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The effect of mutations in the Xis-binding sites on site-specific recombination in coliphage HK022

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Abstract Excisionase (Xis) is an accessory protein that is required for the excision of the related prophages λ and HK022. Xis binds to two tandemly arranged binding sites (X1 and X2) on the P arm of the recombination sites *attP* and *attR*. Gel-retardation analyses and site-specific recombination assays were conducted on derivatives bearing site-directed mutations in the X1 and X2 sites of phage HK022. The results confirm the cooperative binding of Xis to its sites, showing that binding to X1 stimulates further binding to X2. The results also show that mutants affected in a single site are inactive in excision, whereas mutants affected in both sites, which show a complete absence of Xis binding, display significant excision activity. This restored activity is attributed to the interaction of Xis with Integrase, the protein that catalyzes the site-specific recombination reaction.

Keywords Site-specific recombination · Excisionase · Bacteriophage HK022

Introduction

In their lysogenic cycle, coliphages λ and its close relative, phage HK022, insert their circularized chromosome into the genome of the *Escherichia coli* host by a process of site-specific recombination between the *attP* site of the phage and the *attB* site of the host. Due to the difference in structure between these two *att* sites the resulting prophage is flanked by the recombinant sites *attL* and *attR*, which serve as sites for the reverse reac-

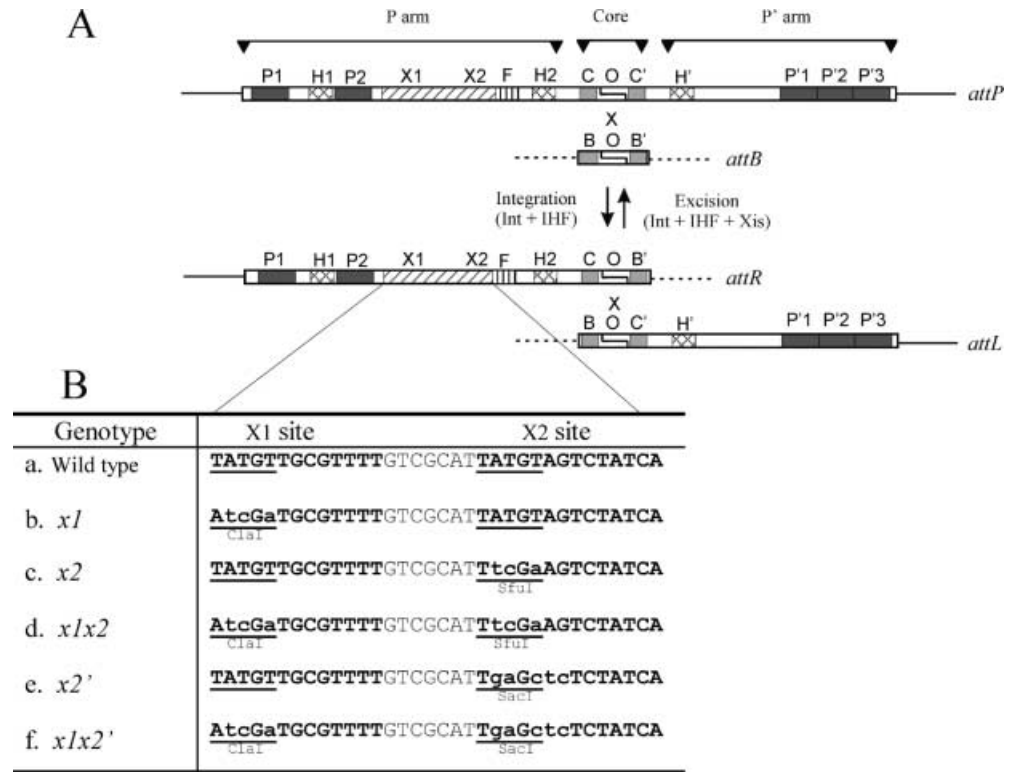
tion that excises the prophage (Fig. 1A). The phage-encoded Integrase (Int) protein catalyzes both integration and excision reactions and requires the assistance of several accessory proteins. The host-encoded Integration Host Factor (IHF) is required for integration and excision, and the phage-encoded Excisionase (Xis) is required for excision only. A third host-encoded protein, Fis, can assist Xis but cannot replace it (Thompson et al. 1987). These accessory proteins are DNA-bending proteins (Thompson and Landy 1988). The bacterial site *attB* is simple: it is composed of 21 bp, of which the central 7 bp form the site of recombination known as the overlap (O). The remaining 7 bp on each side of the overlap (B and B') are inverted repeats that serve as weak binding sites for Int. BOB' is defined as the core. The phage *attP* site is much longer; it is composed of a core (COC') that is similar to *attB* and is flanked by two longer arms, P and P' that carry tight binding sites for Int and IHF. In addition, the P arm carries two tandemly arranged binding sites for Xis (X1 and X2) and one binding site for Fis that overlaps X2 (Fig. 1) (reviewed by Landy 1989, and by Nash 1996).

Phages λ and HK022 are closely related and their mechanisms of site-specific recombination are practically identical. Each phage integrates into a different *attB* site on the *E. coli* chromosome. The Int proteins and the *att* sites of both phages share a strong homology but are specific, i.e. each Int can recognize only its cognate *att* sites. However, the Xis proteins of the two phages are practically identical, they differ only in one amino acid and they are interchangeable. The Xis binding sites are also very similar in both phages (Yagil et al. 1989; Weisberg et al. 1999; Gottfried et al. 2000). In λ , this small accessory protein (72 amino acids long) binds in a cooperative fashion to its sites (X1 and X2) on the P arm of *attR* (Bushman et al. 1984). Xis also interacts with Fis and with Int. Its N-terminal portion carries a domain for DNA binding and interaction with Fis, and the C-terminal portion is responsible for interaction with Int (Wu et al. 1998). In the present study we have constructed site-directed mutations in the

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Fig. 1 **A** Schematic representation of the *att* sites involved in the site-specific recombination of bacteriophages HK022 and λ . COC', BOB', COB', and BOC' are the core sites of *attP*, *attB*, *attR*, and *attL*, respectively. P indicates arm-binding sites for Int, X for Xis, H for IHF and F for Fis. **B** Sequences of the two Xis binding sites of HK022 (X1 and X2, line a) and the various mutants described in the text (lines b–f)



X1 and X2 binding sites of phage HK022 and studied their effect on Xis binding and on site-specific recombination.

Materials and methods

Strains, plasmids and oligomers

Bacteria, phage strains and previously described plasmids used in this study are listed in Table 1. Plasmids that were constructed in the course of this study and details of their construction are specified in Tables 2 and 3.

Site-directed mutagenesis and plasmid construction

Plasmids with mutations in the X sites were each constructed by two PCRs, each using one primer with a mutated sequence

together with one universal primer (forward or reverse) for the vector pUC18 as specified in Table 2. The mutagenic primers used for any single mutation were complementary. The PCR products and the vector were then digested with the restriction enzymes indicated in Table 2, mixed, ligated to the vector specified in the Table and transformed into competent *E. coli* cells. All single and double mutants were confirmed by DNA sequencing. The mutated fragments were then transferred by restriction, fragment isolation and ligation into other suitably digested vectors as specified in Table 3. The primers used are listed in Table 4.

Gel-retardation assay

Gel-retardation assays were performed as described previously (Gottfried et al. 2000) using 4 nM ³²P-labeled *attR* and 0.1–0.8 μM Xis as described in the Results.

Table 1 Bacterial strains, plasmids and bacteriophages used

Strain/plasmid	Relevant genotype	Source
Bacterial strains		
BL21(DE3)	Host for T7 promoter expression vectors	Studier and Moffat (1986)
TAP114	$\Delta(lacZ)M15$	Dorgai et al. (1995)
Plasmids		
pBluescript	Ap ^R , cloning vector	Stratagene
pKY110	<i>attP</i> in pEMBL19	Kolot et al. (1996)
pLD46	vector used to construct pLD205 derivatives	Dorgai et al. (1995)
pLD205	<i>attR-t1t2-attL</i> clone in pLD46	Dorgai et al. (1995)
pMK24	<i>attR</i> in pUC18	Gottfried et al. (2000)
pNR69	<i>int</i> under control of the T7 promoter	Yagil et al. (1989)
pPG1	<i>xis</i> under control of the T7 promoter	Gottfried et al. (2000)
pUC18	Ap ^R , cloning vector	Yanisch-Perron et al. (1985)
Bacteriophage		
OR228	HK022 wild type	Yagil et al. (1989)

Table 2 Plasmids constructed using site-directed mutagenesis by PCR

Plasmid	Cloned site(s) ^a	Mutation	Template	Primers and restriction enzymes used ^b	Vector (insertion site)
pPG101	<i>attP</i>	<i>x1</i>	pKY110	f + oEY137 (<i>EcoRI</i> + <i>ClaI</i>); r + oEY138 (<i>HindIII</i> + <i>ClaI</i>)	pUC18 (<i>EcoRI</i> / <i>HindIII</i>)
pPG116	<i>attP</i>	<i>x2</i>	pKY110	f + oEY139 (<i>EcoRI</i> + <i>SfuI</i>); r + oEY140 (<i>HindIII</i> + <i>SfuI</i>)	pUC18 (<i>EcoRI</i> / <i>HindIII</i>)
pPG102	<i>attP</i>	<i>x1x2</i>	pPG101	f + oEY139 (<i>EcoRI</i> + <i>SfuI</i>); r + oEY140 (<i>HindIII</i> + <i>SfuI</i>)	pUC18 (<i>EcoRI</i> / <i>HindIII</i>)
pPG125	<i>attR-t1t2-attL</i>	<i>x1</i>	pPG123	f + oEY138 (<i>BamHI</i> + <i>ClaI</i>); r + oEY137 (<i>BamHI</i> + <i>ClaI</i>)	pBluescript <i>BamHI</i>
pPG126	<i>attR-t1t2- attL</i>	<i>x2</i>	pPG123	f + oEY140 (<i>BamHI</i> + <i>SfuI</i>); r + oEY139 (<i>BamHI</i> + <i>SfuI</i>)	pBluescript (<i>BamHI</i>)
pPG127	<i>attR-t1t2-attL</i>	<i>x1x2</i>	pPG125	f + pEY140 (<i>BamHI</i> + <i>SfuI</i>); r + pEY139 (<i>BamHI</i> + <i>SfuI</i>)	pBluescript (<i>BamHI</i>)
pPG142	<i>attR-t1t2-attL</i>	<i>x2'</i>	pPG123	f + oEY151 (<i>BamHI</i> + <i>SacI</i>); r + oEY150 (<i>BamHI</i> + <i>SacI</i>)	pBluescript (<i>BamHI</i>)
pPG143	<i>attR-t1t2-attL</i>	<i>x1x2'</i>	pPG125	r + oEY150 (<i>BamHI</i> + <i>SacI</i>)	pBluescript (<i>BamHI</i>)

^a *t1t2* indicates the *rrnB t1* and *t2* terminators (Dorgai et al. 1995)

^b Listed are the pairs of primers used for each of the two PCRs (see Table 4). f and r are the universal M13 forward and reverse primers, respectively (Perkin Elmer)

Table 3 Plasmids constructed by ligation only

Plasmid	Cloned site(s) ^a	Mutation	Fragment(s) inserted	Vector [insertion site(s)]
pET14m ^b	His-tag	None	Fragment formed by annealing of oEY115 and oEY116	pET14b (<i>NcoI</i> / <i>NdeI</i>)
pPG15	<i>xis</i>	None	<i>NdeI-XhoI xis</i> fragment of pPG1	pET14m (<i>NdeI/XhoI</i>)
pPG107	<i>attR</i>	<i>x1</i>	<i>HindIII-AseI</i> fragment of pPG101 and <i>AseI-EcoRI</i> fragment of pMK24	pUC18 (<i>HindIII/EcoRI</i>)
pPG109	<i>attR</i>	<i>x1x2</i>	<i>HindIII-AseI</i> fragment of pPG102 and <i>AseI-EcoRI</i> fragment of pMK24	pUC18 (<i>HindIII/EcoRI</i>)
pPG113	<i>attR</i>	<i>x2</i>	<i>HindIII-AseI</i> fragment of pPG116 and <i>AseI-EcoRI</i> fragment of pMK24	pUC18 (<i>HindIII/EcoRI</i>)
pPG123	<i>attR-t1t2-attL</i>	None	<i>BamHI</i> fragment of pLD205	pBluescript (<i>BamHI</i>)
pPG128	<i>attR-t1t2-attL</i>	<i>x2</i>	<i>BamHI</i> fragment of pPG126	pLD46 (<i>BamHI</i>)
pPG131	<i>attR-t1t2-attL</i>	<i>x1x2</i>	<i>BamHI</i> fragment of pPG127	pLD46 (<i>BamHI</i>)
pPG132	<i>attR-t1t2-attL</i>	<i>x1</i>	<i>BamHI</i> fragment of pPG125	pLD46 (<i>BamHI</i>)
pPG153	<i>attR-t1t2-attL</i>	<i>x1x2'</i>	<i>BamHI</i> fragment of pPG143	pLD46 (<i>BamHI</i>)
pPG154	<i>attR-t1t2-attL</i>	<i>x2'</i>	<i>BamHI</i> fragment of pPG142	pLD46 (<i>BamHI</i>)
pPG186	<i>attR</i>	<i>x2'</i>	<i>BamHI-PstI</i> fragment of pPG142	pUC18 (<i>BamHI/PstI</i>)
pPG187	<i>attR</i>	<i>x1x2'</i>	<i>BamHI-PstI</i> fragment of pPG143	pUC18 (<i>BamHI/PstI</i>)

^a *t1t2* indicate the *rrnB t1* and *t2* terminators (Dorgai et al. 1995)

^b Plasmid pET14m contains six His codons in place of the *NcoI-NdeI* fragment of pET14b (Novagen)

Table 4 List of oligonucleotide primers used

Primer	Sequence (5'→3') ^a
oEY115	CATGGGCCATCATCATCATCA
oEY116	TATGATGATGATGATGATGGCC
oEY137	GACTGGA ^{atc} Ga ^{IGCGTTTTGTCGCATTATG} (<i>ClaI</i>)
oEY138	GCGACAAAACGCA ^{tCgatTCCAGTCACTATG} (<i>ClaI</i>)
oEY139	GTCGCATT ^{tc} Ga ^{AGTCTATCATTTAACCACAG} (<i>SfuI</i>)
oEY140	GGTAAATGATAGACT ^{tCgaAATGCGACAAAACG} (<i>SfuI</i>)
oEY150	GTCGCATT ^{gaGctcTCTATCATTTAACC} (<i>SacI</i>)
oEY151	GGTAAATGATAG ^{agCtcAATGCGAC} (<i>SacI</i>)

^aNewly created restriction sites (in *parentheses*) are *underlined* and introduced mutations are shown in *lower case*

Protein purification

Int-HK022 was purified according to Nash (1983). Xis was purified as described by Gottfried et al. (2000) from an IPTG-induced culture of strain BL(DE3)(pLys) transformed with plasmid pPG15. IHF was kindly given to us by C. Robertson and H. Nash.

In vitro site-specific recombination

In the in vitro excision assay the reaction mixture (10 µl) contained 50 nM KCl, 50 mM TRIS-HCl pH 7.5, 5 mM spermidine, 1 mM EDTA, 1 mg/ml BSA, 0.2 µM Xis, 0.1 µM IHF, 0.1 µM Int and the appropriate plasmid substrate at a concentration of 0.6 nM.

The reaction was incubated at room temperature for 10 min. Reaction products were cleaved with the restriction enzyme *XhoI*. The enzyme was inactivated by incubating for 10 min at 75°C, and the restriction fragments were labeled by filling-in with [α -³²P]dCTP and Klenow enzyme. The products were separated by electrophoresis on a 1% agarose gel and visualized by autoradiography. The in vitro integration assay was carried out as previously described (Kolot and Yagil 1994).

In vivo excision assay

Strain TAP114 was transformed with plasmid pPG123 and its derivatives mutated in the X sites, and each was infected with phage HK022, during the logarithmic phase of growth, at a moi=10. After 40 min the cells were sonicated and assayed for β -galactosidase activity (Miller 1972; Yagil et al. 1995).

Quantitative analyses

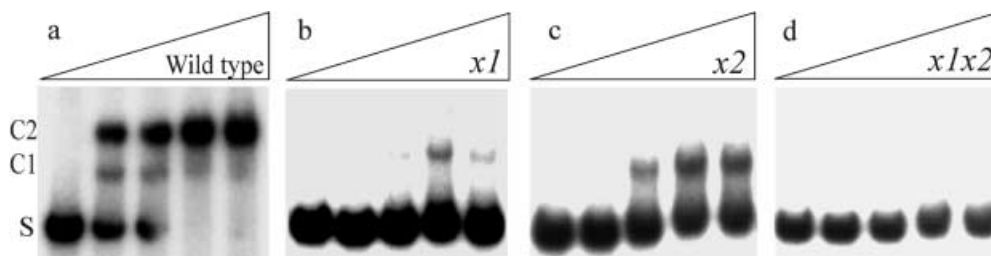
Autoradiographs were evaluated by exposure to a BAS1000 FUJI Phosphorimager screen, and the relative intensities of the bands were calculated using the TINA program package (version 2) from Rayset.

Results

Mutagenesis of the X sites

The positions of the two adjacent binding sites (X1 and X2) for Xis on the P arm of the phage are shown in Fig. 1A, and their sequences are (in bold face) in Fig. 1B, line a. Each site contains the sequence TATGT in its 5' end (underlined in Fig. 1B, line a). This sequence is conserved in λ , HK022 and phage 434 (Numrych et al. 1991). In order to determine the role of these binding sites in the process of site-specific recombination, we mutated them by site-directed mutagenesis and tested the ability of Xis to bind to the mutated substrates and mediate site-specific recombination. The mutations were designed to replace each of the conserved TATGT sequences with a specific restriction site. Three such arm site mutants were constructed: the mutation in the X1 site created a *ClaI* restriction site (*x1*, Fig. 1B, line b), the second was mutated in the X2 site, creating an *SfiI* site (*x2*, Fig. 1B, line c), and the double mutant *x1x2* combined both mutations (Fig. 1B, line d).

Fig. 2a–d Gel-retardation assay of Xis binding to a wild-type *attR* sequence, and to *attR* mutated in the X sites. The wedges represent increasing concentrations of Xis (0, 0.1 μ M, 0.2 μ M, 0.4 μ M and 0.8 μ M). S indicates the labeled substrates, C1 and C2 are Xis-DNA complexes



Xis binding

Using the gel-retardation assay (Garner and Revzin 1981) we first tested the ability of Xis to bind to the various mutated sites. A labeled wild-type *attR* fragment of HK022 of 450 bp and three similar fragments that contained the mutated X sites were challenged with increasing amounts of Xis (Fig. 2). The purified Xis protein included six N-terminal His residues that do not interfere with its activity (Cho et al. 2000; Gottfried et al. 2000). The results show that, in the presence of low concentrations of Xis, the wild-type *attR* (Fig. 2a) forms, as expected, two types of complexes. In the less retarded complex (C1) Xis occupies one or other of the two sites, while the more retarded complex (C2) results from occupation of both sites. The observation that C2 appears at the lowest concentration of Xis used (0.1 μ M) confirms the cooperative binding mode shown in the case of λ (Bushman et al. 1984). The single mutants show binding to one site only (Fig. 2b and c), presumably their single wild-type site. The much stronger binding of Xis to the *x2* mutant implies that Xis binds to the X1 site more efficiently and suggests that binding of Xis to X1 stimulates further binding to X2. Xis is completely incapable of binding to the double mutant (Fig. 2d).

Site-specific excision in vitro

Next, the effect of the site-directed mutations on the excision process in vitro was tested. In the in vitro assay the substrates were four circular plasmids each bearing a 1 kb fragment that carried *attR* and *attL* sites that are separated by \sim 1 kb of an irrelevant sequence (Fig. 3a). Each substrate carried one of the three mutations described above and the control was the wild type *attR*. Each substrate also carried a single *XhoI* restriction site in the vector sequence. In the presence of Int, IHF and Xis, excisive recombination between *attL* and *attR* is expected to result in two smaller circular plasmids (Fig. 3b), one that carries *attB*, the *XhoI* site and the vector sequence, and another that carries *attP* and the intervening sequence. To monitor the *attL* \times *attR* excisional recombination event we cleaved the products of the reaction with *XhoI*, end-labelled the resulting fragments, separated them on a gel, and visualized them by autoradiography.

The *attB* excision product is expected to be a labeled linear product of 3 kb that is shorter than the labeled linearized substrate of 4 kb. The results, presented in

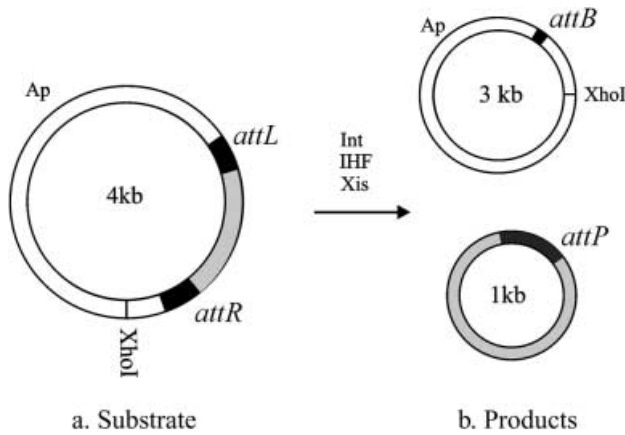


Fig. 3a, b Substrate (a) and expected products (b) of the in vitro assay for Xis activity. Ap, ampicillin resistance marker

Fig. 4A, show that in the wild type the expected product appears only in the presence of Int, IHF and Xis, confirming that it is indeed the product of an excision reaction. The single mutant *x1* showed a weak activity and mutant *x2* was completely inactive. However, the double mutant (*x1x2*), unexpectedly, displayed a significant level of activity. A quantitative analysis revealed that *x1* showed 11% of the wild-type activity, while the double mutant (*x1x2*) had 29% of the wild-type activity (Fig. 4B).

Site-specific excision in vivo

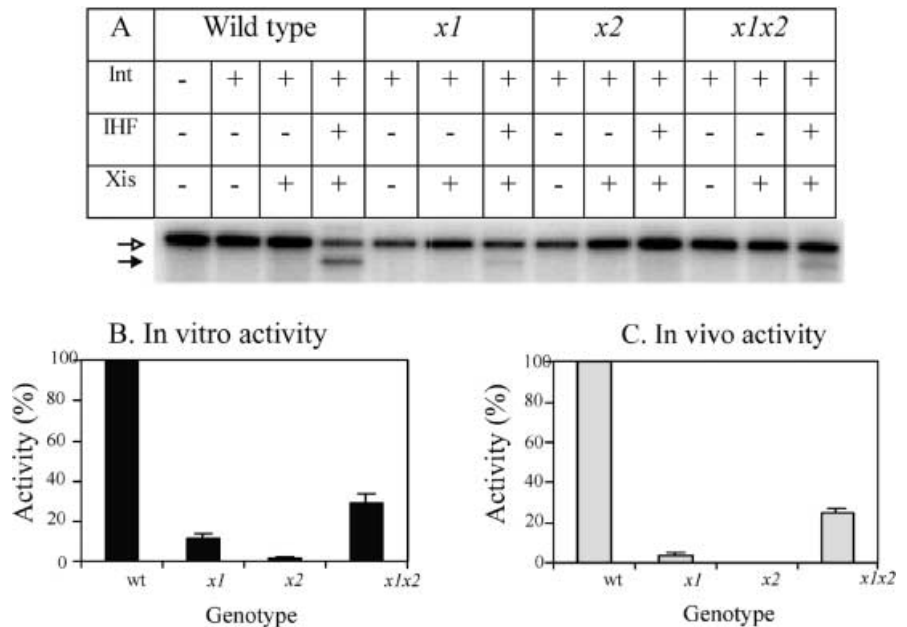
To test the validity of these in vitro observations we carried out an in vivo assay using the same *attL-attR* fragments. In this assay the wild type and the mutated *attL-attR* fragments were cloned on a vector (pLD46), between the *lacZ* gene and its promoter, thus preventing

the expression of β -galactosidase. The intervening sequence between *attL* and *attR* carried the strong *rrnB t1* and *t2* terminators. Excisive recombination (*attL* \times *attR*) removes the DNA that intervenes between the promoter and *lacZ*, leaving an in-frame *attB* of 21 bp, and allowing the expression of β -galactosidase (Dorgai et al. 1995). $\Delta lacZ$ cells (strain TAP114) transformed with these plasmids were infected with wild-type HK022 phage (to supply Int + Xis) and the infected cells were assayed for β -galactosidase activity. The results, shown in Fig. 4C, confirm the in vitro observations, i.e. the *x1* mutant showed 3.4% activity, the *x2* mutant was practically inactive and the *x1x2* double mutant showed 25% of the wild type activity.

Is the excision activity in the *x1x2* mutant due to a direct repeat?

As mentioned above, both wild-type binding sites contain the same 5-bp sequence (TATGT, Fig. 1B, line a). We have noticed that the *x1x2* double mutant carries a different direct repeat of 4 bp (tcGa, Fig. 1B, line d). To test whether the elevated activity of the double mutant is due to the newly formed direct repeat, and to confirm our results, we constructed a new mutation (*x2'*) in the X2 site (Fig. 1B, line e) that created a *SacI* restriction site. We then constructed the double mutant (*x1x2'*) that does not carry a direct repeat (Fig. 1B, line f). The insert in Fig. 5 shows the results of the in vitro excision test of the wild type and mutants *x2'* and *x1x2'*. Again, the single mutant (*x2'*) was inactive (1.5% activity), whereas the double mutant (*x1x2'*) showed 29% of the wild-type activity (Fig. 5, black columns). In the corresponding in vivo test *x2'* was completely inactive and *x1x2'* showed 26% of the wild-type activity (Fig. 5, gray columns). Finally, binding of Xis to the second set of mutants was

Fig. 4A–C Excision tests using *attR* sites mutated in their Xis binding sites X1 and X2. **A** In vitro test. The open arrow points to the substrate and the filled arrow to the product. **B** In vitro activities of the mutants relative to the wild type (wt). **C** In vivo activity measured as a percentage of the β -galactosidase activity of the wild type. The data represent an average of three repeats



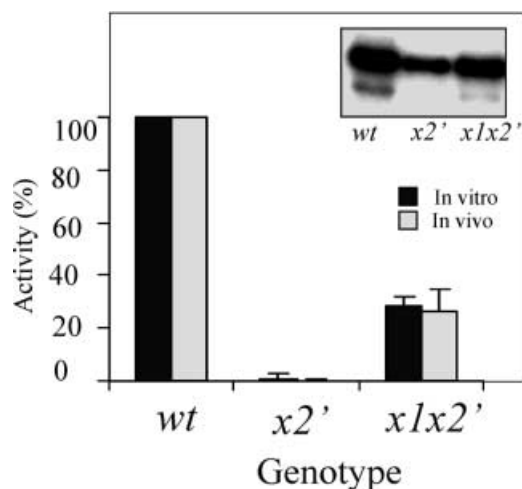


Fig. 5 Relative excision activity on the $x2'$ mutants in vitro (black columns) and in vivo (gray columns). The insert shows the results of the in vitro test

very similar to its behavior with the first set, i.e. significant binding to one site in $x2'$ and no binding to $x1x2'$ (data not shown). Thus, the data agree with those from the first set of mutants, and the elevated activity of the first double mutant ($x1x2'$) is unrelated to the fact that it contains a direct repeat.

Site-specific integration in vitro

Though it is known that Xis does not participate in the process of integration we tested whether our first set of mutants had any effect on the integration process. For that purpose we inserted the three mutations into an *attP*-carrying plasmid and performed an in vitro integration assay, reacting each circular *attP* with a radioactively labeled linear *attB* fragment in the presence of the necessary proteins, Int and IHF. Recombination between the circular *attP* plasmid (3.3 kb) and the labeled linear *attB* fragment (102 bp, S in Fig. 6) is expected to create a linear 3.54-kb labeled fragment (P2 in Fig. 6). As an internal control, each reaction also included a larger plasmid of 4 kb carrying the wild-type *attP*. In this case the expected product of integration is a linear 4.24-kb DNA fragment (P1 in Fig. 6). The results show that none of the mutants inhibited the integration process. On the contrary, the strong P2 products in lanes F and G indicate that the mutations in the $x2$ mutant and the $x1x2$ double mutant may have enhanced the integration.

Discussion

The assays used to assess the binding of Xis to its binding sites (X1, X2) have confirmed previous observations in phage λ that the binding is cooperative (Bushman et al. 1984) and show that binding of Xis to X1 stimulates further binding of Xis to X2. The

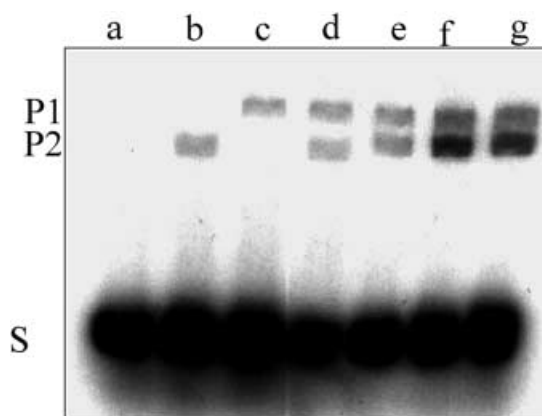


Fig. 6 In vitro integration test using *attP* substrates carrying the X mutations, and the wild-type *attP* from which they were derived. The expected product is P2. A larger wild-type *attP* substrate was included that is expected to give the larger product P1. S, labeled *attB* fragment. Lane a, no Int; lane b, wild-type substrate alone; lane c, larger wild-type control alone; lane d, both wild-type *attPs* together; lane e, $x1$; lane f, $x2$; lane g, $x1x2$. The reactions in lanes d–g all included both *attP* plasmids

cooperativity is so strong that even at the lowest concentration of Xis that showed a gel shift the complex representing Xis bound to a single site is less abundant than that in which the protein is bound to both sites (data not shown).

Mutations in X1 or in X2 abolish Xis binding as well as excision activity in phage HK022, in agreement with similar results obtained in λ (Numrych et al. 1991). The observation that Xis exhibited a significant excision activity with the double mutant substrate (25% or more of wild-type activity), together with the fact that the protein was unable to bind to the P arm, favors previous evidence of an essential interaction between Xis and Int that is required for the excision reaction. The P2 site is a weak binding site for Int and is required only for excision and the 15 C-terminal amino acids of Xis carry residues that are required to promote binding of Int to the P2 site (Wu et al. 1998). This cooperative Xis-Int interaction is probably a DNA-independent protein-protein interaction (Abremski and Gottesman 1981; Bushman et al. 1984; Numrych et al. 1992). In the case of the single X mutants, where little or no activity was evident, we suggest that binding of Xis to a single site causes the formation of an incorrect excision intasome. In the double mutants, where no binding of Xis to *attR* is evident, the partial excision activity becomes possible because the abortive binding of Xis to the P arm is prevented. As a result the available Xis molecules can still interact with Int and allow part of the excision reaction to take place even without arm-binding.

Finally, it is unlikely that Fis is involved in the partial restoration of activity in the double mutants. The cooperative interaction between Xis and Fis occurs when Xis is bound to the X1 site (Thompson et al. 1987). However, the mutations in $x2$ were not in the region where the binding sites of Xis (X2) and of Fis

(F) overlap. Thus, in the *in vivo* tests Fis could have been available to complement the abortive binding of Xis to X2. However, it did not do so, because the $x2$ mutants were not active in excision. Moreover, the *in vitro* tests were done with purified proteins in the absence of Fis.

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