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Bacteriophage P4 Vis protein is needed for prophage excision

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Abstract

Upon infection of its host *Escherichia coli*, satellite bacteriophage P4 can integrate its genome into the bacterial chromosome by Int-mediated site-specific recombination between the *attP* and the *attB* sites. The opposite event, excision, may either occur spontaneously or be induced by a superinfecting P2 helper phage. In this work, we demonstrate that the product of the P4 *vis* gene, a regulator of the P4 late promoters P_{LL} and P_{sid}, is needed for prophage excision. This conclusion is supported by the following evidence: (i) P4 mutants carrying either a frameshift mutation or a deletion of the *vis* gene were unable to excise both spontaneously or upon P2 phage superinfection; (ii) expression of the Vis protein from a plasmid induced P4 prophage excision; (iii) excision depended on a functional integrase (Int) protein, thus suggesting that Vis is involved in the formation of the excision complex, rather than in the excision recombination event per se; (iv) Vis protein bound P4 DNA in the *attP* region at two distinct boxes (Box I and Box II), located between the *int* gene and the *attP* core region, and caused bending of the bound DNA. Furthermore, we mapped by primer extension the 5′ end of the *int* transcript and found that ectopic expression of Vis reduced its signal intensity, suggesting that Vis is also involved in negative regulation of the *int* promoter.

Keywords: Lysogenization; DNA binding; DNA bending; Vis regulator; Bacteriophage P4

Introduction

The temperate bacteriophage λ represents the model system for integration and excision of phage genome into the bacterial chromosome. The site-specific recombination between the *attP* site on the phage DNA and the *attB* site on the bacterial chromosome is promoted by an integrase, the product of the λ *int* gene, that cuts and religates the two DNA molecules (Friedman, 1992; Gingery and Echols, 1967; Nash, 1981, 1990; Swalla et al., 2003). A protein–DNA bond, which involves an integrase active tyrosine, conserves the energy of the phosphodiester bond and an external high-energy source is thus not required. In addition to the integrase, host-encoded proteins such as IHF and FIS assemble into a macromolecular machine known as intasome and participate in the site-specific recombination event (Finkel and Johnson, 1992; Freundlich et al., 1992). The λ *attP* and the bacterial site *attB* share an identical 15-bp core region within which the recombination event occurs. The *attP* site is flanked by regions recognized and bound by the proteins forming the intasome. The recombination event that leads to prophage excision requires, beside the integrase, another phage-coded protein named excisionase. Excisionase binds DNA at *attL* and assists integrase binding and the formation of the intasome (Cho et al., 2002; Numrynch et al., 1992).

The temperate bacteriophage P4 is the prototype of satellite phages (for reviews, see Briani et al., 2001; Lindqvist et al., 1993). P4 lacks all the structural genes necessary for the construction of its viral particle and exploits the genetic information carried by a helper phage such as P2 for the construction of its capsid and for cell lysis. Thus, P4 can enter the lytic cycle only when the helper phage genome is present in the infected *Escherichia coli*

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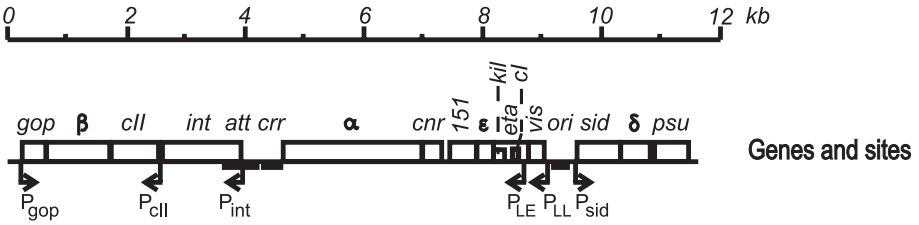

Fig. 1. Physical and genetic map of P4. Coordinates are from the annotated complete nucleotide sequence of P4, GenBank accession No. X51522. The genes and sites are indicated by open and closed boxes, respectively. The arrows beneath the map indicate the promoters.

cell. In the absence of P2, P4 can propagate in the bacterial cell as a high copy number plasmid. Alternatively, both in the presence and in the absence of the helper, P4 can lysogenize the bacterial cell.

The P4 genes essential for lytic or plasmid propagation are encoded in the right part of P4 map (Fig. 1) and are transcribed in two divergent operons. The left operon, which encodes the genes for P4 replication, is transcribed from P_{LE} early after infection and from P_{LL} late after infection and in the plasmid state (Dehò et al., 1988; Sabbattini et al., 1995). The right operon is transcribed from the P_{sid} promoter. Both P_{LL} and P_{sid} are positively regulated by either the P4-encoded δ or the P2-encoded Ogr transcriptional activators. Ogr and δ are structurally related proteins that control the P2 and P4 late promoters by interacting with an upstream region (Dale et al., 1986; Dehò et al., 1988; Grambow et al., 1990; Julien and Calendar, 1995). In addition, P_{LL}, but not P_{sid}, can also be activated by the P2 Cox factor by an independent mechanism (Saha et al., 1989).

Although P_{LL} and P_{sid} share positive regulators, they are differently controlled by the P4 Vis protein (Fig. 2A). The Vis protein, encoded by the first gene downstream of P_{LL}, is

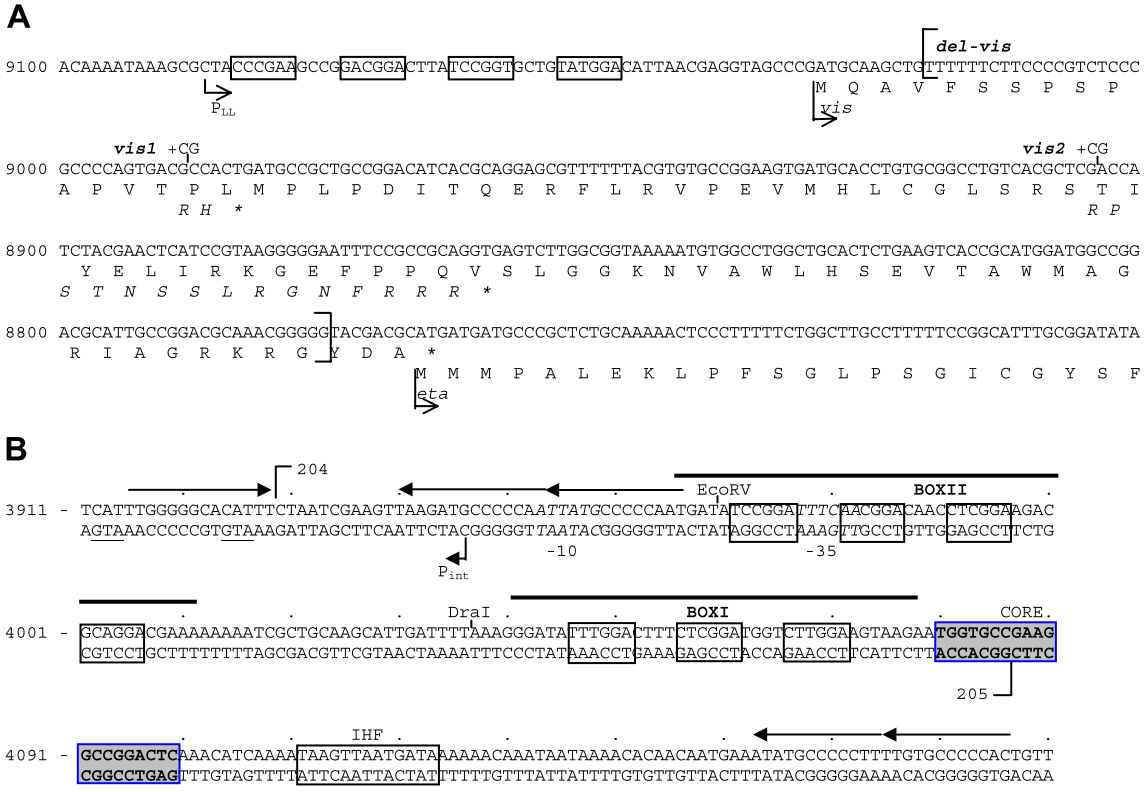

Fig. 2. (Panel A) Sequence of the P4 *vis* gene. The sequence of the P4 8701–9100 sense strand is reported. The transcription start point from P_{LL} and the *vis* and *eta* translation initiation codons are indicated by arrows. The *vis1* and *vis2* frameshift mutations are indicated above the sequence; the DNA fragment deleted in P4 *del-vis* is delimited by brackets. Below the sequence, the amino acid sequence of the Vis protein and of the first codons of *eta* is reported. The polypeptides synthesized by the mutants are indicated below in italics. The proposed Vis binding consensus sequences are boxed. (Panel B) Sequence of the P4 *att* region. The sequence of P4 3911–4180 is reported. The position of the restriction sites used in this work is indicated above the sequence. The transcription start point from P_{int} is indicated by an arrow; the -10 and -35 consensus sequences are in italics. The two potential start codons for *int* translation are underlined. The grey-boxed region is the *attP* core (Pierson and Kahn, 1987). A line above the sequence indicates the sequences protected by Vis in footprinting analysis. Box I and Box II repeats are boxed. Arrows above the sequence indicate the direct and inverted repeats suggested to be the Int binding sites to the *att* arms. The region amplified by PCR using oligonucleotides 204 and 205 is delimited by two bent lines.

Table 1
Growth of P4 *vis* mutants in infection of *E. coli* (P2)[a]

Infecting phage[b]	Cells yielding phage[c] (%)	Phage produced–infected cell[c]	Survivors[c] (%)	Lysogenic clones[d]	
				Immune to P4 *cI405* infection (%)	Releasing P4 phage (%)
P4	38	78	86	76	76
P4 *vis1*	30	20	78	83	0
P4 *vis2*	32	15	71	83	0
P4 *del-vis*	nt[e]	nt[e]	70	92	0

[a] The P2 lysogenic bacterial host was C-339.
[b] A culture of C-339 was grown to 1×10^8 cells/ml in LD broth, 5 mM $CaCl_2$, at 37 °C and infected with the phage at a multiplicity of infection of 5–10. After 5 min, the culture was diluted 100 times in fresh broth and incubated with aeration.
[c] The infected cells yielding phage and the survivors were measured 20 min after infection, and the fraction was calculated on the cell titer measured at the infection time point. The phage titer was assayed 70 min after the infection.
[d] The presence of lysogens among 100 surviving colonies was determined by testing both immunity to P4 *cI405* infection and the presence in the culture of free P4 phage by a replica plating assay (Alano et al., 1986).
[e] Not tested.

an 88 amino acid long polypeptide that presents a helix-turn-helix motif and is able to bind P4 DNA in several specific sites: (i) downstream of P_{LL}; (ii) upstream of P_{sid}; and (iii) upstream of the P4 *int* gene (Polo et al., 1996). The Vis protein was found to repress transcription from P_{LL}, whereas it appears to enhance δ-dependent transcription from P_{sid} (Polo et al., 1996).

P4 lysogenization requires integration of the phage genome into the bacterial chromosome and depends on P4 integrase, encoded by the P4 *int* gene (Fig. 1; Calendar et al., 1981; Pierson and Kahn, 1984, 1987). The P4 *attP* site is located immediately upstream of the *int* gene. The P4 *attB* site is located at 97.2 min of the *E. coli* map, within the *leuX* gene, which encodes a $tRNA^{Leu}$ isoacceptor (Calendar et al., 1981; Pierson and Kahn, 1987). Site-specific recombination occurs between identical 20 nt long G + C-rich core regions present in *attP* and *attB*, thus preserving the integrity of the *leuX* gene sequence upon prophage integration (Pierson and Kahn, 1987; Fig. 2B).

P4 integrase belongs to the tyrosine recombinases, which include the well-characterized λ integrase. The C-terminal half of the protein, which is directly involved in the recombination event, is particularly well conserved, whereas the N-terminal region that specifically binds the arm sequences of the *att* sites is more divergent (Esposito and Scocca, 1997; Grainge and Jayaram, 1999). In the P4 *att* site, a pair of 16-bp direct repeats, present on either side of the core sequence, and an inverted repeat in the left arm are supposed to be bound by Int (Pierson and Kahn, 1987; Fig. 2B). A consensus sequence for binding of IHF is present in the right arm of *attP*, suggesting that this bacterial protein is part of the P4 integration complex.

A potential *int* promoter was suggested based on canonical σ^{70} consensus and it has been proposed that Int autoregulates its own expression by binding to the *attP* left arm sequences that overlap the P_{int} region (Fig. 2B; Pierson and Kahn, 1987).

Excision of P4 prophage occurs spontaneously at low frequency, but can be induced upon P2 infection of a P4 lysogenic strain at much higher frequency (Six and Lindqvist, 1978). One might suppose that a P4 protein is required as excisionase, although such a protein has not been identified.

In this work, we show that Vis, in addition to its regulatory role on P_{LL} and P_{sid}, is essential for P4 excision and negatively regulates P_{int}.

Results

Mutations in the P4 vis gene prevent prophage induction

To better characterize the role of *vis* gene in P4 regulation, we constructed by in vitro mutagenesis two different mutations: *vis1*, an insertion of 2 bp at 8988, and *vis2*, an insertion of 2 bp at 8904 (see Materials and methods). Both mutations generated a premature stop codon (Fig. 2A).

The P4 *vis* mutants formed very turbid plaques on P2 lysogenic *E. coli* indicators. One step growth analysis (Table 1) showed that P4 *vis* mutant phage yields were slightly reduced as compared to $P4^+$. In both wild-type and mutant infections, the largest fraction of cells surviving P4 infection (≥76%) was immune to P4 *cI405* infection, a clear plaque, immunity sensitive mutant, indicating that lysogenization was not impaired. However, none of the P4 *vis* survivors spontaneously released P4, in contrast to the 100% of P4 immune clones obtained upon $P4^+$ infection.

The titer of free P4 phage present in overnight cultures of strains doubly lysogenic for either P2 and P4 *vis1* or P2 and P4 *vis2* was less than 10 pfu/ml as compared to 10^4 pfu/ml in the corresponding strain doubly lysogenic for P2 and wild-type P4.

In a singly lysogenic strain, the P4 prophage may be induced by infection with P2 A^- (Six and Lindqvist, 1978). We therefore measured P4 production after infection of C-1a (P4 *vis1*) and C-1a (P4 *vis2*) lysogens with P2 *Aam129*. No P4 phage was released by the lysogenic strains (<10 pfu/ml compared to about 10^8 pfu/ml released by the P4 wild type lysogenic strain). Therefore, the mutations in the P4 *vis* gene

Table 2
Induction of P4 prophage by the Vis protein in *E. coli* (P2)

Prophage–plasmid[a]	Phage titer (pfu/ml)[b]		
	pGZ119 − Vis	pGM677 + Vis	pGM582 + Int
P4	2×10^4	3.0×10^9	nt[c]
P4 *vis1*	<10	1.7×10^8	nt[c]
P4 *vis2*	<10	6.5×10^5	nt[c]
P4 *del-vis*	<10	5.6×10^8	2×10^2
P4 Hy1	<10	<10	2×10^3
P4 Hy1/pGM677	nt[c]	nt[c]	5×10^5

[a] The strains were C-117 lysogenic derivatives and carried the indicated plasmids: pGZ119 (control vector), pGM677, which expresses Vis, and pGM582, which expresses Int.
[b] Overnight cultures of the bacterial strains, grown at 37 °C in LD broth with either 30 μg/ml chloramphenicol or 100 μg/ml ampicillin, were diluted 20-fold in fresh medium and incubated with aeration. Free P4 phage was assayed after 4 h.
[c] Not tested.

affect both spontaneous and P2-induced release of P4 phage.

Phage release requires both P4 DNA excision and expression of P4 replication genes (Calendar et al., 1981; Gibbs et al., 1973). Unlike most temperate phages, P4 induction does not require inactivation of prophage immunity; rather, P4 immunity is bypassed by activation of the late promoter P_{LL}, which leads to the immunity-independent expression of P4 replication genes (Dehò et al., 1988; Forti et al., 2002; Ghisotti et al., 1992). We have previously observed that both P4 *vis1* and P4 *vis2* mutations exert a strong polar effect, causing a premature arrest of transcription starting at P_{LL} (Forti et al., 1999; unpublished results). Thus, the inability of P4 *vis* mutant prophages to be induced could be dependent on defective expression of left operon replication genes, defective excision, or both. To avoid polar effects, we used P4 *del-vis* that carries a deletion of the 8776–9020 region (K. Reiter and R. Calendar, unpublished). In this phage, the first three codons of *vis* are fused to the downstream *eta* gene (Fig. 2A) so as not to prevent translation of *eta*. Northern analysis of P4 *del-vis* transcription upon infection of *E. coli* C-1a indicated that transcription of the left operon from P_{LL} occurred at a higher level than in wild-type (data not shown), as expected in the absence of the Vis repressor protein (Forti et al., 1999; Polo et al., 1996).

P4 *del-vis* was able to lysogenize both in the presence (C-339) and in the absence (C-1a) of P2 prophage, but the lysogens did not release phage either spontaneously (Table 1) or after P2 *Aam129* infection (data not shown), confirming that prophage induction requires the Vis protein.

Vis expression induces Int-dependent P4 prophage excision

To determine whether prophage induction could be complemented by Vis expressed in trans, the *vis* gene was cloned in the pGZ119 vector, downstream of the *ptac* promoter (pGM677). Strains doubly lysogenic for P2 and P4, P4 *vis1*, P4 *vis2*, and P4 *del-vis* were transformed with either pGM677 or pGZ119, and P4 production was monitored in bacterial cultures of the transformed strains (Table 2). In the presence of pGM677, which expresses Vis, free P4 phage was found in all cultures. Basal expression levels of *vis* from *ptac* in the absence of IPTG appeared to be sufficient to induce P4. Moreover, partial lysis of several independent cultures was observed and in the pGM677 transformants P4 prophage maintenance was unstable: about 10–30% of the cells in the culture were cured, as assessed by the loss of immunity to P4 *cI405* infection (data not shown). Thus, pGM677 appears to cause P4 prophage excision and is at least partially incompatible with P4 prophage maintenance.

It has been demonstrated that P4 integrase is essential for prophage excision because an *E. coli* strain lysogenic for P4 Hy1, which carries a deletion of the *int* region (Souza et al., 1978), does not release phage (Calendar et al., 1981). We tested whether Vis-dependent excision also required P4 integrase (Table 2). Transformation of C-117 (P4 Hy1) with pGM677 did not cause phage production (<10 pfu/ml), confirming the dependence on P4 integrase.

On the other hand, transformation of C-117 (P4 Hy1) with a plasmid expressing Int (pGM582) complemented prophage excision. Expression of both Vis (pGM677) and Int (pGM582) in C-117 (P4 Hy1) increased P4 release about 200-fold (5×10^5 pfu/ml). Interestingly, expression of P4 integrase from pGM582 caused some phage release also in C-117 (P4 *del-vis*), indicating that the Int protein alone is able to cause a low level of prophage excision.

To rule out that P4 excision depended on P2 genes, we transformed C-1a lysogenic for P4 or mutant derivatives with the *vis* plasmid pGM677 and the parent vector pGZ119, and analyzed the maintenance of the prophage DNA in the transformed strains by colony hybridization. As shown in Table 3, C-1a (P4) and C-1a (P4 *del-vis*) were cured from P4 prophage after transformation with pGM677, but not with pGZ119, indicating that excision did not depend on any P2 gene. Moreover, we tested the effect of pGM677 transformation on C-1a (P4 α*am1*), a mutant defective in P4 DNA replication (Gibbs et al., 1973) and C-1a (P4 δ35), defective in late operon activation (Souza et al., 1977). Both lysogenic strains were cured, indicating that

Table 3
Vis-induced curing of P4 lysogenic cells

Bacterial strain	Presence of P4 prophage after transformation with[a]	
	pGZ119 − Vis	pGM677 + Vis
C-1a (P4)	10/10	0/12
C-1a (P4 *del-vis*)	11/11	0/12
C-1a (P4 α*am1*)	68/68	0/166
C-1a (P4 δ35)	10/10	0/30

[a] The strains were transformed with the indicated plasmids and the transformants selected for chloramphenicol resistance (30 μg/ml). Single transformant colonies were purified and the presence of P4 prophage was assessed by colony hybridization with the oligonucleotide complementary to the 3823–3840 P4 region.

P4 replication and P4 late gene expression were not required for P4 excision.

The Vis protein binds P4 DNA in the att region

The Vis protein acts as a transcriptional regulator and was shown to bind P4 DNA in the P_{LL} and P_{sid} regions (Polo et al., 1996). If Vis were an excisionase it would also be expected to bind the P4 *att* region. To test this, we performed electrophoretic mobility shift assays using a GST-Vis fusion protein and DNA fragments of the P4 *att* region. The P4 3927–4084 region (Fig. 2B), which includes the region upstream of the *int* gene and the *att* site, was amplified by PCR in the presence of (α^{32}P)-ATP (Fig. 3A). This fragment was efficiently bound by the GST-Vis protein (Fig. 3B). Several retarded bands were observed and slower bands appeared by increasing the amount of GST-Vis protein suggesting multiple binding of the protein to the DNA. No retarded bands could be observed with the control GST protein. Moreover, the addition of nonspecific competitor DNA up to 1:100 ratio did not alter GST-Vis specific binding.

To define more accurately the Vis binding site(s), we performed mobility shift assays with subfragments of the above region. The DNA fragment was digested either at 3969 with *Eco*RV or at 4034 with *Dra*I. The 115-bp fragment (3969–4084) obtained by *Eco*RV digestion was bound by GST-Vis, whereas the remaining 43-bp fragment (3927–3969) was not (Fig. 3C). On the contrary, both the 108-bp (3927–4034) and the 50-bp (4035–4084) fragments derived by *Dra*I digestion were bound. The two fragments of the *Dra*I digestion were purified and used separately in mobility shift experiments (Fig. 3D). Both were bound and multiple retarded bands were visible in each case. This demonstrates that two different Vis-binding sites are present in the *att* region between the putative *int* promoter and the *att* core, namely Box II, within the 3927–4034 region, and Box I, in the 4035–4084 fragment (Fig. 2B). The efficiency of Vis binding to the two sites was estimated by competition experiments using a 1:1 mixture of the two fragments. Vis bound about 2-fold more efficiently to Box II than to Box I (data not shown). Moreover, Vis affinity to Box II, estimated on the concentration of protein necessary to bind 50% of the input DNA fragment, was about five times higher than its affinity to the P_{LL} region (data not shown; Polo et al., 1996).

To better delimit Vis binding sites, we performed DNase I protection experiments using the 3901–4211 P4 region, labeled at the 3′ end at 3901, and the Vis protein cleaved with PreScission Protease from the GST peptide (Fig. 4). Footprinting analysis indicated that two different regions were protected, one extending from 3965 to 4011 (Box II), the second from 4041 to 4077 (Box I). Box II protection

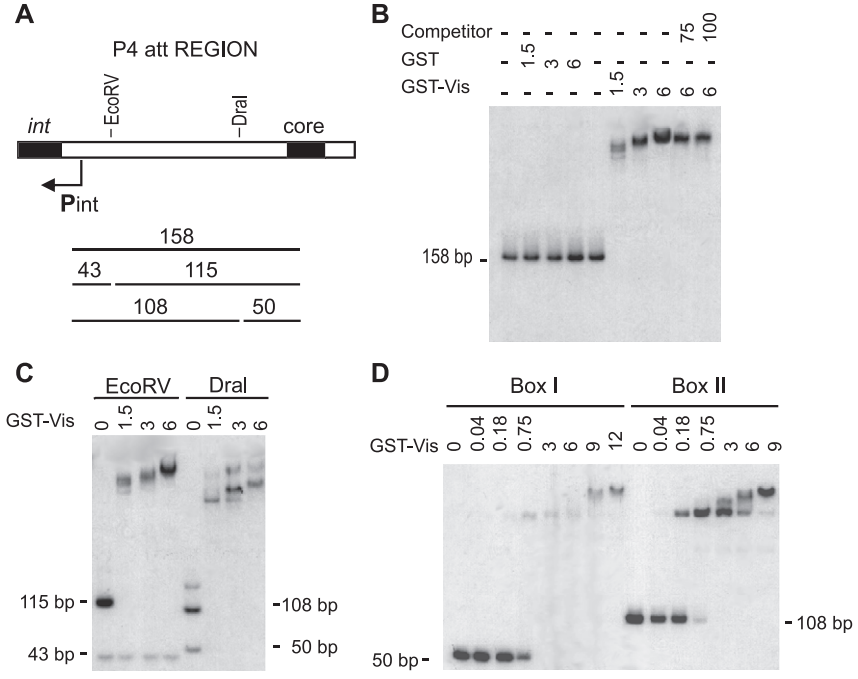

Fig. 3. Binding of Vis to the *attP* region. (Panel A) The 3927–4084 P4 DNA fragment (158 bp) was obtained by PCR amplification in the presence of (α^{32}P)dATP, as indicated in Materials and methods. (Panel B) Binding reaction mixtures contained 0.04 ng of the 158 bp DNA fragment and the concentrations of the GST or the GST-Vis proteins indicated (in ng) at the top of the lanes. A 75- or 100-fold molar excess of competitor DNA was added to the binding reaction, as indicated. Gel electrophoresis was in 6% polyacrylamide gel. (Panel C) The DNA fragments were obtained by *Eco*RV or *Dra*I digestion of the 158-bp fragment of panel A. The dimensions of the resulting fragments are indicated. The *Dra*I digestion was not complete, and the 158 bp band is still visible. The amount of GST-Vis protein (in ng) is indicated at the top of the lanes. (Panel D) The 50-bp (4035–4084) and 108-bp (3827–4034) fragments obtained by *Dra*I digestion were purified and bound with the amount of the GST-Vis protein (in ng), as indicated on top of the lanes.

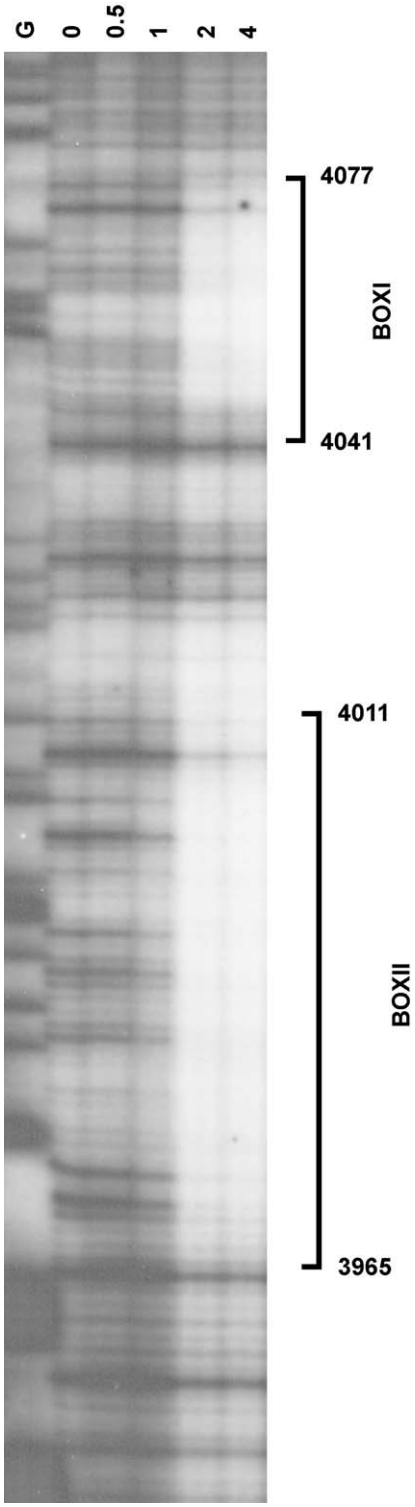

Fig. 4. Footprint of the *attP* region. The first lane contains the G Maxam–Gilbert sequencing reaction mixture. The Vis protein, obtained from GST-Vis by PreScission treatment, was used for the protection experiments in the amounts (in ng) indicated on top of the lanes. The protected regions are indicated to the right and the P4 coordinates are given.

was detectable at lower Vis concentration than Box I (1 and 2 ng of Vis, respectively), in agreement with the mobility shift experiments.

The Vis protein bends the DNA

Excisionase proteins are known to bend DNA. Therefore, we tested whether Vis was also able to bend DNA upon binding. Circular permutations of DNA fragments encompassing the 3958–4031 P4 region, which contains Box II, were obtained by digesting pGM756 with the enzymes indicated in Fig. 5A. The fragments were end-labeled, incubated with the GST-Vis protein, as described for the mobility shift assays, and separated by polyacrylamide gel electrophoresis (Fig. 5B). The retarded bands migrated differently, indicating that the presence of Vis distorted the DNA fragment. The *Pvu*II and *Mlu*I fragments were the most and least retarded, respectively. By comparing the electrophoretic migration of these two fragments, a bending angle of about 90°, centered at coordinate 3996, was calculated, according to Kim et al. (1989).

The P_{int} promoter is negatively regulated by Vis

A putative promoter with a good similarity to the -10 (cATAAT) and -35 (TTGAaA) *E. coli* sequences was found by sequence inspection in the region upstream of the *int* gene (Pierson and Kahn, 1987). By primer extension, we identified an RNA 5′ end (a major signal at C3946 on the coding strand) that was compatible as the transcription start point of this putative promoter (Fig. 6). Minor signals downstream could be due to mRNA degradation. The major signal was present early upon P4 infection of *E. coli* C-1a and decreased in intensity at later times (60 min).

Box II is immediately upstream of P_{int}, overlapping the -35 region (Fig. 2B). This suggested that Vis might regulate P_{int} activity. Indeed, primer extension of the RNAs synthesized after P4 infection of C-1a carrying pGM677 showed that, in the presence of Vis, the P_{int} signal was reduced at early times after P4 infection, suggesting that Vis might negatively regulate transcription from P_{int}.

Discussion

Vis is involved in P4 prophage excision

Many DNA-binding proteins may fulfill multiple physiological roles depending on the position of the binding site relative to other genomic elements and partner proteins they may interact with. Vis protein, a short, basic polypeptide expressed from the P4 late promoter P_{LL}, was first identified as a regulator of late promoters P_{LL} and P_{sid}, and shown to be able to bind to both promoter regions (Polo et al., 1996). Vis binding to P_{LL} represses transcription, thus regulating its own expression. On the contrary, Vis binding to the P_{sid} region appears to enhance and anticipate transcription (Polo et al., 1996; D. Ghisotti, unpublished results). In this work, we demonstrate that Vis binds the *attP* region and is directly involved in prophage excision.

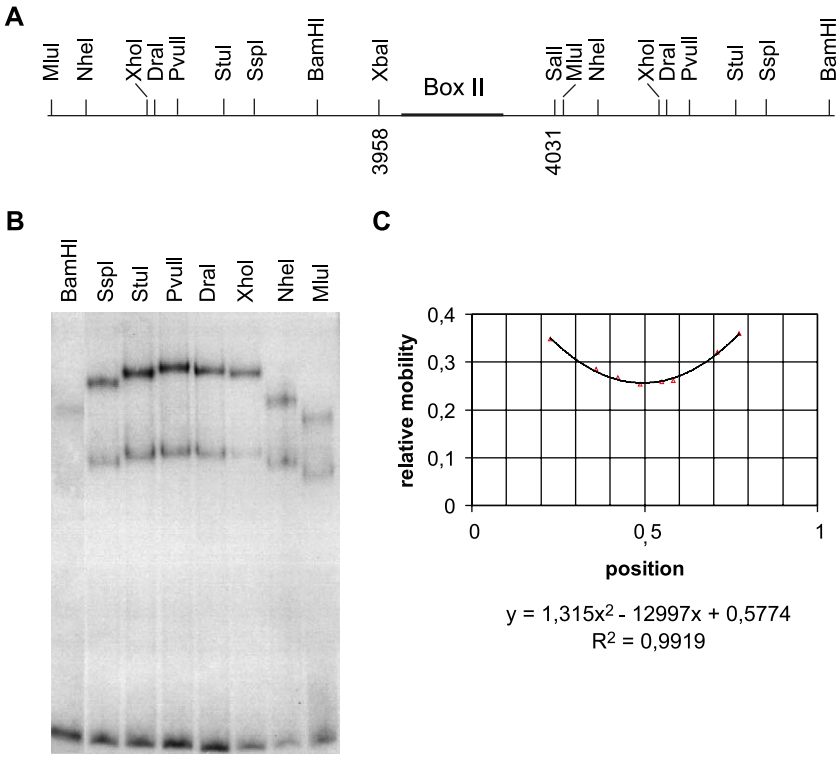

Fig. 5. Vis bends the DNA. (Panel A) Polylinker region of pGM756 carrying the P4 3958–4031 DNA cloned into *Xba*I–*Sal*I. The positions of the symmetric restriction site on either side of the P4 region are shown. (Panel B) Fragments (188 bp) obtained by digestion of pGM756 with the enzymes indicated on top of the lanes were end-labeled, about 0.05 ng was incubated with 3 ng of GST-Vis, and separated on 5% polyacrylamide gel electrophoresis. (Panel C) The migration of each retarded fragment (upper band) relative to the unbound DNA (lowest band) was measured and used for determination of the bending angle. The equation of the parabola is given.

P4 *vis* mutant prophages are defective in both spontaneous (in a P2–P4 double lysogen) and induced (by P2 superinfection of a P4 lysogen) phage production. This phenotype may be the consequence of a defect either in prophage excision or in expression of the lytic pathway. However, consistent with the regulatory roles previously assigned to Vis, the P4 lytic cycle is only slightly impaired by *vis* mutations (Table 1; Polo et al., 1996). Therefore, Vis appears to act at the level of prophage excision.

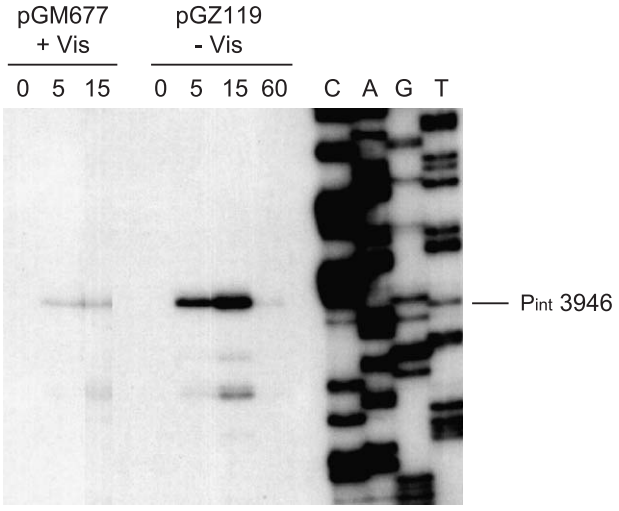

Fig. 6. Primer extension of *int* transcripts. RNA was extracted from C-1a cultures carrying pGZ119 (control plasmid) or pGM677 (expressing Vis) at different times after P4 infection, as indicated in minutes above the lanes. Primer extension and sequencing reactions were performed with an oligonucleotide complementary to the P4 3823–3840 region. The position of the major 5′ end signal is indicated.

Two major hypotheses may then be considered: either Vis is essential for the expression of a gene essential for P4 DNA excision, or it is itself involved in this event. The former seems implausible. We could easily rule out that the Vis-regulated excision function is coded by the two main divergent P_{LL} and P_{sid} operons: first, because Vis represses P_{LL} and only positively modulates, but it is not essential for, transcription of P_{sid}; second, because none of the genes encoded by the *sid* operon appear to be involved in excision. This is further confirmed by data presented in Table 3. It is known that P4 α or δ mutants are defective in the expression of the late operons (Barrett et al., 1976; Christie and Calendar, 1983; Halling and Calendar, 1990); nevertheless, excision of these mutant prophages is induced by ectopic expression of Vis.

The remaining gene is *int*, which is required both for integration and excision (Pierson and Kahn, 1984, 1987). P4 *vis* mutants lysogenize at normal frequency, thus Vis is not required for Int expression upon infection. In the prophage, *int* appears to be transcribed at a low basal level (Fig. 6D;

Ghisotti, unpublished results). It seems unlikely that Vis promotes excision by increasing Int expression, rather our data indicated that, in the presence of Vis, transcription from P_{int} was reduced. Finally, induction of an Int-deficient prophage is 250-fold higher when both Int and Vis are ectopically expressed from *ptac* than if only Int is provided in trans (Table 2). This implies a direct role of Vis in excision.

P4 prophage excision occurs spontaneously at low frequency and, at a much higher efficiency, upon infection of a P4 lysogen by the helper P2 (Six and Lindqvist, 1978). The different frequency of the excision event might be easily correlated to the different expression level of the *vis* gene. Vis is expressed from the P4 late promoter P_{LL} (Dehò et al., 1992; Polo et al., 1996). After P2 infection, the P2 Cox and Ogr proteins activate P_{LL}, and *vis* is immediately transcribed (Dale et al., 1986; Dehò et al., 1984; Halling and Calendar, 1990; Saha et al., 1989). Therefore, the first response of a P4 prophage to P2 infection is to promote excision and enter the lytic cycle. On the other side, in the lysogenic state, transcription from P_{LL}, that depends on P4 Delta, is not active (Dale et al., 1986; Dehò et al., 1984), thus preventing Vis expression and prophage excision.

Vis has a helix-turn-helix motif, common to other excisionase proteins, such as P2 Cox and phage 186 Apl (Dodd and Egan, 1990; Shearwin and Egan, 1996; Yu and Haggård-Ljungquist, 1993). These proteins, like Vis, have been shown to act both as negative regulators of the lysogenic promoter and as excisionases (Dodd et al., 1993; Reed et al., 1997; Yu and Haggård-Ljungquist, 1993). Moreover, the Vis protein shows high homology with AlpA, a protein that induces excision of the cryptic prophage CP4−57 (Kirby et al., 1994). However, contrary to what occurs in P4, AlpA seems to activate transcription of the integrase-coding gene *slpA*.

Vis binds to the attP region

Vis was able to bind the *attP* region in two distinct regions, both located between P_{int} and the *attP* core. Footprinting analysis indicated that the first region extends from 3965 to 4011 (Box II), the second from 4041 to 4077 (Box I).

Polo et al. (1996) identified an 11-bp sequence element, Y.Y.R.T.C.C.G.R.N.R.Y, that is partially conserved at P_{LL} (two copies, in inverted orientation), P_{sid}, and *attP* (Box I and Box II). However, the Vis-protected regions at P_{LL} (Polo et al., 1996) and Box I−Box II extend beyond these sequences. Further examination suggested that Vis may be recognizing a group of imperfect repeats of a 6-bp consensus, T.C.C.R.notC.notT, separated by 4 bp, with a 10-bp periodicity. Four copies of this consensus sequence may be found at Box II and P_{LL}, and three copies at Box I (Figs. 2A and B). Vis binding to the repeats could explain the Vis DNase I footprints at *att* (Fig. 4) and at P_{LL} (Polo et al., 1996). Similar sequences are present in ΦR73, a P4-like *E. coli* retronphage (Inouye et al., 1991; Sun et al., 1991). The Box I−Box II conservation between the P4 and ΦR73 *att* sites is particularly striking because there is little similarity outside these sites in this region of the two phages. There is also a 10-bp repeated sequence element within Box I and Box II of CP4−57 (Kirby et al., 1994), but the sequence is somewhat different from that in P4 and ΦR73, presumably reflecting the divergence of the CP4−57 AlpA protein from the Vis proteins.

The sequence conservation among the repeats is not strong, so to test whether the sequences contain enough information to specify selective Vis binding, we used a weight matrix approach, similar to that used for analysis of binding sites for the P2 and 186 excisionases, Cox and Apl (Dodd et al., 1993). A weight matrix consisting of the base frequencies of the aligned 6-bp sequences (and two positions on each side) from the seven repeats at P4 *att*, the seven repeats at ΦR73 *att*, and the four repeats at P4 P_{LL} was poorly selective. That is, sequences scoring as highly as each of the P4 and ΦR73 *att* and P_{LL} sequences occurred frequently in random DNA sequence. However, strong selectivity was obtained if the weight matrix was repeated three times, so that three consecutive repeats, spaced by 10 bp, were scored. That is, the frequency of 30-bp sequences with such a good match to three tandem repeats was very low in random sequence. In addition, there were no other significant matches in the rest of the P4 or ΦR73 genomes. Thus, the identified sequence element does contain sufficient information to specify selective Vis binding but only if the element is repeated at least three times. A similar result was found with the Cox and Apl sequences, although in these cases two repeated elements were capable of providing reasonable selectivity (Dodd et al., 1993). The sequence identified at P_{sid} by Polo et al. (1996) matches the consensus as well as many of the other sequences but was not detected by the weight matrix because it is only a single copy of the repeat.

Unlike other excisionases, such as P2 Cox and λ Xis, Vis appears to recognize direct repeats in a head to tail arrangement, suggesting that Vis binds as a monomer. A similar situation has been already demonstrated for phage 186 Apl protein, which was found as a monomer in solution and that binds to direct tandem repeats. Shearwin and Egan (1996) proposed that single monomers may bind to the DNA and subsequent oligomerization of the proteins occurs at the DNA level.

Vis bends the DNA

Like most excisionase proteins, Vis binding causes bending of DNA, suggesting that Vis actively participates in the formation of the protein−DNA complex involved in excision. Vis bending at Box II appeared to form an about 90° angle, centered on 3996. However, it must be noted that Vis also binds Box I and is likely to cause bending in this region, and that an A/T tract near 4010 might contribute to the overall DNA curvature in this region. The position of the two boxes in the prophage is between *int* and *attL*, suggestive of an arrangement found for other excisionase proteins,

such as Cox and Apl (Dodd et al., 1993; Yu and Haggård-Ljungquist, 1993).

Materials and methods

Bacteria, phages, plasmids, and cultures media

The bacterial and phage strains and the plasmids used are listed in Table 4. P4 coordinates throughout refer to the complete P4 sequence, GenBank accession number X51522. Bacterial strains were grown in LD broth (Sabbattini et al., 1995) and specific antibiotics were added when required.

Table 4
Bacterial and phage strains

Strain	Relevant genotype	Reference or source
E. coli C and E. coli K12		
C-1a	F^-, prototrophic	Sasaki and Bertani, 1965
C-117	C-1a (P2)	Bertani, 1968
C-117 (P4 Hy1)	P4 Hy1 prophage inserted in *attB*	Calendar et al., 1981
C-339	C-1a (P2 *lg cc*)	Barrett et al., 1976
C-5205	polyauxotrophic	Dehò, 1983
JM-101	polyauxotrophic	Messing et al., 1981
Bacteriophages		
P2	Wild type	Bertani, 1968
P2 *Aam129*	Replication defective	Lindahl, 1970
P2 *lg cc*	Large plaques	Bertani et al., 1969
P4	Wild type	Six and Klug, 1973
P4 *cI405*	C8446T mutation in *cI*; immunity defective, recessive mutant	Calendar et al., 1981; Lin, 1984
P4 *del-vis*	deletion of the P4 8776–9020 region, internal to *vis*	K. Reiter and R. Calendar, unpublished
P4 Hy1	substitution of the P4 220–3631 region with λ DNA; lacks the *gop*, β, *cII* and *int* genes	Souza et al., 1978
P4 *vis1*	frameshift mutation in *vis*	This work
P4 *vis2*	frameshift mutation in *vis*	This work
P4 α*am1*	Replication defective	Gibbs et al., 1973
P4 β35	Transactivation deficient	Souza et al., 1977
Plasmids		
pBend2	DNA bending vector	Kim et al., 1989
pGEX-6P-1	expression vector	Pharmacia
pGM582	P4 4249–2471 cloned into pUC18; *int* gene expression	This work
pGM677	P4 9030–8762 cloned into pGZ119EH; expression of the Vis protein	This work
pGM756	P4 3958–4031 cloned into pBend2	This work
pGM825	P4 8762–9027 cloned into pGEX-6P-1; expression of the GST-Vis fusion peptide	Polo et al., 1996
pGZ119EH	cloning vector; chloramphenicol resistance	Lessl et al., 1992
pUC18	cloning vector; ampicillin resistance	Yanisch-Perron et al., 1985

Construction of the P4 vis1 and P4 vis2 mutants

The 6447–10657 P4 region was cloned in pUC18 and the resulting plasmid DNA was partially digested with either *Aha*II or *Taq*I, end filled, and religated to create the *vis1* and *vis2* mutations, respectively, and strain JM101 was transformed. The DNA extracted from the transformants was sequenced to confirm the presence of the *vis1* mutation (a CG insertion at 8988 (sense strand); Fig. 2A) or the *vis2* mutation (a CG insertion at 8904; Fig. 2A). The *Mlu*I–*Apa*LI fragments (P4 8626–10653) derived from the above plasmids, carrying the *vis* mutations, were ligated to the P4 wild-type 10653-*cos*-8626 fragment and transfected into the C-5205 strain. The presence of the *vis* mutations was confirmed by sequencing the phage DNA.

Colony hybridization

The procedure was performed as described by Sambrook et al. (1990) using as a probe a 17 nt long oligonucleotide complementary to the 3823–3840 P4 sequence.

RNA extraction and primer extension

RNA was extracted from *E. coli* cultures at different times after infection with P4, as described by Dehò et al. (1992). The concentration of the RNA was determined spectrophotometrically and the quality of the RNA was controlled by agarose gel electrophoresis. An oligonucleotide complementary to the P4 3823–3840 region was end-labeled and used both for sequencing the P4 *int* region and in primer extension experiments.

Labeled DNA fragment amplification

The P4 3927–4984 region was amplified by PCR using two oligonucleotides corresponding to the P4 3927–3947 and 4084–4056 sequence. Radioactive amplification with AmpliTaq polymerase (Perkin-Elmer) was performed with 1 ng P4 DNA template in the presence of 30 μCi of (α^{32}-P) dATP (0.1 μM) and 1 μM of each dCTP, dGTP, and dTTP. The labeled DNA fragment was separated by polyacrylamide gel electrophoresis and the band, visualized by autoradiography, was cut out of the gel and eluted overnight in H_2O. Subfragment purification of this DNA region, obtained by *Eco*RV or *Dra*I digestion, was performed in the same way.

Electrophoretic mobility shift assay

Purification of the GST-Vis fusion protein, binding to the labeled DNA fragments, and gel electrophoresis in non-

denaturing 6% polyacrylamide gels were performed as in Polo et al. (1996). Competitor DNA was obtained by PCR amplification of a 200-bp-long portion of *E. coli pnp* region.

DNase I footprinting

GST tag was removed from GST-Vis fusion protein purified from pGM825 by treatment with PreScission Protease (Amersham) as indicated by the manufacturer. DNaseI footprinting was performed as described by Polo et al. (1996). The P4 DNA region used covered 3901–4211 and was end-labeled at 3901.

DNA bending

DNA fragments containing circular permutations of the P4 3958–4031 region were obtained by digestion of pGM756 with different enzymes, purification, and end-labeling either by Klenow filling or with T4 polynucleotide kinase. The electrophoretic migration was tested after binding with the GST-Vis protein, as described above. The relative positions of the retarded bands were compared and the bending angle was calculated according to Kim et al. (1989).

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