

Activity of coliphage HK022 excisionase (Xis) in the absence of DNA binding

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Abstract A mutated excisionase (Xis) protein of coliphage HK022 whose single Cys residue was replaced by Ser does not bind to its two tandem binding sites (X1, X2) on the P arm of *attR*. Despite its DNA-binding inability the protein showed 30% excision activity of the wild type Xis both in vitro and in vivo. This partial activity is attributed to the interaction of Xis with integrase that is retained in the mutant protein. This protein–protein interaction occurs in the absence of DNA binding.

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1. Introduction

The excisionase (Xis) proteins encoded by bacteriophages λ and HK022 differ in one amino acid and are functionally identical [1]. They belong to the family of recombination directionality factors that direct site-specific recombination reactions towards excision by inhibiting integration [2]. The site-specific integration and excision reactions of each phage are catalyzed by the phage-encoded integrase (Int) protein. Integration results from a crossover between two 21 bp long core sites on the *Escherichia coli* DNA (*attB*, or BOB') and a similar core (*attP* or COC') on the phage DNA (Fig. 1). *attP* is much larger than *attB* because its core is flanked by two longer arms (P and P') that carry binding sites for Int and for accessory proteins that are essential for the site-specific recombination reactions. To catalyze the integrative (*attB* \times *attP*) reaction Int requires the host-encoded accessory protein, integration host factor (IHF). As a result, the integrated prophage is flanked by the recombinant *attL* and *attR* sites, which are the sites for the reverse excision reaction. In addition to Int and IHF, the excision reaction (*attL* \times *attR*) requires Xis. Factor for inversion stimulation (FIS) is a third

host-encoded accessory protein that can assist excision when the concentration of Xis is low. All three accessory proteins (IHF, Xis and FIS) are DNA bending proteins that bind to the P and P' arms and thereby facilitate access of arm-bound Int monomers to the core (reviewed by [3,4]).

Xis binds cooperatively to two tandem binding sites (X1, X2) on the P arm, each of 13 bp (Fig. 1). X2 overlaps with F, a single binding site for FIS. Xis plays a dual role in directing the formation of the excisive DNA–protein complex (known as the intasome). In addition to its DNA bending properties Xis interacts with Int monomers to divert their binding from the P1 arm binding site to the P2 site [5,6]. Xis is a small protein of 72 amino acids composed of two domains, an amino-terminal DNA binding domain (amino acids 1–53) and a carboxy-terminal domain (amino acids 54–72) that interacts with Int [7–9]. In a recent work we have shown that in an *attR* site that carries a mutations in both Xis binding sites (X1 and X2) the binding ability of Xis is abolished. Nevertheless this double mutant allows 25% of the wild type excision activity in vivo as well as in vitro [10]. In this work, we confirm this observation and demonstrate that DNA binding of Xis is not required for its interaction with Int.

2. Materials and methods

2.1. Bacteria, phage and plasmids

These are listed in Table 1.

2.2. Construction and cloning of the plasmid that expresses the C28S Xis mutation

The *xis* mutation with the Cys to Ser substitution (*xis*C28S) was constructed by a polymerase chain reaction (PCR) that used plasmid pPG15 as template. One oligomer carried the T7 terminator sequence of the vector pET14b (Novagen) and the other oligomer (α EY141, 5'-GCCTTGAAACAGTTCGTCGATGGGTGCGCGAA-TcCAGG) carried the mutation (in lower case) and a native *XmnI* restriction site (underlined). The PCR product and plasmid pPG15 were each restricted with the enzymes *XmnI* and *XhoI* (the latter site belongs to the linker) and the PCR product was cloned into pPG15 replacing the corresponding wild type sequence. DNA sequencing confirmed the presence of the *xis*C28S mutation and the plasmid that carries it was designated pPG165.

2.3. Isolation of a phage that carries the *xis*Y2C mutation

pLD177 is an excision reporter plasmid that is specific to phage λ . Wild type λ phage forms blue plaques in the presence of Xgal on a Δ lac host (TAP114) transformed with pLD177 [11]. Phage strain Y1096 carries *int* and *xis* of HK022 and does not form blue plaques under these conditions. However, it does form blue plaques on the same host that is lysogenic with the λ prophage B225 (strain EY1604), because the prophage that is mutated in *xis* supplies Int- λ and phage Y1096 supplies Xis. Y1096 was mutagenized by growing the phage on a *mutD* mutator host (strain LE30) and plated on strain EY1604 in

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Abbreviations: FITC, fluorescein-5-isothiocyanate; FRET, fluorescence resonance energy transfer; IHF, integration host factor; Int, integrase; TRITC, tetramethylrhodamine-5- (and 6-) isothiocyanate; Xis, excisionase

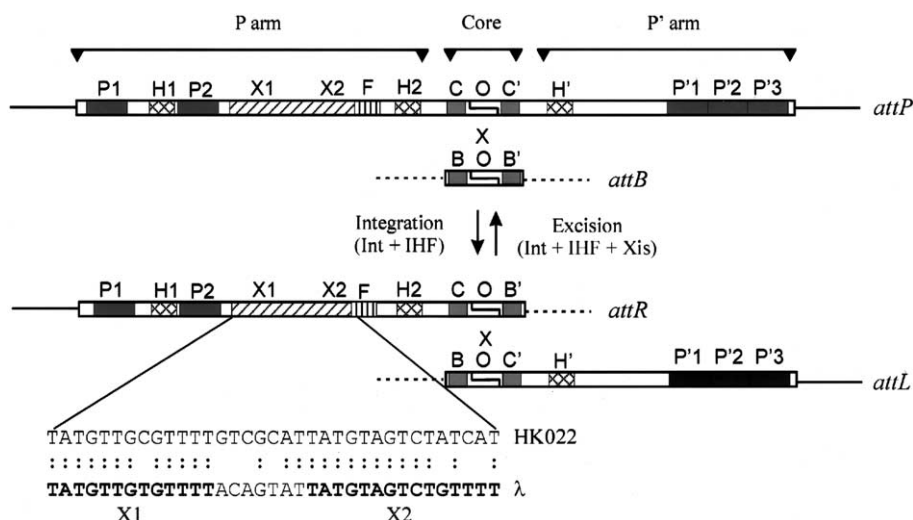


Fig. 1. A: Schematic presentation of the *att* sites involved in the site-specific recombination reaction of bacteriophages λ and HK022. COC', BOB', COB', and BOC' are the core sites of *attP*, *attB*, *attR*, and *attL*, respectively. P indicates arm binding sites for Int, X for Xis, H for IHF and F for Fis. The sequences of the two Xis binding sites X1 and X2 in *attR* of λ and of HK022 are aligned.

the presence of Xgal. Rare white plaques are expected to have resulted from a mutation in *xis* of HK022. Four such plaques were isolated; their region of *xis* was amplified by PCR and the DNA sequence in two of them showed that the Tyr2 codon (UAC) of *xis* has been changed to a codon of Cys (UGC). One of the mutant strains was designated EY261 and the mutation was designated *xisY2C*.

2.4. Protein purification

Wild type and the C28S mutant Xis were purified as described [12] from an isopropyl β -D-thiogalactopyranoside-induced culture of strain BL21(DE3) (pLys) [13] transformed with plasmids pPG15 or pPG165. The N-terminal Int^{1–64} peptide was cloned into the expression vector pET11 [14] (plasmid pVB262), overexpressed and purified using a phosphocellulose column according to Nash [15]. Int^{56–357} was cloned and expressed in vector pET14m [10] (plasmid pMK60) and the His-tagged peptide was purified on a nickel column resin according to the Novagen pET system manual. This fragment showed a high topoisomerase activity [16] (not shown). Purified IHF was a gift of Carol Robertson and Howard Nash.

2.5. Gel shift assay

Reactions (10 μ l) were as previously described [12]. In Fig. 2 the concentrations of Xis were 0.2, 0.4, 0.8 and 1.6 μ M. In Fig. 6 protein concentrations were 0.2 μ M Xis and 0.3, 0.6 and 1.2 μ M of Int.

2.6. In vitro excision assay

This assay was done as previously described [10].

2.7. In vivo excision assay

Lysogenic cells were grown logarithmically in a rich medium to a density of $A_{600} = 1.2$. The cells were centrifuged and the titer of free phage released to the supernatant was determined by a viable count.

2.8. Fluorescence resonance energy transfer (FRET) analysis

Xis protein was labeled with the fluorescence probe fluorescein-5-isothiocyanate (FITC). Int and its derivative peptides Int^{1–64} and Int^{56–357} were each labeled with tetramethylrhodamine-5- (and 6-) isothiocyanate (TRITC) [17]. Prior to the reactions all the proteins were dialyzed against 600 mM KCl, 50 mM phosphate buffer, pH 8, and concentrated up to 2 mg/ml. Fifty μ l of a solution of either 1 mg/ml FITC or 2 mg/ml TRITC were added to 950 μ l of the relevant conjugated protein. The mixture was incubated in the dark at 4°C with constant shaking. After 3 h the mixture was dialyzed against 600 mM KCl, 50 mM K₂HPO₄, 1 mM EDTA and 10% glycerol at pH 7 until no free fluorescence probe could be observed. The amounts of FITC or TRITC bound to proteins were estimated by absorbance at 499 and 544 nm (molar extinction coefficients $\epsilon = 68\,000$ for FITC $\epsilon = 84\,000$ for TRITC). The degree of labeling was 15% for Xis-FITC,

30% for Int-TRITC, 25% for Int^{1–64}-TRITC and 33% for Int^{56–357}-TRITC conjugates.

Energy transfer measurements were carried out using an LS50B Perkin-Elmer luminescence spectrometer. Two μ M Xis-FITC conjugate was mixed with equimolar amounts of the different TRITC conjugates and emission data were collected every 2 min. The excitation wavelength was set at 490 nm and the emission intensity was monitored between 500 nm and 630 nm.

3. Results

3.1. Xis is partially active in the absence of DNA binding

Xis carries a single cysteine residue at position 28 that resides in its DNA binding domain. We have constructed an Xis-overexpressing plasmid (pPG165) whose single Cys resi-

Table 1
Bacteria, phage and plasmids

	Relevant genotype	Source
Bacteria		
EY1659	MG1655 (HK022 <i>xisY2C</i>)	This study
EY1604	pLD177 in TAP114(B225)	This study
EY1662	MG1655 (OR228)	This study
EY1663	RJ1800 (Y1096 <i>xisY2C</i>)	This study
EY1666	RJ1800 (HK022)	This study
RJ1800	MG1655 <i>fis::Km767</i>	[22]
LE30	<i>mutD5</i>	[11]
MG1655	Wild type	[23]
TAP114	$\Delta(lacZ)$ M15	[11]
Phage		
B225	λ <i>int^c xis</i>	[24]
Y1096	<i>attP-int-xis</i> -HK022 <i>imm-λ</i> cI857	[1]
OR228	HK022 wild type	[1]
EY261	Y1096 <i>xisY2C</i>	This study
Plasmids		
pPG15	His-tag <i>xis</i> -HK022 cloned in pET14m	[10]
pLD177	A reporter plasmid for λ excision	[11]
pMK60	Int ^{56–357} cloned in pET14m	This work
pPG123	<i>attR-t1t2-attL</i> cloned in pBluescript	[10]
pPG165	<i>xisC28S</i> -HK022 mutant cloned in pET14m	This work
pVB262	Int ^{1–64} cloned in pET11	This work

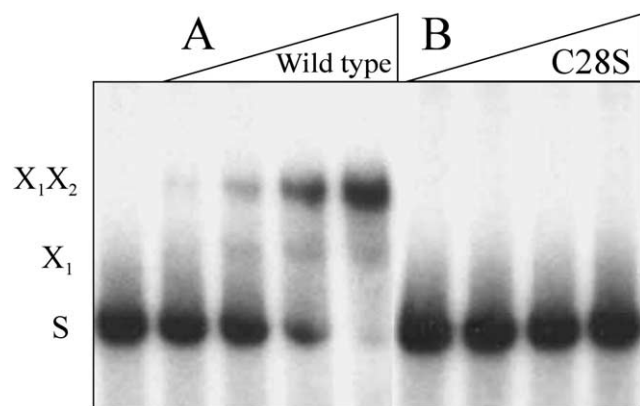


Fig. 2. Gel shifts of an *attR* site by wild type (A) and mutant C28S Xis (B). Triangles represent the increasing concentrations of Xis (0.2 μ M, 0.4 μ M, 0.8 μ M and 1.6 μ M); S indicates the labeled substrates (0.02 μ M); X_1 and X_1X_2 are Xis–DNA complexes.

due was replaced by a Ser residue (C28S). Wild type Xis and the mutant Xis were overexpressed, purified and used in a gel shift assay to observe binding to a labeled HK022 *attR* fragment of 450 bp (Fig. 2). The wild type protein retarded the DNA fragment by forming two slower moving bands (Fig. 2A). In the first band (X_1) Xis is bound to X_1 , and in the second band (X_1X_2) Xis is bound to both sites. Due to a strong binding cooperativity X_1X_2 is the major band at all concentrations of Xis [6,10]. The mutant Xis protein that challenged the *attR* sites at the same concentrations was completely unable to bind (Fig. 2B).

The activity of the C28S mutant protein was tested in vitro and in vivo. In the in vitro reaction the substrate (plasmid pPG123) carried the *attL* and *attR* sites in tandem and a single *XhoI* site that is located on the vector. Excisional recombination between *attL* and *attR* on the same plasmid (reaction in *cis*) is expected to yield two smaller plasmids. Linearization of the reaction products with *XhoI*, followed by radioactive end labeling, is expected to yield a linear product of 3 kb that is shorter than the original substrate of 4 kb. Fig. 3, lane a shows the substrate alone and in lane b Xis was omitted from the reaction. Lane c shows the full reaction (Xis, Int and IHF) using the wild type Xis. Lane d shows

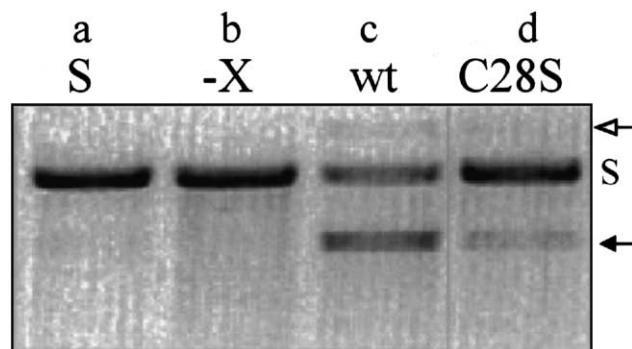


Fig. 3. In vitro excision assay catalyzed by wild type and by C28S mutant Xis. The concentration of Xis was 0.2 μ M. a: Substrate only. b: The reaction mixture in the absence of Xis. c: Full reaction with the wild type Xis. d: Full reaction with the C28S mutant Xis. The black arrow shows the product of the reaction. The open arrow shows the weak 5 kb product of the reaction in *trans*.

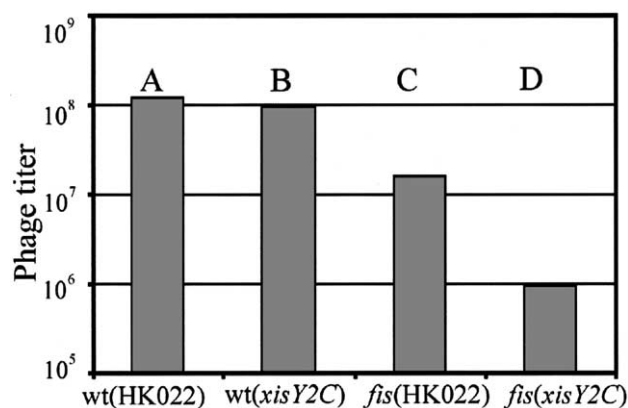


Fig. 4. Titers of free phage in lysogenic strains. A: Wild type lysogen with a wild type HK022 prophage (strain EY1662). B: Wild type lysogen with an HK022*xisC2Y* prophage (strain EY1659). C: *fis::Km* lysogen with a wild type HK022 prophage (strain EY1666). D: *fis::Km* lysogen with an HK022*xisI* prophage (strain EY1663).

the full reaction using a similar concentration of the mutant Xis. The 3 kb product is shown with the black arrow. The weak and longer product (open arrow) that is observed mainly in lane c is the product of a recombination event in *trans* (between two substrates) that is expected to yield two linear products of 5 and 3 kb. Quantitation has shown that the C28S mutant exhibited 30% of the wild type activity.

To test the activity of the C28S mutant in vivo we selected an HK022 phage that was mutated in *xis* (see Section 2). A sequence analysis has shown that in this mutant a Tyr residue at position 2 of Xis was replaced with a Cys residue (*xisY2C*). Viable phage released in a liquid culture of a lysogen that carries the *xisY2C* mutation (strain EY1659) was compared to the phage excised from a lysogen that carried the wild type HK022 prophage (strain EY1662). Fig. 4, columns A and B show that excision of free phage ($\sim 1 \times 10^8$ /ml) was identical in both lysogens. However, when we compared two similar lysogens whose host's *fis* gene was inactivated (*fis::Km*) the *xisY2C* lysogen (strain EY1663, column D) released over one order of magnitude less phage (9.5×10^5 phage/ml) than did the comparable lysogen with the wild type HK022 prophage (strain EY1666, column C, 1.6×10^7 phage/ml). Thus, the

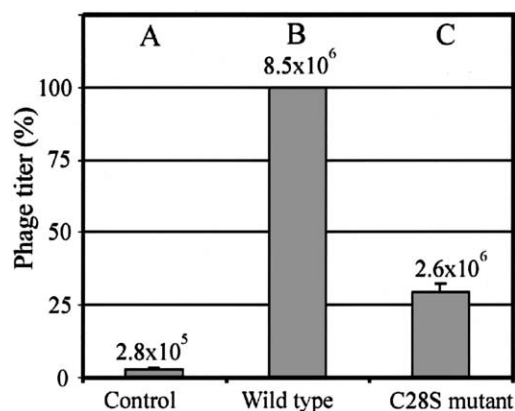


Fig. 5. Relative phage excision by strain EY1663 [*fis::Km*(HK022 *xisY2C*)] transformed with the vector pET14m (column A), with pPG15 (column B) and with pPG165 (column C). The numbers above each column indicate the actual average phage titer/ml of three experiments.

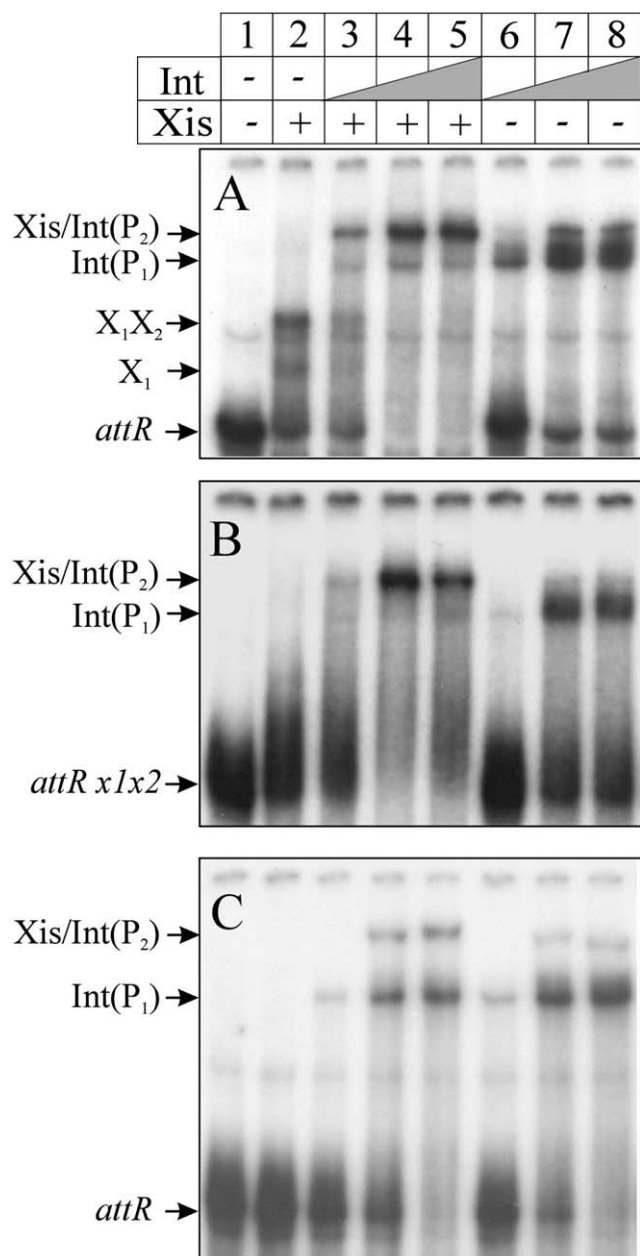


Fig. 6. Gel shifts of *attR* sites (lane 1) challenged with Xis (lane 2), with Xis and increasing amounts (0.3, 0.6 and 1.2 μ M) of Int (columns 3–5) and with the same increasing amounts of Int alone (lanes 6–8). A: Wild type Xis and wild type *attR*. B: Wild type Xis and *attR* mutated in the X1 and X2 sites. C: C28S mutant Xis and wild type *attR*.

xisY2C mutation is expressed only in the absence of FIS. It is also noteworthy that in the *fis::Km* host less wild type HK022 phage was released as compared to the wild type host (compare columns C and A). In order to test to what extent the C28S Xis protein can help the *xisY2C* mutant prophage to excise we transformed the *fis::Km(xisY2C)* lysogen (strain EY1663) with the plasmid that expresses the C28S Xis (pPG165) or with the plasmid that expresses the wild type Xis (pPG15). Phage release of these two lysogenic transformants along with a control strain transformed with the vector plasmid is shown in Fig. 5. The results of three experiments show that the C28S mutant protein (Fig. 5, column C) stimu-

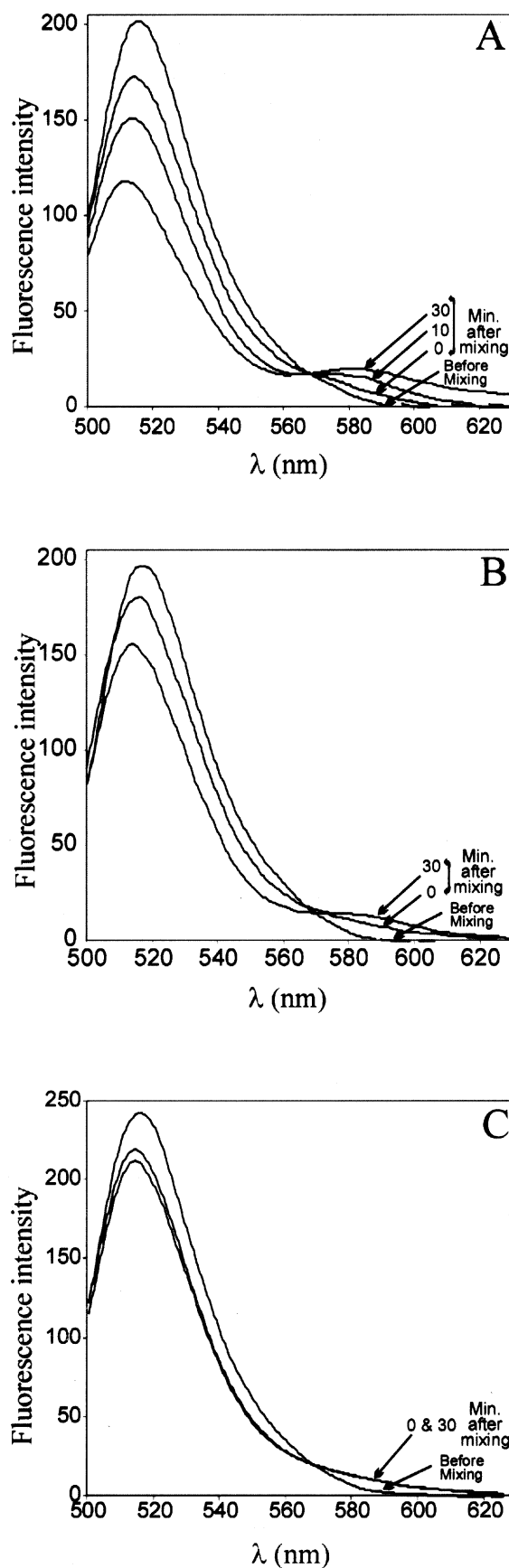


Fig. 7. Xis–Int interactions assayed by FRET analyses. A: Xis–Int. B: Xis–Int^{1–64}. C: Xis–Int^{56–357}.

lated an average of 30% phage release as compared to the wild type (column B). Thus, the *in vivo* assay confirmed the *in vitro* results.

3.2. Xis–Int interaction

The C-terminal domain of Xis is responsible for its interaction with Int to divert Int binding to the P2 arm binding site, thereby favoring the excision reaction [5–8]. We tested if the C28S-mutated Xis, which cannot bind to its site on DNA but shows partial excision activity, can still interact with Int. Fig. 6 shows gel retardation assays of labeled *attR* sites (lane 1) challenged with Xis alone (lane 2), Xis with increasing amounts of Int (lanes 3–5) and increasing amounts of Int alone (lanes 6–8). In Fig. 6A *attR* and Xis were both wild type. As shown above (Fig. 2) Xis alone binds cooperatively to the X1X2 sites (Fig. 6A, lane 2) and in the presence of increasing amounts of Int it forms a predominant slower moving complex (P2, lanes 3–5) that is, presumably, due to a DNA-bound Xis–Int complex that is located on the P2 site. This site is hardly occupied in the presence of Int alone (lanes 6–8). In Fig. 6B the challenged *attR* fragment was mutated in both X1 and X2 sites that prevent binding of Xis (lane 2 and [10]). Nevertheless, in the presence of increasing amounts of Int the wild type Xis again recruits Int to the presumed P2 site that is hardly occupied by Int alone. In Fig. 6C the *attR* was wild type and Xis carried the C28S mutation. Here also the mutant Xis, which is not able to bind to *attR*⁺ (lane 2 and Fig. 2), recruits Int to the P2 site, though less extensively than does the wild type Xis. These results demonstrate that Xis can direct Int to bind to the P2 site independently of its ability to bind to DNA.

3.3. FRET analyses

To test whether Xis and Int can interact in the absence of DNA we used a FRET assay. Since efficient FRET requires close proximity (<100 Å) between a donor and acceptor group, a significant FRET process is consistent with direct physical interaction [18]. Purified Xis was conjugated with FITC and purified Int was conjugated with TRITC (excitation/emission wavelength maxima of 490/518 and 550/580, respectively) and the spectral overlap results in a R_0 value of about 55 Å [17]. The two labeled proteins were mixed at an equimolar ratio to a final concentration of 2 μM and were subjected to an excitation of Xis-FITC at 490 nm. The emission of Xis-FITC at 518 nm and that of Int-TRITC at 580 nm (Fig. 7A) shows that the mixing has resulted in a decline in the emission of the labeled Xis at 518 nm coupled with a corresponding increase in the emission of Int at 580 nm as a result of energy transfer from the conjugated Xis to the conjugated Int. These results are consistent with a direct physical interaction between the two proteins. Recent evidence has indicated that the amino-terminal arm binding domain of Int (residues 1–64 [5,19]) is responsible for the Xis–Int interaction [20]. We cloned and purified separately residues 1–64 of Int and a C-terminal fragment of Int that included residues 56–357 and subjected them to an identical FRET analysis with Xis. FRET is observed between Xis and the Int^{1–64} fragment (Fig. 6B), although the latter carries only one Lys residue for binding to the fluorophore, whereas FRET is not observed between Xis and Int^{55–357} (Fig. 6C), although this fragment contains 24 Lys residues. These results confirm that Xis indeed interacts with the N-terminal part of Int.

4. Discussion

In this work we have demonstrated that the C28S-mutated Xis protein of HK022 is unable to bind to its X1X2 sites on the P arm of *att* (Fig. 2); nevertheless, it shows 30% activity of the wild type Xis, both *in vitro* (Fig. 3) and *in vivo* (Fig. 5). This confirms our previous observation that wild type Xis, when unable to bind to mutated X1X2 sites, also shows a similar activity [10] and indicates that Xis can be partially active in the absence of DNA binding. This partial activity that is independent of DNA binding can be attributed to the Xis–Int interaction that is retained in the C28S mutant protein (Fig. 6). In the gel shift experiments reported above as well as in the λ system [5] the Xis–Int interaction was demonstrated while Int was bound to DNA. This does not exclude the possibility that some kind of DNA binding is a prerequisite for the Xis–Int interaction. However, the FRET experiments (Fig. 7) indicate that interaction exists in the absence of DNA. Mutations and deletions of Xis in its C-terminal domain also show partial excision activity [21,7]. Therefore, each of the two functions of Xis (DNA binding and Xis–Int interaction) contributes independently to the full excision activity.

Sam et al. [21] have constructed a similar C28S mutation in Xis of phage λ that, in contrast to the same mutation in HK022, binds normally to *attR*-λ. This discrepancy is difficult to explain because the Xis proteins of the two phages differ in only one amino acid at position 59 that does not belong to the DNA binding domain and the two proteins are functionally interchangeable [1]. The sequences of the X1 X2 binding sites of HK022 have only been deduced from their homology with the λ sites, and they show some difference between them (Fig. 1). The X1 sites differ in one bp and the X2 sites differ in three bp. Perhaps this is the reason for the differences in the binding ability of the two C28S mutants.

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