Derepression of Prophage P2 by Satellite Phage P4: Cloning of the P4 ϵ Gene and Identification of Its Product

TAO LIU, SARA K. RENBERG, AND ELISABETH HAGGÅRD-LJUNGQUIST*

Department of Genetics, Stockholm University, S-106 91 Stockholm, Sweden

Received 16 December 1996/Accepted 17 March 1997

Escherichia coli phage P4 lacks all of the genetic information necessary for capsid, tail, and lysis functions. P4 is therefore dependent on a helper phage, such as P2, for lytic propagation. During P4 superinfection of a P2 lysogen, the P2 prophage is derepressed by the action of the P4-encoded ϵ gene. We have cloned the ϵ gene and identified the 10-kDa E protein. The ϵ gene product is the only P4 protein required to derepress prophage P2, which leads to in situ P2 DNA replication. A two-plasmid derepression assay system has been developed to examine the derepression activity of E. The reporter plasmid contains the two face-to-face promoters, Pe and Pc, involved in the lysis-lysogeny transcriptional switch of phage P2 and the immunity repressor C. The Pe promoter is coupled to a *cat* reporter gene. In the construct, the C repressor is transcribed from the Pc promoter and represses the Pe promoter, which mimics the in situ-repressed P2 prophage. The E protein is supplied in *trans* from a compatible plasmid in which the ϵ gene is under the control of the T7 promoter. We show here that in the two-plasmid assay system, induction of the E protein derepresses the Pe promoter. The *ash9* mutation, which is located upstream of the ϵ gene, enhances the E-mediated derepression of the Pe promoter. The purified E protein shows no specific DNA binding activity, and the implications of this are discussed.

Satellite phage P4 is a defective phage that relies on a helper, like phage P2 or a P2-related phage, for lytic growth. For reviews, see references 6 and 24. In the absence of a helper, P4 can either form lysogeny (9, 37) or maintain itself in the form of a multicopy plasmid (12, 19). For lytic growth, however, it depends on all late genes of the helper to make the structural components, to package its DNA, and to lyse the host cell (37, 38). P4 even has the capacity to redirect the head assembly into a smaller head that fits only the small 11.6-kb P4 genome, thereby excluding the 33.5-kb P2 helper genome from being packaged into viable particles (1, 15, 27, 36, 39). P4 can utilize its P2 helper both in a mixed infection and in a superinfection (37). When P4 infects a P2 lysogenic host cell, the P2 prophage is derepressed and starts unidirectional DNA replication in situ. The derepression is dependent on the activity of the P4 ϵ gene (16). Derepression in the P2-P4 system is a mutual phenomenon, and P2 infection of a P4 lysogen induces formation of P4 phages. This requires the activity of the P2 *cox* gene (40). The P2 Cox protein has been shown to act as a transcriptional activator of the P4 late promoter PII (33). The P2 Cox protein and the P4 E protein show no amino acid sequence similarity and thus may use different mechanisms for derepression of each other. To study the mechanisms behind the E-promoted derepression of P2, we have cloned the ϵ gene and identified the E protein.

MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. The strains and plasmids used in this study are listed in Table 1.

Media. The media and methods used for propagation of bacteria and phages are described in reference 3. Antibiotics were added as described in reference 33.

Plasmid constructions. All in vitro constructions were performed in accordance with standard procedures (27). *Escherichia coli* C-1a was used as the recipient for transformation. All constructs made from PCR-amplified materials were checked by DNA sequencing (34).

(i) **Construction of pEE801, in which the *ash* ϵ region is under the control of the T7 ϕ 10 promoter.** The *ash* ϵ region of phage P4 was amplified by PCR by using oligonucleotides P4 ϵ -1 and P4 ϵ -2 as primers (Table 2) and P4 wild-type phage DNA as the template. The PCR fragment generated was inserted into the filled-in *Nco*I site of pET-8c.

(ii) **Construction of pEE802, in which the ϵ gene is under the control of the T7 ϕ 10 promoter.** The ϵ gene was amplified by PCR by using oligonucleotides P4 ϵ -4 and P4 ϵ -2 as primers and P4 wild-type phage DNA as the template. The PCR fragment generated was inserted into the filled-in *Nco*I site of pET-8c. The valine start codon of the ϵ gene was then fused in frame with the methionine start codon of the vector.

(iii) **Construction of pEE803, in which the *ash9* *eam104* region is under the control of the T7 ϕ 10 promoter.** pEE803 was constructed as pEE801 with P4 *ash9* *eam104* phage DNA as the template.

(iv) **Construction of pEE675, the reporter plasmid in the E derepression assay system.** The P2 region, encompassing repressor gene *c* and promoters Pe and Pc, was amplified by PCR by using oligonucleotides P2 76.3R and P2 77.4L as primers and P2 Ig DNA as the template. The PCR fragment generated was inserted into the *Sma*I site of pKK232-8, and clones in which the promoterless *cat* gene would be expressed from the Pe promoter were selected after sequencing.

(v) **Construction of pEE804 by subcloning of the *ash* ϵ region, controlled by the T7 ϕ 10 promoter from pEE801, into pACYC177.** The filled-in *EcoRV*-*Bgl*II fragment from pEE801 with the *ash* ϵ region controlled by the T7 promoter was inserted into the *Hinc*II site of pACYC177. Clones with the *EcoRV*-*Bgl*II fragment in the direction opposite to that of the ampicillin promoter were selected after sequencing.

(vi) **Construction of pEE805 by subcloning of the ϵ gene, controlled by the T7 ϕ 10 promoter from pEE802, into pACYC177.** The *EcoRV*-*Bgl*II fragment containing the ϵ gene under the control of the T7 promoter was subcloned into pACYC177 as described above.

(vii) **Construction of pEE806 by subcloning of the *ash9* *eam104* region, controlled by the T7 ϕ 10 promoter from pEE803, into pACYC177.** The *EcoRV*-*Bgl*II fragment containing the *ash* ϵ region under the control of the T7 promoter was subcloned into pACYC177 as described above.

(viii) **Construction of pEE807 by replacement of *ash9* with wild-type *ash* in pEE806.** *Bgl*II digestion of pEE806 results in one large fragment with *eam104* and one small fragment with *ash9*. The large fragment with *eam104* was ligated with the small *Bgl*II fragment from pEE804 with *ash*⁺, producing pEE807 (*ash*⁺ *eam104*).

(ix) **Construction of pEE808 by replacement of *eam104* with wild-type ϵ in pEE806.** The small fragment from *Bgl*II digestion of pEE806 with *ash9* was ligated to the large *Bgl*II fragment from pEE804 with the ϵ gene, producing pEE808 (*ash9* ϵ ⁺).

Expression and partial purification of E protein. BL21(DE3) cells containing pEE801 or pEE802 were grown to an optical density of about 2×10^8 /ml, and IPTG (isopropyl- β -D-thiogalactopyranoside) was added to a final concentration

* Corresponding author. Phone: 46 8 161270. Fax: 46 8 164315. E-mail: Elisabeth.Haggard@genetics.su.se.

TABLE 1. *E. coli* strains, bacteriophages, and plasmids used in this study

Strain, phage, or plasmid	Pertinent feature(s)	Reference(s)
<i>E. coli</i> strains		
BL21(DE3)	<i>ompT hsdR hsdM lon E. coli</i> B strain, T7 RNA polymerase under control of <i>lac</i> promoter	41
BL21(DE3)pLysS	Strain BL21(DE3) containing pACYC184 derivative expressing low level of T7 lysozyme	41
BL21(DE3)(P2 _{lg})pLysS	BL21(DE3)pLysS lysogenized by P2 <i>lg</i>	This report
C-1a	F ⁻ prototrophic <i>E. coli</i> C strain	35
Bacteriophages		
P2 <i>lg</i>	Used as wild-type P2 but forms large plaques	4
P4	Wild type	37
P4 <i>ash9</i> <i>eam104</i>	P2 prophage derepression-deficient P4 phage, amber mutation in ϵ gene and <i>ash9</i> upstream mutation	22, 24
Plasmids		
pET-8c	pBR322 derivative containing T7 promoter	42
pACYC177	P15 derivative	10
pKK232-8	pBR322 derivative containing promoterless <i>cat</i> gene	8
pSS27-5	Amp ^S derivative of pACYC177	32
pSS32-1	pKK232-8 derivative in which <i>cat</i> gene is under control of P2 Pe promoter	31
pEE675	pKK232-8 derivative with P2 nt 25,557–26,006 containing CPePc region; Pe promoter controls <i>cat</i> gene	This report
pEE801	pET-8c derivative with P4 nt 7,871–8,581 containing <i>ash</i> ϵ region under control of T7 promoter	This report
pEE802	pET-8c derivative with P4 nt 7,871–8,181 containing ϵ gene under control of T7 promoter	This report
pEE803	pET-8c derivative with P4 nt 7,871–8,581 containing <i>ash9</i> <i>eam104</i> region under control of T7 promoter	This report
pEE804	pACYC177 derivative with <i>EcoRV</i> - <i>Bgl</i> II fragment from pEE801 containing <i>ash</i> ϵ region under control of T7 promoter	This report
pEE805	pACYC177 derivative with <i>EcoRV</i> - <i>Bgl</i> II fragment from pEE802 containing ϵ gene under control of T7 promoter	This report
pEE806	pACYC177 derivative with <i>EcoRV</i> - <i>Bgl</i> II fragment from pEE803 containing <i>ash9</i> <i>eam104</i> region under control of T7 promoter	This report
pEE807	pEE806 derivative with <i>ash</i> ⁺ <i>eam104</i>	This report
pEE808	pEE806 derivative with <i>ash9</i> ϵ ⁺	This report

of 0.5 mM. Cell growth was continued for 3 to 4 h. Crude extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were stained with Coomassie blue (33). The E protein was purified from strain BL21(DE3) containing pEE802 (Fig. 1). Cells were induced with IPTG as described above. The pellet was resuspended in buffer A (0.3 M potassium phosphate [pH 7.5], 3 mM EDTA) supplemented with 0.5 M KCl and stored at –70°C until use. The frozen bacteria were thawed at room temperature and lysed by sonication. The E protein forms inclusion bodies, the pellet was therefore collected and washed in buffer A plus 0.5 M KCl with 1% Triton X-100 at 30°C for 30 min. The pellet was collected and redissolved in buffer B (10 mM Tris-HCl, pH 8.0) with 6 M urea and stirred for 1 h at room temperature. The remaining pellet was collected and redissolved in buffer B with 8 M urea and stirred for 2 to 3 h at room temperature. After centrifugation, the supernatant was refolded by four dialysis steps as described by Novagen (Madison, Wis.). The E protein thus obtained is about 90 to 95% pure (Fig. 1).

Polyclonal antiserum and Western blotting. The partially purified E protein was subjected to SDS-PAGE. After electrophoresis, the gel was briefly stained with Coomassie blue (0.05% in water), and the protein band corresponding to the E protein was extracted from the gel. The polyclonal rabbit antiserum used was made by MabTech (Stockholm, Sweden).

For Western blotting, the crude cell extracts were subjected to SDS-PAGE and electroblotted onto a Hybond C filter (Amersham, Amersham, England) under standard conditions (33). The staining was carried out with diaminobenzidine and H₂O₂, which are substrates for horseradish peroxidase conjugated with the second antibody, anti-rabbit immunoglobulin G.

Determination of P2 prophage derepression and replication upon induction of E. Strain BL21(DE3)(P2)pLysS, carrying plasmid pET8c or pEE801, was grown at 37°C to the mid-log phase. The cultures were then divided into two parts, and IPTG was added to one of them to a final concentration of 0.5 mM. Growth was continued, optical density at 600 nm was monitored, and samples were collected immediately before and after induction. Total DNA was extracted and digested with restriction endonucleases *Pst*I and *Cla*I. The fragments were resolved by electrophoresis through a 1.0% agarose gel in the presence of ethidium bromide and photographed under UV light. The fragments were then denatured and transferred to a nitrocellulose membrane by standard procedures (33). The membrane was hybridized with ³²P-labelled, denatured P2 DNA and autoradiographed. Labelled P2 DNA was prepared by using the Rediprime DNA labelling system (Amersham) and then purified on a Microspin S-300 column (Pharmacia, Uppsala, Sweden).

Determination of chloramphenicol acetyltransferase (CAT) activity. BL21 (DE3) cells with pEE675 and pEE804, pEE805, or pEE806 were grown to a density of about 2 × 10⁸/ml, and IPTG was added to a final concentration of 0.5 mM. Growth was continued, and samples were collected after 30, 60, 90, 120, and

180 min. The extracts for CAT assays were prepared as described in reference 30. Total protein concentrations were determined by the method of Bradford (7). The extracts were diluted to have the same total protein amount, 0.1 µg, in the CAT reactions, which were performed as described in reference 20, with ¹⁴C-labelled D-threo-dichloroacetyl-1-1-chloramphenicol (Amersham). CAT activities, determined by PhosphorImager analysis, were calculated as the ratio of acetylated chloramphenicol to total chloramphenicol. Total radioactivity varied slightly between samples due to loading differences. This does not interfere with the calculated CAT activity since that depends on the relative amounts of acetylated and nonacetylated chloramphenicol in the individual samples. The activities were normalized by giving the fully derepressed Pe promoter a value of 1, plasmid pSS32-1. The CAT activity directed from the Pe promoter has been shown to be reproducible in several experiments. All of the CAT experiments were repeated with consistent results.

RESULTS

Strategy for gene ϵ cloning. The complete P4 genome, 11,627 nucleotides (nt) has already been sequenced (21) (Gen-

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Position of most 5' base relative to left end of P2 or P4 genome	Sequence
P4 ϵ -1 L	8,581	GTG TGG TGT GTG GTC AGT CG
P4 ϵ -2 R	7,870	ATC TCA CCG GGC TTG GGA AA
P4 ϵ -4 L	8,178	GTG CGT AAT AAA AAA GCC CCT CAG
P4 ϵ -5 R	8,417	ACG CTC GAT GGC GAT AGC CC
P4 ϵ -6 L	8,316	CTG TCC GTC GCG CAG ACC GT
P2 76.3R	25,557	GTA GGC ACT TGT GAT GAA TA
P2 76.3L	25,593	AAC AAT TCA TCG TGA ATA TAT TC
P2 77.3R	25,804	GAA CCC CTG TTA AAT CAG CAA
P2 77.4L	25,606	GTC GTC CTC TAG CCC TCA TG
T7 forward		TAA TAC GAC TCA CTA TAG GG
T7 reverse		GCT AGT TAT TGC TCA GCG G

FIG. 1. Partial purification of P4 E protein. For details of the purification, see Materials and Methods. Lanes: 1, crude extract of an IPTG-induced culture of BL21(DE3) containing plasmid pEE802; 2, same as lane 1 but not induced; 3, supernatant after sonication; 4, pellet after sonication; 5, supernatant after washing in 1% Triton X-100; 6, supernatant after washing in 6 M urea; 7, sample after solubilization in 8 M urea. The molecular mass markers (lane mw) were bovine serum albumin (68 kDa), albumin (45 kDa), chymotrypsinogen A (25 kDa), cytochrome *c* (12.5 kDa), and aprotinin (6.5 kDa).

Bank accession number X51522), and the ϵ gene has been located to an open reading frame between positions 7891 and 8178 (22). This part of the P4 genome can be transcribed from two promoters, the constitutive early Ple promoter and the late PII promoter located 400 nt upstream of Ple (see Fig. 2). The PII promoter can be activated by the P4 δ (13), P2 Ogr (11), or P2 Cox (32) protein. The transcript initiated at PII contains, besides the genes it has in common with the Ple transcript, i.e., ϵ , orf151 (unknown function), *cnr* (control of P4 copy number) (43), and α (primase and helicase) (44), the gene *vis* (late transcription control) (28), and orf199. The first gene of the Ple transcript is *kil*, which causes cell death when overexpressed (2) and overlaps orf199 in frame. The start codon of *kil* is believed to be located about 330 nt downstream of Ple, but its product has not been identified (45). The untranslated leader region of the Ple transcript contains the P4 immunity functions. The P4 immunity factor is a small RNA that acts by premature termination of the Ple transcript (14, 17, 29). The immunity region also contains the *ash* mutations, which were isolated as P4 mutants able to grow on P3 lysogens (*ash* stands for adaptation to a secondary helper) (22).

The presumed ϵ gene starts with a valine codon at position 8178, but the ϵ reading frame is open for about 320 nt upstream of the valine start codon and thus includes the *kil* gene, but in a different reading frame, and the *ash* mutations. We made two ϵ gene constructs in expression vector pET8c, where they are transcribed from the T7 promoter (Fig. 2). One construct, pEE802, contains only the presumed ϵ gene, and the valine start codon is fused in frame to the methionine start codon of the vector. In the other construct, pEE801, the first methionine codon of orf199 contained on the Ple transcript is fused with the methionine codon of pET8c.

The constructs were transformed into strain BL21(DE3), which contains the T7 polymerase under the control of the *lac* promoter, and the proteins made after IPTG induction of the T7 polymerase were analyzed by SDS-PAGE. As can be seen in Fig. 3A, lanes 1 and 2, induction of plasmid pEE802 leads to overexpression of a protein with the expected size (10.8 kDa) of the E protein. Plasmid pEE801, however, overexpresses only a protein of about 26 kDa (Fig. 3A, lanes 3 and 4), which does not fit the size expected from the cloned orf199 region (14.8 kDa). A possible explanation for the 26-kDa protein is that there is a mistake in the published sequence and that the ϵ

FIG. 2. Genetic map of the P4 PII operon showing the ϵ constructs made in this work. The PII and Ple operons of phage P4 are shown at the top, and the pertinent region is highlighted below. The arrows indicate the transcripts initiated at Ple and PII, respectively. The three ϵ gene constructs made in the pET-8c expression vector, where the T7 promoter directs the respective gene region, is shown below the operon. pEE801 contains the ϵ gene with the wild-type valine start codon and the leader region. pEE802 contains the ϵ gene with a methionine start codon. pEE803 is the same as pEE801 but includes the *ash9* and *eam104* mutations. The locations of the mutations used in this work are indicated.

gene extends into orf199, generating the 26-kDa protein seen upon induction. To test this possibility, polyclonal antibodies were raised against the partially purified 10.8-kDa protein obtained after induction of pEE802, and the proteins made after induction of pEE801 were analyzed for reactivity against the antibodies. As can be seen in Fig. 3B, lanes 3 and 4, the 26-kDa protein does not react against the antibodies and thus cannot be a fusion product of orf199 and gene ϵ . The 26-kDa protein was shown by amino acid sequencing to be the product of the β -lactamase gene (data not shown). The induced pEE801 plasmid produces the same 10.8-kDa protein (Fig. 3B, lane 3) as plasmid pEE802, although the induced level is about the same as that obtained before induction of plasmid pEE802. That the level of expression differs can be explained by the facts that the initiation codon in plasmid pEE802 is ATG and in pEE801 it

FIG. 3. Identification of the cloned genes. Lanes 1 to 6 show total cell extracts of BL21(DE3) containing the following plasmids: lanes 1 and 2, pEE802; lanes 3 and 4, pEE801; lanes 5 and 6, pET8c. Lanes M, molecular mass markers (see the legend to Fig. 1). The presence or absence of IPTG is shown below each lane. (A) Coomassie blue-stained SDS-polyacrylamide gel showing the induction of the cloned genes. (B) Western blot with anti-E serum.

FIG. 4. Growth of strain BL21(DE3)(P2)pLysS in the absence of plasmids or in the presence of pET8c (control) or pEE801 (containing the inducible ϵ gene) after induction with IPTG (arrow). The control culture of bacteria lacking plasmids was not affected by IPTG; therefore, the curves obtained with and without IPTG are identical. OD600 nm, optical density at 600 nm.

is GTG and that the ribosome binding sites and the leader regions differ.

E is the only P4 protein required to induce prophage P2. P2 has been classified as a noninducible phage, since inactivation of the P2 repressor is abortive, i.e., the cell dies without phage production (5, 40). Induction of P2 leads to in situ DNA replication, since prophage P2 is unable to excise from the chromosome due to inefficient expression of the P2 integrase from the prophage structure (16, 26). Transformation of plasmid pEE802 into a P2 lysogenic derivative of BL21(DE3) is lethal to the host even if it contains plasmid pLysS (41), which produces T7 lysozyme, a natural inhibitor of T7 RNA polymerase. However, plasmid pEE801, which expresses a low level of E compared to pEE802, can be maintained in the P2 lysogenic derivative of BL21(DE3)pLysS. As can be seen in Fig. 4, induction of ϵ leads to lysis of the cells within 60 min. It should be noted that induction of control plasmid pET8c, which lacks the ϵ gene, reduces the growth rate of the host cells, but in this case the cells do not lyse. To monitor the fate of the P2 prophage DNA upon induction of gene ϵ , samples were collected at different times after induction. After digestion of total DNA with *Cla*I and *Pst*I, the P2-containing fragments were analyzed by the Southern blot technique by using labelled P2 DNA as the probe. As can be seen in Fig. 5A, cleavage of mature P2 DNA generates eight fragments, of which C and F are the cohesive end fragments that are mostly joined under the conditions used. Fragment B contains the P2 *attP* site; thus, in the prophage DNA this fragment is split into two new fragments, X and Y, containing the *attL* and *attR* junctions, respectively. The origin of replication is located within fragment G, and since P2 replication is unidirectional only about 80% of the prophage is expected to be amplified after in situ replication. Thus, the relative amounts of fragments X, H, E, and G are expected to be reduced compared to those of the other fragments, and a new fragment of 810 nt, representing the P2 genome from the origin of replication to the right terminus of fragment G, should appear. However, P2 replication is initiated by a single-stranded cut at the origin and proceeds by the rolling-circle mode. The cleavage reaction is mediated by the P2 A protein, which becomes covalently linked to the 5' end of the cleaved DNA (23, 25). Therefore, the new 810-nt fragment will be covalently linked to the A protein, which will reduce its migration in an agarose gel. As

can be seen in Fig. 5B, induction of control plasmid pET8c does not change the pattern of prophage fragments; they are all present in about equimolar amounts compared to the uninduced culture. Induction of gene ϵ contained on plasmid pEE801, however, leads to amplification of fragments A, C plus F, and D, relative to fragments E, G, and H, 60 min after induction. Furthermore, a new fragment is obtained that migrates faster than fragment E but slower than expected for an 810-nt-long DNA fragment (ori in Fig. 5B), which probably represents the P2 DNA from the origin of replication to the right *Cla*I site covalently linked to the A protein. Upon longer exposure, a weak band at about 800 nt can also be detected. Therefore, induction of the cloned P4 ϵ gene leads to induction and in situ replication of prophage P2.

Biological assay system for ϵ . Since induction of the cloned ϵ gene leads to cell death, an assay system was developed in which the transcriptional switch that controls the lytic versus

A

B

FIG. 5. Depression of prophage P2 replication after induction of P4 gene ϵ . (A) Schematic drawings of the P2 DNA fragments generated after cleavage of P2 phage or prophage DNA with *Cla*I and *Pst*I. The phage cohesive end fragments can appear in joined and free forms after cleavage. The *E. coli* DNA is indicated by boxes at the ends of the P2 prophage, and a restriction cleavage site is indicated within the boxes, but the identity and location of the enzyme closest to the hybrid *att* sites are not known. The fragment lengths are as follows: A, 12,020 nt; B, 4,730 nt; C, 4,590 nt; D, 4,390 nt; E, 3,310 nt; F, 2,890 nt; G, 1,290 nt; H, 370 nt. The lengths of X and Y are unknown. (B) Southern blot of total DNA collected from strain BL21(DE3)(P2)pLysS containing plasmid pET8c (control) or pEE801 (containing the inducible ϵ gene) at the times after induction with IPTG indicated above the lanes. The DNA was cleaved with *Pst*I and *Cla*I, and after separation and blotting it was hybridized against 32 P-labelled P2 DNA. The new fragment, ori, probably constituted the DNA fragment generated by replication from the origin to the first *Cla*I site on the right that is covalently joined to the A protein. Cleaved P2 phage DNA is included as a marker on the right side.

FIG. 6. Schematic drawing of the lytic-lysogeny transcriptional switch of phage P2. The genes required to establish lysogeny, *int* and *c*, are controlled by the *Pc* promoter that faces the *Pe* promoter. *Pe* controls the genes involved in lytic propagation. The two divergent transcripts overlap by about 30 nt. The *C* immunity repressor blocks *Pe* (thick arrow) and regulates its own promoter (thin arrow). *Cox* is a repressor of *Pc* (thick arrow), and at high concentrations it represses its own promoter, *Pe* (thin arrow).

lysogenic transcriptional mode could be quantitated. The transcriptional switch of P2 consists of two face-to-face promoters (the early *Pe* promoter and the *Pc* promoter which controls immunity repressor gene *c* and integrase gene *int*) and two repressor proteins, *C* and *Cox* (Fig. 6). The promoters are located so that the transcripts overlap by about 30 nt. The first gene of the *Pe* transcript is *cox*, which codes for a repressor of *Pc* and which also at high concentration down-regulates its own promoter, *Pe* (31). The first gene of *Pc* codes for P2 immunity repressor *C*, which blocks *Pe* and regulates its own promoter positively as well as negatively (30). Since the target for the *E* protein is not known, the whole *C-Pe-Pc* region was cloned in front of a promoterless *cat* gene so that the *cat* gene was under the control of *Pe* (pEE675). Under these conditions, the *cat* gene will be turned off since it mimics the lysogenic condition under which the *C* protein has turned the *Pe* promoter off. To provide the *E* protein in *trans*, the cloned ϵ gene from plasmid pEE802, including the T7 promoter, was moved to a compatible plasmid, pACYC177, generating plasmid pEE805. Transformation of strain BL21(DE3), which contains the T7 polymerase under control of the *lac* promoter, with plasmids pEE675 and pEE805 gave full induction of the *cat* reporter gene even before induction of the T7 polymerase (data not shown). This implies that a very low level of *E* is enough to turn the transcriptional switch from the lysogenic to the lytic mode. Since the level of *E* produced is much lower in plasmid pEE801, it is not detectable by *E*-specific antibodies before induction (Fig. 3), the ϵ gene, including its upstream region and the T7 promoter, was moved from plasmid pEE801 to pACYC177, generating plasmid pEE804. As can be seen in Fig. 7, lanes 1 and 2, the level of *cat* expression in strain BL21(DE3) transformed with plasmids pEE675 and pEE804 is as low before induction as that obtained with a fully repressed *Pe* promoter. Induction of the T7 polymerase leads to an increase in *cat* activity, and 180 min after induction it has reached 40% of that obtained with the fully expressed *Pe* promoter.

Prevention of the derepression activity of *E* by the ϵ am104 mutation. The ϵ gene is defined by an amber mutation, ϵ am104 (16, 22). Since turning of the transcriptional switch could be mimicked after induction of plasmid pEE804 and as the cloned fragment has the capacity to code for at least one more protein, the wild-type ϵ gene region was exchanged with ϵ am104 as described in Materials and Methods, generating plasmid pEE807. As can be seen in Fig. 8, induction of ϵ am104 does not lead to activation of the *cat* reporter gene; thus, the activity detected is a consequence of the active ϵ gene product.

Enhancement of expression of gene ϵ by the *ash9* mutation. The ϵ gene product is not detectable after wild-type P4 infec-

FIG. 7. Kinetics of CAT activity in extracts from the two-plasmid derepression assay. CAT activities were determined as described in Materials and Methods. The locations of chloramphenicol, 1-acetate chloramphenicol and 3-acetate chloramphenicol are shown. Lane 1 (leftmost lane) is a fully repressed *Pe* promoter. The bacteria contain reporter plasmid pEE675, including the *C-Pe-Pc* region, with the *Pe* promoter directing the *cat* gene, and pSS27-5, an ampicillin-sensitive derivative of pACYC177. Lanes 2 to 7 are samples taken from bacteria containing reporter plasmid pEE675 and plasmid pEE804, which encodes the inducible *P4* ϵ gene and the upstream region. Lane 2 is a sample taken before and lanes 3 to 7 are samples taken at different times after IPTG induction of *E*. Lane 8 is the fully expressed *Pe* promoter. The bacteria contain only pSS32-1, in which the *cat* gene is under *Pe* control.

tion. However, 20 min after P4 *ash9* infection, as opposed to P4 *ash9* ϵ am104 infection, a 10-kDa protein has been detected, which implies that the *ash9* mutation affects the expression of ϵ (22). During P4 lytic growth, transcription from the constitutive promoter *Ple* occurs only for a short time, since this transcript contains the *trans*-acting immunity factor that controls premature transcriptional termination of the *Ple* transcript (18). The upstream *Pll* promoter is activated instead, and as the *Pll* transcript is insensitive to the termination mechanisms, it proceeds through the whole α operon, allowing P4 replication (13, 14). The *Pll* transcript, as opposed to the *Ple* transcript, does not have an untranslated leader region, which may account for its insensitivity to the immunity factor (18). The P4 fragment cloned into plasmid pEE804 mimics the transcript from *Pll*, since the ϵ leader sequence is translated. Since the *ash* mutants, as opposed to wild-type P4, are able to grow

FIG. 8. Effects of the ϵ am104 and *ash9* mutations on CAT activity in the two-plasmid derepression assay. Lane 1 (leftmost lane) is a fully repressed *Pe* promoter (see lane 1 in Fig. 7). Lanes 2 and 3 are samples taken before and 180 min after IPTG induction of cells harboring reporter plasmid pEE675 and plasmid pEE804, which contains the inducible wild-type ϵ gene and the wild-type upstream region. Lanes 4 and 5 are samples taken before and after IPTG induction of cells harboring pEE675 and pEE807, containing the inducible ϵ am104 gene and the wild-type upstream region. Lanes 6 and 7 are samples taken before and 180 min after IPTG induction of cells harboring pEE675 and pEE808, containing the inducible wild-type ϵ gene and the upstream region with the *ash9* mutation. Lane 8 is a fully expressed *Pe* promoter (see lane 8 in Fig. 7).

FIG. 9. Kinetics of CAT activity in extracts from the two-plasmid derepression assay when the *ash9* mutation is present. Lane 1 (leftmost lane) is a fully repressed *Pe* promoter (see lane 1 in Fig. 7). Lanes 2 to 7 are samples taken from bacteria containing the reporter plasmid pEE675 and plasmid pEE808, containing the inducible wild-type ϵ gene and the upstream region with the *ash9* mutation. Lane 2 is a sample taken before and lanes 3 to 7 are samples taken at different times after IPTG induction of E. Lane 8 is a fully expressed *Pe* promoter (see lane 8 in Fig. 7).

on a P3 lysogen, their derepression activity should be altered. To test this, the wild-type ϵ leader region in plasmid pEE804 was exchanged with that of *ash9*, generating plasmid pEE808, and the kinetics of the derepression was measured after induction of the T7 polymerase in strain BL21(DE3) containing *cat* reporter plasmid pEE675 and plasmid pEE808 or pEE804. As can be seen in Fig. 9, derepression of the P2 early promoter by E is more efficient in the presence of the *ash9* mutation. To test if this correlates with increased production of E, the level of E produced after induction of cells containing plasmid pEE805 (wild-type ϵ leader) was compared with the level obtained in cells containing plasmid pEE808 (*ash9* leader region) in crude extracts by using E-specific antibodies. However, the level of E produced in both extracts was too low for detection with E-specific antibodies.

Lack of specific DNA binding activity of partially purified E protein. Bacteriophages P2 and P4 show mutual derepression. Derepression of prophage P4 by P2 requires the P2 Cox protein, which has been shown to act as a transcriptional activator on the late P4 promoter PII (32). Even though the P2 Cox and P4 E proteins show no sequence similarities, they might act by similar mechanisms. Therefore, the DNA binding capacity of crude extracts of cells expressing the cloned E protein or the partially purified E protein was analyzed by the gel shift technique. The migration of 32 P-end-labelled P2 DNA fragments containing the early control region of wild-type P2 or virulent P2 mutants containing deletions of the whole early control region were analyzed. However, no specific shifts in the migration of the labelled fragments could be demonstrated (data not shown). At high protein concentrations, an unspecific shift of the labelled fragments was obtained. Footprint analysis under the same conditions was undertaken, but no specific binding to the P2 early control region was detected (data not shown).

DISCUSSION

Upon infection of a P2 lysogenic cell, unrelated defective phage P4 is able to derepress the resident prophage, thereby getting access to the late P2 gene products required for P4 lytic growth. The derepression of prophage P2 requires the activity of P4 gene ϵ . In this study, we cloned the ϵ gene and identified its product. The E protein very efficiently turns the transcriptional switch of P2 from the lysogenic mode to the lytic mode.

The mechanism behind this derepression is not known, but there are at least three ways by which E may turn the P2 transcriptional switch, (i) as a transcriptional repressor for *Pe*, which controls the synthesis of immunity repressor C, (ii) as a transcriptional activator for *Pe* or an alternative promoter controlling the early genes, or (iii) by direct interaction with the P2 C repressor function. To act as a transcriptional repressor or activator, the E protein should have specific DNA binding activity. The purified E protein did not show any specific DNA binding to the P2 *Pe*-Pc region by gel retardation assays or footprint analysis. Since a computer-assisted analysis did not reveal any DNA binding motifs in the DNA sequence either, there is no support for alternative 1 or 2; instead, we favor the hypothesis that P4 E inactivates the P2 C protein by direct protein-protein interaction. The possibility that the partially purified E protein did not regain its biological activity after renaturation cannot be excluded, but this does not seem very likely, as it is a small protein containing only one cysteine.

The expression of the α operon is quite complex (18), and in this work we addressed only the question of whether the *ash9* mutation affects the expression of ϵ under conditions in which the leader region is translated. The *ash* mutants, which are clustered in the immunity region (11), have pleiotropic effects; (i) they were originally isolated as P4 mutants able to grow on P3 lysogens, and (ii) they all affect the capacity of P4 to lysogenize. We found that the *ash9* mutation enhances the capacity of E to turn the P2 transcriptional switch to the lytic mode. The *ash* region has the capacity to code for a 15-kDa protein, but as some of the *ash* mutations do not change the amino acid residues of this potential polypeptide, *ash* probably affects expression of ϵ by some other mechanism (22). Since the transcript containing the immunity region has a complex secondary structure and is efficiently processed, generating the stable RNA immunity factor (14, 17, 29), a more likely explanation is that it either affects the stability of the transcript or facilitates the initiation of ϵ gene translation by affecting the secondary structure of the RNA.

ACKNOWLEDGMENTS

This work was supported by grant 72 from the Swedish Medical Research Counsel and by the Carl Trygger Foundation. T.L. and S.K.R. both were supported by the Sven and Lilly Lawsky Foundation. T.L. and S.K.R. contributed equally to this work.

REFERENCES

- Agarwal, M., M. Arthur, R. D. Arbeit, and R. Goldstein. 1990. Regulation of icosahedral virion capsid size by the in vivo activity of a cloned gene product. *Proc. Natl. Acad. Sci. USA* 87:2428–2432.
- Alano, P., G. Dehó, G. Sironi, and S. Zangrossi. 1986. Regulation of the plasmid state of the genetic element P4. *Mol. Gen. Genet.* 203:445–450.
- Bertani, G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J. Bacteriol.* 62:293–300.
- Bertani, G., E. Haggård-Ljungquist, K. Jagusztyn-Krynica, and S. Jupp. 1978. Defective particle assembly in wild type P2 bacteriophage and its correction by the *lg* mutation. *J. Gen. Virol.* 38:251–261.
- Bertani, L. E. 1968. Abortive induction of bacteriophage P2. *Virology* 36:87–103.
- Bertani, L. E., and E. W. Six. 1988. The P2-like phages and their parasite, P4, p. 73–143. In R. Calendar (ed.), *The bacteriophages*, vol. 2. Plenum Publishing Corporation, New York, N.Y.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
- Brosius, J. 1984. Plasmid vectors for selection of promoters. *Gene* 27:151–160.
- Calendar, R., E. Ljungquist, G. Dehó, D. C. Usher, R. Goldstein, P. Youderian, G. Sironi, and E. W. Six. 1981. Lysogenization by satellite phage P4. *Virology* 113:20–38.
- Chang, A. C. Y., and S. N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. *J. Bacteriol.* 134:1141–1156.

11. Christie, G. E., and R. Calendar. 1990. Interactions between satellite bacteriophage P4 and its helpers. *Annu. Rev. Genet.* **24**:465–490.
12. Dehò, G., D. Ghisotti, P. Alano, S. Zangrossi, M. G. Borrello, and G. Sironi. 1984. Plasmid mode of propagation of the genetic element P4. *J. Mol. Biol.* **178**:191–207.
13. Dehò, G., S. Zangrossi, D. Ghisotti, and G. Sironi. 1988. Alternative promoters in the development of bacteriophage plasmid P4. *J. Virol.* **62**:1697–1704.
14. Dehò, G., S. Zangrossi, P. Sabbattini, G. Sironi, and D. Ghisotti. 1992. Bacteriophage P4 immunity controlled by small RNAs via transcription termination. *Mol. Microbiol.* **6**:3415–3425.
15. Diana, C., G. Dehò, J. Geisselsoder, L. Tinelli, and E. Goldstein. 1978. Viral interference at the level of capsid size determination by satellite phage P4. *J. Mol. Biol.* **126**:433–445.
16. Geisselsoder, J., P. Youderian, G. Dehò, M. Chidambaram, R. Goldstein, and E. Ljungquist. 1981. Mutants of satellite virus P4 that cannot derepress their bacteriophage P2 helper. *J. Mol. Biol.* **148**:1–19.
17. Ghisotti, D., R. Chiaramonte, F. Forti, G. Sironi, and G. Dehò. 1992. Genetic analysis of the immunity region of phage-plasmid P4. *Mol. Microbiol.* **6**:3405–3413.
18. Ghisotti, D., F. Briani, F. Forti, F. Piazza, S. Polo, P. Sabbattini, T. Sturniolo, S. Terziano, S. Sangrossi, M. Zappone, G. Sironi, and G. Dehò. 1995. Multiple regulatory mechanisms controlling phage-plasmid P4 propagation. *FEMS Microbiol. Rev.* **17**:127–134.
19. Goldstein, R., J. Sedivy, and E. Ljungquist. 1982. Propagation of satellite phage P4 as a plasmid. *Proc. Natl. Acad. Sci. USA* **79**:515–519.
20. Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2**:1044–1051.
21. Halling, C., R. Calendar, G. E. Christie, E. C. Dale, G. Dehò, S. Finkel, J. Flensburg, D. Ghisotti, M. L. Kahn, K. B. Lane, C.-S. Lin, B. H. Lindqvist, L. S. Pierson III, E. W. Six, M. G. Sunshine, and R. Ziermann. 1990. DNA sequence of satellite bacteriophage P4. *J. Bacteriol.* **172**:3549–3558.
22. Lin, C.-S. 1984. Nucleotide sequence of the essential region of bacteriophage P4. *Nucleic Acids Res.* **12**:8667–8684.
23. Lindahl, G. 1970. Bacteriophage P2: replication of the chromosome requires a protein which acts only on the genome that coded for it. *Virology* **42**:522–533.
24. Lindqvist, B. H., G. Dehò, and R. Calendar. 1993. Mechanisms of genome propagation and helper exploitation by satellite phage P4. *Microbiol. Rev.* **57**:683–702.
25. Liu, Y., and E. Haggård-Ljungquist. 1994. Studies of bacteriophage P2 DNA replication: localization of the cleavage site of the A protein. *Nucleic Acids Res.* **22**:5204–5210.
26. Ljungquist, E., and L. E. Bertani. 1983. Properties and products of the cloned *int* gene of bacteriophage P2. *Mol. Gen. Genet.* **192**:87–94.
27. Nilssen, Ø., C. G. Fossdal, B. Johansen, and B. H. Lindqvist. 1996. Mutational analysis of the bacteriophage P4 capsid-size determining gene. *Virology* **219**:443–452.
28. Polo, S., T. Sturniolo, G. Dehò, and D. Ghisotti. 1996. Identification of a phage-coded DNA-binding protein that regulates transcription from late promoters in bacteriophage P4. *J. Mol. Biol.* **257**:745–755.
29. Sabbattini, P., F. Forti, D. Ghisotti, and G. Dehò. 1995. Control of transcription termination by an RNA factor in bacteriophage P4 immunity: identification of the target site. *J. Bacteriol.* **177**:1425–1434.
30. Saha, S., B. Lundqvist, and E. Haggård-Ljungquist. 1987. Autoregulation of bacteriophage P2 repressor. *EMBO J.* **6**:809–814.
31. Saha, S., E. Haggård-Ljungquist, and K. Nordström. 1987. The Cox protein of bacteriophage P2 inhibits the formation of the repressor protein and autoregulates the early operon. *EMBO J.* **6**:3191–3199.
32. Saha, S., E. Haggård-Ljungquist, and K. Nordström. 1989. Activation of prophage P4 by the Cox protein and the sites of action of the Cox protein on the two phage genomes. *Proc. Natl. Acad. Sci. USA* **86**:3973–3977.
33. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
35. Sasaki, I., and G. Bertani. 1965. Growth abnormalities in Hfr derivatives of *Escherichia coli* strain C. *J. Gen. Virol.* **40**:265–276.
36. Shore, D., G. Dehò, J. Tsipis, and R. Goldstein. 1978. Determination of capsid size by satellite bacteriophage P4. *Proc. Natl. Acad. Sci. USA* **75**:400–404.
37. Six, E. W., and C. A. C. Klug. 1973. Bacteriophage P4: a satellite virus depending on a helper such as prophage P2. *Virology* **51**:327–346.
38. Six, E. W. 1975. The helper dependence of satellite bacteriophage P4: which gene functions of bacteriophage P2 are needed by P4. *Virology* **67**:249–263.
39. Six, E. W., M. G. Sunshine, J. Williams, E. Haggård-Ljungquist, and B. H. Lindqvist. 1991. Morphopoietic switch mutations in bacteriophage P2. *Virology* **182**:34–46.
40. Six, E. W., and B. H. Lindqvist. 1978. Mutual derepression in the P2-P4 bacteriophage system. *Virology* **87**:217–230.
41. Studier, F. W., and B. A. Moffat. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**:113–130.
42. Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**:60–89.
43. Terzano, S., R. Christian, F. H. Espinoza, R. Calendar, G. Dehò, and D. Ghisotti. 1994. A new gene of bacteriophage P4 that controls DNA replication. *J. Bacteriol.* **176**:6059–6065.
44. Ziegelin, G., E. Scherzinger, R. Lurz, and E. Lanka. 1993. Phage P4 α protein is multifunctional with origin recognition, helicase and primase activities. *EMBO J.* **257**:745–755.
45. Ziermann, R., E. W. Six, B. Julien, and R. Calendar. 1993. Bacteriophage P4, p. 171–174. In S. J. O'Brian (ed.), Genetic maps, ed. 6. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.