Alternative Promoters in the Development of Bacteriophage Plasmid P4

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Infection of *Escherichia coli* with the satellite virus P4 without its helper bacteriophage P2 leads either to the immune integrated state or to the nonimmune multicopy plasmid condition. We analyzed the transcription pattern of the phage plasmid P4 early and late after infection and during the stable plasmid or lysogenic condition. The early postinfection phase is characterized by the leftward transcription of an operon including the genes *cI* (P4 immunity) and α (replication). This early transcript starts from the promoter P_{LE} , which shows a good homology with the *E. coli* σ^{70} promoter. At later times, the transcription of this operon starts from a different promoter, P_{LL} , located 400 base pairs upstream of P_{LE} and sharing little homology with the canonical *E. coli* promoter sequence; a longer transcript encoding an additional open reading frame is thus produced. P_{LL} shares two boxes of homology with the P4 late promoter P_{SID} , positively regulated by the P4 δ gene product, and depends on δ function for its full activation. In the multicopy plasmid state, the transcription pattern is similar to that observed at late times after infection. Since in the plasmid state not only is P4 immunity not expressed but its establishment is prevented, even though the P4 *cI* gene is transcribed, the P4 *cI* function may be regulated at the posttranscriptional level. In the immune state, transcription starts from P_{LE} but does not continue to cover the P4 α gene. This suggests that P4 immunity acts by prematurely terminating transcription initiated at P_{LE} .

The genetic element P4 (for reviews, see reference 18 and L. E. Bertani and F. W. Six, in H. F. Fraenkel-Conrat and R. R. Wagner, ed., *The Viruses*, in press) can be propagated in its bacterial host *Escherichia coli* as a lytic bacteriophage, as a multicopy plasmid, or as an integrated provirus.

For its lytic development, P4 requires all the morphogenetic genes of a P2 or a P2-like helper bacteriophage (31); the provirus condition may be maintained both with and without the helper (7, 31); the multicopy plasmid state may be established without a helper phage genome (7, 12, 16, 27).

After infection of *Escherichia coli* without a helper phage genome, P4 enters a phase of uncommitted replication, followed by commitment to either the immune prophage condition or the nonimmune multicopy plasmid mode of propagation; all these phases correspond to different regulatory conditions (1).

The early postinfection phase is characterized by the synthesis of protein 1, the product of gene α involved in P4 DNA replication priming, and protein 2, a 45-kilodalton polypeptide of unknown function (2, 15, 21). Leftward transcription (according to the orientation shown in Fig. 3) of a region including gene α has been detected (19).

At later times, leftward transcription and α protein production decrease, the rightward transcription of the *sid*- δ -*psu* operon is activated from its own promoter P_{SID} (10, 19), and the products of *sid* and *psu* genes (proteins 3 and 4) may be detected (3, 29; D. M. Kurnit, R. L. Neve, F. S. Lee, R. Lagos, and R. Goldstein, Gene, in press).

Expression of the *sid*- δ -*psu* operon is dependent on either a functional δ gene or the helper *ogr* function (3, 10, 19, 24, 33).

In this report we analyze the P4 transcription pattern without a helper phage, upon P4 infection, in the lysogenic and in the plasmid conditions. (The term lysogenic is used as

a synonym for immune integrated even though, without the helper, the prophage P4 cannot enter the lytic cycle and lyse the bacterial host.)

MATERIALS AND METHODS

Bacteria, phages, and plasmids. The *E. coli* C strain C-1a (28) was used as the non-P2 lysogenic P4 host. P4 prophage- or P4 plasmid-carrying clones either are described by Dehò et al. (12) and Alano et al. (1) or were obtained as described by these researchers. The P4 lysogenic strains are indicated by the host strain number, followed by the prophage name in parentheses; for the plasmid-carrying clones, the host and the plasmid names are separated by a slash. Phages and plasmids are shown in Table 1.

RNA extraction from *E. coli*. The cells were grown in LC at 37°C to a density of 40 Klett units (approximately 10^8 cells per ml). A 5-ml sample was quickly chilled in centrifuge tubes precooled at -20°C and containing 20 μ l of 2 M sodium azide and was spun for 10 min at 6,000 rpm; the sediment was suspended in 0.35 ml of a solution consisting of 50 mM Tris hydrochloride (pH 6.8), 2 mM EDTA, and 1% sodium dodecyl sulfate. The RNA was then extracted as described by Hagen and Young (17).

To prepare RNA from P4-infected cells, the culture was grown as described, $CaCl_2$ was added to 2.5 mM to allow phage adsorption, and aeration was interrupted. The phage was then added, aiming at a multiplicity of infection of 10, and after 10 min, the unadsorbed phage was measured and aeration was resumed. Every 20 min or immediately before taking a sample, the turbidity was monitored and the culture was diluted to 40 Klett units with prewarmed LC. At the appropriate time, samples were taken and processed as described above.

Northern blot (RNA blot) hybridization. Agarose gel electrophoresis of glyoxalated RNA, transfer to nitrocellulose filters (Schleicher & Schuell, Inc.) by blotting, and subse-

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TABLE 1. Phages and plasmids

Bacteriophage or plasmid	Source or reference	Construction
Bacteriophages		
P4 ⁺	31	
P4 cI405	7	
P4 cI405 <i>kil1</i>	1	
P4 cI405 <i>psu1</i>		From a cross ^a of P4 cI405 × P4 <i>psu1 vir1</i> .
P4 cI405 <i>sid1</i>		From a cross ^a of P4 cI405 × P4 <i>sid1</i> .
P4 cI405 α am105		From a cross ^a of P4 cI405 × P4 α am105.
P4 cI405 835		From a cross ^b of P4 cI405 α am105 × P4 <i>vir1</i> 835 ϵ am104.
P4 <i>psu1 vir1</i>	29	
P4 <i>sid1</i>	30	
P4 <i>vir1</i>	23	
P4 α am105	14	
P4 835 ϵ am104	Obtained by P. Youderian	
Plasmids		
pBR325	6	
pGM2		A pBR325 derivative. The <i>AvaI</i> - <i>Bam</i> HI smaller fragment was replaced in vitro by the P4 <i>vir1</i> fragment <i>AvaI</i> -5790- <i>Bam</i> HI-7377. ^c
pGM4		A pUC8 derivative. Carries the P4 cI405 <i>kil1</i> <i>SmaI</i> -5792- <i>Bam</i> HI-7377 fragment.
pJGHy83	Obtained by R. Goldstein	A pBR325 derivative. The <i>AvaI</i> - <i>Bam</i> HI smaller region was replaced in vitro by the <i>AvaI</i> -2983- <i>Bam</i> HI-7377 fragment of P4 <i>vir1</i> .
pUC8	35	

^a Crosses were performed as described by Dehò (11). To increase the recombination frequency, the parental phages were UV irradiated as described by Geisselsoder et al. (14). The progeny was plated on a P3 lysogenic strain which is nonpermissive for the cI⁺ parent (22), and the double mutant recombinant was scored for the presence of the unselected marker by replica plating as described by Dehò (11).

^b The cross was performed as described by Dehò (11). The *am*⁺ recombinants were selected.

^c The number after the name of the restriction enzyme indicates the position of the cut in the P4 sequence (13, 22; Halling, personal communication).

quent hybridization to DNA labeled with ³²P by nick translation were performed as described by Thomas (34).

S1 nuclease mapping. Double-stranded DNA fragments labeled at a single 5' end were used as probes. The DNA was digested with the appropriate restriction enzyme, dephosphorylated with calf intestinal alkaline phosphatase, end labeled with polynucleotide kinase and [γ -³²P]ATP, and digested with a second restriction enzyme. The probe fragment was separated by acrylamide gel electrophoresis and recovered by electroelution (25).

DNA-RNA hybridization and digestion with nuclease S1 were carried out as described by Barry et al. (4).

RESULTS

P4 transcription patterns in the different regulatory conditions. We analyzed the transcription pattern of P4 in its different regulatory states by Northern blot hybridization. The RNAs extracted from P4 lysogenic cells, from clones carrying P4 as a plasmid, and from nonlysogenic cells infected with P4 were fractionated by agarose gel electrophoresis, blot transferred onto a nitrocellulose filter, and hybridized to a radioactive P4 DNA probe as described in Materials and Methods.

At least three P4-coded transcripts were differently expressed at the different time periods after infection and in the plasmid condition (Fig. 1). A transcript of approximately 4 kilobases (kb), named the 4.1-kb transcript, appeared early after infection with wild-type P4 and was no longer detectable 60 min after infection. At this time point and in the plasmid state, two new RNAs were present, one estimated to be 4.2 to 4.5 kb long (designated the 4.5-kb transcript) and the other about 1.7 to 2 kb (named the 1.9-kb transcript). Two additional hybridization signals at the positions corresponding to 2.8 and 1.5 kb may be due at least in part to nonspecific binding of P4 DNA to host RNA (probably the

23S and 16S rRNA, respectively), since the signals were observed even with RNA extracted from *E. coli* in the absence of any P4 genome. Another RNA of about 1.3 kb was observed both in the P4-infected cells at all time points and in the plasmid condition (Fig. 1A).

A similar pattern is observed with the immunity-defective mutant P4 cI405 (Fig. 1B and C). However with this mutant, the 4.1-kb transcript appeared to be more abundantly expressed at early times after infection and did not completely disappear at late times and in the plasmid condition. Also, the signal relative to the 4.5-kb transcript at late times after infection was stronger in the cI mutant than in the wild type. P4 cI405 *kil1*, a derivative of P4 cI405 which, unlike the parent, establishes the plasmid state in the large majority of the infected cells (1), exhibited a transcription pattern indistinguishable from that of P4 cI405.

On the other hand, the immunity-insensitive mutant P4 *vir1* did not produce the 4.1-kb mRNA at detectable levels and even at early times after infection produced the 4.5-kb transcript (Fig. 1A). None of the 4.5-, 4.1-, 1.9-, or 1.3-kb transcripts were detectable in P4 lysogenic cells.

To determine from which region of the P4 genome these P4-coded RNAs are transcribed, we performed multiple Northern blot hybridizations using different P4 DNA regions as probes (Fig. 2). Since both the 4.5- and the 4.1-kb transcripts hybridized with probes c and d but not with probe b, we may conclude that the left end of such RNAs is located between restriction sites *SmaI*-5792 and *EcoRI*-8480 (the number after the restriction enzyme indicates the position of the cut in the P4 sequence); therefore, the 4.5 and the 4.1 transcripts cover the α gene and possibly ϵ and cI. By the same token, the 1.9-kb late transcript, which hybridized with probes f and g but not with b, c, d, and e, appears to be coded by the P4 region to the right of *AvaI*-2983, thus including the *sid*- δ -*psu* operon. The length of such an RNA

FIG. 1. P4 transcription analysis by Northern blot hybridization. Total RNA was extracted as described from the cultures listed below. A 20- μ g sample of glyoxalated RNA was loaded into the wells of a 1.5% agarose gel, except for the first well, which was loaded with 10 ng of glyoxalated *Eco*RI-digested P4 DNA as a molecular weight marker. Electrophoresis, blotting, and hybridization were performed as described in Materials and Methods. (A) Lanes: 1, molecular weight markers; 2, C-1a; 3, C-1a(P4⁺); 4, C-1a(P4⁺ vir1); 5, C-1a(P4⁺ vir1); 6, C-1a/P4 vir1; 7, C-1a/P4 cI405; 8, 9, and 10, C-1a infected with P4⁺, with samples taken 20, 60, and 120 min postinfections, respectively; 11, 12, and 13, C-1a infected with P4 vir1, 20, 60, and 120 min postinfection, respectively. (B) Lanes: 1, 2, 3, and 4, C-1a infected with P4 cI405, with samples taken 10, 20, 40, and 60 min postinfection, respectively; 5 and 6, C-1a infected with P4 cI405 *kil*1, 20 and 60 min postinfection, respectively; 7 and 8, C-1a infected with P4 cI405 *sid*1, 20 and 60 min postinfection, respectively; 9 and 10, C-1a infected with P4 cI405 835, 20 and 60 min postinfection, respectively; 11 and 12, C-1a infected with P4 cI405 *psu*1, 20 and 60 min postinfection, respectively; 13, 14, 15, and 16, RNA extracted from wild-type P4- and P4 vir1-infected cells, added as an internal control. (C) RNA extracted from plasmid-carrying clones. Lanes: 1, C-1a/P4 vir1; 2, C-1a/P4 cI405; 3, C-1a/P4 cI405 *kil*1; 4, C-1a/P4 cI405 *sid*1; 5, C-1a/P4 cI405 835; 6, C-1a/P4 cI405 *psu*1. The numbers to the right of the panels indicate the molecular size (in kilobases) of the transcripts or of the DNA molecular weight markers.

accounts for the full transcription of this operon from its promoter *P*_{SID} (10). More puzzling is the hybridization pattern relative to the 1.3-kb transcript; since it hybridized with probes b, f, and g but not with probes c and d, it appears that its transcription runs through the P4 cohesive ends. However, it also hybridized with probe e, whose right end is farther than 1.3 kb from the *cos* site. Several hypotheses come to mind. (i) The 1.3-kb signal is due to nonspecific hybridization. (ii) RNA species from different P4 regions have similar molecular weights. (iii) The 1.3-kb RNA is the product of a splicing reaction. We did not investigate this problem further.

Transcription initiation points for the *cI*- α operon. To assess the differences between the two overlapping early- and late-plasmid transcripts, which are 4.1 and 4.5 kb long, respectively, we determined the initiation points of these transcripts by S1 nuclease protection experiments (5).

Harris and Calendar (19) have shown that P4 early transcription occurs from right to left in the central right region of the P4 genome. We therefore prepared an *Mlu*I-3020-*Dra*I-2452 P4 DNA fragment 5' end labeled at the *Mlu*I extremity (Fig. 3, bottom), performed the S1 protection assay after hybridization with RNA extracted from cells carrying wild-type P4 in the different regulatory conditions, and fractionated the products of the reactions by acrylamide-urea gel electrophoresis. To determine the length of the protected DNA fragments, the same DNA probe was sub-

PROBE	P4 DNA REGION IN THE PROBE							P4 TRANSCRIPT			
	kb	10	8	6	4	2	0	4.5	4.1	1.9	1.3
a.								+	+	+	+
b.								-	-	-	+
c.								+	+	-	-
d.								+	+	-	-
e.								+	+	-	+
f.								+	+	+	+
g.								+	+	+	+

FIG. 2. Hybridization of P4 transcripts to different regions of P4 genome. RNAs from P4 cI405-infected cells (20 and 60 min postinfection) and from plasmid P4 cI405 carriers were Northern blotted and hybridized to different P4 probes. In some cases, the same filter was reused for hybridization with a different probe. The presence (+) or absence (-) of hybridization signal on the autoradiograph after prolonged exposure is indicated to the right. The following DNA probes were used: a, total P4 DNA; b, P4 *Eco*RI-8481-*Eco*RI-11300 restriction fragment isolated by agarose gel electrophoresis and electroelution (25) (in another experiment λ gt-P4 *Eco*RI:C [33, 33a] was used); c, P4 DNA *Sma*I-5792-*Eco*RI-8480 restriction fragment; d, pGM2; e, pJGHy83; f, pGM4; g, P4 *Sma*I-5791-*cos*, right arm of P4 genome obtained by *Sma*I digestion of mature P4 DNA.

FIG. 3. Genetic and physical map of phage plasmid P4. In the upper part (18), transcripts are indicated by the arrows. In the lower part, restriction fragments used for S1 mapping are shown.

jected to chemical sequencing reactions (26) and was run on the same gel. Two main groups of S1-protected fragments resulted (Fig. 4). The lowest-molecular-weight group is well resolved and is composed of several fragments differing by one nucleotide in length. The position of the most abundant fragment corresponds, after correction for the difference in migration between S1-cleaved and chemically cleaved DNA

FIG. 4. S1 mapping of P4 promoters P_{LE} and P_{LL} . The P4 *MluI*-*DraI* restriction fragment shown in Fig. 1, 5' end labeled at the *MluI* extremity, was obtained from pGM4. The RNA extracted from the cultures indicated below was hybridized to the labeled probe, S1 digested, fractionated by 6% acrylamide-7 M urea gel electrophoresis, and autoradiographed. Lanes: 1, C-1a; 2, C-1a($P4^+$); 3, C-1a/ $P4^+$; 4 and 5, C-1a infected with $P4^+$, 20 and 90 min postinfection, respectively; 6, 7, and 8, chemical sequencing reactions of the same *MluI*-*DraI* fragment. The number to the right of the gel indicates the nucleotide coordinate in the P4 sequence.

(32), to a P4 DNA fragment terminating at nucleotide 2950 of the P4 sequence (22; C. Halling, personal communication). To determine precisely the length of the higher-molecular-weight fragment, we performed a similar S1 mapping experiment using the *TaqI*-2738-*DraI*-2452 P4 DNA fragment labeled at the *TaqI* 5' end as a probe (Fig. 3, bottom). From the results shown in Fig. 5, we may conclude that the protected fragment ends at nucleotide 2555.

These results indicate that two different initiation points for transcription from right to left, spaced approximately 400 base pairs apart, are located in the region between bases 2500 and 3000 of the P4 genome. The downstream transcription initiation point appears to be actively used in the lysogen and at early times after infection; the intensity of its signal decreased at late times after infection and was much reduced in the plasmid condition (Fig. 4).

The upstream initiation point appears to be efficiently used in the plasmid condition and late after infection. The intensity of its signal at early times after infection varied from experiment to experiment but was clearly lower than at late times after infection and was virtually nil in the lysogen.

We suggest that the downstream and the upstream transcription initiation points represent the starting points of the 4.1- and 4.5-kb transcripts, respectively, observed by Northern blot hybridization. We shall call the promoter regions for the 4.1- and 4.5-kb transcripts P_{LE} (promoter left early) and P_{LL} (late), respectively.

FIG. 5. S1 mapping of P_{LL} . The *TaqI*-*DraI* restriction fragment shown in Fig. 1, 5' end labeled at the *TaqI* extremity, was obtained from pGM4. The probe was hybridized to the RNA extracted from the cultures listed below, S1 digested, analyzed by 5% acrylamide-7 M urea gel electrophoresis, and autoradiographed. Lanes: 1, C-1a; 2, C-1a($P4^+$); 3, C-1a/ $P4^+$; 4 and 5, C-1a infected with $P4^+$, 20 and 90 min postinfection, respectively; 6, C-1a($P4^+$ *vir1*); 7, C-1a/ $P4^+$ *vir1*; 8 and 9, C-1a infected with $P4^+$ *vir1*, 20 and 90 min postinfection, respectively; 10, 11, and 12, chemical sequencing reactions of the *TaqI*-*DraI* fragment; 13, *TaqI*-*DraI* fragment, not S1 treated; 14, C-1a/ $P4^+$ *cI405*; 15, C-1a/ $P4^+$ *cI405 kil*. The number to the right of lane 12 indicates the nucleotide coordinate in the P4 DNA sequence. After prolonged exposure of the autoradiograph, a protection signal was visible in lanes 4 and 5 but not in lanes 1 and 2.

FIG. 6. Transcriptional activity at P_{LE} and P_{LL} in P4 mutants. The S1 mapping was performed with the *MluI-DraI* P4 DNA fragment as described in the legend to Fig. 4, and the products were analyzed by 6% acrylamide-7 M urea gel electrophoresis. The RNA was extracted from the infected cells described below. (A) Lanes: 1 and 2: C-1a infected with P4 cI405 at 20 and 90 min postinfection; 3, 4, 5, and 6: C-1a infected with P4 cI405 835 at 20, 60, 90, and 120 min postinfection; 7 and 8: C-1a infected with P4 cI405 *kil1*, 20 and 60 min postinfection; 9 and 10: C-1a infected with P4 *vir1*, 15 and 60 min postinfection. (B) Lanes: 1, C-1a(P4 *vir1*); 2, C-1a/P4 *vir1*; 3, C-1a(P4 *wt*); 4, C-1a/P4 *wt*; 5, C-1a/P4 cI405 *sid1*; 6, C-1a/P4 cI405 835; 7, C-1a/P4 cI405 *psu1*; 8, C-1a/P4 cI405; 9, C-1a/P4 cI405 *kil1*; 10, C-1a; 11, DNA probe not digested. The numbers to the left of the gel indicate the length (in nucleotides) of the DNA fragments. The 569-nucleotide fragment is an undegraded DNA probe; the 465- and 71-nucleotide bands are the fragments protected by the transcripts initiating at P_{LL} and P_{LE} , respectively.

The protection patterns obtained with the P4 cI405 mutant in the early, late, and plasmid regulatory conditions were similar to that of wild-type P4 (Fig. 5 and 6). In contrast, P4 *vir1* showed a strong protection signal at the upstream initiation start point even early after infection and exhibited a less-intense one in the lysogenic condition (Fig. 5 and 6). This protected fragment was indistinguishable from the one originated by wild-type P4 at late times after infection and in the plasmid condition (Fig. 5).

Dependence of transcription of late 4.5-kb RNA on P4-coded functions. Transcription from P_{LL} is fully activated only late after infection and in the plasmid condition; this suggests that a P4-coded function may be necessary to promote transcription of the 4.5-kb mRNA.

It is known that the late expression of the P4 *sid-δ-psu* operon from its promoter P_{SID} requires DNA replication and the functional product of P4 gene δ (3, 10, 19). In addition, P_{LL} and P_{SID} share two 6-base-pair boxes of homology (Fig. 7; see Discussion). These facts prompted us to test whether transcription of the 4.5-kb RNA depends on a functional δ gene product. As a control, we also tested the *sid* and *psu* mutations, since expression of the P4 *sid* and *psu* genes depends on δ .

We first analyzed the ability to establish the plasmid condition of the P4 *sid*, δ , or *psu* mutants. To avoid the establishment of the lysogenic condition and to score the plasmid-carrying clones among the survivors more easily, the effects of the *sid*, δ , and *psu* mutations were observed in the immunity-defective mutant P4 cI405. We therefore infected *E. coli* C-1a with the double mutants P4 cI405 *sid1*, 835, or *psu1* and analyzed the surviving clones for the presence of the P4 genome and its immune condition by the *cox* test (12). P4 cI405 *sid*, δ , and *psu* mutants were able to establish the plasmid state, as indicated by the stable maintenance of the P4 genome in the nonimmune condition (Table 2). All the clones stably carrying P4 as a plasmid exhibited the typical colony morphology (rosette; 12). We also extracted and analyzed the DNA of several plasmid-carrying clones by agarose gel electrophoresis. The intensity of the band of plasmid P4 cI405 *sid*, δ , or *psu* DNA after ethidium bromide staining was similar in intensity to the P4 cI405 control (data not shown). Thus the *sid*, δ , or *psu* mutations do not prevent the establishment of the multicopy plasmid state.

The transcription analysis by Northern blot hybridization of these P4 mutants upon infection and in the plasmid condition is shown in Fig. 1B and C and is summarized in

FIG. 7. Comparison between P4 promoter sequences. Common sequences in P_{SID} (10) and P_{LL} are underlined. Homologies to *E. coli* σ^{70} promoter consensus sequence (■) and inverted repeats (→) are indicated. The C in P_{LL} (*) is substituted by a T in P4 *vir1* (22). *E. coli* σ^{32} (9) and P2 late (8) promoter consensus sequences are shown.

Table 3. From the transcription analysis, the following observations can be made. (i) The overall level of P4 transcription in the multicopy plasmid state of the P4 *sid*, δ , and *psu* mutants was severely reduced. Since the *psu* gene does not seem to be expressed by P4 *sid* or δ mutants (10, 20), it appears that the lack of the *psu* function determines the reduced level of the P4 transcripts in the plasmid condition but does not affect the ability to produce both the 4.5- and 1.9-kb transcripts. (ii) Neither the 4.5- nor the 1.9-kb transcript was detected in P4 *sid* and δ mutants.

Since the *sid1* mutation appears to exert a polar effect on δ gene expression (20), these data suggest that the late 4.5- and 1.9-kb transcripts are coregulated and that both depend on the P4 δ function for their transcription.

However, S1 protection analysis, which is a more sensitive technique than Northern blotting, revealed a low transcriptional activity from P_{LL} of the P4 *sid* and δ mutants in the plasmid condition (Fig. 6B); in addition, transcription initiation from P_{LL} of the P4 δ mutant after infection did not increase at late time points (Fig. 6A [P4 *sid* not tested]).

DISCUSSION

Transcription of the *cI*- α operon. We have shown that P4 late transcription involves not only the activation of the rightward *sid*- δ -*psu* operon from promoter P_{SID} (3, 10, 19) but also a different mode of transcription of the *cI*- α operon, resulting in the production of a mRNA longer than that expressed early after infection (4.5 and 4.1 kb, respectively); we also showed that in the multicopy plasmid condition the transcription pattern is similar to that observed late after infection.

We suggest that the 4.1- and the 4.5-kb transcripts initiate at P_{LE} and P_{LL} , respectively, and that both terminate at the strong *rho*-independent terminator α situated between nucleotides 7013 and 7037 (13) as a result of the following observations. (i) The length of the transcripts estimated by agarose gel electrophoresis and Northern blotting conformed to the expected value (about 4.1 and 4.5 kb, respectively). (ii) Production of the 4.1- or 4.5-kb mRNAs was observed only when the promoter P_{LE} or P_{LL} , respectively, was active, although the opposite did not always hold (for example, in the lysogen or late after infection, transcription started at P_{LE} , but the 4.1-kb mRNA was not detected by Northern blot hybridization; conversely, in the plasmid state established by P4 cI405 δ 35 or *sid1*, transcription started at P_{LL} , but no 4.5-kb mRNA was detected). (iii) The P4 *vir1*

TABLE 2. Establishment of the plasmid condition upon infection of *E. coli* with P4 mutants^a

Infecting phage	MOI ^b	% of surviving cells ^c	% of P4-carrying clones among survivors ^d
P4 cI405	8.7	0.6	93.0
P4 cI405 <i>kil1</i>	10.5	129.0	99.8
P4 cI405 <i>sid1</i>	8.3	0.67	97.8
P4 cI405 δ 35	8.5	1.9	97.3
P4 cI405 <i>psu1</i>	8.3	0.97	78.0

^a The experiment was performed as described by Alano et al. (1).

^b Multiplicity of infection.

^c Viable count was made after 40 h of incubation at 37°C.

^d Surviving clones were tested for ability to release P4 upon superinfection by P2 and P2 *cox*, as described by Dehò et al. (12). Several independent clones were also tested for the presence of plasmid P4 DNA as described previously (1).

TABLE 3. Transcription pattern of P4 mutants^a

P4 genotype	Regulatory condition	Transcript size ^b			
		4.5	4.1	1.9	1.3
Wild type	Infection, early	—	+	—	+
	Infection, late	+	—	+	+
	Plasmid	+	—	+	+
	Lysogen	—	—	—	—
<i>vir1</i>	Infection, early	++	+	—	+
	Infection, late	+	—	+	+
	Plasmid	+	—	+	+
	Lysogen	±	—	—	—
cI405	Infection, early	—	++	—	+
	Infection, late	++	+	+	+
	Plasmid	+	+	+	+
cI405 <i>kil1</i>	Infection, early	—	++	—	+
	Infection, late	++	+	+	+
	Plasmid	+	+	+	+
cI405 <i>sid1</i>	Infection, early	—	+	—	+
	Infection, late	—	+	—	+
	Plasmid	—	±	—	±
cI405 δ 35	Infection, early	—	+	—	+
	Infection, late	—	+	—	+
	Plasmid	—	±	—	±
cI405 <i>psu1</i>	Infection, early	—	+	—	+
	Infection, late	±	±	±	+
	Plasmid	±	±	±	±

^a Data presented in Fig. 1, plus observations obtained by different exposures of the same autoradiographs and by repetitions of similar experiments, are summarized.

^b Indicated by the presence (+) or absence (—) of hybridization signal. When stronger (++) or fainter (±) signals are indicated, P4 wild type in the different regulatory conditions is taken as a reference.

mutation, a promoter up mutation (22) within P_{LL} , led both to an early high level of transcription initiation from P_{LL} and to early production of the 4.5-kb mRNA.

Promoters for the leftward transcription. In Fig. 7, the sequences upstream of the transcription initiation points so far identified in P4 are compared. P_{LE} , identified by Lin (22) and in the present study, exhibited a strong homology with the consensus sequence of *E. coli* RNA polymerase σ^{70} (5 of 6 at the -10 region and 4 of 6 at the -35 region).

P_{LL} showed a lower degree of homology, but the single base substitution in the -10 region carried by P4 *vir1* (22; Fig. 7) appeared to increase its strength considerably. In fact, the 4.5-kb late transcript was produced by P4 *vir1* early after infection, and the S1 protection by the transcript starting at P_{LL} was stronger in P4 *vir1* than that in cells infected with P4⁺ or P4 cI at early times. Moreover, transcription from P_{LL} and production of the 4.5-kb mRNA was detectable even in cells carrying P4 *vir1* in the immune state. Therefore, the partial insensitivity to P4 immunity exhibited by P4 *vir1* (7, 23) is probably due to the higher activity of the mutated promoter P_{LL} , as suggested by Lin (22).

Most of the previous studies on the temporal pattern of P4 gene expression have been conducted using the mutant P4

vir1. Since in this mutant the late leftward mRNA is transcribed early after infection, the pattern of P4 gene expression should be reinvestigated. However, our data suggest that the early production of the 4.5-kb transcript does not modify the temporal pattern of activation of the *sid-δ-psu* operon.

Comparison between P_{LL} and P_{SID} , the late promoter for the rightward transcription of the *sid-δ-psu* operon (10; Fig. 7), revealed that two 6-base-pair boxes, located approximately at the -10 and -35 regions, respectively, were identical in the two promoters although differently spaced; additional homologies were at the -60 region, where an eight-nucleotide palindrome required for activation of P_{SID} , partially conserved in the P2 late promoters, is located (10). The -60 region of P_{LL} contained an imperfect palindrome; six of seven of the complementary bases in the palindrome were conserved in P_{SID} (Fig. 7).

Lin (22) has observed a further transcription initiation point downstream of P_{LE} , but no obvious homology with the other P4 promoters has been observed. Our S1 mapping data confirm the earlier finding (19) that transcription in this region proceeds from right to left; using 5'-right-end-labeled DNA probes from this region, we did not observe any protection against S1 digestion (data not shown).

Dependence of the 4.5-kb mRNA transcription on P4-coded functions. The 4.5-kb transcript was detected by Northern blotting only late after infection and in the plasmid condition. In addition, the P4 *sid* and δ mutants did not synthesize such an mRNA in detectable amounts. These facts suggest that transcription of the 4.5-kb mRNA depends on a P4-coded function(s). Since the *sid1* mutation exerts a polar effect on δ (20), the P4 δ gene product seems to be the best candidate for such a regulatory role. Also, the homology between P_{LL} and P_{SID} , which is positively regulated by the δ gene product (10), suggests that these two promoters may share common regulatory elements. In fact, P_{LL} is fully activated late after infection and in the plasmid condition only in the presence of a functional δ gene product.

However, P_{LL} appears to be less dependent on P4 δ function than P_{SID} . In fact, it is possible to detect by S1 mapping a low transcriptional activity at P_{LL} in all cases tested in which the 4.5-kb transcript is not detectable by Northern blotting, namely, early after infection and in the *sid* and δ mutants.

The P4 *psu* gene function, which is known to suppress the polar effect of amber mutations in P2 late genes (29), does not seem to be involved in activating P_{LL} , nor does it seem to be indispensable for transcription of the 4.5-kb mRNA. However, a drastic effect of *psu* is observed on the amount of P4 mRNA in the plasmid condition, as detected by Northern blotting. Therefore, the P4 *psu* gene product might affect either transcription elongation, possibly by preventing premature termination, or mRNA stability.

Establishment of the multicopy plasmid condition and activation of P_{LL} . Expression of the late rightward *sid-δ-psu* operon is not necessary for the establishment of the multicopy plasmid condition, at least in the presence of the P4 *cI405* mutation, since the mutants in each of these genes can establish the plasmid state. Therefore, establishment of the plasmid condition depends on the expression of the leftward operon. Transcription from P_{LL} appears to be involved in establishing the multicopy plasmid condition. In fact, P4 *vir1*, which produces the 4.5-kb transcript early after infection, establishes the multicopy plasmid condition with high efficiency (12).

The 4.5-kb transcript has the coding capacity for an

additional protein of 88 amino acids (*orf88*; Fig. 3); this hypothetical gene product might play a role in establishing or maintaining the multicopy plasmid condition. Alano et al. (1) detected a polypeptide of approximately 8 kilodaltons produced by P4 in the plasmid condition and by P4 *vir1* but not by P4 *vir*⁺ early after infection. Studies are in progress to determine whether this polypeptide is the product of *orf88* and its role in the plasmid state.

The hypothetical role of *orf88* in the establishment of the plasmid condition is not ruled out by the finding that in the plasmid state of the P4 *sid* and δ mutants, the 4.5-kb transcript is not detected by Northern blotting and that only the 4.1-kb transcript is present. In fact in this situation, P_{LL} is active, although at a low level, and the region covering *orf88* is transcribed, as shown by S1 mapping. We therefore suggest that at least when P4 immunity is prevented due to a mutation in *cI*, a low level of P_{LL} transcription is sufficient for the establishment and the maintenance of the plasmid condition.

Transcription in the P4-immune condition and expression of P4 immunity. In the lysogenic condition, transcription from P_{LE} starts at a substantial level, as shown by S1 protection experiments; however, we could not detect the 4.1-kb transcript by Northern blotting. In addition, a *BstEII*-5157-*Bam*HI-988 probe 5' end labeled at the *BstEII* end was not protected by the RNA extracted from P4 lysogenic cells (data not shown), thus indicating that the transcript initiating at P_{LE} does not cover that *BstEII* site, located in the α gene. These facts suggest that the P4 prophage immunity may not rely simply on a transcription repression mechanism; we suggest that in the immune condition the transcript starting at P_{LE} terminates prematurely, thus preventing expression of the α gene that is necessary for P4 replication. Alternatively, the 4.1-kb transcript in the lysogen might be very unstable.

The immunity determinant *cI* belongs to the same transcription unit as α and it is transcribed even in the plasmid state when P4 immunity is not detectable (1), thus suggesting that P4 *cI* gene expression is posttranscriptionally regulated. Transcription from the further downstream promoter located within *cI* described by Lin (22) might also play a part in preventing immunity. Experiments serving to determine the mechanism by which P4 expresses its immunity are in progress.

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