Journal of General Virology (2006), **87**, 2423–2431 DOI 10.1099/vir.0.81875-0

Expression of phage P4 integrase is regulated negatively by both Int and Vis

D. Piazzolla,† S. Calì,‡ E. Spoldi,§ F. Forti, C. Sala, F. Magnoni, G. Dehò and D. Ghisotti

Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy

Correspondence
D. Ghisotti
Daniela.Ghisotti@unimi.it

Phage P4 *int* gene encodes the integrase responsible for phage integration into and excision from the *Escherichia coli* chromosome. Here, the data showing that P4 *int* expression is regulated in a complex manner at different levels are presented. First of all, the P_{int} promoter is regulated negatively by both Int and Vis, the P4 excisionase. The N-terminal portion of Int appears to be sufficient for such a negative autoregulation, suggesting that the Int N terminus is implicated in DNA binding. Second, full-length transcripts covering the entire *int* gene could be detected only upon P4 infection, whereas in P4 lysogens only short 5′-end covering transcripts were detectable. On the other hand, transcripts covering the 5′-end of *int* were also very abundant upon infection. It thus appears that premature transcription termination and/or mRNA degradation play a role in Int-negative regulation both on the basal prophage transcription and upon infection. Finally, comparison between P_{int}–*lacZ* transcriptional and translational fusions suggests that Vis regulates Int expression post-transcriptionally. The findings that Vis is also an RNA-binding protein and that Int may be translated from two different start codons have implications on possible regulation models of Int expression.

Received 27 January 2006
Accepted 3 April 2006

INTRODUCTION

Temperate bacteriophages may choose between the lytic and the lysogenic cycle for their propagation in the bacterial host. In the lysogenic state, most phages, such as λ, P2 and P4 integrate their genome into the host chromosome by site-specific recombination between the phage *attP* and the bacterial *attB* sites (Nash, 1981; Pierson & Kahn, 1984, 1987; Richet *et al.*, 1988; Yu *et al.*, 1989; Saha *et al.*, 1990; Cho *et al.*, 2002; Frumerie *et al.*, 2005). In the case of λ, the recombination event is mediated by a site-specific recombinase, the Int phage integrase. Recombination initiates with the pairing of two specific DNA sequences by a tetramer of recombinase molecules. A Holliday junction is formed by cleavage, exchange and ligation of one pair of strands, and is resolved by the exchange of the second pair of strands by isomerization of the recombinase tetramer (Biswas *et al.*, 2005; Radman-Livaja *et al.*, 2006). A DNA–protein complex named intasome is responsible for site-specific recombination, in which *attP* and *attB* sites, phage integrase, as well as bacterial factors, such as integration host factor (IHF) and Fis, are involved (de Moitoso & Landy, 1991; Patsey & Bruist, 1995; Swalla *et al.*, 2003). The reverse excision event requires, in addition, a phage encoded excisionase that is part of the intasome complex (Numrych *et al.*, 1991; Wu *et al.*, 1998; Cho *et al.*, 2002).

Bacteriophage P4 Int recombinase belongs to the large family of tyrosine recombinases which includes over 100 members that show very little sequence identity; however, a C-terminal region of P4 integrase that can be aligned with other tyrosine integrases presents typical motifs (Esposito & Scocca, 1997; Nunes-Düby *et al.*, 1998). In particular, in P4 integrase two different DNA-recognition motifs for binding to the core and arm sites that are present in the *attP* site were identified (Fig. 1a; Argos *et al.*, 1986; Pierson & Kahn, 1987; Nunes-Düby *et al.*, 1998; Grainge & Jayaram, 1999). Upon integration, the phage genome is linearly integrated into the bacterial chromosome and the *int* gene is proximal to the *attL* site in the prophage as conventionally defined (Nash, 1981). Contrary to what occurs in λ and P2, P4 *int* is transcribed starting from *attL* towards the centre of the P4 genome (Pierson & Kahn, 1984, 1987; Ghisotti *et al.*, 1990).

The P4 *attB* region is within the $tRNA^{leu}$ gene. P4 carries a region identical to the 3′-end of the tRNA so that part of the tRNA is duplicated upon integration and a complete copy of the gene is maintained (Pierson & Kahn, 1987; Kita *et al.*, 1999; Bishop *et al.*, 2005). Excision of the prophage not only

†Present address: Max F. Perutz Laboratories at the Vienna Biocenter, Department of Microbiology and Genetics, University of Vienna, 1030 Vienna, Austria.

‡Present address: Keryos, Via della Filanda 5, 20060 Gessate (MI), Italy.

§Present address: Molmed SpA, Via Olgettina 58, 20132 Milano, Italy.

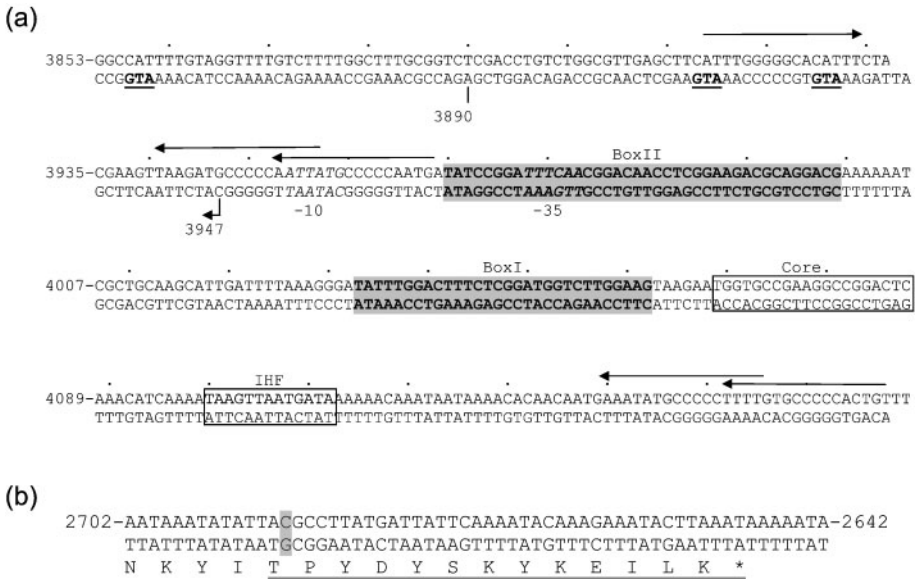

Fig. 1. P4 *attP–int* region. (a) Nucleotide sequence of the P4 *attP–int* region is from GenBank sequence X51522, with the coordinates corrected +1 because of a G : C insertion at 2687. The arrows above the sequence indicate direct and inverted repeats thought to be bound by P4 Int (arm sides; Pierson & Kahn, 1987). The core and IHF-binding sites are boxed. The Vis-binding sites are shaded in grey (BoxI and BoxII; Calì *et al.*, 2004). The bent arrow at 3947 indicates the 5′-end of *int* mRNA. P_{int} −10 and −35 consensus sequences are in italic. Potential ATG start codons for Int are underlined. The position 3890, in which the Vis-induced 5′-end was located, is indicated. (b) Base pair insertion at 2687 within *int* coding sequence. The terminal portion of the P4 *int* sequence, in inverted orientation with respect to the P4 sequence, is reported with the C : G base insertion highlighted. Below the nucleotide sequence, the amino acid sequence of the integrase protein is reported with residues different from that of the GenBank sequence X51522 underlined.

requires the integrase protein, but also depends on the P4 Vis protein, which binds and bends DNA at the *attP* site. Vis is thought to favour the formation of the DNA–protein complex necessary for prophage excision (Calì *et al.*, 2004).

In all temperate phages, expression of integrase is regulated according to the phage developmental pathways (Grainge & Jayaram, 1999; Radman-Livaja *et al.*, 2003). In this work expression of P4 *int* gene has been analysed and its negative regulation by both Int and Vis is suggested.

METHODS

Bacterial strains, plasmids, oligonucleotides, media and chemicals. The *Escherichia coli K-12* and *E. coli C* derivatives, and the plasmids used in this work are listed in Table 1. The oligonucleotides used are listed below, with the upper-case letters corresponding to P4 sequence (coordinates are reported), the lower-case letters are sequences not corresponding to P4 (casual), R indicates complementary strand, the underlined sequences indicate the *Eco*RI or *Bam*HI restriction sites: no. 524 (5′-ccaaggatccCGAAGTTAAGATGCCCC-3′; P4 3935–3952); no. 525 (5′-ggctgaattcTCGTCCTGCGTCTTCCG-3′; P4 4010–3994 R); no. 526 (5′-gcgcgaattccTTACTTCCAAGACCATC-CG-3′; P4 4076–4057 R); no. 527 (5′-ccgcgaattcTTCCGATTTATTGA-TGGGCT-3′; P4 4219–4200 R); no. 546 (5′-cacaggatccCCATCGGCC-ATTTTGTAGG-3′; P4 3848–3866); no. 547 (5′-cacaggatccGCGTTGA-GCTTCATTTGG-3′; P4 3902–3919); no. 573 (5′-ccccggatcCATTTC-TAATCGAAGTTAAGATGC-3′; P4 3925–3948); no. 663 (5′-cgcggaat-tcATCTTAACTTCGATTAGAAATGTGC-3′; P4 3922–3947 R). All the fragments obtained by PCR amplification were sequenced. LD broth was described by Ghisotti *et al.* (1992); ampicillin (100 μg ml^{-1}) and chloramphenicol (30 μg ml^{-1}) were added when necessary.

RNA analysis. Northern blot hybridization was performed as described previously (Dehò *et al.*, 1992). In brief, total RNA (20 μg) extracted from *E. coli* C-1a and from P4-infected cells was fractionated on either 1·5 % formamide/formaldehyde agarose or 10 % polyacrylamide/urea denaturing gels, transferred to Hybond-N filter membranes (Amersham) and hybridized to the ^{32}P-labelled RNA probe Int1, which covers the P4 nt 4035–3690 region. Hybridization and primer-extension analysis were performed as described by Briani *et al.* (1996) and by Boorstein & Craig (1989), respectively.

RNA electrophoretic mobility shift assay. Glutathione *S*-transferase (GST)-Vis and GST purification were performed as described in Polo *et al.* (1996). ^{32}P-labelled *int* RNA was synthesized by *in vitro* transcription with T7 RNA polymerase and pGM823 DNA as template, as described in Regonesi *et al.* (2004). The probe corresponds to the 3947–3848 P4 *int* transcript. Then, 1 pg (equivalent to 0·03 fmol) of the purified *int* RNA was incubated in the presence of either GST-Vis or GST in binding buffer [100 mM KCl, 10 mM Tris/HCl, 1 mM EDTA, 0·1 mM DTT, 5 % glycerol (v/v), 50 μg BSA ml^{-1}] in the presence of RNasin (0·4 U) in a final volume of 8 μl at room temperature for 20 min. The samples were fractionated by native 5 % polyacrylamide gel electrophoresis at 4 °C.

***β*-Galactosidase assay.** *β*-Galactosidase activity was assayed in DH10B strains harbouring the reporter plasmids. Bacteria were grown in LD up to OD_{600} = 0·2–0·4 at 37 °C. *β*-Galactosidase activity was measured using the method of Miller (1972). Each assay was repeated at least three times with independent extracts.

Table 1. Bacterial strains, plasmids and phages used

Strain or plasmid	Relevant genotype	Reference
***E. coli* strains**		
C-1a	*E. coli C* prototroph	Sasaki & Bertani (1965)
C-1a(P4)	*E. coli C* lysogenic for P4	Dehò *et al.* (1984)
C-1a/pP4	*E. coli C* carrying plasmid P4	Dehò *et al.* (1984)
DH5α	*E. coli K-12 F⁻ araD139* Δ(*ara, leu*)*7697* Δ*lacX74 galU galK rpsL deoR* Φ*80dlacZ*Δ*M15 endA1 nupG recA1 mcrA* Δ(*mrr hsdRMS mcrBC*)	Hanahan (1983)
DH10B	*E. coli K-12 F⁻* Φ*80dlacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi1 gyrA96 relA1*	Grant *et al.* (1990)
Plasmids		
pGEM3Z	Amp^R	Promega
pGM331	Carries the $tRNA^{Gly}$ reporter gene in pGZ119EH	Briani *et al.* (1996)
pGM597	P4 *Eco*RV3970–*Sph*I3690 in pUC18	This work
pGM603	P4 *Bam*HI4250–*Hin*dIII3690 in pUC18	This work
pGM604	P4 *Bam*HI4250–*Sph*I3594 in pUC18	This work
pGM605	P4 *Bam*HI4250–*Sal*I3044 in pUC18	This work
pGM606	P4 *Bam*HI4250–*Sph*I2475 in pUC18	This work
pGM677	P4 9031–8763 in pGZ119EH; expresses Vis	Calì *et al.* (2004)
pGM791	P4 4010–3935 was amplified by PCR with the oligonucleotides no. 524 and 525, digested with *Eco*RI and *Bam*HI and cloned into pRS415 digested with the same enzymes	This work
pGM792	P4 4076–3935 was amplified by PCR with the oligonucleotides no. 524 and 526, digested with *Eco*RI and *Bam*HI and cloned into pRS415 digested with the same enzymes	This work
pGM793	P4 4219–3935 was amplified by PCR with the oligonucleotides no. 524 and 527, digested with *Eco*RI and *Bam*HI and cloned into pRS415 digested with the same enzymes	This work
pGM796	P4 4010–3902 was amplified by PCR with the oligonucleotides no. 547 and 525, digested with *Eco*RI and *Bam*HI and cloned into pRS414 digested with the same enzymes	This work
pGM798	P4 4010–3848 was amplified by PCR with oligonucleotides no. 546 and 525, digested with *Eco*RI and *Bam*HI and cloned into pRS414 digested with the same enzymes	This work
pGM805	P4 4010–3848 was amplified by PCR with the oligonucleotides no. 573 and 525, digested with *Eco*RI and *Bam*HI and cloned into pRS414 digested with the same enzymes	This work
pGM823	P4 3947–3925 was amplified by PCR with the oligonucleotides no. 546 and 663, digested with *Eco*RI and *Bam*HI and cloned into pGEM3Z digested with the same enzymes	This work
pGZ119EH	Cam^R	Lessl *et al.* (1992)
pRS414	Amp^R, plasmid for translational fusions with *lacZ*	Simons *et al.* (1987)
pRS415	Amp^R, plasmid for transcriptional fusions with *lacZ*	Simons *et al.* (1987)
pUC18	Amp^R	Yanisch-Perron *et al.* (1985)
Phage		
P4	Wild type	Six & Klug (1973)

RESULTS

Correction of the P4 *int* gene sequence

The P4 *int* gene was cloned and sequenced and a G:C insertion at 2687 was identified (Fig. 1b; GenBank accession no. X51522). The presence of the insertion was confirmed by sequencing P4 *int* genes of different origin, such as P4 DNA extracted from *E. coli* C-1a/pP4, C-1a(P4) and three P4 DNA preparations from our phage collection. The presence of the insertion causes a frameshift in the terminal part of the Int polypeptide, creating a nonsense codon at 2649. The last 13 aa of the protein thus differ from the published sequence. The overall similarity with tyrosine-integrases of other phages was not modified, since the C-terminal end of P4 integrase is not significantly conserved.

Transcriptional profile of the *int* gene

Northern analysis of *int* transcripts after infection of *E. coli* C-1a with P4, using the Int1 probe (4035–3690), proximal to the P_{int} promoter, is reported in Fig. 2. In the first 40 min after infection, two transcripts covering the whole *int* gene (2·2 and 1·3 kb) were observed. A major signal was visible at low molecular masses ($<0·6$ kb). Upon hybridization of the same filter with either Int2 (3690–3216) or Int3 (3216–2622) probes, the 2·2 and 1·3 kb transcripts were still present, whereas the shorter RNAs disappeared (data not shown). Thus, the short transcripts mapped to the 5′-end of the *int* gene and may be due to premature transcription termination and/or transcript degradation. In the lysogenic state, the only transcripts identified were short transcripts; 2·2 and 1·3 kb RNAs were not visible.

The 2·2 kb transcript was present with all three probes, suggesting that it extends beyond the *int* coding sequence. This analysis does not discriminate whether it protrudes 5′ or 3′ from the *int* gene. The presence of *attP*-α upstream and *cII* downstream of *int*, followed by a Rho-independent transcription termination site about 2·2 kb from P_{int} (Ghisotti *et al.*, 1990), favours the latter hypothesis, suggesting that the 2·2 kb transcript encompasses *int* and *cII*.

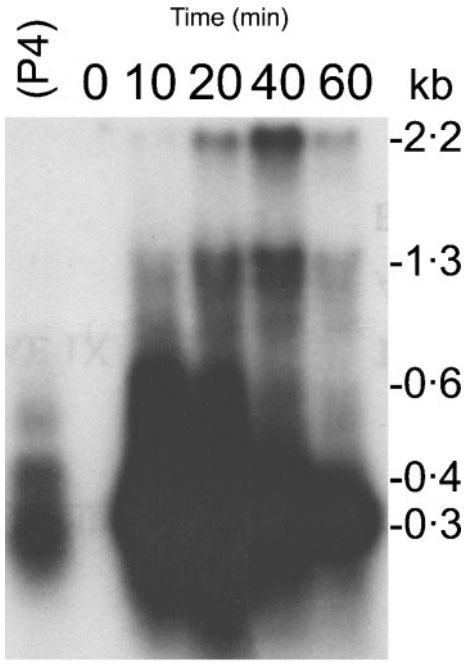

Fig. 2. Northern blot analysis of P4 *int* gene transcription. RNA was extracted from cultures of strain C-1a lysogenic for P4 (P4), before P4 infection (0) or infected with P4 at the times indicated in minutes on top of the lanes. The RNA was fractionated on a denaturing 1·5% agarose gel, blotted and the filter hybridized to the Int1 riboprobe (P4 4035–3690) as indicated in Methods.

P4 integrase negatively regulates P_{int} activity

Calì *et al.* (2004) identified the 5′-end of P4 *int* mRNA upon phage infection at 3947 (3946 in Calì *et al.*, 2004) by primer extension. Canonical −10 (cATAAT) and −35 (TTGAaA) promoter consensus sequences located immediately upstream were proposed as the P_{int} promoter (see Fig. 1a).

In order to characterize the function of P_{int}, the RNA expressed from a set of plasmids harbouring different portions of the P4 *int* region was analysed by primer extension using an oligonucleotide complementary to the 3823–3840 P4 region, proximal to P_{int} (Fig. 3a). In pGM603, which carries the P4 4250–3690 region, a strong band was visible at

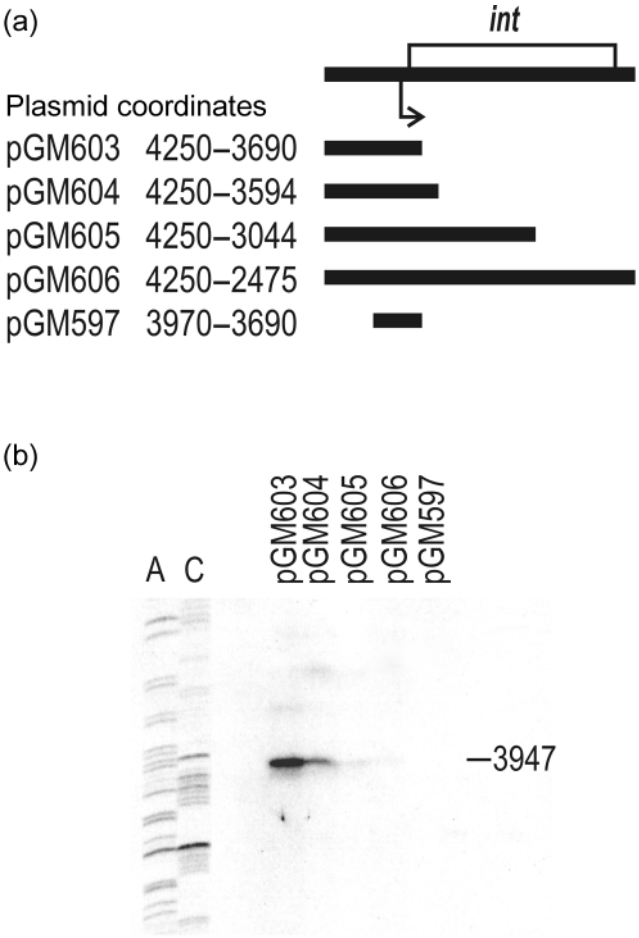

Fig. 3. Analysis of P_{int} transcription in the presence of Int. (a) Plasmids carrying P_{int} DNA regions cloned into plasmid pGM331. The coordinates of the cloned regions are reported. (b) Primer-extension analysis of P_{int}. Strain DH5α was transformed with the indicated plasmids, RNA extracted and primer-extension reactions performed with an oligonucleotide complementary to the 3840–3823 P4 region of *int* mRNA. The extended molecules were run in a denaturing 6% polyacrylamide gel. A and C DNA sequence reactions obtained with the same oligonucleotide were run in the same gel.

3947 (Fig. 3b). The same signal was less intense in pGM604 (4250–3594), and progressively fainter bands were observed in pGM605 (4250–3044) and pGM606 (4250–2475). Densitometric quantification indicated that the signal decreased to 42 % in pGM604, 16 % in pGM605 and 6 % in pGM606. Thus, when the P4 DNA fragment contained the whole *int* gene (pGM606) or at least 60 % of it (pGM605), the *int* mRNA abundance was greatly reduced, suggesting that Int negatively regulates transcription initiation at P_{int}. No band at 3947 was present with pGM597, which carries the 3970–3690 P4 DNA, lacking the −35 P_{int} sequence.

Control of P_{int} by P4 Vis

In a previous study (Calì *et al.*, 2004), we reported that P4 Vis binds to the *attP* region and is able to repress P_{int} activity. To characterize further the effect of Vis on P_{int}, we constructed several plasmids carrying P_{int} fragments upstream of the reporter *lacZ*. The plasmids differed in the 5′-extension of the P4 region that included either BoxII (from coordinate 4010; pGM791 in Fig. 4), BoxI (from coordinate 4076; pGM792) and the core region (from coordinate 4219; pGM793). *E. coli* DH10B strains carrying pGZ119EH (control plasmid) or pGM677 (expressing Vis) were transformed with the plasmids and β-galactosidase activity was measured. The results are reported in Fig. 4.

All plasmids expressed a high level of β-galactosidase in the absence of Vis. The highest activity was seen with the shortest fragment, carried by pGM791, suggesting that the upstream regions have a negative effect on P_{int} activity. This negative regulation might be caused by DNA conformation of the upstream region and/or binding of some bacterial factor.

In the presence of Vis, β-galactosidase activity was reduced. In all three plasmids the amount of reduction was around 23–29 %, without a clear effect due to the presence of BoxI and the core region. Thus, BoxII appears to be sufficient for full repression by Vis.

Identification of P4 integrase start codon

The translation initiation codon of P4 integrase has not been experimentally determined. The *int* coding sequence presents three ATG codons in the first 90 nt downstream of the 5′-end of the *int* mRNA, none preceded by a potential ribosome-binding site (Fig. 1a). In order to determine which ATG serves as the *int* start codon, we cloned P_{int} and fragments extending to the first, second and third ATG, creating translational fusions with *lacZ* (Fig. 4). Strains DH10B/pGZ119EH and DH10B/pGM677 were transformed with the plasmids and β-galactosidase activity was measured. Fusion with the first ATG (pGM805) did not express β-galactosidase (6 Miller units), indicating that this codon is not used for translation initiation. On the other hand, fusions with the second ATG (pGM796) expressed β-galactosidase weakly (802 Miller units), whereas the highest level of β-galactosidase expression was obtained in fusions with the third ATG (pGM798; 3355 Miller units). These results suggest that both the second and the third ATG may contribute to integrase translation in P4. It should be noted that the fusions with the second and third ATG also include the first three codons downstream. It has been shown that not only ATG, but also codons in +2 to +5 positions may alter translation efficiency (Gonzalez de Valdivia & Isaksson, 2004, 2005). Therefore, the constructs pGM796 and pGM798 reflect the translation initiation efficiency of

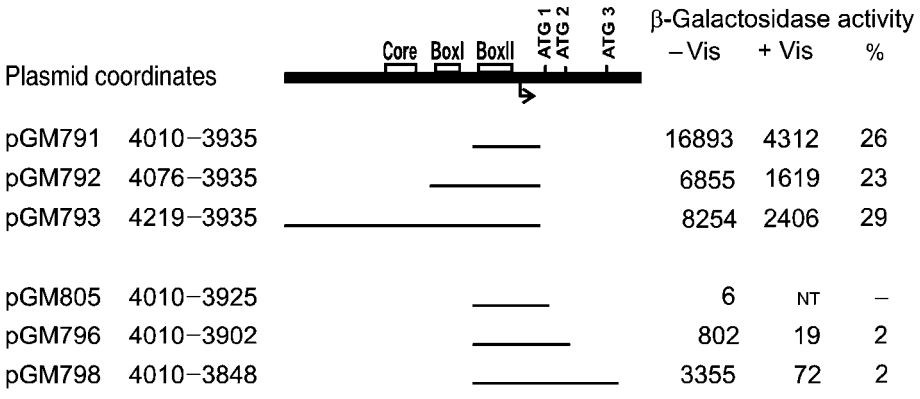

Fig. 4. β-Galactosidase activity expressed from P_{int}–*lacZ* fusions. Different P4 DNA fragments were cloned into either pRS415 (pGM791, 792 and 793; transcriptional fusions) or pRS414 (pGM805, 796 and 798; translational fusions) upstream of the reporter *lacZ*. The map of the P4 region, with the indication of the relevant sites, is shown. The bent arrow corresponds to 3947, the 5′-end of the *int* mRNA. The first three ATG are also indicated. *E. coli* DH10B carrying the pGZ119EH vector (−Vis) or pGM677, which expresses the Vis protein (+Vis), were transformed with the plasmids and β-galactosidase activity was measured in the extracts, as indicated in Methods. The mean of three independent assays is given. Standard deviation was less than ± 10 %. The fraction of β-galactosidase expressed in the presence and absence of Vis is indicated (%). NT, Not tested.

a larger initiation region, and suggest that the third ATG substantially contributes to *int* translation.

All these plasmids were regulated negatively by the presence of Vis: the level of β-galactosidase expression was reduced to 2 %. This effect is stronger than that observed with plasmids carrying a transcriptional fusion with *lacZ*, in which about 25 % of the activity was still present. Thus, it appears that Vis has a dual negative effect on both transcription and translation.

Vis appears to favour *int* mRNA processing

The effect of Vis on P_{int} was analysed further by performing primer-extension experiments in the presence and absence of Vis on RNA extracted from *E. coli* DH10B/pGZ119EH and DH10B/pGM677 transformed with pGM798, which carries a translational *int–lacZ* fusion. A strong band at 3947 and weaker downstream signals were present in the absence of Vis (Fig. 5). In the presence of Vis, the signal at 3947 was reduced to about 30 %, but a downstream band at 3890 was intensified. This band was barely visible in the absence of Vis. Thus, Vis appears to intensify a specific 5′-end downstream of the transcription start site. This new 5′-end could be due to Vis-induced mRNA processing or, less likely, to a new transcription initiation point.

P4 Vis is able to bind RNA

To test whether Vis binds RNA, we performed an RNA mobility shift assay of 3947–3848 *int* RNA in the presence of the purified GST-Vis protein (Fig. 6a and b). By increasing the amount of GST-Vis, the RNA fragment was shifted, indicating that Vis is able to bind the transcript. The binding ability essentially depended on Vis, since the effect of GST alone on RNA mobility was barely detectable. The addition of increasing amounts of poly(A) efficiently competed Vis binding. Moreover, we also tested Vis binding to a different RNA fragment (the *lacZ* RNA) and found a similar binding efficiency for Vis (data not shown).

Sequence analysis with CLUSTAL W (Higgins *et al.*, 1994) of the Vis polypeptide identified a putative S4 RNA-binding motif in the protein, between residues 28 and 64, as shown in Fig. 6(c).

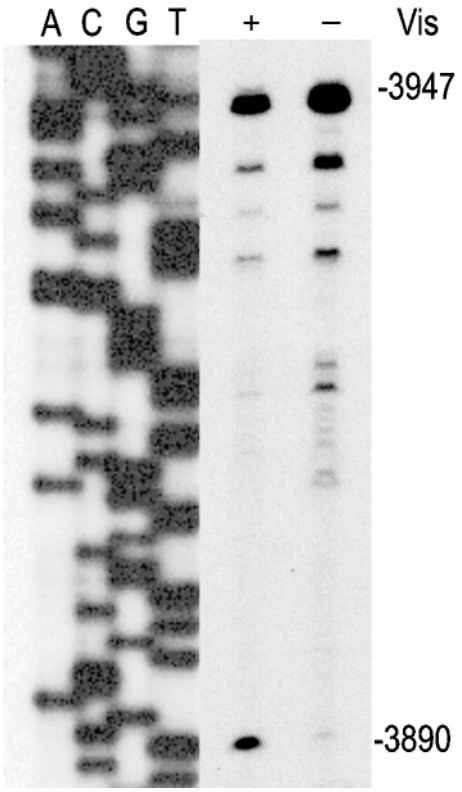

Fig. 5. Primer extension of *int* mRNA in the presence or absence of Vis. DH10B/pGM677 (expressing Vis, +) and DH10B/pGZ119EH (control vector; –) were transformed with pGM798; RNA was extracted and primer-extension analysis performed with an oligonucleotide complementary to the downstream *lacZ* mRNA (5′-GGTTTTCCCAGTCACGACG-3′). DNA sequence reactions of pGM798 obtained with the same oligonucleotide were run in the same gel.

DISCUSSION

P4 integrase autoregulates its own expression

Expression of P4 integrase appears to be modulated during the P4 life-cycle. First, regulation of *int* expression occurs by negatively regulating transcription initiation at P_{int}. This negative regulation may be achieved by P4 Int itself, probably by binding to specific sites upstream and downstream of P_{int} (Pierson & Kahn, 1987). The effect of the negative control was confirmed by monitoring P_{int} activity in different constructs, carrying either the whole *int* coding sequence (pGM606) or about 60 % of it (pGM605). In both plasmids, the P_{int} signal was severely decreased. P4 integrase protein and its N-terminal portion have not been identified, thus nothing is known about the stability of either protein. Nevertheless, our results indicate that the truncated N-terminal portion of P4 integrase is sufficient for repression of P_{int}.

The main products of *int* transcription are short RNAs that cover the 5′-end of the gene. These transcripts may be produced by processing of longer transcripts and/or by premature transcription termination. In any case, the presence of these short RNAs in all phases of the P4 life-cycle, including the lysogenic condition, indicates that P_{int} basal level activity is always substantial and thus post-transcription initiation regulatory mechanisms may play a relevant role in control of P_{int} expression.

P4 Vis is involved in P_{int} regulation

Our data indicate that Vis, the P4 excisionase (Calì *et al.*, 2004), modulates Int expression both at transcription initiation and post-transcriptional levels. Expression of the *lacZ* reporter gene in plasmids that carry either a transcriptional or a translational fusion downstream of P_{int}

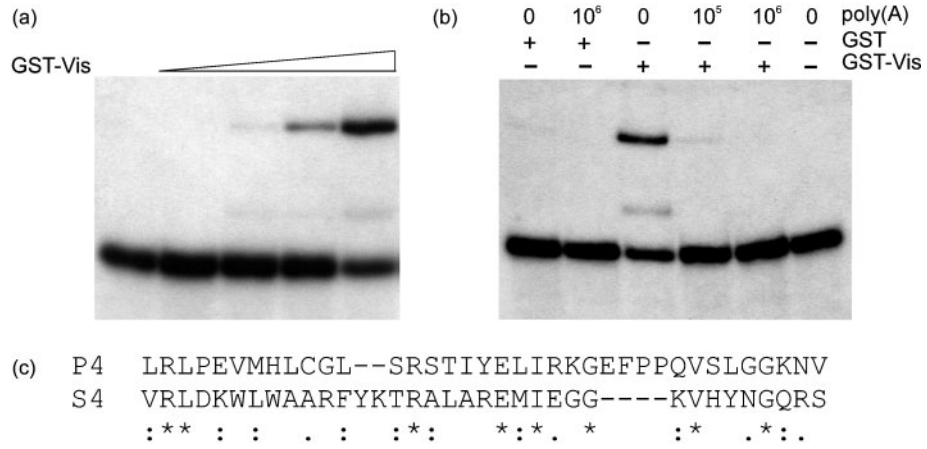

Fig. 6. Vis binding to *int* RNA. (a) Electrophoretic mobility shift analysis was performed with a 100 nt long RNA corresponding to the initial part of the *int* transcript (3947–3848). RNA (1 pg, equivalent to 0·03 fmol) and increasing amounts of GST-Vis (molar ratio of 3×10^2, 9×10^2, 3×10^3 and 4×10^3, respectively) were incubated for the binding reaction. Reactions were run in a 5 % non-denaturing polyacrylamide gel. (b) The same RNA used in (a) was incubated in the presence of either GST (molar ratio of 10^4) or GST-Vis (molar ratio of 4×10^3). Poly(A) RNA was added, as competitor, in the amount indicated above the lanes (in pg). (c) S4 RNA-binding domain in Vis. Vis was aligned with the 30S ribosomal protein S4 motif with CLUSTAL W (Higgins *et al.*, 1994) multiple sequence alignment. (*) represents that nucleotides are identical; (:) represents that conserved substitutions have been observed; and (.) represents that semi-conserved substitutions have been observed. The position of the domain within the Vis protein is between aa 28 and 64.

decreased in the presence of Vis. In transcriptional fusions, the β-galactosidase activity was reduced to less than 30 % and the presence of BoxII was sufficient for observing the effect. The Vis-binding site at BoxII overlaps the −35 sequence of P_{int} and this may account for the negative effect on P_{int} activity. Further upstream regions do not seem to be implicated in this regulatory mechanism.

When the plasmids carry a translational *int–lacZ* fusion, Vis had a much greater effect on the level of β-galactosidase activity, reducing it to about 2 %. This suggests that Vis is also involved in post-transcriptional regulation of *int* expression. We demonstrated that Vis binds an *int* RNA, and found an S4 RNA-binding motif within the protein. Moreover, in the presence of Vis, the abundance of a 5′-end at 3890, within the *int* coding sequence, is enhanced. It may be suggested that Vis binds *int* mRNA, preventing *int* translation and promoting an endonucleolytic cut of *int* RNA that functionally inactivates the *int* mRNA. Vis-dependent negative control on *int* synthesis is likely to be relevant for preventing *int* expression upon prophage excision and avoid reintegration of P4 in the chromosome.

On the other hand, P4 prophage genome excision requires the presence of both Int and Vis (Calì *et al.*, 2004). A basal level of Int is likely to be continuously expressed, as suggested by repression of transcription from P_{int}. Expression of Vis from P_{LL} is the first event that occurs upon P4 prophage de-repression (Polo *et al.*, 1996). This may provide the amount of Vis required for the formation of the excision complex and the production of a free circular P4 genome. Expression of *vis* will then be autoregulated by Vis repression of P_{LL}.

In conclusion, we have shown a dual effect of Int and Vis on P4 *int* expression that might modulate the amount of integrase present in the cell and adapt it to the different conditions in P4 life-cycle.

P4 integrase: one or two proteins?

It appears that two different ATG codons at 3915 and 3858 (pGM796 and pGM798) are used for Int translation. The two open reading frames are 422 and 403 aa long and the two proteins differ by 19 aa at their N-terminal end. Our data suggest that P4 integrase may be expressed in two forms. Whether these proteins have a different functional role in P4 lysogenization was not established. In Tn*5*, the two proteins differing by 55 aa at the N-terminal end are the transposase and its inhibitor, respectively (de la Cruz *et al.*, 1993; Davies *et al.*, 1999; Reznikoff, 2003; Steiniger-White *et al.*, 2004). Translation of the inhibitor utilizes a distinct initiation site relative to the transposase, and, *in vivo*, the inhibitor protein is a transdominant negative regulator of transposition and acts presumably by forming heteromultimers with transposase. A similar role could be performed by the two forms of P4 integrase, in which the longer protein may be functional in integration, whereas the shorter could be involved in inhibition of integrase activity.

ACKNOWLEDGEMENTS

This work was supported by grant no. 2002053757-001 from the Ministero dell'Istruzione, dell'Università e della Ricerca, Rome, Italy,

by grant no. 01-0786 of INTAS, and by grants from the Ministero dell'Istruzione, dell'Università e della Ricerca Scientifica e Tecnologica, Rome, Italy.

REFERENCES

Argos, P., Landy, A., Abremski, K. & 9 other authors (1986). The integrase family of site-specific recombinases: regional similarities and global diversity. *EMBO J* **5**, 433–440.

Bishop, A. L., Baker, S., Jenks, S. & 8 other authors (2005). Analysis of the hypervariable region of the *Salmonella enterica* genome associated with tRNA(leuX). *J Bacteriol* **187**, 2469–2482.

Biswas, T., Aihara, H., Radman-Livaja, M., Filman, D., Landy, A. & Ellenberger, T. (2005). A structural basis for allosteric control of DNA recombination by lambda integrase. *Nature* **435**, 1059–1066.

Boorstein, W. R. & Craig, E. A. (1989). Primer extension analysis of RNA. *Methods Enzymol* **180**, 347–369.

Briani, F., Zangrossi, S., Ghisotti, D. & Dehò, G. (1996). A Rho-dependent transcription termination site regulated by bacteriophage P4 RNA immunity factor. *Virology* **223**, 57–67.

Calì, S., Spoldi, E., Piazzolla, D., Dodd, I. B., Forti, F., Dehò, G. & Ghisotti, D. (2004). Bacteriophage P4 Vis protein is needed for prophage excision. *Virology* **322**, 82–92.

Cho, E. H., Gumport, R. I. & Gardner, J. F. (2002). Interactions between integrase and excisionase in the phage lambda excisive nucleoprotein complex. *J Bacteriol* **184**, 5200–5203.

Davies, D. R., Mahnke Braam, L., Reznikoff, W. S. & Rayment, I. (1999). The three-dimensional structure of a Tn*5* transposase-related protein determined to 2·9-Å resolution. *J Biol Chem* **274**, 11904–11913.

Dehò, G., Ghisotti, D., Alano, P., Zangrossi, S., Borrello, M. G. & Sironi, G. (1984). Plasmid mode of propagation of the genetic element P4. *J Mol Biol* **178**, 191–207.

Dehò, G., Zangrossi, S., Sabbattini, P., Sironi, G. & Ghisotti, D. (1992). Bacteriophage P4 immunity controlled by small RNAs via transcription termination. *Mol Microbiol* **6**, 3415–3425.

de la Cruz, N. B., Weinreich, M. D., Wiegand, T. W., Krebs, M. P. & Reznikoff, W. S. (1993). Characterization of the Tn*5* transposase and inhibitor proteins: a model for the inhibition of transposition. *J Bacteriol* **175**, 6932–6938.

de Moitoso, V. & Landy, A. (1991). A switch in the formation of alternative DNA loops modulates lambda site-specific recombination. *Proc Natl Acad Sci U S A* **88**, 588–592.

Esposito, D. & Scocca, J. J. (1997). The integrase family of tyrosine recombinases: evolution of a conserved active site domain. *Nucleic Acids Res* **25**, 3605–3614.

Frumerie, C., Sylwan, L., Ahlgren-Berg, A. & Haggård-Ljungquist, E. (2005). Cooperative interactions between bacteriophage P2 integrase and its accessory factors IHF and Cox. *Virology* **332**, 284–294.

Ghisotti, D., Finkel, S., Halling, C., Dehò, G., Sironi, G. & Calendar, R. (1990). Nonessential region of bacteriophage P4: DNA sequence, transcription, gene products, and functions. *J Virol* **64**, 24–36.

Ghisotti, D., Chiaramonte, R., Forti, F., Zangrossi, S., Sironi, G. & Dehò, G. (1992). Genetic analysis of the immunity region of phage-plasmid P4. *Mol Microbiol* **6**, 3405–3413.

Gonzalez de Valdivia, E. I. & Isaksson, L. A. (2004). A codon window in mRNA downstream of the initiation codon where NGG codons give strongly reduced gene expression in *Escherichia coli*. *Nucleic Acids Res* **32**, 5198–5205.

Gonzalez de Valdivia, E. I. & Isaksson, L. A. (2005). Abortive translation caused by peptidyl-tRNA drop-off at NGG codons in the early coding region of mRNA. *FEBS J* **272**, 5306–5316.

Grainge, I. & Jayaram, M. (1999). The integrase family of recombinase: organization and function of the active site. *Mol Microbiol* **33**, 449–456.

Grant, S. G., Jessee, J., Bloom, F. R. & Hanahan, D. (1990). Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. *Proc Natl Acad Sci U S A* **87**, 4645–4649.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.

Higgins, D. G., Thompson, J. D. & Gibson, T. J. (1996). Using CLUSTAL for multiple sequence alignments. *Methods Enzymol* **266**, 383–402.

Kita, K., Tsuda, J., Kato, T., Okamoto, K., Yanase, H. & Tanaka, M. (1999). Evidence of horizontal transfer of the EcoO109I restriction-modification gene to *Escherichia coli* chromosomal DNA. *J Bacteriol* **181**, 6822–6827.

Lessl, M., Balzer, D., Lurz, R., Waters, V. L., Guiney, D. G. & Lanka, E. (1992). Dissection of *IncP* conjugative plasmid transfer: definition of the transfer region Tra2 by mobilization of the Tra1 region in trans. *J Bacteriol* **174**, 2493–2500.

Miller, J. H. (1972). In *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Nash, H. A. (1981). Integration and excision of bacteriophage lambda: the mechanism of conservation site specific recombination. *Annu Rev Genet* **15**, 143–167.

Numrych, T. E., Gumport, R. I. & Gardner, J. F. (1991). A genetic analysis of Xis and FIS interactions with their binding sites in bacteriophage lambda. *J Bacteriol* **173**, 5954–5963.

Nunes-Düby, S. E., Kwon, H. J., Tirumalai, R. S., Ellenberger, T. & Landy, A. (1998). Similarities and differences among 105 members of the Int family of site-specific recombinases. *Nucleic Acids Res* **26**, 391–406.

Patsey, R. L. & Bruist, M. F. (1995). Characterization of the interaction between the lambda intasome and *attB*. *J Mol Biol* **252**, 47–58.

Pierson, L. S., III & Kahn, M. L. (1984). Cloning of the integration and attachment regions of bacteriophage P4. *Mol Gen Genet* **195**, 44–51.

Pierson, L. S., III & Kahn, M. L. (1987). Integration of satellite bacteriophage P4 in *Escherichia coli*. DNA sequences of the phage and host regions involved in site-specific recombination. *J Mol Biol* **196**, 487–496.

Polo, S., Sturniolo, T., Dehò, G. & Ghisotti, D. (1996). Identification of a phage-coded DNA-binding protein that regulates transcription from late promoters in bacteriophage P4. *J Mol Biol* **257**, 745–755.

Radman-Livaja, M., Shaw, C., Azaro, M., Biswas, T., Ellenberger, T. & Landy, A. (2003). Arm sequences contribute to the architecture and catalytic function of a lambda integrase-Holliday junction complex. *Mol Cell* **11**, 783–794.

Radman-Livaja, M., Biswas, T., Ellenberger, T., Landy, A. & Aihara, H. (2006). DNA arms do the legwork to ensure the directionality of lambda site-specific recombination. *Curr Opin Struct Biol* **16**, 42–50.

Regonesi, M. E., Briani, F., Ghetta, A., Zangrossi, S., Ghisotti, D., Tortora, P. & Dehò, G. (2004). A mutation in polynucleotide phosphorylase from *Escherichia coli* impairing RNA binding and degradosome stability. *Nucleic Acids Res* **32**, 1006–1017.

Reznikoff, W. S. (2003). Tn*5* as a model for understanding DNA transposition. *Mol Microb* **47**, 1199–1206.

Richet, E., Abcarian, P. & Nash, H. A. (1988). Synapsis of attachment sites during lambda integrative recombination involves capture of a naked DNA by a protein-DNA complex. *Cell* **52**, 9–17.

Saha, S., Haggård-Ljungquist, E. & Nordstrom, K. (1990). Integration host factor is necessary for lysogenization of *Escherichia coli* by bacteriophage P2. *Mol Microbiol* **4**, 3–11.

Sasaki, I. & Bertani, G. (1965). Growth abnormalities in Hfr derivatives of *Escherichia coli* strain C. *J Gen Microbiol* **40**, 365–376.

Simons, R. W., Houman, F. & Kleckner, N. (1987). Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene* **53**, 85–96.

Six, E. W. & Klug, C. A. (1973). Bacteriophage P4: a satellite virus depending on a helper such as prophage P2. *Virology* **51**, 327–344.

Steiniger-White, M., Rayment, I. & Reznikoff, W. S. (2004). Structure/function insights into Tn*5* transposition. *Curr Opin Struct Biol* **14**, 50–57.

Swalla, B. M., Cho, E. H., Gumport, R. I. & Gardner, J. F. (2003). The molecular basis of co-operative DNA binding between lambda integrase and excisionase. *Mol Microbiol* **50**, 89–99.

Wu, Z., Gumport, R. I. & Gardner, J. F. (1998). Defining the structural and functional roles of the carboxyl region of the bacteriophage lambda excisionase (Xis) protein. *J Mol Biol* **281**, 651–661.

Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

Yu, A., Bertani, L. E. & Haggård-Ljungquist, E. (1989). Control of prophage integration and excision in bacteriophage P2: nucleotide sequences of the *int* gene and *att* sites. *Gene* **80**, 1–11.