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Mechanisms of Genome Propagation and Helper Exploitation by Satellite Phage P4

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INTRODUCTION

The genetic element P4 (214, 217) enjoys multiple options for its propagation. Extracellularly, P4 may be found as a virion capable of injecting its own DNA into *Escherichia coli* and other gram-negative bacteria (90, 119, 177, 178, 182, 217). Most of the proteins that make up its capsid and tail, however, are encoded by a helper bacteriophage of the P2 family, since the P4 genome lacks information for these structural proteins and for the lysis of the host cell (215, 217).

When P4 infects a sensitive *E. coli* host harboring the genome of the helper phage P2, it may enter either the lysogenic or the lytic pathway, being dependent on all the morphopoietic and lysis functions encoded by the helper to accomplish the latter mode of replication (215, 217). In the absence of the helper phage, infection of *E. coli* by P4 may lead either to the immune-integrated condition, analogous to the lysogenic state (26, 217), or to the establishment of the multicopy plasmid mode of maintenance (45, 84, 177) (Fig. 1).

The P2-P4 system was extensively reviewed in 1988 by Bertani and Six (12) and recently by Christie and Calendar (35) and Egan and Dodd (57). Kahn et al. (120) stress the more technical aspects of working with P2 and P4 and their use in genetic manipulations. This review will emphasize recent advances in the areas of P4 immunity, the structure and function of the transactivators of P2 and P4, and the morphopoiesis of P2 and P4 with special reference to capsid size determination.

P2-P4 Phage System

Bacteriophage P2 was isolated from the *E. coli* Lisbonne strain, which also harbors phages P1 and P3 (10). A number of P2-related phages have been described, including 186, PK, 299, HK239 (12), and a P2-like phage (PSP3) obtained from *Salmonella potsdam* (24). The P2-like phages cross-react serologically, and they all show the same morphology—an icosahedral capsid or head with a contractile tail

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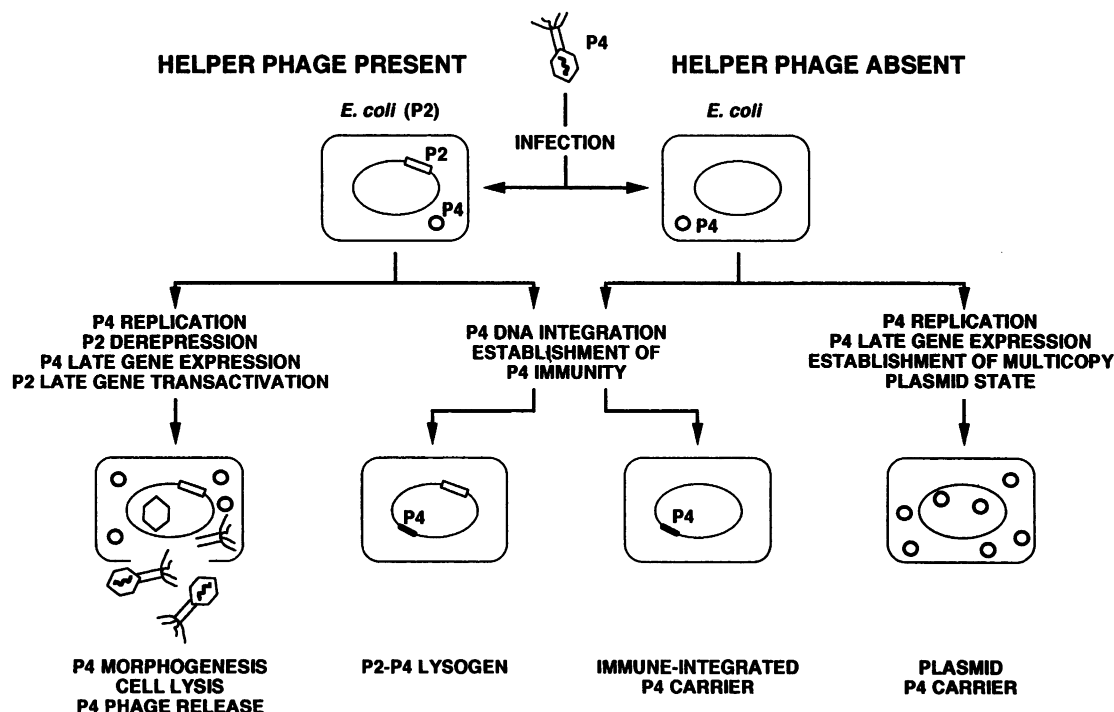

FIG. 1. P4 life cycle. See the text for explanation. Modified from reference 44 with permission.

containing six side tail fibers that extend from the base plate as well as one that extends straight down (Fig. 2). P2 and 186 are the most extensively studied among the P2 family, and they both can serve as helpers for the helper-dependent phage P4 (217), although P4 is unable to derepress the 186 prophage (206).

Bacteriophage P4 was isolated from *E. coli* K-235, a colicin K-producing strain which also harbors phage PK (117), as a PFU on a P2 lysogenic strain (214). Electron micrographs of P4 show particles with icosahedral capsids that are smaller than those of P2 and with tails identical to those of P2 (55, 111) (Fig. 2). A P4-encoded protein is responsible for this capsid size reduction, which results in a capsid designed to fit the P4 genome rather than the P2 genome, which is three times as large (1, 51, 210, 221). As a prophage, P4 is integrated in the bacterial chromosome (26, 217) and expresses superinfection immunity, which operates at the level of transcription termination mediated by a small RNA molecule (44, 78). As a plasmid, P4 replicates autonomously and maintains itself as a multicopy plasmid in the absence of helper functions (45, 84, 159). This property, as well as its genetic organization, suggests that P4 may be considered an episomal element that evolved the ability to exploit a "helper" bacteriophage for horizontal propagation via a novel specialized transduction mechanism. Colony hybridization tests with P4 DNA have indicated the presence of P4 DNA sequence similarities in one of every four strains of a clinical collection of *E. coli* (3, 59). Recently, a P4-like phage carring a retron element has been described. This retronphage (ϕR73) can use P2 as a helper, and the virion appears identical to P4 (112, 228). *E. coli* K-12 and B strains contain functional P2 *ogr* genes (5, 222), and, in the case of the *E. coli* B strain, the *ogr* gene is probably part of a cryptic prophage (39).

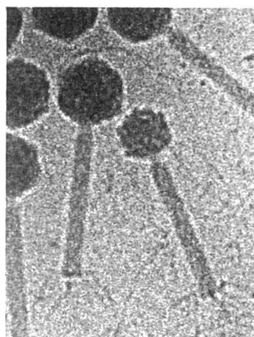

FIG. 2. Cryoelectron micrograph of P2 (left) and P4 (right) particles. The micrograph was provided by T. Dokland and prepared as described in reference 55.

Genome Organization of P2 and P4

The P2 genome contains approximately 33.8 kbp (152), whereas the P4 genome, which is fully sequenced, consists of 11,624 bp (96, 250). The *cos* sites of P2 and P4 DNA share the same 55-bp DNA sequence, of which 19 bp make up the cohesive ends (249). Both genomes are double-stranded linear molecules in the virion, and they circularize after injection into the host (154, 159).

The P4 genome contains two operons essential for DNA replication and particle formation (Fig. 3), which are transcribed in opposite directions from p_{sid} and p_{LE} or p_{LL} (41, 48, 99). The *int*, *c*II, *gop*, and β genes, present in the left part of the genome, are all nonessential for lytic growth (79). The leftward operon contains the DNA replication gene α (81, 130, 131); the ε gene encoding the P2 derepression function (76); the *kil* gene, whose overexpression is lethal to the

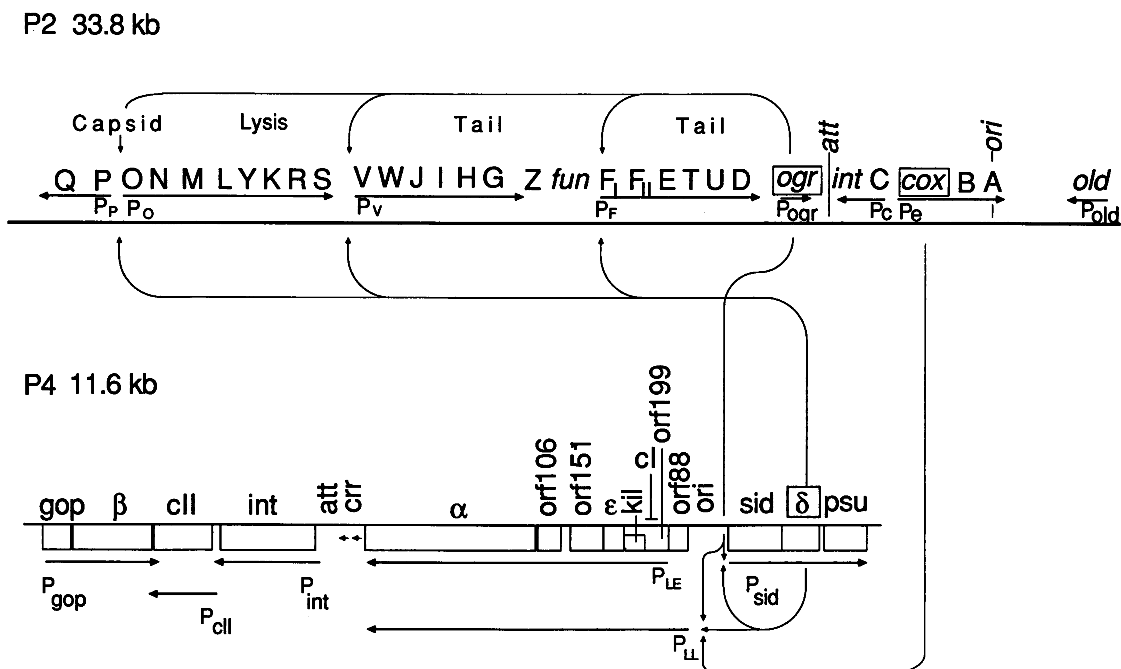

FIG. 3. Genetic maps of P2 and P4 and transactivation pathways. The maps shown here focus on the Ogr- and δ-initiated expression of the P2 late genes, the P4 Sid operon involved in capsid size determination, and the p_{LE}/p_{LL} promoters controlling the operon involved in genome propagation. For more detailed information, see recently updated maps of P2 and P4 (152, 250). The straight and somewhat thicker arrows represent transcription units, and the curved arrows show transactivation pathways of Ogr, δ, and Cox. The two small arrows under *crr* represent two direct repeats of a 120-bp sequence required for P4 DNA replication. The boxes under the P4 map represent the actual sizes of the genes; otherwise, the maps are not drawn to scale.

bacterial host (2, 62, 78); and open reading frames of unknown function (62, 78, 96, 144). This operon may be transcribed from two tandem promoters: the constitutive promoter p_{LE} (46, 144) and p_{LL} 400 nucleotides (nt) upstream, positively regulated by the P4 δ gene (48), as well as the P2 *cox* and *ogr* genes (32, 43, 92, 202). The prophage attachment site (*att*) and the integration (*int*) gene, necessary for the site-specific integration of the prophage, are found to the left of *crr* (*cis* replication region) (26, 180, 181, 217). The *crr* element is required for P4 DNA replication and consists of two directly repeated sequences of 120 bp (61). The region between *int* and the *cos* site may be deleted and/or substituted without affecting the lytic, plasmid, or lysogenic responses of P4 and has therefore been named the nonessential region (26, 42, 79, 112, 118, 156, 157, 192, 225). The rightward operon contains the capsid size determination gene *sid* (41, 157, 210) and the polarity suppression gene *psu* (134, 151, 207, 230), as well as the δ gene, whose product transactivates transcription of the P2 late genes (41, 224). The *psu* gene encodes an antiterminator for Rho-dependent transcription termination (207) and also functions as a decoration protein of the P4 capsid (114). The *sid* gene, which controls capsid size in the P2-P4 system, and the *psu* gene are the only morphopoietic genes present in the P4 genome.

All the other morphopoietic functions are provided by the helper genome (215). As can be seen in the genetic map of P2 (Fig. 3), the left 74% of the genome contains six genes (*Q*, *P*, *O*, *N*, *M*, and *L*) responsible for capsid synthesis, including packaging of DNA, and 14 genes for tail synthesis (19–21, 142, 145, 147, 148, 153, 232, 233). Twelve of the tail genes are located in the *VWJIHG* and *F₁F₂ETUD* operons. Tail genes *R* and *S*, which are part of the *ONMLYKRS*-operon, are thought to control tail length (142), and *K* and *Y* are part of a lysis gene complex (248). The *ONMLYKRS* operon may also contain a function which negatively controls the mRNA levels of the *VWJIHG*, *F₁F₂ETUD* and *PQ* operons (34). Phage 186 has a similar set of genes encoding the capsid and tail proteins (103). By using P2-186 hybrids, *E*, *U*, *T*, and *D* have been ruled out as genes coding for the tail fibers on the basis of the host range of the hybrid phages (104, 243). Therefore the *VWJIHG* operon must contain genes for the tail fibers. On the basis of similarity to other phage tail fiber proteins, Haggård-Ljungquist et al. (91) proposed that gene *H* encodes the side tail fiber protein of P2 and that gene *G* plays a role in the assembly of tail fibers. This and observations on P1-like phages (205) have led to the discovery that the original strain of lambda phage carries nonessential side tail fibers (102).

The remaining 26% of the P2 genome contains two genes (*A* and *B*) involved in P2 DNA replication (29, 68, 73, 94, 146), a repressor gene (*C*) (163, 165, 204, 240), and an *int* gene required for prophage establishment (31, 162, 244, 245). In addition, the integration host factor is needed for lysogenization of *E. coli* by P2 (203). Three more genes have been identified in this region: the *ogr* gene controlling P2 late-gene expression (231), the *old* gene (212), and the *cox* gene (94). The Cox protein is involved in (i) excision of the integrated P2 prophage genome (150, 243a); (ii) transcriptional repression of the P2 p_C promoter, which controls the synthesis of the immunity repressor C and the integrase (50, 201); and (iii) transcriptional activation of the p_{LL} promoter of P4 (202). The P2 nonessential *old* gene product kills *E. coli recB* and *recC* mutants and interferes with the growth of phage lambda (212, 213); this latter property is used for the selection of λ

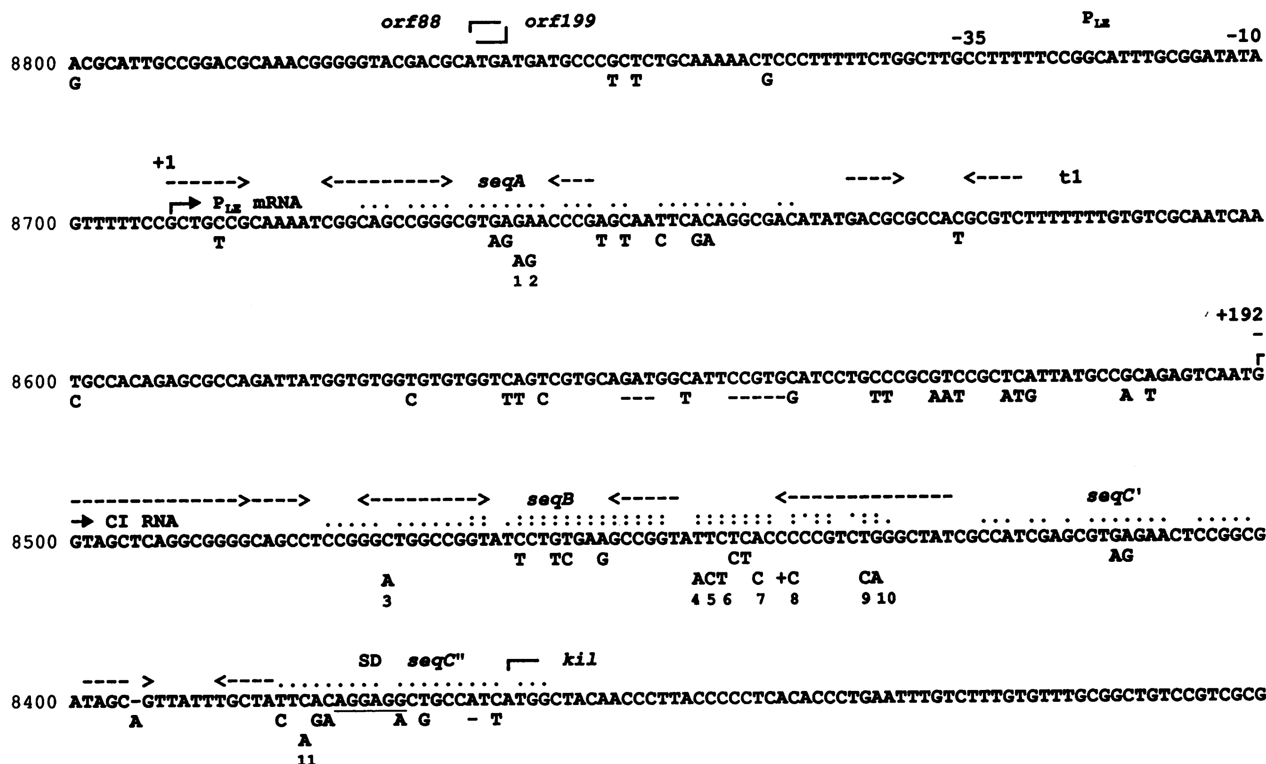

FIG. 4. Immunity region of bacteriophage P4. The complementary strand of P4 from nt 8300 to 8800 (96) is reported. On the first line under the P4 sequence are indicated the bases which are changed in the homologous region of retronphage φR73 (228); on the second line are reported the P4 mutations affecting the immunity. The P4 mutations are numbered underneath for reference: 1, *seqA405*; 2, *seqA8* (the substituted bases are complementary to *c*I405 and *ash8*, respectively [200]); 3, *ash3*; 4, *ash9*; 5, *ash8*; 6, *c*I405; 7, *ash7*; 8, *ash2*, *ash4*, *ash10*, *ash28*; 9, *ash23*; 10, *ash29* (144, 216); 11, *seqC30* and *seqC31* (200). Above the P4 sequence, ⌐┘ and ┌— represent the stop codon and start codon, respectively, of genes or open reading frames indicated; ┌→ represents the 5′ end of the transcript from p_{LE} and CI RNA; dashed arrows indicate inverted repeats; *seqA* and *seqC* are indicated by dots; in *seqB* the bases complementary to *seqA* and to *seqC* are indicated by the upper and lower sets of dots, respectively; SD, ribosome-binding site for *kil*.

transducing phages (60, 149). On the basis of its DNA sequence (93), the *old* gene product appears to belong to the superfamily of UvrA-related ATPases, and it may be an ATPase component of a nuclease (128).

P4 IMMUNE-INTEGRATED CONDITION

Lysogenization by bacteriophage P4 requires integration of the phage genome in the bacterial chromosome and the establishment of the immune condition; this may occur in either the presence or absence of the helper, and thus P4 encodes all the functions necessary for lysogenization (26, 217).

In most known prophages, immunity is elicited by a repressor protein which prevents transcription initiation at promoters controlling expression of lytic functions. This simple model, adopted for example by lambda (89), Mu (108), and P2 (12), is sometimes complicated by the presence of an "antirepressor" function(s) that may prevent expression of the repressor activity. Thus, immunity maintenance may require an additional repressor that inhibits expression of the antirepressor. This additional control of the immunity repressor by an anti-immunity repressor is found in P1 (241), P22 (185), and 186 (136). It is interesting that the anti-immunity repressor of 186 is not phage encoded but is the host *lexA* gene product (135).

The immunity system of P4 deviates from the above model in that (i) no repressor protein is encoded by the phage, but, rather a small RNA is the immunity factor; and (ii) expression of the replication functions is not prevented by repression of transcription initiation but by premature termination of transcription initiated at a constitutive promoter (and/or by processing of the primary transcripts).

Immunity Region

The identification of the P4 region responsible for the establishment and maintenance of P4 immunity and the analysis of the underlying mechanisms has been made by studying immunity-defective mutants, cloning of the genetic determinants of P4 immunity, and analyzing P4 transcription during the establishment and maintenance of immunity.

The structure of the P4 region containing all the known *cis*- and *trans*-acting determinants of P4 immunity is shown in Fig. 4. The P4 immunity region includes the constitutive promoter p_{LE} and a downstream nontranslated region about 330 nt long, rich in direct and inverted repeats that may allow the formation of multiple mutually exclusive secondary structures in the corresponding RNA. Notably, a region called *seqB* shares complementarity with *seqA* and *seqC* (Fig. 4 and 5A), which are located on either side of *seqB*. *seqC* is split in two portions, *seqC′* and *seqC″*, by a 17-nt sequence which has the potential for a stem-loop structure. *seqC″* overlaps the ribosome-binding site and the start codon of *kil*, the first translated gene downstream of p_{LE}. All the

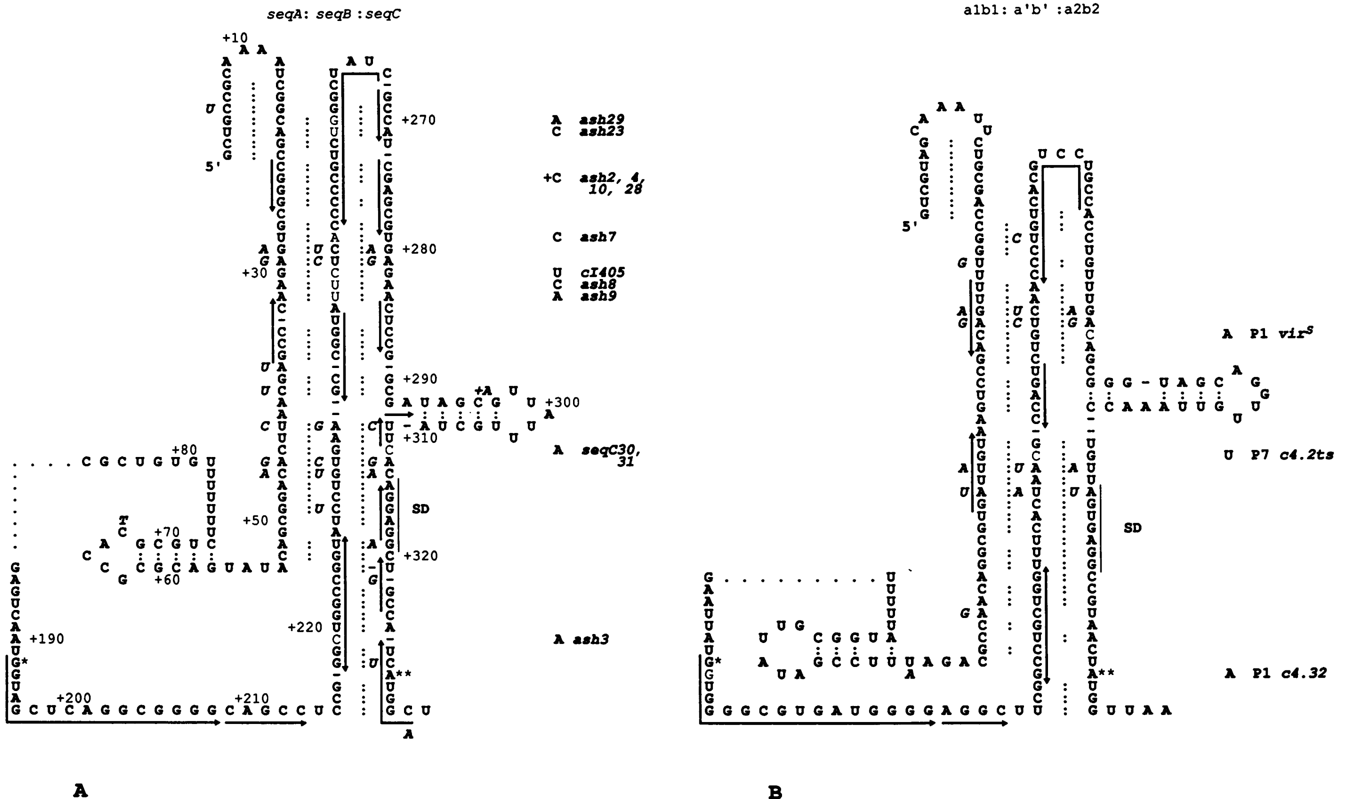

FIG. 5. Palindromic sequences in P4 and P1 immunity control regions. The main palindromic sequences on the primary transcript of the P4 immunity region are indicated either by colons or by arrows. The figure has been drawn so as to align the bases of *seqA-seqC* complementary to *seqB* in P4 (A) and the a1b1-a2b2 bases complementary to a'b' in P1 (B; indicated above the sequence). The sequence variations in φR73 (relative to P4) and P7 (relative to P1) are in italics. On the right are reported point mutations known to affect P4 immunity or P1-P7 anti-immunity; the corresponding bases in the sequence are in regular type on the same line. *, 5' end of P4 CI or P1 C4 RNA; **, start codon of the first open reading frame in the regulatory region of P4 and P1 (*kil* and *orfX*, respectively). Information used in drawing panel B is from reference 37, but additional palindromic sequences are shown in this figure, and the a1b1/a2b2 region complementary to a'b' has been extended. The exact start point of the P1 transcript has not been determined.

spontaneous immunity-defective (recessive) mutations so far sequenced are clustered within the *seqB* region (see below).

The immunity region is covered by *orf199*, whose start codon is located upstream of p_{LE}. Therefore this region may be translated only when transcription starts at p_{LL}, 400 nt upstream of p_{LE}, because of the coupled translation of *orf88* and *orf199* (78, 183) (Fig. 4).

A striking feature in the overall P4 genome organization is that the *c*I locus, i.e., the putative immunity determinant, is located downstream of the two promoters that control the expression of the P4 replication genes, at the 5' end of the operon it is supposed to repress. Moreover, one such promoter, p_{LE}, responsible for the early expression of the P4 replication genes, is constitutive (48, 144). This suggested that transcription termination could be involved in P4 immunity and prompted a detailed transcriptional analysis of the P4 leftward operon.

P4 Transcription during Establishment and Maintenance of the P4 Lysogenic Condition

Earlier studies of P4 gene expression led to the identification of three main regulatory states: (i) the immune-integrated (lysogenic) state (26, 217); (ii) the early transient uncommitted phase, occurring immediately after P4 infection of sensitive cells, characterized by transcription of the P4 leftward operon from the constitutive promoter p_{LE} (8, 48, 99, 144); and (iii) the late transcription phase, characterized by the activation of the late, positively regulated promoters p_{LL} and p_{sid}. This regulatory phase is characteristic of both the lytic and the plasmid conditions (41, 48, 99). The nonessential region appears to be transcribed from the two convergent promoters p_{gop} and p_{cII} in all the above conditions (8, 79).

The temporal control of the onset of the late mode of transcription and the developmental decision between lytic-plasmid and immune states are strongly influenced by the presence of the helper genome in the infected cells (26, 45, 217). On infection of a sensitive host in the absence of the helper, P4 lysogenizes the great majority of the cells while the plasmid state is established in 1% or less of the infected bacteria (45). This has allowed detailed study of P4 transcription during the establishment of lysogeny in a rather homogeneous cell population.

P4 early transcription starts from p_{LE} at nt G8692 (Fig. 4) (48). The two longest mRNAs produced are 4.1 and 1.3 kb long, and these cover the left operon from p_{LE} through α and from p_{LE} through *orf151*, respectively. Additional transcripts between 0.3 and 0.5 kb long are abundantly produced during this phase. Thus, transcription of the left operon appears to be prone to terminate (or the early transcripts are

degraded) and the encoded genes seem to be differentially expressed (49, 234).

At intermediate times and late after infection, the 4.1- and 1.3-kb mRNAs are no longer detectable while transcripts of 0.3 to 0.5 kb persist and new RNA species between 45 and 80 nt long are produced. This last transcription pattern is observed in the lysogenic condition and in cells in which the P4 immunity region has been cloned (48, 49). Not all of these short RNA molecules start at p_{LE}: a family of RNAs slightly heterogeneous in length (about 75 and 76 nt long) has its 5′ end at nt 8501 (+192) and covers *seqB* (SeqB RNA [49, 62]). The other short (50-, 70- to 80-, and 300-nt) RNAs start at p_{LE}. As discussed below, the SeqB RNA appears to be produced by processing of an RNA transcribed from p_{LE}.

The transcription pattern observed during the establishment and maintenance of the immune-integrated condition clearly shows that transcription initiation at P_{LE} is not repressed. Therefore, transcription termination and/or RNA degradation must prevent expression of the left operon from p_{LE}.

P4 Immunity Determinants

Identification of the P4-encoded factor responsible for eliciting immunity in *trans* has been performed through the analysis of P4 immunity-defective recessive mutants and the cloning of the minimal region still capable of expressing immunity. P4 mutants that are recessive and unable to lysogenize define two complementation groups, *c*I and *c*II (26). The *c*II gene is located in the nonessential region and is not involved in the P4 immunity process (26, 78).

The *c*I mutations map between *orf88* and ε and define the P4 immunity locus (26, 48, 118, 144). P4 mutants selected for their ability to grow on P3 lysogens (*ash* mutants [adaptation to a secondary helper]) also make clear plaques, and the *c*I mutants are Ash$^-$ (12, 144). Although producing clear plaques, several *ash* mutants are not absolutely defective in the maintenance of immunity (77, 137, 138); their clear-plaque phenotype may be due to a less efficient or delayed establishment of the immune transcription mode.

All the *ash* and *c*I mutations sequenced so far are clustered in a short region between p_{LE} and *kil* (62, 137, 138, 144). The longest open reading frame downstream of p_{LE} and covering this region could code for a 137-amino-acid polypeptide (*orf137* [144]); it has therefore been suggested that this polypeptide is the product of the *c*I gene. However, there is evidence against this hypothesis (78); most important, as discussed below, P4 immunity may be expressed from a 90-nt P4 fragment (nt 8507 to 8418) internal to *orf137* (62).

To identify the genetic determinant eliciting P4 immunity, Ghisotti et al. (78) cloned progressively shorter P4 DNA fragments around the *c*I-*ash* region. The shortest fragment conferring immunity on the cell and complementing *c*I mutants was 357 nt (nt 8774 to 8418) and contained p_{LE} and the region from +1 to +275. Transcription of this latter region is necessary for expression of P4 immunity, and it produces a transcript pattern similar to the one observed in lysogenic cells (49, 78). To define more precisely the region encoding the immunity factor, the above fragment has been subcloned under control of the inducible promoter p_{lac}. A DNA fragment as short as 91 bp (nt 8418 to 8508) is sufficient to express immunity, produce the SeqB RNA, and complement P4 *c*I405 in the presence of the inducer (62). This suggests that the SeqB RNA is the *trans*-acting P4 immunity factor and represents the product of the P4 *c*I gene (henceforth it will be called CI RNA). No promoter could be identified to account for the 5′ end of CI RNA; this suggests that the P4 immunity factor is processed from longer transcripts starting at p_{LE} (49).

In agreement with the hypothesis that the CI RNA is the P4 immunity factor, the appearance of CI RNA correlates with the turning off of the 4.1- and 1.3-kb leftward transcripts and with the transition from uncommitted growth to the immune condition (2, 49). The CI RNA is extremely stable, with an estimated half-life of more than 2 h (246). The secondary structure predicted for this RNA (Fig. 6) may be related to its stability: it presents a major stem-loop structure interrupted by a minor stem-loop structure and an 8-nt single-strand bulge (62). *c*I405 and other *ash* mutations are located in the bulge, whereas the *ash* mutations with the weakest CI phenotype are located in the major stem. The *ash3* mutation, which exhibits a strong CI phenotype, is located in the minor stem, outside of *seqB*. The predicted secondary structure of the *ash3* CI RNA would be disrupted by the C8474A transversion (62).

The most obvious target for an RNA effector is a complementary RNA molecule. As described above, there are two regions exhibiting complementarity with the *seqB* sequence of CI RNA: *seqA* and *seqC*. In the next paragraphs we discuss evidence indicating that complementarity of *seqA* and *seqC* with *seqB* is involved in the immunity mechanism of P4.

Clear-plaque-dominant (virulent) mutants, which are insensitive to prophage immunity, often identify the target for the immunity factor. However, all P4 virulent mutants so far isolated, such as P4 *vir1*, turned out to contain promoter up mutations in p_{LL} (137, 199, 217, 235). The *vir1* mutation makes expression of P4 replication genes from p_{LL} independent of the positive regulator δ (46, 48, 137, 217). The longer transcript made from p_{LL} contains *orf88* and *orf199*, whose translation may interfere with the usual immunity mechanism. Recently, a second class of virulent mutants (VirII) have been isolated from P4 *ash29*; the *virII* class contains a C8382A transversion, within *seqC″* (*seqC30*; Fig. 4 and 5) (199). The corresponding base in *seqB* is located in the CI RNA major loop. Transcription analysis of one such mutant shows that it makes long (4.1- and 1.3-kb) transcripts from p_{LE}, even when P4 immunity is expressed from a multicopy plasmid (199). Thus the VirII mutants point to *seqC* as a site where CI RNA may act to elicit immunity.

Involvement of *seqA* in P4 immunity is suggested by the following observation: transcription of a cloned P4 DNA fragment containing *seqA* but not *seqB* prevents lysogenization by an infecting P4; inactivation of the promoter as well as partial deletion of *seqA* in the plasmid relieves the interference with P4 immunity (78, 200). This suggests that the RNA transcribed from the plasmid competes with RNA from the infecting phage for the immunity factor produced by the phage itself, thus indicating a possible interaction between the complementary regions of *seqA* and *seqB* (78, 200).

It might be suggested that SeqA acts as an antagonist of P4 immunity not only when expressed from a plasmid but also in the phage itself, for example by competing with SeqC for the CI RNA. This does not seem to be the case, however, since (i) transcription from p_{LE} is terminated prematurely in a P4 cloned fragment from p_{LE} to *seqB* and expression of a downstream reporter gene is greatly reduced (49), so that a normal amount of SeqA in *cis* to *seqB* appears to allow substantial transcription termination in the absence of SeqC; and (ii) P4 *seqA* mutants, obtained by site-directed mutagen-

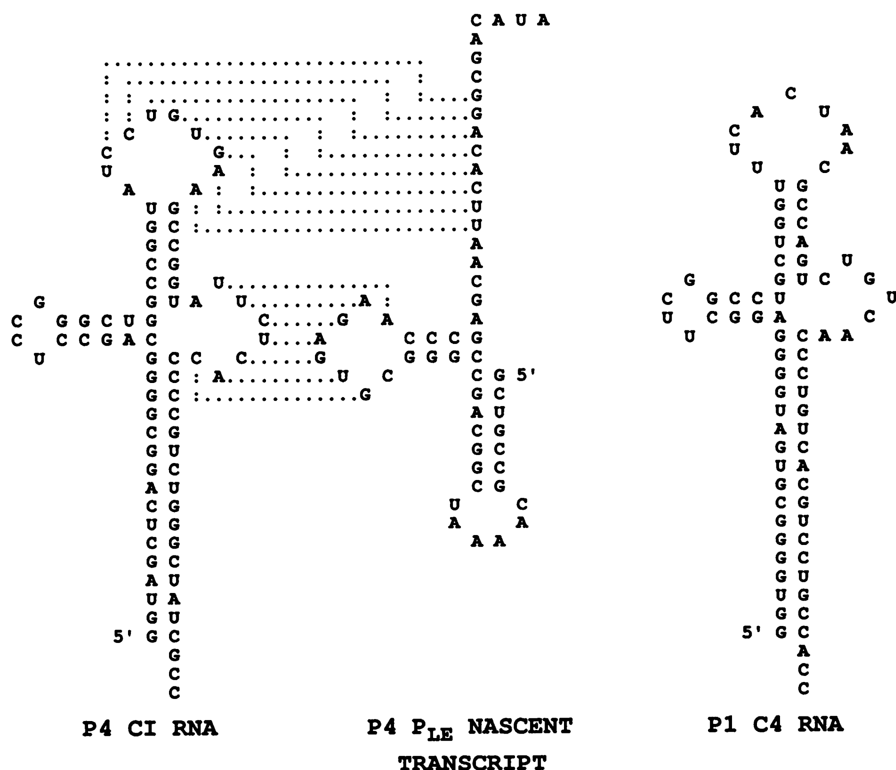

FIG. 6. Predicted secondary structure of P4 CI RNA, P1 C4 RNA, and the P4 p_{LE} nascent transcript. The secondary structures and the free energy have been obtained by using the program FOLD (252). The complementary bases in single-stranded regions *seqA* and *seqB* are connected by dotted lines. The precise 3′ end of P4 CI RNA has not been determined. P1 C4 RNA is modified from reference 38 with permission. P4 CI RNA, $-\Delta G$ = 36.0 kcal/mol; P4 p_{LE} nascent transcript, $-\Delta G$ = 14.6 kcal/mol; P1 C4 RNA, $-\Delta G$ = 34.6 kcal/mol (1 kcal = 4.184 kJ). Complementary bases in single-stranded regions of p_{LE} nascent transcript and CI RNA are connected by dotted lines.

esis, give clear plaques, indicating that SeqA-SeqB interaction is required to give immunity. These mutants, however, are still sensitive to P4 immunity when they superinfect a lysogenic strain (200), suggesting that *seqA* may not be the only target of immunity.

Model for P4 Immunity

A model for P4 immunity is based on the notion that complementary sequences may allow both intra- and intermolecular pairing of RNA molecules and that these interactions may have profound effects on transcription, stability, or translation of the target RNA (for reviews, see references 58 and 211). The proposed model suggests that (i) the CI RNA, which contains *seqB*, is the immunity factor; (ii) this RNA interacts with the target RNAs, i.e., the transcripts originating at p_{LE}, which exhibit complementarity with *seqB* at the level of *seqA* and *seqC*; (iii) because of this interaction, expression of the downstream genes is prevented by premature transcription termination of the primary transcript; and (iv) intramolecular and intermolecular pairing of the above complementary sequences may be involved in the immunity mechanism.

The first question that should be addressed is whether transcription termination or rapid mRNA degradation is the main event preventing expression of genes distal to p_{LE}. Although no conclusive evidence has yet been obtained, transcription termination more simply accounts for the available data (49). It would be unique if a 4.1-kb DNA region was all transcribed while the RNA was quickly degraded without being translated. For an adequate description of the P4 immunity mechanism, one should know how transcription termination is elicited by CI RNA. Because of its complementarity with two regions both apparently involved in bringing about immunity, the P4 immunity factor may act either independently or cooperatively at these two sites.

On the basis of the observation that the cloned p_{LE}-*seqB* region is sufficient to prevent expression of downstream genes by causing transcription termination, Ghisotti et al. (78) and Dehò et al. (49) proposed a model for P4 immunity based on *seqA-seqB* interaction. The *seqB* sequence on the CI RNA may interact with *seqA* on the nascent p_{LE} transcript and promote transcription termination. How this interaction may occur has yet to be elucidated. However, the CI RNA secondary structure (Fig. 6) and the position of *c*I405 and other *ash* mutations (Fig. 5A) suggest that pairing may occur between the bulge (and possibly the main loop) on the CI RNA and a loop (and the unfolded "tail") on the nascent transcript (Fig. 6). The identification of the actual transcription termination sites is complicated by the processing events leading to the production of CI RNA. Thus, the 50- and 80-nt transcripts containing *seqA* produced by the prophage might be derived from transcription termination or by processing of the 300-nt transcripts. Last, but most important, is how the *seqB-seqA* interaction causes transcription termination. Several hypotheses may be proposed, such as imposing an alternative secondary structure on the nascent RNA that unmasks potential transcription termination sites or promoting the nucleation of a transcription

termination complex. The in vitro reconstruction of the P4 immunity system should help elucidate these aspects.

The recent isolation of VirII mutants, the comparison with the homologous retronphage φR73, and the analogy with phage P1 anti-immunity regulation (see next section) have drawn attention to *seqC* as a second possible site of action for the P4 immunity factor. *seqC*" overlaps the Shine-Dalgarno sequence and the start codon of *kil*, the first translated gene of the p_{LE} mRNA. A testable hypothesis is that CI RNA binding to *seqC* prevents translation initiation of *kil* and that this would promote transcription termination because of a polar effect. *seqC* may represent a "safety" block for RNA polymerase molecules that escape transcription termination controlled by the immunity system operating upstream.

Comparison with Retronphage φR73 and Phage P1

The retronphage φR73 (112, 228) is heteroimmune to P4 (216) and thus provides the opportunity to compare similar immunity systems with altered specificity. The putative φR73 CI RNA sequence presents six base changes, all in *seqB*, two in the bulge and four in the major loop. It is noteworthy that for five of these changes there is a complementary base substitution both in *seqA* and in *seqC* (Fig. 4 and 5A), whereas the sixth is a C-to-U transition in the major loop, still compatible for pairing with the G in *seqA* and *seqC*. The sequence of p_{LE} mRNA from +128 to +173 exhibits the highest divergence, including some small deletions (Fig. 4). It will be interesting to determine whether these changes are involved in the modified specificity of φR73 immunity.

The P4 immunity system is strikingly similar to bacteriophage P1 anti-immunity control system, both at the level of organization and structure of the regulatory region and, presumably, for at least some of the underlying mechanisms. The P1 *immI* region (98, 100) encodes (listed 5′ to 3′) the *c4* repressor gene, an open reading frame of unknown function (*orfX*), and *ant*, coding for the antirepressor. *orfX* is preceded by a ribosome-binding site, whereas translation of *ant* appears to be coupled to *orfX*. Transcription of the *immI* region depends on two tandem promoters upstream of *c4*. Citron and Schuster (37, 38) have shown that the transcribed region between the promoters and *orfX* is not translated and that *c4* is a 77-nt RNA generated by processing of the untranslated region. The secondary structure of C4 RNA is almost identical to that of the P4 CI immunity factor (Fig. 6). The single-stranded sequences in the bulge and in the major loop (a′ and b′, respectively) are complementary to sequences a1b1 and a2b2, which are repeated 5′ and 3′ of a′b′, respectively (Fig. 5B). b2, which is separated from b1 by a stem-loop structure, overlaps the *orfX* ribosome-binding site and start codon. P1 C4 repressor both inhibits translation of *orfX* and prevents transcription of *ant* by inducing premature Rho-dependent transcription termination (13). This suggests that C4 RNA may act as an antisense RNA interacting with the complementary region covering the *orfX* ribosome-binding site, thus preventing translation and causing transcription termination as a polar effect (13, 37). Further insight into P1 antiimmunity control comes from the heteroimmune phage P7, whose different immunity is due only to the *immI* region. The P7 *c4* gene shows four base changes in the a′b′ sequence which are compensated both in a1b1 and in a2b2 (37) (Fig. 5B). It would thus appear that in P1 the complementary sequence upstream of *c4* may also play some role in controlling P1 anti-immunity; its role, however, has not yet been investigated.

Prophage Integration

In the lysogenic condition the P4 genome is integrated in the bacterial chromosome at a preferential site (*attB*) corresponding to the 3′ end of *leuX*(*supP*), the gene encoding a $tRNA^{Leu}$ isoacceptor (26, 181, 217, 242). The integration reaction requires the P4 *attP* site, at which the recombination takes place, and the *trans*-acting integrase protein encoded by the P4 *int* gene (26, 180). The P4 *attP* site is contained in a 0.5-kb restriction fragment (180). Site-specific recombination occurs within a G+C-rich core region 20 bp long, identical to the 3′ end of *leuX*; thus, P4 integration via site specific recombination preserves the integrity of the tRNA gene (181). The core region is flanked by two pairs of 16-bp direct repeats; a fifth repeat in the opposite orientation is present on the left side of *attP* (181). The involvement of such arm repeats in the integration process has not been investigated.

The region encoding the P4 integrase has been identified by functional and deletion analysis of cloned P4 fragments (180). An open reading frame 439 codons long is the putative *int* gene (181). According to the predicted amino acid sequence, P4 integrase belongs to a family of rather diverged but still related site-specific recombinases (4). P4 integrase is necessary for both integration and excision, and no other P4-encoded functions necessary for either event have been found (180, 181).

The P4 *int* gene is located at the left of *attP* and is transcribed leftward (180, 181). By sequence inspection, two promoters have been identified (181): p_I, which resembles the *E. coli* σ^{70} promoter consensus sequence and overlaps the *attP* left pair of direct repeats, and p_{II}, which is located about 100 bp downstream of p_I, and shares similarity with P2 and P4 late promoters. When cloned on a plasmid upstream of *lacZ*, p_I directs expression of β-galactosidase; P4 integrase provided in *trans* by a compatible plasmid strongly represses p_I activity. This indicates that the P4 *int* gene is autogenously repressed and suggests that the 16-bp repeats may represent integrase-binding sites (181). By analogy with the other P4 and P2 late promoters with which it shares homology, it has been suggested that p_{II} may be activated by P2 Ogr and/or P4 δ positive regulators, thus providing Int upon induction of P4 prophage by P2 (see below); the *leuX* promoter might also drive P4 prophage *int* gene transcription (181).

Integration within known or putative tRNA genes appears to be common among both prokaryotic and eukaryotic mobile elements, including coliphage 186 (56); retronphage φR73 (112, 228); *Salmonella* phage P22 and the P22-like cryptic prophage DLP12 (160, 181); *Haemophilus influenzae* phage HP1 (106, 107); the UV-inducible archaebacterial element SSV1; the integrative plasmids pMEA100, pSAM2, and SLP1, harbored by *Nocardia mediterranei*, *Streptomyces ambofaciens*, and *Streptomyces coelicolor*, respectively (169, 193); and the putative mobile elements of the slime mold *Dictyostelium discoideum* (166). This may reflect structural or mechanistical features (such as dyad symmetry elements, structure/sequence repetition within a genome, and/or conservation among species) offered by tRNA genes that may be exploited for evolution of integration systems (107, 193).

REGULATION OF PLASMID STATE

P4 DNA Replication

Autonomous replication of P4 DNA, which occurs both in the plasmid state and during the lytic cycle (159), requires the functional products of P4 α and the host *dnaE* (DNA polymerase III α subunit) genes, whereas it does not depend on the host *dnaA* (initiator), *dnaB* (helicase), *dnaC* (DnaB complex), and *dnaG* (primase) genes (22, 81). P4 DNA replication starts bidirectionally at the *ori* site. In addition, the *crr* region, located between *att* and α, is required in *cis* for this replication to occur (61, 130). P4 α protein (777 amino acid residues) is endowed with primase activity in its amino-terminal half (6, 7, 129, 130, 227). Alignment with other bacterial, phage, and plasmid primases has shown six common sequence motifs, including an amino-terminal zinc finger that is thought to be involved in the recognition of the priming sites on the DNA template and two regions resembling the Mg^{2+}-mediated nucleoside triphosphate (NTP)-binding motifs of ATPases and other RNA and DNA polymerases (110, 227). The sequence of the carboxy-terminal part of the α protein suggests that it contains the helicase activity and encompasses a type A nucleotide-binding site showing similarities with NTP-binding domains of several DNA helicases, such as *E. coli* protein UvrA, helicase IV, Rep, UvrD, and PriA; phage T4 gp41; and phage T7 gp4 (85, 86, 227). These features of P4 α protein may account for the independence of P4 replication from the host primase and helicase functions (22, 227). Particularly significant is the homology between the primase domain of P4 α protein and the primase domain of conjugative plasmids ColIb-P9 (IncI1), RP4, and R751 (IncP), associated with common functional features (227). An amino acid substitution in a conserved motif (EGYATA→QGYATA) eliminates primase activity of both P4 and RP4 primases and cause P4 phage to grow poorly and to depend on the host *dnaG* primase (227). The above motif appears to be part of a more extended amino acid sequence conserved in several prokaryotic primases (110, 179). Mutants with both amber and temperature-sensitive conditional-lethal mutations in the α gene have been isolated (6, 76, 81); such mutations are not suppressed by the host primase, indicating that other functions encoded by the α gene are essential for P4 replication. Recent studies (139, 247) have shown that the P4 α gene product is a multifunctional protein endowed with primase, helicase, and DNA-binding activity.

Early studies of P4 gene expression showed that production of α protein is modulated during the P4 life cycle, with a burst of α protein production occurring within the first 20 min followed by a decrease to about 25% of the peak production rate (8). These studies, however, were biased by the use of the P4 *vir1* mutant, which is partially insensitive to P4 immunity and starts transcription of the α operon from the mutated p_{LL} promoter earlier than the wild type does. Transcription studies with wild-type P4 (43, 46, 48, 49) indicate a two-stage regulation of α expression. A burst of α gene transcription occurs during the early uncommitted phase of replication, starting from p_{LE}, and is turned off readily by the P4 immunity termination mechanism. Transcription from p_{LL} activated by P4 δ and/or P2 Cox and Ogr proteins allows late expression of the left operon. A transcription termination region between *orf151* and orf106 modulates the differential expression of the α (and *orf106*) gene(s) relative to the upstream genes in the P4 left operon (49, 234).

Multicopy Plasmid State

In addition to the immune-integrated condition, P4 infection of a cell that is nonlysogenic for P2 may lead to the establishment of the high-copy-number-plasmid mode of replication (26, 45, 84, 177). The choice between the immune-integrated and multicopy-plasmid pathways occurs after a transient phase of uncommitted replication and appears to be equivalent to the choice between lysis and lysogeny that is made when the helper phage genome is present (2). The multicopy-plasmid state may be established at low frequency by wild-type P4 (1% of survivors or less [45]). This frequency may be increased by mutations affecting (*cI*, *ash*) or bypassing (*vir1*) P4 immunity (45, 77, 84, 133, 177, 216). The average number of P4 genomes per bacterial chromosome is about 30 to 50; an integrated copy of P4 may be also found but is not essential for the plasmid condition (45). Bacterial clones carrying P4 as a plasmid may be recognized by their typical colony morphology (rosette) due to the presence of a high proportion of long filamentous cells. Thus plasmid P4 appears to interfere with cell division and also affects the growth rate of the plasmid-carrying clone (45).

The plasmid state is characterized by transcription of the two operons of the essential region from the positively regulated late promoters p_{LL} and p_{sid} (Fig. 3) (46, 48), whose activation depends on P4 δ protein (41, 46, 48) and the host RNA polymerase (126). An additional promoter, p_{LL}*, partially overlapping p_{LL}, is responsible for the δ-independent low level of transcription of the left operon (46).

Since δ is transcribed from p_{sid}, the P4 rightward operon is autogenously regulated. How transition to the plasmid mode of transcription occurs has not been investigated in detail. It is conceivable that a low basal level of δ expression may occur during the uncommitted phase of P4 replication and that this could prime p_{sid} and p_{LL}. Alternatively, other P4 function(s) encoded in the left operon and expressed from either p_{LE} or p_{LL}* may help activate p_{sid}.

In the plasmid state, p_{LE} is still active: the transcripts characteristic of the lysogenic condition are abundantly produced and only a small amount of full-length transcripts (1.3 and 4.1 kb) may be detected (48, 49). Thus the immune and the plasmid modes of transcription are not mutually exclusive; rather, the plasmid mode of transcription is superimposed on the immunity regulation, the plasmid state being epistatic to the immune state. In the plasmid state, transcription from p_{LL} is not subject to the strong transcription termination mechanism caused by P4 immunity and operating on the RNA starting at p_{LE} (49, 184). This could be due to translation of *orf88* and *orf199* and/or to differences in the secondary structure of the RNA transcribed from p_{LL}, which may prevent the interaction between CI RNA and the SeqA/SeqC targets.

It therefore appears that, for an infecting P4 phage, immunity is the default regulatory condition that is always activated. To enter the alternative life-styles (plasmid state or lytic cycle), P4 simply bypasses the immunity mechanism, which is not turned off. In contrast, in most known bacteriophages such as lambda and P2, the immune and lytic patterns of gene expression are mutually exclusive, the former preventing the latter and vice versa.

P2 AND P4 LYTIC CYCLES AND RECIPROCAL INTERACTIONS BETWEEN SATELLITE PHAGE AND HELPER

P2 Lytic Cycle and Control of P2 *ogr* Gene Expression

Immediately after infection, P2 DNA circularizes through its cohesive ends (154, 239) and the early genes *cox*, *B*, and *A* are transcribed from the early promoter p_e (67, 158, 204). Cox prevents transcription of the P2 immunity repressor gene *C* from the divergent promoter p_c, thus favoring the choice of the lytic cycle. Expression of *C* would, in turn, prevent transcription from p_e and determine the choice of the lysogenic cycle (11, 201, 204).

The products of genes *A* and *B* are necessary for P2 DNA replication (146, 148), together with the host functions DnaB (helicase), DnaE (polymerase), DnaG (primase), and Rep (helicase) (22, 25). gpA acts in *cis* by introducing a strand-specific nick at the origin of replication *ori*, located within the *A* gene sequence itself (29, 68, 73, 146, 161). DNA synthesis proceeds unidirectionally on the nicked strand (209), whereas the circular strand is conserved and functions as a template in a rolling-circle mode of replication (121, 132). Infection with *B* mutants, which are defective in DNA synthesis, results in DNA circles with a single-stranded tail, which corresponds to the strand nicked by gpA. Hence, the *B* gene product appears to participate in lagging-strand synthesis (68). *sub* (suppression of *B* requirement) is an *E. coli* host mutation that specifically allows growth of replication-deficient *B* mutants of P2 (229).

Transcription from P2 late promoters requires the host RNA polymerase, the P2-encoded positive regulator Ogr (15, 34, 155, 231), and P2 DNA replication (74, 95, 141, 146, 154). Under certain conditions, however, phage 186 gene product B (the Ogr analog) has been shown to activate the late *V* operon of phage 186 in the absence of DNA replication (52).

ogr gene expression is initiated from p_{ogr} at an intermediate time between the onset of early and late transcription. When late-gene expression is in progress, the *ogr* gene is cotranscribed with the late F_IF_2ETUD operon (17). Since Ogr activates this transcription, Ogr positively controls its own synthesis. In addition, the *ogr* gene appears to be controlled autogenously at p_{ogr} (Fig. 3). Furthermore, transcription of the *ogr* gene in the prophage of a P2 heteroimmune lysogen is stimulated after infection with P2, suggesting that p_{ogr} is under indirect immunity control and that it is activated by an unidentified P2 early-gene product during infection (14, 17). In the case of phage 186, its repressor (CI) of lytic transcription directly represses *B* gene expression (53), even though the genetic organization and transcription patterns of the *c*I and *B* genes of phage 186 are very similar to those of the corresponding genes (*C* and *ogr*) in P2 (187, 195, 201, 204).

P4 Lytic Cycle and Reciprocal Interactions with Helper Phage P2

The P4 lytic cycle requires efficient exploitation of the helper genetic information to obtain the morphogenetic gene products. This is accomplished both by utilizing the P2-encoded regulatory mechanisms normally acting on the helper and by carrying out new P4-encoded functions that bypass or enhance P2 control systems. These interactions are depicted in Fig. 3.

P4 lytic replication may occur within different scenarios: P4 and P2 coinfecting a nonlysogenic host; P4 infecting a P2-repressed lysogen; a P4 lysogen being infected by P2. In each of these situations, the satellite phage senses the presence of the helper and responds by activating its own functions that will in turn modify the pattern of gene expression of the helper. Reciprocal regulatory interactions developed in the P4-P2 system involve lifting of the immunity mechanisms (mutual derepression) and direct activation of the late operons of both phages (reciprocal *trans*-activation).

The P4 lytic cycle in a P2 lysogenic host requires derepression of the prophage helper (76, 219). Derepression of P2 prophage is controlled by the P4 ε gene, which is required for the lytic cycle only when P4 infects a P2 lysogen but not in mixed infection with P2 (51, 76). Although the mechanism has not yet been elucidated, ε causes P2 early-gene expression and P2 DNA replication in situ without prophage excision (218, 219). P2 late-gene expression ensues via the normal P2 DNA replication and Ogr-dependent activation mechanisms, and this is sufficient for the completion of P4 lytic cycle (76, 95, 225).

P4, however, may efficiently *trans*-activate P2 late-gene expression, bypassing the need for P2 replication and the positive regulator, Ogr; this process requires the product of the P4 δ gene (95, 215, 225), which activates P2 transcription from the same promoters used by Ogr (33, 34).

The P4 transcription pattern observed on infection of a P2 lysogen or in a P2-P4 mixed infection is similar to that occurring in a P4 solo infection, except for the timing of late promoter activation and the relative amount of transcripts produced. In the presence of P2, P4 late genes are expressed about 20 min earlier and 5 to 10 times more abundantly (8, 41, 46, 48, 77, 99). The higher expression level of late transcripts may also reflect the higher proportion of cells entering the lytic pathway relative to those establishing the plasmid state (45, 46, 48). The activation of P4 p_{sid} and p_{LL} late promoters is effected by P2 Ogr protein (32, 41, 43, 99). P2 Ogr and P4 δ proteins act independently to activate both P2 and P4 late promoters (33, 34, 36, 41, 46, 48, 95). Sequence similarities between promoters, as well as the regulatory factors of the two phages (96, 237), provide the basis for the reciprocal *trans*-activation of P2 and P4 late operons (see below).

Infection of a P4 lysogenic strain by P2 induces the P4 lytic cycle; efficient induction requires a functional P2 *cox* gene to derepress P4 (45, 202, 219), but Cox is not needed to induce the P4 lytic cycle in plasmid P4 carriers (45).

The P2 Cox protein activates transcription at p_{LL} (47, 92, 201, 204). This protein has been shown to bind in vitro both to P2 p_C (the promoter for P2 repressor transcription) and upstream of P4 p_{LL}; a putative Cox-binding consensus sequence has been derived by comparison of the two regions (202). This suggests that Cox may act as an additional *trans*-activator of p_{LL}, thus promoting immunity-insensitive transcription of the P4 left operon. The Cox-binding consensus sequence is not found upstream of p_{sid}.

Structure and Function of P2 and P4 Late Promoters

P2 morphopoietic genes are expressed from four late operons (145, 147, 232). The four P2 late promoters differ from a standard *E. coli* σ^{70} promoter (33, 34). Sequence comparison of the P2 promoters has revealed three regions of similarity located upstream of the −35 region, centered around nt −50, −65, and −88. The conserved sequences at nt −50 and −65 constitute an imperfect element of dyad symmetry. Mutational analysis has demonstrated a need for a sequence between nt −69 and −64 with a critical break

point at nt −65 from the start point of transcription. That sequence is part of the imperfect dyad symmetry element (32, 87). The *E. coli* σ^{70} factor is necessary but not sufficient for transcription of the late P2 promoters (126, 155). Initiation of transcription from these promoters requires the P2-encoded transcriptional regulatory Ogr protein as well as concurrent P2 DNA replication (126, 145, 154, 231). The phage 186 *B* gene product and the P4 δ gene product are also able to activate transcription from the P2 late promoters (23, 95, 104). The P4 p_{sid} promoter has similar structural features to the four P2 late promoters (237), and in a coupled transcription-translation system as well as in vivo, either δ or Ogr proteins can activate transcription at the p_{sid} promoter in the absence of any other phage-encoded factor (126, 237).

δ cannot be an alternative σ subunit, because antibodies against σ^{70} prevent transcription from p_{sid} in vitro (126). The Ogr protein and its functional analogs, the B protein of phage 186 (122, 123) and the δ protein of P4, may, rather, function in a transcription initiation complex composed of the promoter upstream dyad symmetry element and the host RNA polymerase.

Structural Requirements for P2 Ogr and P4 δ Function

The P2 *ogr* gene encodes a basic protein of 72 amino acids (16, 36). Additional studies have shown that the C terminus of Ogr can be shortened by 21 amino acids without loss of function (70). Sequence conservation of 4 cysteines among the Ogr-like proteins suggested Zn binding. Purified Ogr binds Zn, and results of cobalt substitution experiments are consistent with cysteinyl coordination of the zinc (140). It has recently been shown by directed mutational analyses that the Cys residues, believed to be involved in Zn coordination, are required for late-gene transcription (70). However, studies of the ability of the mutants to bind zinc have been inconclusive because of the finding that the Zn blot technique used is unreliable (70).

It is believed that Ogr interacts with the dyad symmetry element upstream of the four late P2 operons it controls. In a similar fashion, the Ogr protein is thought to interact at the dyad symmetry upstream of the promoter controlling the *sid*-δ-*psu* operon of phage P4 (Fig. 3) (96). Genetic evidence indicates an interaction between Ogr and the host RNA polymerase. The *rpoA109* allele of *E. coli* specifically blocks P2 late transcription (231). The *rpoA109* mutation is located in the gene coding for the α subunit of RNA polymerase and results in a His-to-Leu substitution in its C-terminal domain (66). This block can be suppressed by mutations in the P2 *ogr* gene (231). The *ogr* mutations so far analyzed are all at position 42 and are replacements of Tyr by Cys, Ala, or Gly (16, 71, 127). P4 growth is also blocked in the *rpoA109* strain, and P4 *org* mutants which have mutations in the δ gene and can grow on *E. coli rpoA109* can be isolated (97).

The δ protein consists of 166 amino acids, but a closer inspection has revealed two Ogr-like domains in its amino acid sequence (97). In both domains the cysteine residues are conserved relative to Ogr and the B protein of phage 186. The δ protein may be considered an "Ogr dimer." The P4 *org* mutations, which allow P4 to grow on *E. coli rpoA109*, cause a Thr-to-Ala change at amino acid 127, in the second Ogr domain of the δ protein (97). Surprisingly, the transactivator gene product of the P4-like retronphage φR73 contains 81 amino acids and is similar to P2 Ogr; i.e., it has only a single domain (112).

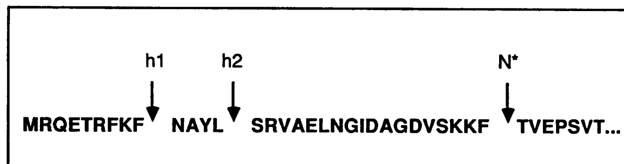

FIG. 7. Processing sites in the P2 capsid protein gpN. The N-terminal part of gpN (357 amino acids) is shown with the processing sites of h1, h2, and N*. The arrows indicate the processing sites.

CAPSID STRUCTURES OF P2 AND P4 AND CAPSID SIZE DETERMINATION BY P4

Icosahedral virus capsids consist of 12 pentamers of capsid protein (one pentamer per vertex), as well as hexameric units of the capsid protein subunit in larger viruses. In this case the protein subunits are positioned in different environments. Therefore they cannot be perfectly identical and may give rise to nonequivalent interactions. Caspar and Klug (28) proposed rules for a "quasi-equivalent" bonding between the subunits in icosahedral viruses, which meant that each of the 20 triangular faces related by icosahedral symmetry could be subdivided into smaller triangles. This was called triangulation and allows the virus to accommodate $60T$ quasi-equivalent subunits, where T is the triangulation number. In tail-containing bacteriophages, one of the vertices must be occupied by a portal protein rather than a pentamer. The portal protein—here refered to as the portal vertex protein—is necessary for DNA packaging and tail connection. During assembly of the viruses, an additional scaffold protein is found in proheads but is absent in the mature capsid (27).

Capsid Gene Products of P2 and P4

P2 gene *N* codes for the major capsid protein (N*), which is a processed version of gpN (357 amino acids). In the presence of gpO, 31 N-terminal amino acids of gpN are removed to give rise to N* (143, 188, 196). In addition, partial processing of gpN by removal of 9 and 13 amino acids gives rise to components h1 and h2, respectively (Fig. 7). These products are present in less than one copy per P2 particle (196). Two other minor proteins of the head, h6 and h7, appear not to be gpN-derived products, since they do not react with antibodies specific to N* (196). In fact, h7 has now been shown to be the N-terminal part of gpO (called gpO*; ca. 17 kDa) (63, 167). The function of gene product Q is not known, but gpQ has a molecular mass (39 kDa) roughly corresponding to that of h6, which could be the postulated portal protein of P2 and P4 capsids. gpL is said to be a capsid finishing protein because it protects DNA in P2 heads from the action of pancreatic DNase (190). Furthermore, the *V* gene is of interest for the capsid maturation, since it might be involved in the capsid tail connection (34, 80).

The P4 capsid, like that of P2, is assembled from gpN. As in P2, gpN is processed during maturation by gpO-dependent cleavage. In the case of P4, however, gpN is not fully processed, and significant amounts of h1 and h2, in addition to N*, appear in the mature P4 capsid. The h1- and h2-specific amino acids are exposed on the surface of the mature P4 capsid as revealed by antibodies specific to the h1/h2 N-terminal epitope (113). Rishovd and Lindqvist (196) have shown a content of about 20 h1 and 40 h2 subunits per P4 capsid, assuming a triangulation number (28) of $T = 4$

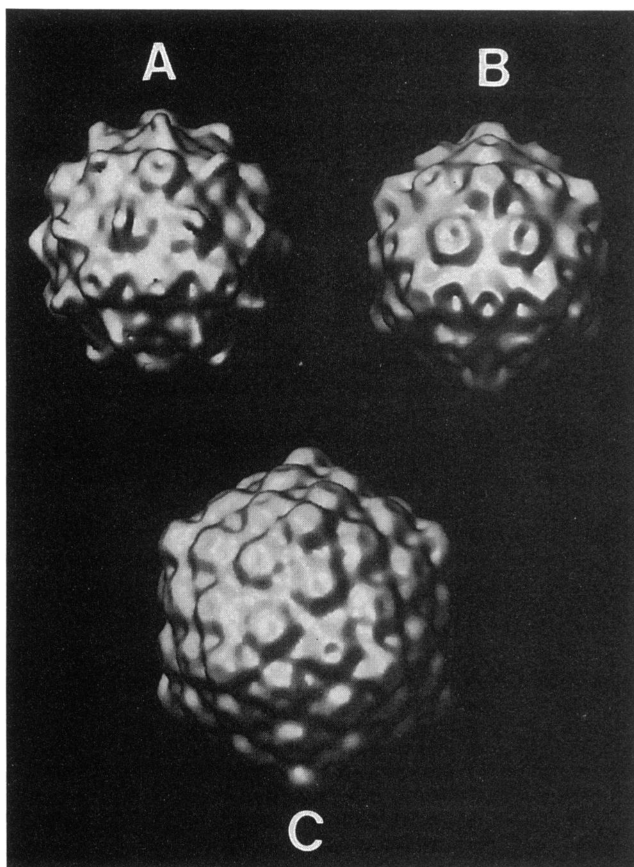

FIG. 8. Image reconstructions of P2 and P4 capsids. The capsids are displayed in the following order: (A) P4 Psu$^+$ (B) P4 Psu$^-$, and (C) P2. The image reconstructions were performed by Terje Dokland (54, 55).

(55). Assuming a structural role for these proteins, there appear to be two possibilities: either h1 and h2 together make up the pentamers, or two molecules of h1 and/or h2 might form hexamers together with four molecules of N*. The latter possibility has been favored (196) since image reconstruction from cryoelectron micrographs shows that the hexamers appear different in P2 and P4, whereas the pentamers do not (55).

Image Reconstruction of P2 and P4 Capsids

To address the problem of capsid size determination, it has been important to define the structures for both P2 and P4 capsids. Geisselsoder et al. (75) proposed a triangulation number of $T = 4$ for P4 and $T = 9$ for P2, whereas Walker and Andersen (238) had suggested $T = 7$ for P2. Using cryoelectron microscopy in combination with image reconstruction, Dokland et al. (55) have recently demonstrated that the correct values are $T = 7$ for P2 and T = 4 for P4. Examples of image reconstructions of P2 and P4 capsids are shown in Fig. 8. The P2 capsid consists of 420 subunits of N*, of which 60 constitute the pentamers and 360 are the hexamers. In the case of P4, its capsid contains 240 subunits of gpN-derived protein of which 60 make up the pentamers and 180 are the hexamers. In the mature P2 and P4 capsids, however, one of the vertices should contain a portal vertex protein component instead of a pentamer.

The image reconstruction analysis further revealed that N* has a two-domain configuration in both P2 and P4 (55). The two domains of N* are connected by a lower-density hinge region, whose angle varies in the different N* subunit conformations. The major domain forms the capsomer proper, whereas the other domain forms trivalent contacts between the capsomers. When the hexamers of P2 and P4 are compared, the intercapsomer distance (12 nm) of P4 is the same as in P2, although the P4 hexamers appear not to protrude so much from the surface, and they have a larger hole in the middle (55).

Protein Processing in Capsid Formation

Protein processing has been observed during the morphogenesis of several bacteriophages including T4 (18), T5 (253), λ (27, 101) and generally occurs in the maturation of prohead-like structures. The proposed rationale for its widespread occurrence is to confer irreversibility on the morphogenetic process and to stabilize the resulting structures. In P2 the interaction between gpO and gpN appears to establish the complex processing machinery responsible for several specific cleavages (three sites in gpN and at least one site in gpO). There is a striking similarity between the cleavage site of h1 and that giving rise to N*, both having the sequence Lys-Phe-/-polar-hydrophobic (Fig. 7). If this is the sequence recognized by a specific proteolytic enzyme, it seems improbable that this protease would recognize the h2 site (Tyr-Leu-/polar-charged). The cleavage of gpO has recently been clarified to some extent. As mentioned above, the N-terminal half of gpO, called O*, is the h7 component of P2 and P4 (63, 167). Previous studies have estimated h7 (i.e., O*) to be present in 95 and 33 copies per P2 and P4 particle, respectively (8). The role, if any, of this truncated gpO in the particle remains to be elucidated, but the different numbers of O* in the capsids seem to reflect the difference in size of the P2 and P4 capsids. This result and the fact that P4 is unable to produce viable progeny on a P2 O$^-$ lysogen (215) are difficult to reconcile with the report that Sid can compete with and substitute for gpO during P4 capsid assembly (1). In this study, Sid-expressing cells, containing a P4 cosmid and infected with a P2 O$^-$ amber mutant, gave rise to a very low level (much less than one particle per infected cell) of transducing particles of P4 capsid size. This yield of transductants was 2,000-fold higher than in a control infection in which Sid was not expressed. This transduction assay may then detect a low level of gpO-independent but functional P4 capsid assembly, which has little to do with the normal productive P4 capsid assembly.

At what stage does processing occur? In other phage systems, assembly precedes processing (27). This appears also to be the case for P2 and P4, since both P2- and P4-sized procapsid-like structures containing completely unprocessed gpN have been observed (167). This result supports the notion that assembly precedes the processing and favors the idea that gpN and gpO associate prior to capsid assembly. Whether this occurs at the monomeric level or at the penta/hexamer stage is an open question. We favor the possibility that gpN and gpO coassemble at the monomeric level, as appears to be the case for phage P22, for which it has been shown in vitro that the scaffolding protein regulates the polymerization of P22 coat subunits into icosahedral shells (186). Thus, gpO may be considered a scaffolding protein, which controls the efficiency and precision of the assembly process of gpN into P2 procapsid. When this is completed, gpO finds itself in a structural context in the procapsid, where it elicits the processing of gpN. gpO may

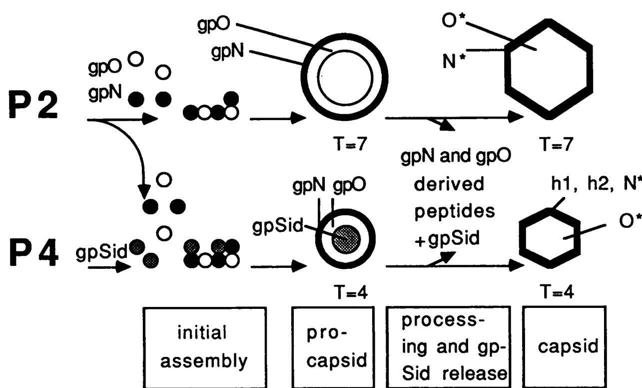

FIG. 9. Outline of capsid size determination by P4. The portal vertex component has not been included in this outline. Open circles, gpO; solid circles, gpN; shadowed circles, gpSid.

itself function as a protease in that process, or it could simply assist an unknown protease to carry out the processing of gpN. At the same time, gpO is itself cleaved to O*, which is retained in the mature P2 particle. The fate of the C-terminal part of gpO is not known, but it is probably degraded.

P4 Capsid Size Determination

The presence of the P4 *sid* gene product is sufficient to direct the assembly process into P4-size capsids (1, 83, 173, 210). The difference in processed gpN between the P2 and P4 capsids raised the possibility that the mechanism of gpSid action involves differential processing of gpN, resulting in capsid size reduction. This seems unlikely, however, since the assembly of both P2- and P4-size capsids appears to precede the processing of gpN. Therefore, the presence of h1 and h2 in the P4 capsids appears to be a consequence of the smaller capsid assembly rather than the cause of it.

gpSid has been reported to be present in the P4 procapsid, but few or no Sid molecules are present in the mature P4 particle (8). Using polyclonal antibodies directed against Sid, Fossdal (63) was unable to detect Sid in P4 particles purified by CsCl density gradient centrifugation, whereas procapsid structures, obtained from the same gradient, contained substantial amounts of gpSid. Hence gpSid does not seem to be a required component of the P4 particle but, rather, functions during the procapsid formation and then is released. gpSid may be considered an effector of conformational variation of gpN, which satisfies the needs of P4 capsomer formation. Although a hinge region has been suggested to confer the necessary flexibility on gpN (221), Dokland et al. (55) have suggested that size determination by Sid involves a major change of the whole hexamer complex (the distance across the hexamer is 12% greater in P4) rather than just restricting the angular variation or allowing extreme hinge angles to occur. In that context, gpSid may function as a chaperone-like component (105, 221) by promoting correct folding and positioning of gpN to yield the more extended P4 hexamer form.

An outline of capsid size determination in the P2-P4 phage system is shown in Fig. 9. By analogy with other phage systems such as phage ϕ29 (88), a portal vertex protein is believed to be required for functional capsid assembly of P2 and P4. The presence of a portal vertex protein appears, however, not to be obligatory for initiating capsid formation, since P2-size procapsid-like structures can be formed in cells expressing cloned *N* and *O* genes alone (167). This seems also to be the case for phage P22, in which capsid shell initiation can take place in the absence of the portal vertex protein (9).

Mutations That Prevent Small-Capsid Formation

Six et al. (221) isolated P2 mutants which are nonresponsive to the action of P4 Sid (the mutations are called *sir* [sid responsive]). It has been shown that all the mapped mutations are located within a 38-codon segment in the middle of the *N* gene of P2. The existence of the *sir* mutations indicates that gpN is a target of the Sid protein. Relatively conservative changes at the amino acid level (e.g., tyrosine to phenylalanine at residue 207 of a total of 357 residues) render the *N* gene product nonresponsive to Sid. Genetic analysis of P4 mutants able to grow under nonpermissive conditions in the presence of a P2 *ts*V mutant suggest that Sid may also interact with gpV (220), which may be bound to the portal vertex protein.

By expanding the P4 chromosome to a P2 size and propagating it on a P2 Sir$^-$ lysogen, phage stocks can be made (221) from which P4 Sid mutants can be isolated by plating the stock on P2 Sir$^+$ lysogens. DNA sequence analyses of a large number of Sid mutants have revealed missense as well as deletion mutations, which cluster in two regions of the *sid* gene. One set of the mutations cluster in the N-terminal part of the 244-amino-acid gpSid, whereas the other mutations are confined to the C-terminal part. This distribution may reflect two functional domains of the Sid protein (174).

Stabilization of Small Capsids by Psu as a Decoration Protein

The product of the polarity suppressor gene (*psu*), which functions as an antiterminator of transcription (41, 134, 153, 207), also associates with the P4 virions (8). Recently it has been shown that Psu is specifically associated with the P4 capsid (114). This association can occur when Psu is (i) provided in vivo from the P4 genome or from a plasmid or (ii) provided in vitro by mixing viable Psu$^-$ P4 particles with Psu protein. P2 capsids are unable to interact with Psu. In this study it was found that Psu$^-$ P4 particles are less heat stable than P4 wild-type particles and that Psu$^-$ mutants treated in vitro with Psu regain their stability. The presence of P4 DNA in the capsid appears not to be required for Psu association, and, when associated, Psu is found on the surface of the capsid (113). Hence Psu appears to function as a decoration protein, similar to the T4 phage Hoc and Soc proteins (115, 116) and to some extent to the phage λ D protein (226). Image reconstructions from cryoelectron micrographs of Psu$^-$ and Psu$^+$ particles show the Psu protein sitting on the top of the hexamers in a fixed orientation (54) (Fig. 8). The h1/h2-specific N-terminal amino acids, which are exposed on the surface of the P4 capsid, appear not to be involved in Psu binding, since antibodies directed against the h1/h2-specific amino acid sequences fail to block Psu association (113).

The presence of Psu on the hexamers may help to prevent P4 DNA from escaping through the capsid shell during heat inactivation (54, 113). Thus Psu appears to function as a capsid stabilizer, in addition to being an antiterminator of transcription in *E. coli* (151). The interconnection, if any, of these two very different properties remains to be worked out. A similar but not identical case of bifunctionality exists

in phage T4, whose RNA ligase also can function as a morphopoietic factor by attaching the tail fibers to the T4 base plate (198, 223).

DNA Packaging

Contrary to phage lambda, which packages its DNA from linear multimers (concatemers), P2 uses monomeric circular DNA as a substrate for packaging (154, 189–191, 194). Purified P2 proheads, gpM and gpP, mixed with covalently closed P2 DNA in the presence of ATP result in cutting out of the cohesive ends (20, 21). gpP is associated with a DNA-dependent ATPase activity (21), and gpP and gpM may correspond to the gpA and nu1 terminase components of lambda, respectively. In a crude in vitro system, linear P2 DNA can be packaged in the absence of gpM (21, 189) but closed-circular monomers are the preferred substrate in vivo, and thus only one *cos* site is needed (191). P4 uses the packaging machinery of P2 to package its own genome. One of the cohesive ends of the packaged chromosomes of P2 and P4 appears to interact with the tail knob of the phage (30).

CONCLUDING REMARKS

Mechanisms based on control of transcription termination are commonly used by bacteriophages to modulate expression of lytic genes, and the study of such phage systems has been of great value in analyzing the bacterial transcription elongation-termination process (for a review, see reference 65). P4, however, is unique among known bacteriophages for having adopted transcription termination as the mechanism for direct maintenance of prophage immunity. Interestingly, the P4-encoded immunity factor is a small RNA, which controls transcription termination by interacting with complementary regions of the nascent mRNA. Involvement of complementary RNA in controlling gene expression has been widely documented in both prokaryotes and eukaryotes and has been also used to repress gene expression (58, 211, 236). In a few cases the cRNA has been recognized as playing a role in causing transcription termination, as in the control of the *E. coli crp* operon (176), the *ant* operon of coliphage P1 (13, 37), and the *repC* control in *Staphylococcus* plasmid pT181 (175). P4 immunity provides a new example of such regulatory mechanisms and may be used as a probe to further elucidate both bacterial transcription termination and cRNA interactions in the regulation of gene expression.

The P2 Ogr gene product shares the ability to bind zinc with several other transcription factors such as TFIIIA, which coordinates the zinc binding with two cysteines and two histidines as ligands; the glucocorticoid and estrogen receptors, which use four cysteine ligands; and the yeast GAL4 protein, which uses six cysteines to bind two zinc atoms (40). These proteins contain a loop of 9 to 13 amino acids, which separate the zinc-binding domains, whereas Ogr contains a loop of 22 amino acids (71). *E. coli* aspartate transcarbamoylase regulatory subunit (208), *E. coli* UvrA nuclease (171), and E6 transforming protein of papillomavirus (124), however, bind zinc with a similar spacing to that found in Ogr (70). In aspartate carbamoyltransferase the zinc-binding domain mediates interaction between the regulatory and catalytic subunits of the enzyme (170). The Zn finger containing protein Mig1, which is responsible for glucose repression of the *gal* genes in yeast cells (172), contains an Ogr loop of 24 residues, with 23% of the amino acids identical to those found in Ogr (216).

In Ogr the zinc binding could be necessary for the presumptive sequence specificity for the dyad symmetries found upstream of the late P2 and P4 promoters. It could also be involved in the interaction with the α subunit of the RNA polymerase or necessary for forming a zinc-bridged dimer, as in the case of the human immunodeficiency virus Tat protein (64). Zinc binding has not been tested in the case of P4 transactivation δ protein, but the structural prerequisite is present in the two Ogr-like parts of the protein. δ could thus form a hairpin dimer by the use of both parts. Both Ogr and δ proteins have low solubilities (140), and future progress in determining the structure and function of these proteins requires improved biochemical access.

The Ogr and δ proteins appear to belong to a family of gene activators in enteric bacteria which interact with the α subunit of the RNA polymerase to initiate transcription of positively regulated genes (72). These include positive regulators required for normal expression of *cysA*, *melAB*, and *araBAD* (82, 197), as well as the EnvZ-OmpR regulator complex for outer membrane proteins in *E. coli* (69, 168) and the OxrA protein involved in the expression of the anaerobically regulated *oxd* genes in *Salmonella typhimurium* (164). Interaction between the gene activators and the α subunit of the RNA polymerase is supported by biochemical data which show that the catabolite repression protein of *E. coli* must interact with the C-terminal part of the α subunit of the RNA polymerase to activate the *lac* P1 promoter (109, 251).

P1 phage is known to assemble into more than one capsid size (238). Certain lambda gene *E* mutants form small $T = 4$ capsids rather than the normal $T = 7$ (125). The capsid size determination in the P2-P4 system, however, is different, since P4 controls the capsid size by its *sid* gene product. It is not understood how P4 Sid gives rise to a different gpN hexamer arrangement in P4 capsids from that in P2 capsids before disappearing. Biochemical studies in combination with genetic analysis of the Sid interactions with gpN and possibly gpO as well as gpV should increase our understanding of that process. To fully understand the problem of capsid size determination by P4, the structures of the P2 and P4 capsids must be determined at the level of atomic resolution and the pathways of folding of gpN must be worked out.

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