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 PLe promoter and the regulated PLL promoter, about 400 nucleotides upstream of PLe. A strong Rho-dependent termination site, timm, is located downstream of both promoters. When P4 immunity is expressed, transcription starting at PLe is efficiently terminated at timm, whereas transcription from PLL is immunity insensitive and reads through timm. 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 PLe promoter, and its expression is controlled by P4 immunity. Overexpression of kil causes cell killing. This gene is the terminal part of a longer reading frame, eta, which begins upstream of PLL. The eta gene is expressed when transcription starts from the PLL 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 timm site. By cloning kil upstream of a tRNA reporter gene, we demonstrated that translation of the kil region prevents premature transcription termination at timm. This suggests that P4 immunity might negatively control kil translation, thus enabling transcription termination at timm. Transcription starting from PLL proceeds through timm. Mutations that create nonsense codons in eta caused premature termination of transcription starting from PLL. Suppression of the nonsense mutation restored transcription readthrough at timm. Thus, termination of transcription from PLL 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 PLe promoter; within 15 min, the P4 immunity control is established and transcription from PLe is subject to strong premature termination at a Rho-dependent termination site, timm, 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 timm (7, 38). How the CI RNA elicits transcription termination is still unexplained.

Mutations either in the cI gene or in the seqA or seqC target sequences may impair the immunity control. In these mutants, transcription from PLL is not subject to efficient termination at timm, 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 PLL promoter, located about 400 bp upstream of PLL (Fig. 1 and 2) (11, 13, 28, 39). This promoter is under the control of both positive (v gene product [11]) and negative (vis gene product [33]) P4-encoded regulators. Although transcription from PLL covers the timm region, it is not subject to premature termination. In particular, when P4 establishes the plasmid state, both the PLL and PLe promoters are active but only transcription from PLL can read through timm. Thus, the immunity control acts only on transcription starting at PLL (7, 14, 38). The P4 virulent mutant, P4 vir1, carries a promoter-up mutation in PLL (Fig. 2) that enables it to bypass the immunity control by allowing early expression of the left operon from the mutated promoter (13).
P4 clI 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, 25). Several P4 Ash− mutants have been sequenced and found to carry a base substitution in clI (6, 28) (Fig. 2). Conversely, the clI405 mutation is also found to exhibit the Ash phenotype (28). The mutant phages are affected in lysogenization ability, and premature transcription termination at clI 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 clI (28). This mutation suppresses the virulence conferred by the vir1 mutation, as shown by the inability of P4 virI ash10 to plate on a P4 P2 double lysogen (6, 28). A possible explanation for this phenotype is reported below.

Several P4 clI mutants (clI405, 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 clI mutants. The isolation of a P4 clI405 derivative, P4 clI405 kilI, which is unable to establish the immune lysogenic state but does not cause cell death after infection, supported this hypothesis (1). The kilI mutation is recessive and linked to clI405.

In this paper, we describe two nested genes, kil and eta, whose coding sequences cover the clI region and demonstrate that their translation prevents transcription termination at clI. Moreover, the clI gene is also nested within the eta gene. Thus, the clI 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 (prototroph) (42), C-8 (polyauxotrophic, 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 MJ101 (50). The phages used were P2 (3); P3 (5); P4 (44); P4 ash7 (from the Six collection); P4 ash23 (27); P4 clI405 (6); P4 clI405 kilI (1); P4 virI30 (39); P4 virI40 (39); P4 virI ash4 (from the Six collection); P4 virI ash28, P4 virI ash31, P4 virI ash32, and P4 virI ash33 (reference 26 and this work), and P4 vs2 (from the Milan collection). The P4 genome coordinates are from the updated P4 DNA sequence (GenBank accession no. X51524; 21).

#### Plasmids

The plasmid vectors used were pUC8, pUC18, and pUC19 (49, 50) and pGM3, a pGZ191EH derivative carrying the trNA105 reporter gene downstream of the puc promoter (7). pGM26 was constructed by insertion of the nt 8130 to 8626 DNA fragment (in the ActC-UVa fusion in the pUC19 vector. pGM26 carriers the nt 6447 to 10657 P4 region cloned in the BamHI-Vdel sites of pUC18. pGM260 carries the P4 nt 9023 to 8657 inserted into the Smal site of the pUC8 vector. The resulting plasmid contains the puc promoter, the Shine-Dalgarno sequence and the first 11 codons of the ActC-UVa fusion 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 EcoRI digestion, filling in, and religation, thus creating a nonsense mutation in codon 6 of lacZ. pGM672, pGM673, and pGM674 are pGEM31 derivatives in which the nt 8401 to 8130 region from P4+, P4 clI405 kilI, and P4 kilI34, respectively, obtained by PCR amplification, has been cloned between puc and the trNA105 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 [TGGAGATCC CTATGGCGTACGAGCA]G plus 264 [GCCGATCCACTGGCCGTC TTGTTAACAC5]G) (see Table 1). The amplified fragments differ from each other for 1 or 2 bp at the 5′ end of the lacZ reading frame. 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 clI (28). This mutation suppresses the virulence conferred by the vir1 mutation, as shown by the inability of P4 virI ash10 to plate on a P4 P2 double lysogen (6, 28). A possible explanation for this phenotype is reported below.

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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 las operon 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).
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 kil 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 32P-labeled RNA probes PLE-t2 and PLL 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 tRNAGly expression (45) was 5′-end labeled with T4 polynucleotide kinase in the presence of [γ-32P]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 PLE and the downstream 570 nt (Fig. 3). The same

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 PLL and PLE and the vis, eta, kil, and e 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, ash9, ash26, and ash28 mutations. The ash7 mutation was sequenced by Lane (26). The ash3, ash5, 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.

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

To map the putative $kil$ gene, we cloned the nt 8626 to 8130 and nt 8470 to 8130 DNA fragments of $P4\ kil1$, $P4\ kil1$, and $P4\ kil343$, 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 $P4\ kil1$ caused cell death whereas the fragments derived from either $P4^{+}$ or $P4\ kil1$ did not. Moreover, the cloned $kil$ nt 8421 to 8130 fragment caused cell killing after induction of $plac$ whereas the $kil$ did not (in such fragments, part of the immunity region including the $c405$ locus is deleted [28]). Thus, we concluded that (i) the killing function is encoded within the nt 8421 to 8130 region; (ii) the $kil$ mutation, which inactivates the killing function, maps in this region; and (iii) the $c405$ 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 $P4\ kil1$ was cloned in pGM216 (see Materials and Methods). Both the $c405$ and the $kil$ mutations were rescued after infection with $P4^{+}$ of $E.\ coli$. The $C-1a$ strain was infected with the $c405$ 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$^{Glu}$ reporter gene downstream of the $kil$ region and analyzed translation be-
yond tmm, 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 tmm, 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 tmm.

The kil gene is the terminal segment of a longer ORF expressed from PLL. By sequence inspection, we found that the kil gene is the terminal part of a longer ORF that extends upstream of PLL (Fig. 2). This ORF could be translated when transcription starts from PLL. Four ATG codons are found upstream of PLL: one is at nt 8814, within vis, the first gene downstream of PLL, 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 tmm. Since eta covers the tmm region, it might be supposed that its translation prevents transcription termination at this site. This might explain why transcription starting from PLL reads through tmm. To verify the above hypothesis, we tested whether mutations that create a stop codon in eta induce premature transcription of transcription starting from PLL. 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 cl 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 PLL (13). The RNAs were hybridized with the PLL-lacZ probe, which covers the PLL proximal region and identifies transcripts starting from both PLL and PLL. The same filters were hybridized with the PLL riboprobe, specific for transcripts from PLL. 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 PLL and the appearance of new RNA species of about 0.5 to 0.7 kb synthesized from PLL. These data suggest that the ash29 and ash10 mutations cause premature termination of transcription from PLL.

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 PLL. 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 PLL persisted for a long time, suggesting that transcription from this promoter is not efficiently terminated at tmm. 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 PLL. 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 PLL transcripts were normally synthesized, but almost all
cells infected with P4 cI405 kil1 approximately 17.5 kDa, which might represent the causes cell death when overexpressed. Good Shine-Dalgarno sequence. It encodes a 65-amino-acid stream e.

\[ \text{transcription from } \text{P LL. It must be noted that the overall expected, the } \text{eta 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.}

\[ \text{The kil gene frameshift mutation creates a fusion with the downstream } \epsilon \text{ reading frame. Accordingly, a protein of approximately 17.5 kDa, which might represent the kil} \epsilon \text{ fusion protein, was observed in cells infected with P4 cI405 kil1 but not in cells infected with P4 cI405 or P4+ (1).}

\[ \text{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).}

\[ \text{The eta gene can be expressed only from P LE. 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.}

\[ \text{In the plasmid condition and after infection with P4 vir1, when transcription starts at the upstream promoter P LE (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.}

\[ \text{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.}

\[ \text{Control of transcription termination at t inm by translation of kil. When the kil-t inm region is cloned on a plasmid, the presence of the kil343 nonsense mutation causes premature termination of transcription at t inm whereas the kil 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 inm.}

\[ \text{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 inm. Consistent with this hypothesis, the Shine-Dalgarno sequence and the ATG codon of kil fall within the seqC \textsuperscript{+} target sequence, complementary to seqB in the CI RNA (Fig. 2).}
Control of transcription termination at \( t_{\text{termin}} \) by translation of \( \text{eta} \). Transcription starting from \( P_{\text{LL}} \) proceeds through \( t_{\text{termin}} \), covering the downstream part of the operon (13, 38). Our data indicate that this is due to translation of \( \text{eta} \), whose start codon is upstream of \( P_{\text{LE}} \), and is not under P4 immunity control. Indeed, mutations that stop \( \text{eta} \) translation caused premature termination of transcription starting at \( P_{\text{LL}} \), and suppression of the P4 \( \text{ash29 amber} \) mutation in a \( \text{supD} \) host restored transcription through \( t_{\text{termin}} \). Premature transcription termination was also caused by nonsense mutations in the upstream \( \text{vis} \) gene, to which \( \text{eta} \) appears translationally coupled.

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

When \( \text{eta} \) translation is blocked by a nonsense mutation upstream of \( \text{kil} \), the \( \text{kil} \) 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 \( P_{\text{LL}} \) might be under the control of P4 immunity and might terminate prematurely at \( t_{\text{termin}} \). Thus, antitermination of transcription from \( P_{\text{LL}} \) 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 \( t_{\text{termin}} \).

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

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

**Possible role of \( \text{kil} \) and \( \text{Eta} \).** Overexpression of \( \text{kil} \) 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 \( \text{Kil} \) protein may interfere with cell division.

The \( \text{Kil} \) 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, \( \text{Kil} \) expression is not harmful to the host; hence, the lethal effect appears to be a consequence of deregulation.

A possible role of \( \text{Kil} \) 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 \( \text{kil} \) controlled by multiple mechanisms in the different developmental phases of P4 but also \( \text{Kil} \) itself is part of the mechanism controlling the P4 life cycle.

Immediately after infection of a sensitive host, \( \text{Kil} \) can be transcribed from \( P_{\text{LE}} \) as the first gene of the operon essential for P4 replication. Translation of \( \text{Kil} \) 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 \( \text{Kil} \) be inhibited, according to our model, but also its transcription may be inhibited, due to termination at \( t_{\text{termin}} \). 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 \( \text{Kil} \) function. These two conflicting demands are met by translation of the \( \text{eta} \) gene.

Blast database searches (2) found several matches for the \( \text{eta/kil} \) 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, \( \Phi R73 \) (23, 47), contains a sequence very similar to the whole \( \text{Eta} \) sequence. Other less extensive sequence matches were found for the bacteriophage N15 \( \text{cAgene} \) region (21) and for the \( \text{Shigella flexneri} \) 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 \( \text{Kil} \), 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 \( \text{eta} \).

Shorter though statistically significant matches were found for the \( \text{eta} \) region immediately upstream of \( \text{Kil} \) 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, \( \Phi R73 \), and N15, a gene encoding a small RNA is nested within the ORF extending upstream of \( \text{Kil} \) (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 \( \text{ci} \) region in ColIb-P9, R64, and SFW and found predicted secondary structures identical to that of P4 CI RNA (data not...
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 *l* (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 the left operon. 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.

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