt 8343 [Fig. 2]) was obtained by PCR amplification of the P4 nt 8421 to 8130 region by using the "overlap extension" technique (22). The resulting DNA fragment was cloned in pUC18 and sequenced.

Isolation of the P4 Ash mutants that suppress P4 virulence. It has been reported (6, 28) that a P4 *vir1 ash10* mutant, unlike P4 *vir1*, cannot be plated on a P2-P4 double lysogen (virulence suppression phenotype of the *ash10* mutation). Based on the hypothesis that virulence suppression could be due to stop codons in *eta*, we searched for other virulence-suppressing P4 Ash$^-$ mutants, both spontaneous and hydroxylamine induced. The Ash$^-$ mutants were screened to identify those whose virulence-suppressing phenotype could be reversed by nonsense suppressors.

(i) Isolation of spontaneous mutants. Five independent lysates of P4 *vir1* were plated (approximately 10^7 PFU) on C-283, a P3 lysogenic strain. The *ash* mutants appeared at a frequency of 10^{-6}. Among such P4 *vir1 ash* mutants, some formed semiturbid plaques on the P2 lysogen C-295 and did not plate on the P2 P4 double lysogen C-236. Three independent mutants (P4 *vir1 ash2*, P4 *vir1 ash4*, and P4 *vir1 ash28*), unable to be plated on a P2-P4 double lysogen (virulence suppression phenotype), were isolated and sequenced. An insertion of a C at nt 8438 to 8442 was found in all three mutants; thus, the mutation was identical to *ash10*.

(ii) Hydroxylamine mutagenesis. A P4 *vir1* stock at 2×10^{11} PFU/ml in 0.075 M $MgCl_2$ was mixed with 1 volume of 2 M hydroxylamine and incubated at 37°C. Samples were removed after 4, 8, and 20 h, hydroxylamine was inactivated with 10% acetone, the phage surviving the mutagenic treatment was assayed, and P4 *vir1 ash* mutants were selected by plating on C-283. The frequency of *ash* mutants was increased from 10^{-6} to 10^{-4} by the mutagenic treatment. The ability of the P4 *ash* mutants to form plaques on C-236 was tested. The P4 *vir1 ash* mutants that exhibited virulence suppression were spotted on C-5580 to identify the *amber* mutants. Four independent mutants (P4 *vir1 ash29*, P4 *vir1 ash31*, P4 *vir1 ash32*, and P4 *vir1 ash33*) were isolated. Restriction analysis showed that all four mutations created a *Mae*I site in the nt 7041 to 8655 region. P4 *vir1 ash29* was sequenced and found to carry a G-to-A transition at 8433. The *ash29* mutation suppressed P4 virulence on a wild-type *E. coli* host, but did not do so on both *supD* and *supF amber* suppressor strains; on the other hand, the Ash$^-$ phenotype (ability to grow on a P3 lysogen) was not noticeably affected by the *amber* suppressor (data not shown).

Marker rescue. *E. coli* C-5205(P2) carrying pGM216, in which the P4 *cI405 kil1* nt 8626 to 8130 region is cloned, was infected with P4$^+$, and the phage produced was analyzed. The phages that formed clear plaques were tested for the ability to grow on P3 lysogens (Ash phenotype) and for causing cell death upon infection of C-1a (killing phenotype). P4 *cI405* kills the infected cells and is Ash$^-$ (8, 28), whereas P4 *cI405 kil1* does not kill the cells and is Ash$^+$ (1). Of 31 clear plaques analyzed, 17 were Ash$^-$ and caused cell killing after infection (like P4 *cI405*) and were Ash$^+$ and did not cause cell killing (like P4 *cI405 kil1*). Thus,

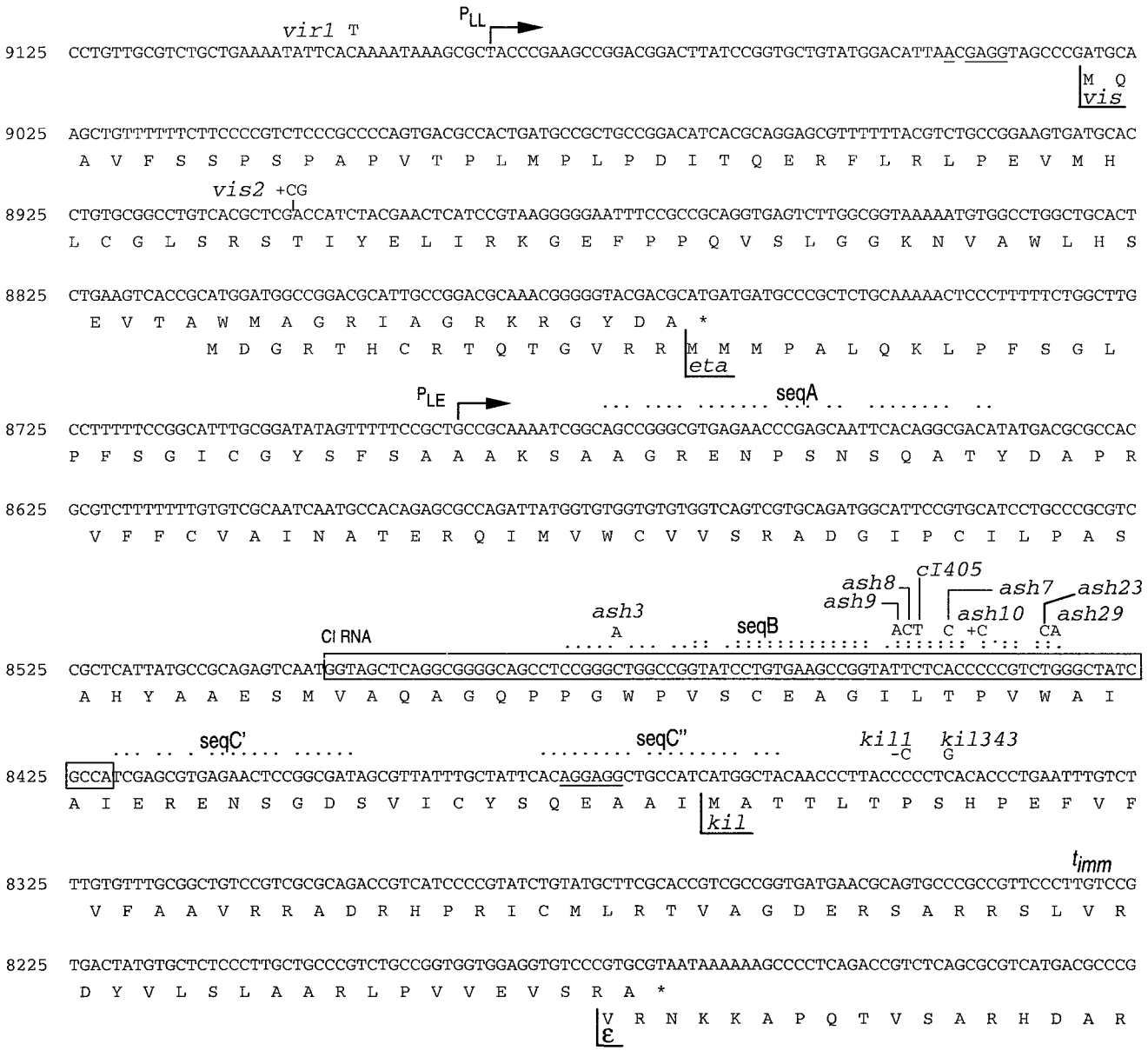

FIG. 2. Sequence of the 5′ region of the P4 left operon. The coding strand of the P4 nt 8126 to 9125 region and the amino acid sequence (in single-letter code) are shown. The transcription start points from P_{LL} and P_{LE} and the *vis*, *eta*, *kil*, and ε initiation codons are indicated. The stop codons are indicated by asterisks. The ribosome binding sites of *vis* and *kil* are underlined. The region encoding the CI RNA is boxed (the 3′ end was modified as described in reference 16). The *seqA*, *seqC′*, and *seqC″* regions are indicated by dots; the bases of the *seqB* region complementary to *seqA* and *seqC* are indicated by the upper and lower sets of dots, respectively. The positions of the mutations are indicated above the sequence. The indicated *ash10* mutation is identical to the *ash2*, *ash4*, *ash26*, and *ash28* mutations. The *ash7* mutation was sequenced by Lane (26). The *ash3*, *ash8*, *ash9*, *ash10*, and *cI405* mutations had been sequenced previously (28). All other *ash* mutations shown were sequenced as part of a thesis project (26) and of the work reported here. They include two mutations, *ash7* and *ash23*, which do not suppress P4 virulence.

both the *cI405* mutation and the *cI405 kil1* double mutations could be rescued from the 8626 to 8130 region at a similar frequency. P4 cI^+ *kil1* recombinants could not be identified.

Northern blot hybridization. RNA was extracted from *E. coli* and from P4-infected cells, fractionated on either 1.5% formamide–formaldehyde agarose or 10% polyacrylamide–urea denaturing gels, and transferred to Hybond N filter membranes (Amersham) as described previously (14). The ^{32}P-labeled RNA probes P_{LE}-t_2 and P_{LL} cover the P4 nt 8418 to 8774 and nt 8774 to 9023 regions, respectively, and were prepared and used for the hybridization as described previously (14). The oligonucleotide used for Northern analysis of $tRNA^{Gly}$ expression (45) was 5′-end labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP as described by Sambrook et al. (40). Hybridization was performed as described by Briani et al. (7).

Computer sequence analysis. For sequence analysis, we used several programs of the Wisconsin package versions 9 and 10, Genetics Computer Group (GCG), Madison, Wis., in particular Bestfit, Pepsort, Pileup, and FoldRNA. For database searches, we used the Blast programs (2).

RESULTS

Identification of the P4 *kil* gene. Infection of *E. coli* with P4 *cI405* leads to cell killing, whereas no death occurs after infection with either $P4^+$ or P4 *cI405 kil1* (1). In attempts to clone P4 *cI405* DNA fragments in the pUC18 vector, we were unable to isolate viable transformants with plasmids carrying the nt 8774 to 8130 fragment, which includes the constitutive promoter P_{LE} and the downstream 570 nt (Fig. 3). The same

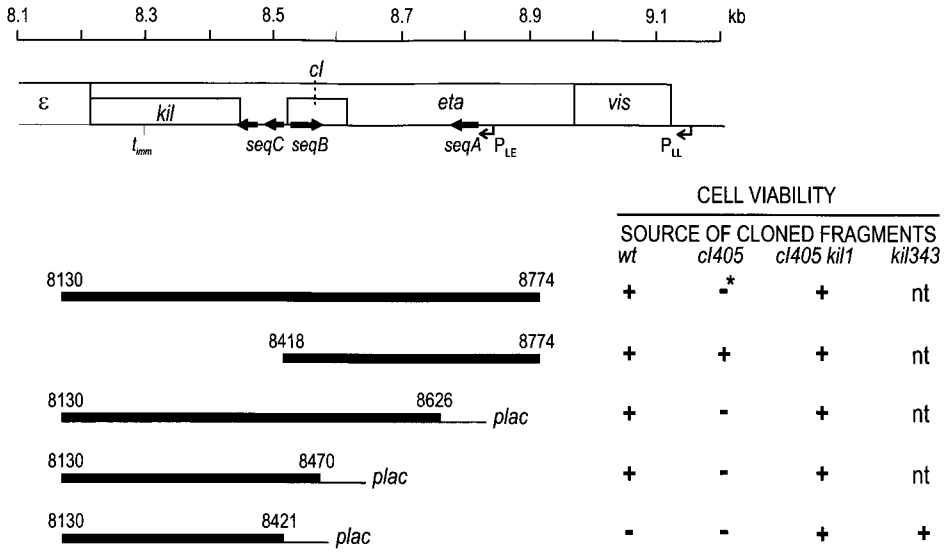

FIG. 3. Effect on cell viability of cloned P4 fragments. The map of the P4 region from kb 8.1 to 9.2 is shown. The genes are boxed. The *seqA*, *seqB*, and *seqC* inverted and direct repeats are indicated by arrows. The positions of the P_{LE} and P_{LL} transcription start points and of the t_{imm} terminator are indicated. The coordinates of the fragments derived from P4 wild type (*wt*), P4 *cI405*, P4 *cI405 kil1*, and P4 *kil343* cloned in the pUC8 vector are indicated. Strain JM101 was transformed, and the viability of the clones carrying the above constructs was tested. +, viable; −, nonviable; −*, not obtained; nt, not tested. For the constructs lacking the P_{LE} promoter, viability was measured in the presence of IPTG (40 μg/ml) to induce the *plac* promoter.

fragment derived from either P4$^+$ or P4 *cI405 kil1* could be readily cloned, as well as the smaller nt 8774 to 8418 fragment of P4 *cI405*. This suggested that the P4 nt 8774 to 8130 region encodes the function responsible for cell killing upon P4 *cI405* infection.

To map the putative *kil* gene, we cloned the nt 8626 to 8130 and nt 8470 to 8130 DNA fragments of P4$^+$, P4 *cI405*, and P4 *cI405 kil1*, in which the P_{LE} promoter region is deleted, downstream of the *plac* promoter. All fragments could be cloned under noninducing conditions. However, after induction of transcription from *plac*, the fragments derived from P4 *cI405* caused cell death whereas the fragments derived from either P4$^+$ or P4 *cI405 kil1* did not. Moreover, the cloned *kil*$^+$ nt 8421 to 8130 fragment caused cell killing after induction of *plac* whereas the *kil1* did not (in such fragments, part of the immunity region including the *cI405* locus is deleted [28]). Thus, we concluded that (i) the killing function is encoded within the nt 8421 to 8130 region; (ii) the *kil1* mutation, which inactivates the killing function, maps in this region; and (iii) the *cI405* mutation is not directly responsible for cell death but, rather, might alter the control of the lethal function.

The nt 8626 to 8130 region of P4 *cI405 kil1* was cloned in pGM216 (see Materials and Methods). Both the *cI405* and the *kil1* mutations were rescued after infection with P4$^+$ of *E. coli*(P2) carrying this plasmid. By sequencing pGM216 we found, on the DNA strand shown in Fig. 2, in addition to the *cI405* mutation (a C to T substitution at nt 8446), a deletion of a C in a series of five C's at nt 8345 to 8349 (Fig. 2 and data not shown). Thus, *kil1* appears to be a frameshift mutation in an open reading frame (ORF) that starts upstream of nt 8345. To define the possible start codon and frame of translation, we created a fusion of the nt 8401 to 8342 P4 DNA fragment and the *lacZ* gene in the three possible frames (see Materials and Methods). In these constructs, expression of β-galactosidase activity depends on translational control sequences present in the P4 fragment. Light blue colonies were found only in the construct, in which *lacZ* is in frame with the ATG codon at nt 8365 (Fig. 2). In P4, this ORF is preceded by a good potential ribosome binding site and encodes a 65-amino-acid polypeptide (*orf65*). The *kil1* deletion causes a frameshift in codon 7 of *orf65*.

To confirm that *orf65* encodes the killing function, we constructed by PCR mutagenesis a C-to-G base substitution at 8343 (*kil343* [Fig. 2]), creating a stop codon (UAG) in codon 8 of the ORF. A plasmid carrying the nt 8421 to 8130 P4 region with the *kil343* mutation did not cause cell killing when transcription of this region was induced from the *plac* promoter (Fig. 3). Thus, *orf65* is the P4 *kil* gene.

Cell death caused by *kil* expression. To analyze the effects of *kil* expression on the viability of the bacterial host, a culture of strain C-1a was infected with P4 *cI405* and the effects on cell growth were monitored. The turbidity of the culture increased exponentially for at least 5 h after the infection; however, microscopic observation of the infected cells showed that after 3 h about 45% of the cells appeared as filaments about 10 times the length of a normal *E. coli* cell and after 5 h, most cells were long, aggregated filaments (data not shown). All the macromolecular syntheses (DNA, RNA, and protein) of the host continued at a normal rate up to 3 h after infection, whereas a 50% decrease was observed after 5 h (data not shown). On the other hand, the fraction of cells surviving the infection, as measured by colony formation, was 0.2%. Unviable microcolonies containing filamentous cells were visible at low magnification.

Similarly, upon induction of transcription of the cloned *kil* gene, arrest of cell division and consequent filamentation were observed and the increase of the cell mass stopped after 3 h (data not shown); viable counts were less than 0.1% of the noninduced cells.

Translation of the *kil* gene interferes with transcription termination at t_{imm}. The Rho-dependent termination site t_{imm} is located within the *kil* gene (7), suggesting that translation of *kil* would interfere with transcription termination at t_{imm}. Thus, we tested whether *kil* translation and transcription termination were inversely correlated. We cloned a tRNA^{Gly} reporter gene downstream of the *kil* region and analyzed transcription be-

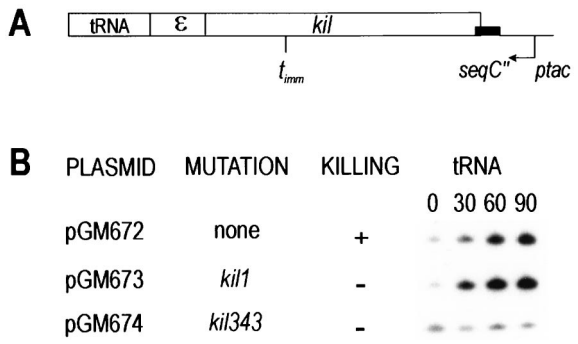

FIG. 4. Correlation between *kil* translation and transcription termination at t_{imm}. (A) The nt 8130 to 8401 P4 region was cloned in the pGM331 vector, upstream of the $tRNA^{Gly}$ reporter gene. (B) The DNA was derived from P4 wild type, P4 *kil1* (frameshift) and P4 *kil343* (nonsense) in pGM672, pGM673, and pGM674, respectively. Expression of the *kil* gene was monitored by measuring cell death in the presence of 40 μg of IPTG per ml, to induce transcription from *ptac*. The production of $tRNA^{Gly}$, measured by Northern blotting, is shown: RNA was extracted at the times indicated (in minutes) after the induction with IPTG, from C-1a cultures carrying the plasmids indicated, fractionated by electrophoresis on a 10% acrylamide gel, and hybridized to the $tRNA^{Gly}$ specific oligonucleotide, ^{32}P labelled at the 5′ end.

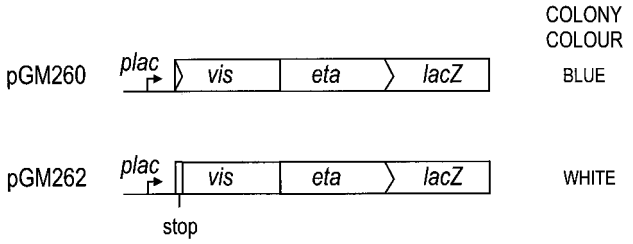

FIG. 5. Translation of the P4 *eta* gene. A schematic representation of pGM260 and pGM262, in which the P4 nt 9023 to 8659 region is cloned, creating an *eta-lacZ* fusion, is shown. pGM262 contains a translational stop codon at the 5′ end of *vis*. The plasmids were carried by JM101. The colors of the colonies grown in the presence of IPTG (40 μg/ml) and X-Gal (40 μg/ml) are reported.

yond t_{imm} by monitoring tRNA production. Constructs carrying the wild-type *kil* gene (pGM672), the *kil343* nonsense mutation (pGM674), or the *kil1* frameshift mutation (pGM673) were obtained (Fig. 4). RNA was extracted from cultures of C-1a carrying the above plasmids at different time points after the addition of the IPTG inducer, and the presence of $tRNA^{Gly}$ was monitored by Northern analysis (see Materials and Methods). Cell killing was also measured. We found that induction of wild-type *kil* expression (pGM672) leads to cell killing and tRNA production. In pGM674, the *kil343* nonsense mutation not only prevented cell killing but also caused premature transcription termination at t_{imm}, as indicated by the lack of production of the reporter tRNA. In pGM673, the *kil1* frameshift mutation, which does not generate a translational stop codon downstream, prevents cell killing but does not affect tRNA production. These results indicate that translation of the *kil* region, but not the presence of the Kil protein, is required for override of t_{imm}.

The *kil* gene is the terminal segment of a longer ORF expressed from P_{LL}. By sequence inspection, we found that the *kil* gene is the terminal part of a longer ORF that extends upstream of P_{LE} (Fig. 2). This ORF could be translated when transcription starts from P_{LL}. Four ATG codons are found upstream of P_{LE}: one is at nt 8814, within *vis*, the first gene downstream of P_{LL}, and three consecutive ATG codons partially overlap the stop codon of *vis*. No good ribosome binding sites are found immediately upstream of these ATG codons, suggesting that this ORF might be translationally coupled to *vis*.

To test whether the ORF would be translated, the P4 nt 9023 to 8659 region, carrying the *vis* gene and the 5′-terminal part of the downstream ORF, was cloned in pUC8, creating a fusion with the *lacZ* gene (pGM260) (Fig. 5). Strain JM101 transformed with the above plasmid formed blue colonies when plated on medium containing IPTG and X-Gal. A similar construct (pGM262), in which a stop codon upstream of the cloned fragment prevents *vis* translation, gave rise to white colonies. This indicates that translation is coupled to the upstream *vis* gene translation. The translational coupling favors the hypothesis that translation initiates at an ATG codon partially overlapped with the *vis* stop codon, thus encoding a 199-amino-acid protein. We named this gene *eta* (for "enables transcription antitermination" [see below]).

Translation of *eta* prevents premature transcription termination at t_{imm}. Since *eta* covers the t_{imm} region, it might be supposed that its translation prevents transcription termination at this site. This might explain why transcription starting from P_{LL} reads through t_{imm}. To verify the above hypothesis, we tested whether mutations that create a stop codon in *eta* induce premature termination of transcription starting from P_{LL}. Two mutations of this type were tested: *ash10*, a base insertion at nt 8438 that creates a stop codon at 8414 (Fig. 2) (28), and *ash29*, a base substitution that creates an *amber* codon at 8433 (Fig. 2) (see Materials and Methods). It should be noted that both mutations are located in the segment of *eta* that contains the *cI* gene. Thus, the mutations not only affect *eta* translation but also produce a defective CI RNA.

We used Northern blotting to analyze the transcripts synthesized by P4 *vir1 ash10* and P4 *vir1 ash29* after infection of strain C-1a (Fig. 6A and data not shown). The phages carried the *vir1* promoter-up mutation to increase the amount of transcription starting from P_{LL} (13). The RNAs were hybridized with the P_{LE}-t_2 probe, which covers the P_{LE} proximal region and identifies transcripts starting from both P_{LE} and P_{LL}. The same filters were hybridized with the P_{LL} riboprobe, specific for transcripts from P_{LL}. Comparing the transcription pattern of the mutants with that of P4 *vir1*, the major effect of the mutations was the lack of the 4.5- and 1.7-kb RNAs starting from P_{LL} and the appearance of new RNA species of about 0.5 to 0.7 kb synthesized from P_{LL}. These data suggest that the *ash29* and *ash10* mutations cause premature termination of transcription from P_{LL}.

Upon P4 *vir1 ash29* infection of the C-520 strain, which carries the *supD amber* suppressor, the synthesis of the 4.5- and 1.7-kb transcripts was restored (Fig. 6B). Thus, suppression of the *amber* stop codon in *eta* prevents premature termination of transcription from P_{LL}. On the other hand, after infection with the mutant phages of both the sup^+ and the *supD* hosts, the 4.1- and 1.3-kb transcripts starting from P_{LE} persisted for a long time, suggesting that transcription from this promoter is not efficiently terminated at t_{imm}. These results indicate that the *ash29* and *ash10* mutations alter the P4 immune response. This latter phenotype is not suppressed by *supD*.

Polar effect of a mutation in the P4 *vis* gene. Since translation of *eta* appears coupled to *vis*, we supposed that mutations which stop *vis* translation might also cause premature termination of transcription from P_{LL}. A frameshift mutation, *vis2* (2-bp insertions at nt 8904 [Fig. 2] [9]), creates a stop codon in *vis* at nt 8861. The transcriptional profile of P4 *vis2* after infection of C-1a was analyzed by Northern blotting (Fig. 6C). The P_{LE} transcripts were normally synthesized, but almost all

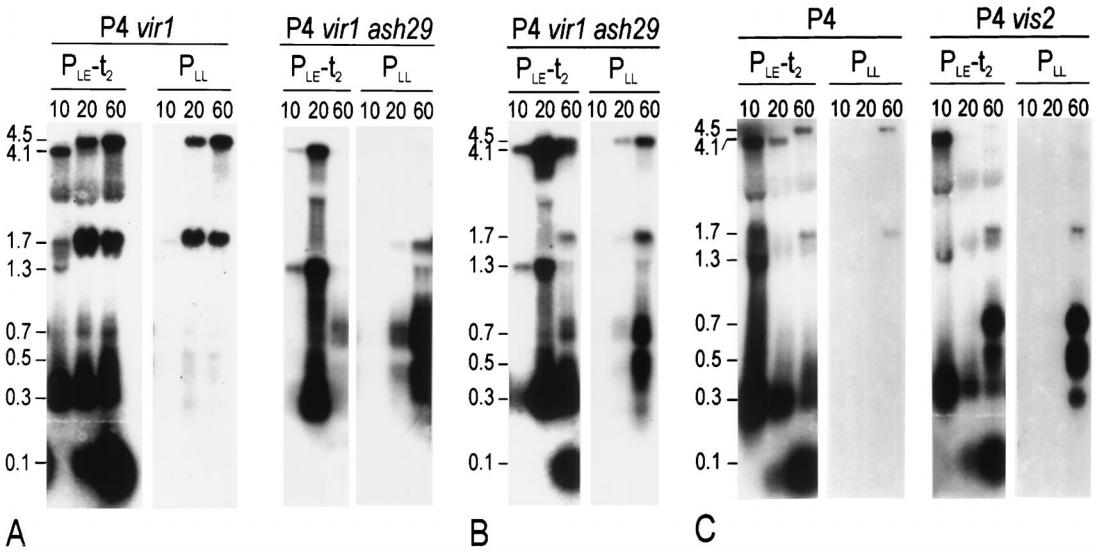

FIG. 6. Transcription of the P4 left operon in P4 mutants. The P4 infecting phage is indicated at the top. RNA extracted from P4-infected cells at the times indicated (in minutes) after infection was fractionated in 1.5% agarose and hybridized to the riboprobe P_{LE}-t_2 (P4 nt 8418 to 8774 region) or P_{LL} (P4 nt 8774 to 9023 region), as indicated (see Materials and Methods). The size of P4 transcripts is indicated on the left in kilobases. (A and C) Infection of C-1a. (B) Infection of C-520 (*supD*).

the RNA synthesized from P_{LL} was 0.5 to 0.7 kb long. Thus, as expected, the *vis2* mutation causes premature termination of transcription from P_{LL}. It must be noted that the overall amount of transcription from P_{LL} was increased in P4 *vis2* compared to P4 wild type, which is consistent with the lack of the Vis repressor (33).

DISCUSSION

P4 *kil* and *eta* genes. We have identified two nested genes, *kil* and *eta*. The start codon of *kil* lies within, and is in frame with, the *eta* coding sequence; thus, the Kil protein is identical to the C-terminal part of Eta. Both genes are located in the P4 left operon proximal to the P_{LL} promoter. However, the *kil* region is transcribed both from P_{LE} and P_{LL} whereas *eta*, whose start codon is upstream of P_{LE}, is expressed only from P_{LL}.

The *kil* gene maps at nt 8365 to 8169 and is preceded by a good Shine-Dalgarno sequence. It encodes a 65-amino-acid polypeptide (7,322 Da) with a calculated pI of 11.87, which causes cell death when overexpressed.

The *kil1* frameshift mutation creates a fusion with the downstream ε reading frame. Accordingly, a protein of approximately 17.5 kDa, which might represent the *kil1*-ε fusion protein, was observed in cells infected with P4 *cI405 kil1* but not in cells infected with P4 *cI405* or $P4^+$ (1).

Expression of the *kil* gene from P_{LE} is controlled by P4 immunity. This is shown by the following findings: (i) host killing after P4 infection was observed when the phage carried a mutation which affects the immunity system (1, 8, 38), (ii) cell killing upon P4 *cI405* infection did not occur in a P4 lysogenic host (1), and (iii) the presence of a wild-type immunity region upstream of a cloned *kil* gene impaired expression of the killing function, whereas the presence of either the *cI405* mutation or a deletion of the immunity region upstream of *kil* led to *kil* expression. The killing function is also controlled in the plasmid state, since P4 *cI405* can propagate as a multicopy plasmid without severely affecting cell viability (1).

The *eta* gene can be expressed only from P_{LL}. The start codon of *eta* has not been defined exactly, since several possible ATG codons are present in frame upstream of P_{LE} (Fig. 2). Accordingly, the Eta protein is expected to be 197 to 199 amino acids long. It appears that *eta* translation is not efficiently initiated per se, probably for the lack of a good ribosome binding site, and it is coupled to translation of the upstream *vis* gene. In fact, if translation of a cloned *vis* gene is prevented, *eta* is not expressed.

In the plasmid condition and after infection with P4 *vir1*, when transcription starts at the upstream promoter P_{LL} (13), the *kil* region is transcribed as the distal part of *eta*. Nevertheless, cell killing is not observed in such conditions. This suggests that *eta* translation prevents translation of the *kil* gene; alternatively, or in addition, the Eta protein might counteract the lethal effect of Kil.

The translational stop within *eta* caused by the *ash29* and *ash10* mutations appears not to interfere with P4 production in lytic infection. In fact, the P4 *vir1* and P4 *vir1 ash29* burst sizes in C-295 infection were quite similar (153 and 138 PFU/infected cell, respectively [43]). Hence, a complete Eta protein appears not to be essential for P4 propagation. That the N-terminal segment of Eta, still present in the truncated forms produced by the *ash29* or *ash10* mutants, might contribute a function for P4 production remains a possibility.

Control of transcription termination at t_{imm} by translation of *kil*. When the *kil*-t_{imm} region is cloned on a plasmid, the presence of the *kil343* nonsense mutation causes premature termination of transcription at t_{imm} whereas the *kil1* frameshift mutation, which does not create stop codons in the *kil* region, does not affect transcription. This rules out a direct role of the Kil protein in antitermination and suggests that translation of the *kil* region per se inhibits transcription termination at t_{imm}.

These data lend further support to the hypothesis that P4 immunity may induce premature transcription termination of the transcripts starting from P_{LE} by impeding *kil* translation (7, 38): interaction of the CI RNA with the complementary target sequences on the nascent transcript might prevent initiation of *kil* translation, thus inducing Rho-dependent transcription termination at t_{imm}. Consistent with this hypothesis, the Shine-Dalgarno sequence and the ATG codon of *kil* fall within the *seqC″* target sequence, complementary to *seqB* in the CI RNA (Fig. 2).

Control of transcription termination at t_{imm} by translation of *eta*. Transcription starting from P_{LL} proceeds through t_{imm}, covering the downstream part of the operon (13, 38). Our data indicate that this is due to translation of *eta*, whose start codon is upstream of P_{LE}, and is not under P4 immunity control. Indeed, mutations that stop *eta* translation caused premature termination of transcription starting at P_{LL}, and suppression of the P4 *ash29 amber* mutation in a *supD* host restored transcription through t_{imm}. Premature transcription termination was also caused by nonsense mutations in the upstream *vis* gene, to which *eta* appears translationally coupled.

Premature transcription termination from P_{LL} generates 500 to 700-nt RNAs. The P_{LL} promoter is located about 850 nt upstream of t_{imm}. It is likely that the P_{LL} transcripts terminated at t_{imm} are subsequently processed, similarly to the P_{LE} transcripts that are terminated at t_{imm} and processed to 0.1- to 0.3-kb RNAs (14, 38).

When *eta* translation is blocked by a nonsense mutation upstream of *kil*, the *kil* gene is not translated, as deduced from the absence of killing and from the occurrence of premature transcription termination. This suggests that, in the mutants, the transcripts starting from P_{LL} might be under the control of P4 immunity and might terminate prematurely at t_{imm}. Thus, antitermination of transcription from P_{LL} in wild-type P4 appears to be due to the presence of ribosomes that might inhibit both the RNA-RNA interactions between CI RNA and the target sequences that control P4 immunity and transcription termination at the Rho-dependent terminator t_{imm}.

This also can explain the virulence suppression phenotype exhibited by some *ash* mutations. The virulence of P4 *vir1* results from a promoter-up mutation in P_{LL} that makes transcription from this promoter independent of positive regulators (13, 28). Since transcription from P_{LL} is not controlled by P4 immunity, the virulent phages can grow on P4 lysogenic hosts. The *ash10* and *ash29* mutations, which suppress P4 virulence, interrupt *eta* translation, thus causing premature termination of transcription at t_{imm} and impairing P4 growth in an immune host.

It should be noted that the *ash29* and *ash10* mutations affect two P4 genes, *eta* and *cI* (Fig. 2), and confer two phenotypic traits: virulence suppression and Ash^- (i.e., the ability to exploit a P3 prophage as helper). The former depends on the translational stop in *eta*, as discussed above, whereas the latter appears to be a consequence of the change in the CI RNA: in fact, for P4 *ash* mutants, transcription starting at P_{LE} is not efficiently terminated at t_{imm}, leading to protracted expression of the downstream genes of the operon. In P4 *ash29*, the nonsense mutation for *eta* is suppressed in a *supD* or *supF* host, as expected, whereas the mutational change for the CI RNA is not suppressed, as demonstrated by the persistence of the 4.1- and 1.3-kb transcripts at late times after infection. Accordingly, the Ash^- phenotype of P4 *vir1 ash29* persists in a *supD* or *supF* host (data not shown). These data suggest that the Ash^- phenotype is correlated with overexpression of one or more genes of the P4 left operon. A possible candidate is the P4 ε gene product, which is required for derepression of P2 prophage (18, 31). It might be hypothesized that greater production of the ε protein may be required to derepress a P3 prophage.

Possible role of Kil and Eta. Overexpression of *kil* both in P4 *cI405* infection and from a plasmid leads to cell filamentation, inhibition of macromolecular syntheses, and production of nonviable microcolonies, suggesting that the Kil protein may interfere with cell division.

The *kil* gene is the first P4 gene expressed after infection, and its expression, regulated by several mechanisms, is confined to the early phase, preceding the onset of the immune system control. Under these conditions, *kil* expression is not harmful to the host; hence, the lethal effect appears to be a consequence of deregulation.

A possible role of *kil* in P4 biology could be to transiently inhibit cell division at early times after infection, thus enabling replication of the phage genome before the cell divides. This might increase the chance that the P4 genome will be inherited, either as an integrated prophage or as a multicopy plasmid, when inhibition of division will be relieved. Moreover, in the presence of P2, the lytic cycle may be more efficient, since inhibition of division may provide both an increased cell size and a higher dosage of helper prophage genomes.

Our results indicate that not only is expression of *kil* controlled by multiple mechanisms in the different developmental phases of P4 but also *kil* itself is part of the mechanism controlling the P4 life cycle.

Immediately after infection of a sensitive host, *kil* can be transcribed from P_{LE} as the first gene of the operon essential for P4 replication. Translation of *kil* may not be efficient, since both readthrough and prematurely terminated transcripts are produced. As soon as the mature CI RNA is produced, not only may translation of *kil* be inhibited, according to our model, but also its transcription may be inhibited, due to termination at t_{imm}. These events appear to be central to the establishment of prophage immunity (7, 38).

However, both in the lytic cycle and under the plasmid conditions, P4 must bypass the transcription termination mechanism controlled by the immunity to express the replication genes, but at the same time it must avoid the expression of the lethal *kil* function. These two conflicting demands are met by translation of the *eta* gene.

Blast database searches (2) found several matches for the *eta*/*kil* region, at both the DNA and protein sequence levels. Some of them have been previously reported (15, 21, 47). Figure 7 shows a multiple alignment of the matching protein sequences. The close relative of P4, ΦR73 (23, 47), contains a sequence very similar to the whole Eta sequence. Other less extensive sequence matches were found for the bacteriophage N15 *cA*/*gene 32* region (21) and for the *Shigella flexneri* prophage-related sequences SFS and, in particular, SFW, which encodes p179 (15). For N15, it was demonstrated that expression of a 57-amino-acid polypeptide, homologous to Kil, causes cell death (34), whereas no effect on cell growth was observed by expressing the less homologous region of SFW (15). All these genes are nested in a longer ORF, which, if translated, might play a role similar to P4 *eta*.

Shorter though statistically significant matches were found for the *eta* region immediately upstream of *kil* with segments of two closely related IncI1 plasmids, ColIb-P9 (41) and R64 (24, 25). Interestingly, these two plasmids, as well as N15, encode primases that are related to that of P4 (32, 46), suggesting evolutionary connections.

It should be noted that in P4, ΦR73, and N15, a gene encoding a small RNA is nested within the ORF extending upstream of *kil* (17, 28, 37). Such small RNAs exhibit remarkable sequence homology that may impose constraints on the codons in the overlapping reading frames. Thus, the alignment for amino acid sequences derived from such a region (overlined in Fig. 7) might not necessarily have a functional relevance at the translational level. This might be especially true for the IncI1 plasmid sequences, for which no evidence for their translation has been reported. We have analyzed by FoldRNA the RNA sequences corresponding to the P4 *cI* region in ColIb-P9, R64, and SFW and found predicted secondary structures identical to that of P4 CI RNA (data not

```
P4 res          1                                                          60
                1        .         .         .         .         .         60
P4 eta/kil      MMMPaLQKlPFSGLPFSGICGYSFSAaAKSAAGReNPsNSqATYDAPrVFFCVAINATER
ΦR73 eta/kil    MMMPvLQKvPFSGLPFSGICGYSFSAvAKSAAGRrNPcNSkATYDAPcVFFCVAINATER
N15 cA-32       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
SFW p179        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~mlNvaienqngwnysapAphkTga
ColIb-P9        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
R64             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

P4 res          61                                                        119
                61       .         .         _________._________._________120
P4 eta/kil      QIMVWcVVsRAdGipcILpAsAHYAaESMVAQAGQPPGWPVScEAGIlTPVWAIa.IERe
ΦR73 eta/kil    QIMVWrVVcRA.Gm..ILfAiAcYAtESMVAQAGQPPGWPVffEAGIPTPVWAIa.IERR
N15 cA-32       ~~~~~~~~~~~~~~~~~~~~~~~~~~~MVAQmGptsvgPVSsctGvenPVWAtTpIEil
SFW p179        gIatptmttahnraqavflcvkHshiqiMVgrAGQPqGWPVSvvtGcsnPV.rlTthEia
ColIb-P9        ~~~~~~~~~~~~~~~~~~~~~~~~~~~MVAQAGQalaWPVSddAGIPTPVWAIT.nERR
R64             ~~~~~~~~~~~~~~~~~~~~~~~~~~~MVdQAGQalaWPVSDdAGIPTPVWAIT.nERR

P4 res          120                                                       172
                121      .         .         .         .         .       180
P4 eta/kil      NSGdSvicysqeaAIMATTLTPSHPE.......FVFVFAAVRRADRhPrICMLRTvAGDE
ΦR73 eta/kil    NSGdSSyLlleGdglMATTLTPSHPE.......FVFVFAAVRRADRhPrICMLRTvAGDE
N15 cA-32       NSGgSt.LykIG..........mHtm.......FkFkFAAVvRtDkKshIhrLsTiAssE
SFW p179        tSGgeSFkltIeaAIMATiLTlSHPdatiengraVttsvAVaeffRKmhknviqkietlE
ColIb-P9        NS.viaFLvvIGdlcyvTstdtetPg.......hhsVgrhVsRAinKPa~~~~~~~~~~~
R64             N~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

P4 res          173                     199
                181      .         .         .   216
P4 eta/kil      RSAR.RSLVRDYVLSLAARLPVVEVSRA~~~~~~~~
ΦR73 eta/kil    RSAR.RSLVRDYVLSLAARLPVVEVSRA~~~~~~~~
N15 cA-32       ReAR.RqfasrfVLvLsARiPVsEVia~~~~~~~~~
SFW p179        cSpefnrLnfkpVtytdAkaknaqctkspktasfsw
ColIb-P9        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
R64             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
```

FIG. 7. Multiple sequence alignment for P4 Eta/Kil and homologs. Multiple alignment by the GCG Pileup program for the translated DNA sequences of P4 Eta/Kil, ΦR73 Eta/Kil (23, 47) (GenBank accession no. M64113), N15 *cA/gene 32* region (21) (AF064539 and U63086), *S. flexneri* SFWp179 (15) (Z23101), ColIb-P9 (41) (AB021078), and R64 (24, 25) (AB027308) is shown. Conserved amino acids are in capitals. In P4 and ΦR73, the first amino acid of the Kil protein is in boldface type, and in the N15 sequence, the first amino acid of the peptide that causes cell killing when expressed is in boldface type (28). The region corresponding to the P4 *cI* gene is overlined.

shown). It would be interesting to analyze whether these elements also express a small regulatory RNA.

Genes that kill the host when overexpressed have been reported for other phages, such as *icd* for P1 (36), *kil* for λ (35), and *kil* for the defective prophage Rac (10). In all these cases, inhibition of cell division appears to be responsible for cell death. Even though P4 Kil shares with other Kil proteins a small size (<100 amino acids) and a positive net charge, no relevant homology could be found between the above proteins with the BestFit program of the GCG software package. This suggests that for these bacteriophages the mechanism underlying inhibition of cell division might have evolved independently.

P4 *vis2* mutant and the control of P_{LL}. The *vis* gene encodes the P_{LL} repressor. The Vis protein binds immediately downstream of P_{LL} and blocks transcription of the left operon from this promoter (33). Thus, the Vis protein negatively autoregulates its own expression. The P4 *vis2* mutant synthesizes a truncated Vis protein, which lacks the helix-turn-helix DNA binding motif. Accordingly, transcription from P_{LL} is greatly increased. It should be noted that the *vis2* mutation does not influence the timing of P_{LL} activation. Thus, P4 *vis2* differs from P4 *vir1* in that in the latter, the promoter-up mutation makes transcription independent of activators (13) but still repressible by the Vis protein, whereas in P4 *vis2*, δ-dependent activation of P_{LL} is not repressed. Although in P4 *vis2* the negative control on the P_{LL} promoter is absent, no overexpression of the left operon genes is observed, due to the polar effect of the *vis2* mutation on *eta*.

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