

Phosphorylation of the integrase protein of coliphage HK022

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Abstract

The integrase (Int) proteins of coliphages HK022 and λ , are phosphorylated in one or more of their tyrosine residues. In Int of HK022 the phosphorylated residue(s) belong to its core-binding/catalytic domains. Wzc, a protein tyrosine kinase of *Escherichia coli*, is not required for Int phosphorylation *in vivo*, however, it can transphosphorylate the conserved Tyr³⁴² catalytic residue of Int *in vitro*. Int purified from cells that overexpress Wzc has a reduced activity *in vitro*. *In vivo*, the lysogenization of wild type HK022 as well as of λ is not affected by the overexpression of Wzc. However, the *nin5* mutant of λ , which lacks a protein-tyrosine phosphatase gene, shows a significantly reduced lysogenization. It is suggested that phosphorylation of Int by Wzc down regulates the activity of Int.

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Introduction

The integrase (Int) protein of the temperate coliphage HK022 (Int-HK022) catalyses the site-specific integration and excision of the bacteriophage in the lysogenic cycle. The mechanism of the site-specific recombination system of phage HK022 is similar, if not identical, to its close relative coliphage λ . Their *int* genes share a 70 percent homology and each is preceded by an *int* promoter (Weisberg et al., 1999; Yagil et al., 1989). In λ the transcription of *int* is regulated by the CII and CIII proteins, CII is a transcriptional modulator that stimulates the transcription of Int- λ and of the CI repressor (Shimatake and Rosenberg, 1981). The CIII protein stabilizes CII thereby promoting the lysogenic pathway (Altuvia and Oppenheim, 1986). The many similarities between the regulatory elements of λ and HK022 indicate that the transcriptional regulation of the *int* gene of HK022 is identical to that of λ . These also

include the promoters of Int, terminators and RNA processing sites *sib/tI* (Weisberg et al., 1999).

Post-translational phosphorylation and dephosphorylation of proteins by protein kinases and protein phosphatases play important roles in regulatory processes in the eukarya (Mukherji, 2005; Pawson and Scott, 2005). The hydroxyl amino acids serine, threonine and tyrosine are the substrates of phosphorylation and dephosphorylation. Protein kinases and phosphatases were later discovered in bacteria, however, their biological role is much less clear (Kennelly, 2002; Grangeasse et al., 2007). Wzc/Wzb is a pair of protein tyrosine kinase/phosphatase, respectively, that was characterized in *Escherichia coli* strain K12; they play a role in the production of colanic acid (Grangeasse et al., 2002; Doublet et al., 2002). Etk/Etp is homologous to Wzc/Wzb which was characterized in a pathogenic strain of *E. coli*; it is involved in the production of group 4 capsular exopolysaccharides (Ilan et al., 1999). Both kinases are autocatalytic enzymes, as they catalyze the phosphorylation of their own tyrosine residues. Few substrates that can be phosphorylated by these kinases are known. The autophosphorylated Wzc protein is able to transphosphorylate UDP-glucose dehydrogenase, an enzyme that is involved in the metabolism of acidic

Abbreviations: Int, integrase; IPTG, β -D-1-thiogalactopyranoside; anti-Ph-Tyr, monoclonal antibody against phospho-tyrosine.

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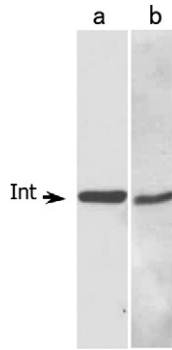


Fig. 1. Immunoblots of purified Int treated with anti-Int antibodies (lane a) and with anti-Ph-Tyr antibodies (lane b).

polysaccharides and colanic acid (Grangeasse et al., 2003). The autophosphorylated Etk can transphosphorylate RpoH and RseA, a sigma factor and an antisigma factor, respectively, that regulate the transcription of heat shock genes (Klein et al., 2003).

The presence of genes that encode protein kinases and phosphatases has also been reported in bacteriophages of the lambdoid family. Phage λ and some of its relatives encode a protein phosphatase (Cohen and Cohen, 1989), and phage 933W, which resides in a pathogenic *E. coli* strain, encodes an autophosphorylating tyrosine protein kinase (Stk) that can transphosphorylate tyrosine residues of artificial substrates (Tyler and Friedman, 2004). Coliphage T7 carries a serine/threonine protein kinase (Fp0.7) that can phosphorylate the β' subunit of the RNA polymerase of its host (Severinova and Severinov, 2006).

In the present work we show that the Int proteins of phages HK022 and λ are phosphorylated in tyrosine residues and that the active Tyr³⁴² residue of Int-HK022 can be transphosphorylated by the Wzc kinase and dephosphorylated by the Wzb phosphatase. The effect of the transphosphorylation of Int by Wzc on site-specific recombination reactions has been examined.

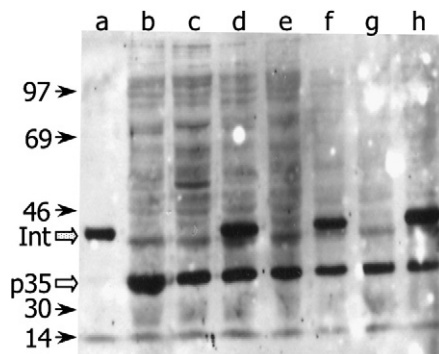


Fig. 2. Immunoblot of cell extracts of Int-overexpressing strains treated with anti-Ph-Tyr. Lane a — purified Int; lane b — induced cells with vector (pT7-3) alone; lanes c, d — uninduced and induced cells that over express the wild type Int-HK022; lane e, f — uninduced and induced cells that over express the Int-HK022 Y342F mutant; lanes g, h — uninduced and induced cells that over express Int- λ . Numbers show molecular weight markers in kDa.

Results

Int-HK022 and *Int*- λ are phosphorylated in vivo

Int protein, purified from cells that express it from a plasmid, was immunodetected by the antibody, raised against Int (anti-Int) (Fig. 1, lane a) as well as by monoclonal antibody against phospho-tyrosine (anti-Ph-Tyr) (Fig. 1, lane b). The immunodetection has indicated that Int is phosphorylated in one or more of its tyrosine residues. To gain further insight into this modification, extracts of *E. coli* cells that express different isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible plasmid-borne *int* genes were analyzed by an anti-Ph-Tyr immunoassay (Fig. 2). The induced Int-HK022 strain, shows a clear band that is identical to the purified Int (Fig. 2, lanes a and d, dotted arrow).

Int belongs to the tyrosine family of site specific recombinases and its Tyr³⁴² residue is the active amino acid and is conserved within the entire family (Azaro and Landy, 2001). We have tested if this residue might be the only one that is phosphorylated. Lanes e and f in Fig. 2 show, respectively, extracts of uninduced and induced cells that overexpress an inactive Int-HK022 mutant whose Tyr³⁴² residue has been mutated to Phe (the IntF mutant). The results indicated that both the wild type and Y342F mutant forms of Int-HK022 contained comparable levels of phosphorylated Tyr. Thus, in Int-HK022, Tyr residues other than Tyr³⁴² were phosphorylated although the possibility that Tyr³⁴² is also subject to phosphorylation was not excluded. Since the Int proteins of phages HK022 and λ are closely related, the induced wild type Int of λ is, as expected, likewise phosphorylated (Fig. 2 lanes g, h). In addition to Int, all cell extracts in Fig. 2 show a background of several other phosphorylated proteins that could include the 79.3 kDa *E. coli* Wzc protein tyrosine kinase. The identity of the prominent cellular

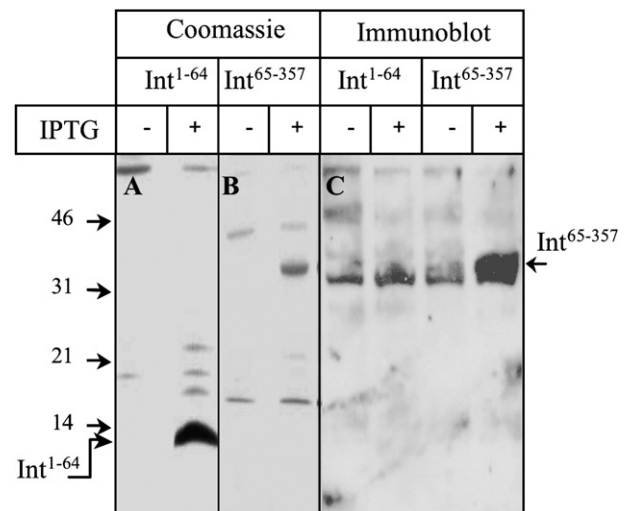


Fig. 3. A. Coomassie-stained gel of cell extracts that have overexpressed uninduced and IPTG-induced Int¹⁻⁶⁴. B. Coomassie-stained gel of cell extracts that have overexpressed uninduced and induced Int⁶⁵⁻³⁵⁷. C. Immunoblot of the cell extracts shown in A and B treated with anti-Ph-Tyr. Numbers show molecular weight markers in kDa.

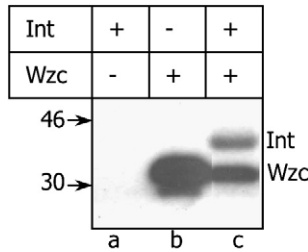


Fig. 4. Transphosphorylation of purified Int by purified Wzc's kinase domain in the presence of ATP (γP^{32}). Lane a — Int alone; lane b — Wzc's kinase domain alone; lane c — Int+Wzc. Numbers show molecular weight markers in kDa.

phospho-Tyr protein of ~ 34 kDa, also observed in all lanes (Fig. 2, open arrow), was not determined.

The arm-binding domain of Int is not phosphorylated

Amino terminal residues 1–55 of Int-HK022 and Int- λ are completely identical and are known to carry the domain of Int that binds tightly to the arms of the recombination (*att*) sites. In Int-HK022 this domain holds four Tyr residues and it has been shown that in λ this domain folds and functions autonomously (Biswas et al., 2005; Sarkar et al., 2001). The sequence variability between the two Ints resides in the remaining carboxyl terminal residues. In Int- λ , these C-terminal residues are separated from the arm binding domain (residues 1–64) by a short linker (residues 65–74) and its C-terminal peptide is composed of a core binding domain (residues 75–169) and a catalytic domain (residues 170–357) (Sarkar et al., 2002). In Int-HK022 this carboxyl-terminal peptide, carries eight Tyr residues. We have cloned and expressed the N-terminal arm-binding peptide of Int-HK022 (residues 1–64, Fig. 3A), as well as the remaining C-terminal portion of Int (residues 65–357, Fig. 3B). Coomassie-stained extracts of *E. coli* cells transformed with each of these clones (Figs. 3A, B) and a corresponding Ph-Tyr immunodetection of these extracts (Fig. 3C) show that none of the Tyr residues that belong to the 1–64 N-terminal peptide of Int is phosphorylated, and that one or more of the remaining 8 Tyr residues in the remaining C-terminal portion of Int is phosphorylated. The molecular weight of the 65–357 peptide is 34.8 kDa and we assume that the slightly smaller band that appears in all four lanes is the unidentified ~ 34 kDa phosphorylated protein also seen in Fig. 2.

Wzc transphosphorylates Int and Wzb dephosphorylates it

Wzc and Wzb are, to date, the best characterized protein Tyr kinase/phosphatase pair in *E. coli*-K12. Wzc is an autocatalytic enzyme, that can transphosphorylate UDP-glucose dehydrogenase, and Wzb can dephosphorylate it (Grangeasse et al., 2003). To test if Int can likewise be a substrate of this protein kinase/phosphatase pair, we have cloned, overexpressed and purified the C-terminal domain of Wzc, known to carry the autophosphorylation and transphosphorylation activities (Grangeasse et al., 2002). Likewise, we have cloned, overexpressed and purified the Wzb protein phosphatase. Next, we have incubated a mixture of Int-HK022 and the Wzc kinase domain

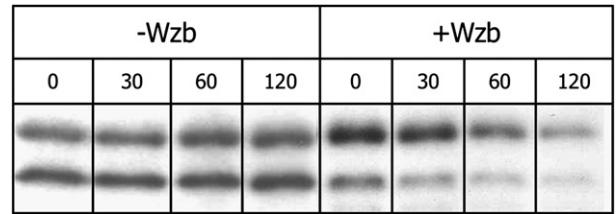


Fig. 5. Dephosphorylation of purified Int and Wzc by Wzb phosphatase. Numbers indicate minutes of incubation time.

and each protein alone with radioactive ATP (γP^{32}). The treated proteins were subjected to gel electrophoresis and an autoradiogram shows that Int alone in the presence of ATP (γP^{32}) is not labeled (Fig. 4, lane a), indicating that it has no autophosphorylation activity. As expected, Wzc alone was autophosphorylated (lane b), and when the mixed proteins were incubated with ATP (γP^{32}) both were labeled (lane c), indicating that Int had been transphosphorylated by Wzc. The mixture of both labeled proteins (Fig. 4, lane c) was treated with purified Wzb for different times (Fig. 5). In the absence of Wzb, both proteins remained stable whereas in the presence of Wzb they were dephosphorylated, though the dephosphorylation of Int was less extensive than that of Wzc. Next, each protein was degraded by a total acid hydrolysis, and the products were separated by a two-dimensional thin-layer electrophoresis along with unlabeled Phospho-Tyr, Phospho-Ser and Phospho-Thr that were used as stained markers (dotted circles in Fig. 6). As

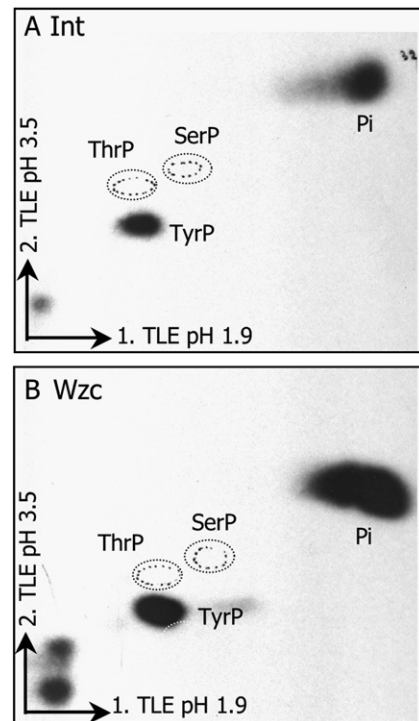


Fig. 6. 2D chromatography of transphosphorylated Int (A) and autophosphorylated Wzc (B), both digested to complete hydrolysis. Dotted circles show the location of the phospho-amino acids. Arrows indicate direction of migration during the thin-layer electrophoresis (TLE).

expected, the autoradiograms of these gels have revealed that both Int (Fig. 6A) as well as Wzc (Fig. 6B) were only labeled in Tyr.

Int phosphorylation *in vivo* does not require Wzc

In order to test the role of Wzc in the phosphorylation of Int *in vivo*, an extract of a *wzc::Km* mutant strain (EY1752) of *E. coli* that was transformed with the inducible Int-HK022 expressing plasmid underwent a Ph-Tyr immunodetection (Fig. 7, lane c). The extent of phosphorylation of the induced Int in the mutant, is similar to that of Int induced in the *wzc*⁺ wild type (compare to Fig. 2, lane d). Thus, if Wzc does phosphorylate overexpressed Int *in vivo*, it is not the only protein kinase that can do so.

In vivo, Tyr³⁴² is not the major phosphorylated residue of Int

Our observation that the IntF mutant protein (Y342F) has reacted with anti-Ph-Tyr (Fig. 2, lane f) indicates that if the active site Tyr³⁴² is phosphorylated *in vivo*, it is not the only one phosphorylated. A comparison of the Wzc-mediated transphosphorylation reaction on identical amounts of overexpressed wild type Int and IntF extracts (Fig. 8) has revealed that the IntF protein was poorly transphosphorylated by the Wzc kinase domain. This indicates that Tyr³⁴² is not phosphorylated in wild type Int *in vivo* because it is the major residue that is transphosphorylated by Wzc *in vitro*. If it would be phosphorylated it would not be an efficient substrate for Wzc-catalyzed transphosphorylation *in vitro*.

Overexpression of Wzc *in vivo* reduces Int activity

To test if the transphosphorylation of Int by Wzc has any effect on the ability of Int to catalyze site-specific recombination, we have purified Int from cells that have overexpressed Int, but still carry the chromosomal *wzc* gene (Fig. 9A lane a), and from cells that have overexpressed Int and the Wzc kinase domain, carried on two compatible expression vectors (Fig. 9A, lane d). The enzymatic activity of each purified Int protein was tested in an *in vitro* integration reaction using identical declining

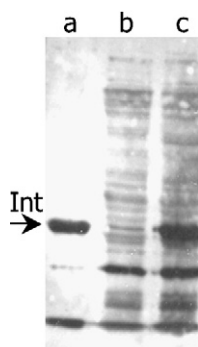


Fig. 7. Anti-Ph-Tyr immunoblots of cell extracts of a *wzc::Km* mutant strain transformed with the Int-overexpressing plasmid. Purified Int (lane a), uninduced cells (lane b), IPTG induced cells (lane c).

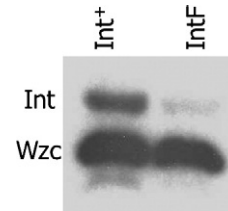


Fig. 8. Transphosphorylation by Wzc of wild type Int and mutant IntF.

amounts of each purified protein. The results (Figs. 9B and C) have revealed that Int generated in the presence of excess Wzc was less active in all concentrations tested, the difference becoming more apparent as the equal concentrations Int decreased (Fig. 9C and insert); at 0.15 $\mu\text{g/ml}$ of each protein the Int generated in the presence of Wzc showed less than 30% activity than did Int purified in the absence Wzc. Assuming that the overexpressed Wzc kinase domain transphosphorylates Int in excess also *in vivo*, it is likely that the Int purified from cells that have overexpressed both proteins is composed of a heterogeneous Int population with a larger ratio of phosphorylated/unphosphorylated Tyr³⁴². In the higher concentrations the active (unphosphorylated) form is able to catalyze the recombination almost like the control, whereas the effect of the inactive (phosphorylated) form becomes more apparent in the lower concentrations of Int.

Attempts to compare the ability of the wild type HK022 to form lysogens in wild type cells, Wzc overexpressing cells and the *wzc::Tn5* mutant did not show any differences (not shown). However, phage λ is known to carry a gene (*orf221*) that encodes a protein phosphatase (Cohen and Cohen, 1989) that is capable to hydrolyze phosphotyrosin-containing protein substrates (Zhuo et al., 1993), and an analogous gene might exist in HK022 as well (see Discussion). We have speculated that dephosphorylation of Int by this PPase may account for our failure to observe any effect of Wzc on HK022 lysogenization *in vivo*. In λ , the *nin5* deletion covers the gene that encodes its PPase (Daniels et al., 1983). Therefore, we have tested if the overexpression of the Wzc kinase domain has a specific effect on the ability of the λ *nin5* mutant to form lysogens. Fig. 10 shows that the lysogenization of the wild type strain (Y1) of λ is not affected by the overexpression of Wzc whereas its isogenic *nin5* mutant (Y1090) shows a 5–6 fold reduction in its ability to lysogenize a host that overexpresses Wzc. With the assumption that the overexpressed Wzc partially transphosphorylates the active Tyr³⁴² of Int we interpret these results to mean that in the absence of the counteracting protein phosphatase this transphosphorylated Int is inactive in lysogenization.

Discussion

Evidence was presented that the Int-HK022 and Int- λ proteins, when expressed in *E. coli*, are phosphorylated in Tyr residues (Figs. 1–2). In Int-HK022 one or more of its seven Tyr residues located between positions 65 and 346 are phosphorylated *in vivo* (Fig. 3). The catalytic Tyr³⁴² is only partially or not at all phosphorylated because it can be transphosphorylated by Wzc *in vitro* whereas the *intF* mutant is poorly transphosphorylated by Wzc

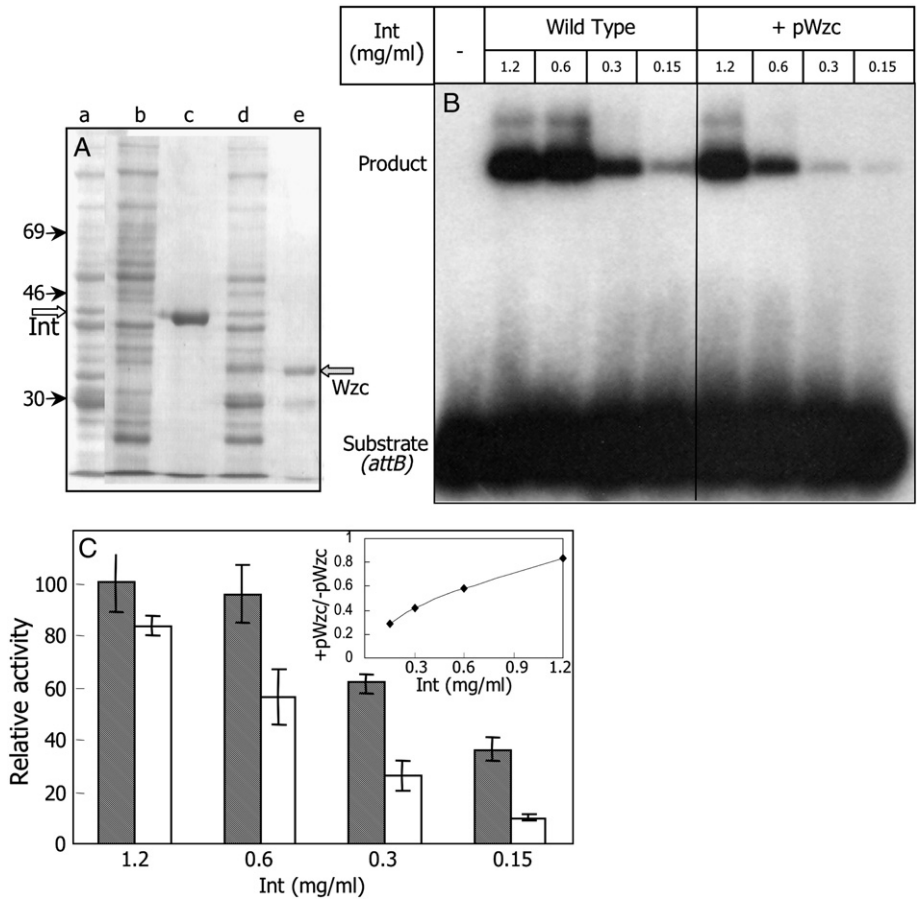


Fig. 9. A. Coomassie-stained gel of induced cells extracts. Lane a — extract of cells overexpressing Int; lane b — extract of cells carrying the cloning vector; lane c — purified Int; lane d — extract of cells overexpressing Int and Wzc’s kinase domain; lane e — purified Wzc’s kinase domain. B. *In vitro* site-specific recombination reactions at decreasing concentrations of Int purified from a wild type host (left) and of Int purified from a Wzc’s kinase domain-overexpressing host (right). C. A histogram of the relative activities of each of the Int used. Hatched bars — Int purified from the wild type host; blank bars — Int purified from the Wzc-expressing host. The insert shows the relative activity of the latter Int as a function of the protein concentration. Numbers in (A) show molecular weight markers in kDa. Data are average of three experiments.

(Fig. 8). Wzc is not required for the phosphorylation of the other Tyr residues that reside in the C-terminal core binding/catalytic domains of Int (Fig. 7). Apparently one or more yet uncharacterized tyrosine protein kinases of *E. coli* must be involved. Indeed, the presence of several additional phospho-Tyr proteins has been observed in the wild type (Fig. 2), as well as in the *wzc::Km* mutant (Fig. 7); these could include additional autocatalytic kinases.

Though most experiments reported here were done with Int of HK022, due to the close similarities of the site-specific recombination mechanisms between phages HK022 and λ it is reasonable to assume that Int of λ shares the same properties.

Does the phosphorylation regulate Int activity? We do not know yet whether the Tyr residues other than Tyr³⁴² play any role in regulation. Purified Int-HK022 from a Wzc overexpressing host showed a significantly reduced site-specific recombination activity *in vitro* (Fig. 9), presumably because of the partial phosphorylation of its active site Tyr³⁴² residue. However, we cannot rule out the possibility that the overexpression of Wzc hinders Int activity in a different manner. Moreover, the experiments were performed under overexpression conditions of Int and only the kinase domain of Wzc which

may not necessarily reflect normal physiology. Nevertheless, the reduced ability of the λ *nin5* mutant, whose protein phosphatase gene is missing, to form lysogens in the Wzc-overexpressing strain (Fig. 10) supports the notion that the host-encoded post-translational phosphorylation of Int inhibits its activity, but may be counteracted by the phage-encoded protein

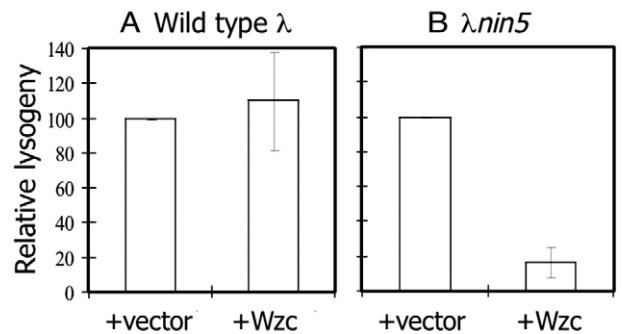


Fig. 10. Histogram showing percent of lysogenization of *E. coli* (strain TAP114) transformed with the Wzc expressing plasmid (pMK472) infected by wild type λ (A) and by λ *nin5* (B) relative to the lysogenization of *E. coli* transformed with the cloning vector (pQE80). Data are average of three experiments.

SVLFFVCLGNICRSP- (91) -DIEDP	Low MW consensus PTP
RKLWACLGDVSRQV- (82) -RWGDR	NinB of HK022

Fig. 11. Alignment between the consensus sequence of a low MW PTP motif (Shi et al., 1998) with the sequence of phage HK022 protein NinB (NP_597901). : — identical residues; + — similar residues.

phosphatase. Earlier studies have shown that the *byp* mutation of λ is located downstream of $\Delta nin5$ but is still located within the open reading frame of the protein phosphatase [*orf221*, (Daniels et al., 1983)]. This mutation causes the inactivation of the phosphatase (Barik, 1993). An earlier observation that the *byp* mutation also causes a 3–5 folds reduction in λ lysogenization supports our interpretation (Sternberg and Enquist, 1979). However, it still remains unclear why the $\Delta nin5$ mutant showed the reduced lysogeny only when Wzc was overexpressed (Fig. 10). The host-encoded Wzb phosphatase may also play a role, but according to our results its activity on Int was rather weak (Fig. 5). In a similar case, the *in vitro* phosphorylation of RpoH by Etk inhibits its ability to transcribe heat-shock genes and *in vivo* a mutation in its cognate protein phosphatase Etp does the same, presumably due to the excess phosphorylation of RpoH (Klein et al., 2003). No protein phosphatase gene has yet been identified in HK022. However, its gene designated *ninB* (position 33876–34316, NCBI access number NP_597901) includes a sequence that resembles the consensus motif of low MW prokaryotic protein phosphatases (Shi et al., 1998) (Fig. 11).

The lysogenization process of temperate phages seems to be of benefit for the survival of both the host and the phage; the counteracting phosphorylation activity of the host vs. the dephosphorylation activity of the phage may control balanced lysogenization rate that is beneficial for both organisms. Another example of phage-host interaction through protein phosphorylation is the protein kinase (0.7) encoded by phage T7. This phage encodes its own RNA polymerase and, upon infection, it destabilizes the host RNA polymerase by phosphorylating its β' subunit (Severinova and Severinov, 2006). In the eukaryotes protein kinases and phosphatases play important regulatory functions in antagonist virus-host interactions. These include regulation of mRNA translation in animal and human viruses that are dependent on the protein synthesis machinery (Mohr, 2006) as well as the regulation cell to cell viral trafficking in plants (Lee and Lucas, 2001).

Materials and methods

E. coli strains and growth conditions

E. coli strain DH5 α (Fermentas) was used to propagate plasmids in cloning experiments and as host for the induction and overexpression of proteins Wzc and Wzb. Strain BL21 (DE3) (Novagen) was used to overexpress Int and IntF. Strain ER06 is a derivative of strain MG1655 (Blattner et al., 1997); it is lysogenic for the defective $\lambda cI857 \Delta(cro-bioA)$ prophage and was used for recombineering (Yu et al., 2000). Strain EY1752 is an ER06 *wzc::Km* derivative constructed by recombineering. Strain NS833 is BL21(DE3) *wzc::Km* constructed by P1 transduction from strain EY1752. Strain TAP114 is $\Delta(lacZ)M15$ (Dorgai et al., 1995); it

was used as host for HK022 and λ growth. Cells were grown in rich Luria–Bertani (LB) media at 37 °C with the proper antibiotic. For protein induction logarithmic cells at a density of $A_{600}=0.4$ were treated with 0.1 mM β -D-1-thiogalactopyranoside (IPTG) and grown for two more hours prior to harvest.

Phage strains

The wild type HK022 strain is OR228 (Yagil et al., 1989), strain Y1 of λ is *cI*⁸⁵⁷ (Roberts and Devoret, 1983) and Y1090 is *nin5 cI*⁸⁵⁷ (Daniels et al., 1983). Strain W248 is λh - $\phi 80 attP$ - $\phi 80 \Delta 9[att-red]$ *imm λ c* (Yagil et al., 1989).

Plasmids

Plasmids are listed in Table 1.

Cell extracts

To obtain clear lysates of cells extract bacterial pellets were resuspended in 10 mM Tris buffer pH8, 1 mM EDTA, followed by adding one fourth volume of a 4 \times electrophoresis Laemmli sample buffer (Sambrook et al., 1989).

Construction of the Wzc and Wzb expressing plasmids

These were performed exactly as described in (Vincent et al., 1999; Vincent et al., 2000). In the case of *wzc*, only its C-terminal kinase domain (residues 447–720) was cloned.

Construction of the HK022 IntF (Y342F) mutant expression plasmid

The *intY342F* mutation was constructed by three PCRs using plasmid pKH70 (Kolot et al., 1999) as template as described (Higuchi et al., 1988).

Table 1
List of plasmids

Name	Relevant genotype	Source
pACYC234	Expression vector	(Peleg et al., 2005)
pETI-1	Expression vector	(Gottfried et al., 2003)
pGEM-T Easy	Cloning vector	Promega
pMK129	IntF expressing on pETI-1	This work
pMK60	Int ^{65–357} expressing plasmid	(Gottfried et al., 2003)
pNR169	Int- λ expressing plasmid	(Nagaraja and Weisberg, 1990)
pNR69	Int-HK022 expressing plasmid	(Yagil et al., 1989)
pNS472	Wzc's kinase domain expressing on pQE80	This work
pNS607	<i>wzc::Km</i> on pGEM-T Easy	This work
pNS493	Wzb expressing on pQE80	This work
pNS507	Wzc's kinase domain expressing on pACYC234	This work
pQE80	Expression vector	Qiagen
pT7-3	Expression vector	(Tabor and Richardson, 1985)
pVB262	Int ^{1–64} expressing plasmid	(Gottfried et al., 2003)

Protein purification

Wzc and Wzb proteins were purified from an (IPTG)-induced cultures of *E. coli* transformed with plasmids pNS472 or pNS493 on a nickel column resin according to the Novagen pET system manual.

The C-terminal Int^{65–357} subunit was purified as described (Gottfried et al., 2003). HK022 wild type Int and the N-terminal Int^{1–64} peptide (Gottfried et al., 2003) were purified using phosphocellulose column according to Nash (Nash, 1983).

Analysis of the phosphoamino acid content of proteins

Acid hydrolysis of immobilized proteins on PVDF membranes was carried out as described previously (Kamps and Sefton, 1989). Two-Dimensional Thin-Layer Electrophoresis (TLE) was carried out using the Hunter thin-layer electrophoresis system (HTLE-7000) as described (Boyle et al., 1991).

Immunoblots

These were done as previously described using polyclonal Int antiserum (Kolot et al., 1999) or Phospho-Tyrosine monoclonal antibody P-Tyr-100 (New England Biolabs).

Phosphorylation and dephosphorylation assays

Assays were performed as described for UDP-glucose dehydrogenase (Grangeasse et al., 2003).

In vivo site-specific recombination reactions

Reactions were performed as previously described (Kolot and Yagil, 1994).

Construction of the *wzc::Km* mutant

The mutant was constructed by the recombineering method (Yu et al., 2000) using strain ER206 as host. Plasmid pNS607, that served as template, carries a Km cassette from plasmid pUC4-K (Vieira and Messing, 1982) inserted into the NruI site of the catalytic C-terminal domain of *wzc* gene on a plasmid. The *wzc::Km* mutation was confirmed by DNA sequencing.

Lysogenization

Lysogens were selected by infecting IPTG-induced logarithmic cells with the relevant phage at a multiplicity of infection of ~5. Following 15 minutes incubation at room temperature the infected cells were diluted and plated on LB plates spread earlier with ~10⁹ clear-plaque forming λ phage strain W248 (Yagil et al., 1989). Cells were plated overnight at 30 °C for single colony growth. Control cells were similarly treated but plated without phage W248. Lysogeny was calculated as percent of survivors on the selective plates. HK022 lysogens are selected this way because its Nun protein protects lysogen killing by λ (Oberto et al., 1989).

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