BINDING SPECIFICITY IN THE SITE-SPECIFIC RECOMBINATION SYSTEMS OF COLIPHAGES λ AND HK022 AND THE ROLE OF THE XIS PROTEIN

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BY

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THIS WORK WAS CARRIED OUT UNDER THE SUPERVISION OF

PROF. EZRA YAGIL
This work is dedicated to
my parents, Rivka and
Salo, with love.
I am greatly thankful:

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ABSTRACT

The three major proteins involved in the site specific recombination reaction of bacteriophages λ and HK022 are Integrase (Int), Integration Host Factor (IHF) and the Excisionase (Xis). Together with the recombining DNA attachment sites (att) these three proteins form a complex, known as the Intasome. Both phages share an identical mechanism of site-specific recombination but one phage cannot integrate into the site of the other. Using the gel-retardation technique, DNA fragments that carry attL and attR sites of each phage were challenged with Xis and IHF and with increasing amounts of each of the two integrases. The results presented in this work have shown that each integrase can form a complete intasome complex only with its cognate att sites suggesting that the specificity difference between the two integrases is due to their binding properties to the core of the att sites.

Xis is a 72 amino acids accessory protein, that is required for excision in the site-specific-recombination mechanism of the related coliphages λ and HK022. It is a DNA-binding protein, that has two adjacent tandem binding sites (X1 and X2) on the phage attachment sites attP and attR. This work describes the cloning and purification of Xis of phage HK022 and the development of both in vivo and in vitro assays that monitor its activity. Preliminary crystallization attempts for the future determination of the three dimensional structure of Xis, are also presented.

The X1 and X2 binding sites of Xis were each mutated at their conserved sequence by site directed mutagenesis. Gel retardation assays of attR sites have indicated that Xis binds to both X sites of the wild type attR and only to one site in the single mutants. The double mutant did not show any binding. The differences in binding intensity of the single mutants suggests a cooperative mode, i.e. that binding of Xis to
X1 stimulates the binding to X2. In vitro and In vivo excision tests have shown little or no excision for the single mutants but, surprisingly, the double mutant has restored about 25% of the wild type activity. It is suggested that under these conditions Xis acts through a protein-protein interaction with Int.

Finally, cross-linking experiments and gel filtration chromatography have shown that Xis forms non-covalent dimers both in vivo and in vitro and that substituting the single Cysteine residue with Serine inactivates the protein though it can still form dimers.
INTRODUCTION

Genetic recombination is a general concept which includes a variety of processes that result in a rearrangement of DNA molecules. The three major processes of genetic recombination are homologous (or general) recombination, transposition, and site specific recombination. Each is catalyzed by a different recombinase protein. Homologous recombination is a reciprocal event in which any two DNA sequences that share an extended homology can recombine at various points. In transposition, homology is usually not required, it is not a reciprocal event and it involves DNA synthesis. Unlike the other processes, site specific recombination is a reciprocal event that occurs between unique short homologous sequences and whose strand exchange is precise and does not involve DNA synthesis (9).

Site specific recombination occurs both in prokaryotes and eukaryotes and fulfills several functions in various biological systems. It is used to reduce circular DNA dimers to monomers e.g. the Cre-lox system of bacteriophage P1 (18) and the Xer-cer system of *Escherichia coli* (8;54). It is used for chromosomal rearrangement and gene expression as in the immunoglobulin gene family (43) and in the gin system of bacteriophage Mu (61). It is also used in the integration and excision processes of some of the lambdoid bacteriophages e.g. λ, phage 21 and HK022 (9;29;39;62). In recent years, site specific recombination became an important tool for the insertion and deletion of genes into and out of the genomes of higher organisms (12;20;23;27;31;50).

The recombinases that catalyze site specific recombination reactions belong to two groups, the integrase family (3;42) and the resolvase/invertase family (57). The latter is characterized by the use of a conserved Serine residue as the attacking nucleophile.
during strand exchange, as well as by the restriction to catalyze only intramolecular recombination reactions. The orientation of the target sites is also of importance, each family member can only recombine either direct or inverted repeats. For example, the Tn3 resolvase can only recombine direct repeats (55;56) whereas the Gin invertase exclusively recombines inverted repeats (61). Unlike the resolvases and invertases, the integrases can carry out both inter and intra molecular reactions regardless of the orientation of their target sites (3;13;28;42;45). Recombination systems of the integrase family can be classified according to their complexity. The simple systems consist of identical recombination sites and an independently acting recombinase e.g. the Cre/lox system of bacteriophage P1 (18) and the Flp-frt system of yeast (29;44). The more complex systems consist of recombination sites that are not completely identical and they are assisted by accessory proteins. The best known of these is the integration and excision system of the λ coliphage and its relative phages like HK022, 21, 434 and others (62;63).

Bacteriophage λ is a temperate phage. During the infection of its host, E.coli, it injects its 49.5kb linear DNA into the cell. The DNA circularizes and the phage can then proceed either in a lytic cycle or to a lysogenic cycle (32). During the lytic cycle, the circularized DNA molecule of the phage replicates in the host cytoplasm and directs the production of new viral particles that eventually cause cell lysis. The newly released phages can then attack other bacterial cells. During the lysogenic cycle the circularized DNA of the phage is inserted into the E.coli chromosome by a site specific single crossover between the attachment site of the circularized phage genome (attP) and the attB site on the bacterial chromosome (Fig. 1). Since attB and attP are not identical (Fig. 1B), recombination between them results in the creation of the recombinant attachment sites attL and attR that flank the inserted prophage. The
lysogenic state is relatively stable and lysogenic cells are resistant to further infection by the same phage. However certain physiological instabilities, such as DNA damage induced by UV light or by chemicals, can promote prophage excision. The excision is the result of a recombination event between \textit{attL} and \textit{attR} (7).

\textbf{Figure 1.} A. Integration and excision of bacteriophage \textit{\lambda}. As a result of a crossover between \textit{attP} (POP') and \textit{attB} (BOB') a prophage is formed which is attached at its end to the lysogenic host chromosome with the recombinant sites \textit{attL} (BOP') and \textit{attR} (POB'). Excision is a Xis-dependent reverse process. B. Schematic presentation of the att sites. COC', BOB', COB', and BOC' are the core sites of \textit{attP}, \textit{attB}, \textit{attR}, and \textit{attL}, respectively. P indicates arm-binding sites for Int, X for Xis, H for IHF and F for Fis.

The recombinase that catalyzes these site specific recombination reactions is the phage-encoded integrase protein (Int). Int is assisted by three accessory DNA bending proteins, the phage encoded excisionase (Xis) and the two host proteins, integration host factor (IHF) and factor for inversion stimulation (Fis) (Fig. 1). Integrative recombination (\textit{attB} x \textit{attP}) requires Int and IHF. Excisive recombination (\textit{attL} x \textit{attR}) requires, in addition, Xis and Fis. Fis however, is dispensable if sufficient amounts of
Xis are present. Each of the four att sites contains a similar sequence of 23 base pairs (bp) known as the core. The core consists of a central 7bp site that is identical in all four att sites. This sequence, known as the overlap region (O), is the site of the genetic exchange. O is flanked by two imperfect inverted repeats of 8bp each, that are weak binding sites for Int (C, C’, B, and B’ in Fig. 1, B). Of the four att sites, attB is the simplest, consisting only of the 23bp core (BOB’). attP is more complex, flanking its core are two arms of 157bp and 85bp designated P and P’ respectively. Both arms carry strong binding sites for Int and binding sites for IHF. The P-arm also contains binding sites for Xis and Fis (Fig. 1B). attR and attL carry the recombinant core sites (COB’ and BOC’, respectively) and one of the arms, P or P’ (reviewed in 28;29;39;63).

Int, is the only catalytic protein involved in the site specific recombination reactions. It is a heterobivalent DNA-binding protein composed of 356 amino acids. Int consists of three functional domains, a 64 amino acids N-terminal domain that binds to the P and P’ arms of the att sites with a high affinity (Fig. 3), a low affinity core binding domain extending from position 65 to 169, and a C-terminal catalytic domain extending from position 170 to 356 (36;60). The three-dimensional structure of the catalytic domain of Int has been resolved (25). The Integrase family is characterized by four perfectly conserved amino acids at its catalytic domain, Arginine 212, Histidine 308, Arginine 311 and Tyrosine 342 (Fig. 3). The conserved Tyrosine 342 is the active site and acts as a nucleophile during strand exchange.

Not much is known about the high order complexes that are formed between Int, IHF and the att sites (and Xis in the case of excision). These complexes are generally termed intasomes. Kim and Landy (21) have proposed a model for the excisive intasome complex (Fig. 2A). According to this model, monomeric Int molecules bind
first to the high affinity arm sites with their N-terminal arm-binding domain. Then, by a bridging mechanism mediated by the DNA-bending accessory proteins, their C-terminal catalytic domain is introduced to the core sites, where the crossover reaction occurs. Four Int monomers are involved in the excision intasome. This bridging mechanism is facilitated by the DNA bending proteins IHF and Xis. For the sake of simplicity Figure 2B presents separate models of attR and attL sites and their interactions with the proteins. The sites H1 and P1 of attR and P’)3 of attL are not occupied since they have a role only in integration (21). No model has been proposed for the integration intasome complex, however, according to Richet et al. (48) Int monomers bind first tightly the arms of attP and with the assistance of IHF as a DNA bending protein, the bound Int monomers are delivered to the core of attP to form an intasome. The attP intasome then recruits the attB from solution to initiate the recombination process.

Recombination occurs at the 7bp overlap region through a staggered cut in each of the recombining sites. First a nucleophilic attack of the OH moiety of the conserved Tyr342 of Int breaks the phosphodiesteric bond in the 5’-end of the top strands of the recombining DNA molecules at their left end of the overlap region. Consequently, a covalent bond is formed between the Tyr342 of Int and the 3’ end of the broken DNA molecules. This event is followed by strand transfer and ligation, leading to the formation of an intermediate Holliday structure. This structure is subsequently resolved by a second exchange between the two bottom strands at the other end of the overlap region.
Figure 2. A. A model for the excision intasome complex. It shows 4 Int monomers. The small circles (light green) represent the arm binding domains of Int and the large circles (dark green) signify the core binding domain. Bends in the attR and attL DNA are caused by bound IHF (pink) and Xis (orange). The Int protein forms inter and intermolecular bridges by binding to the high-affinity arms sites (P'1, P'2 and P2) and to the low-affinity core sites (B, B', C, C'). Adapted from (21). B. An illustration of the DNA-protein interactions, for attL and attR separately.
Coliphage HK022 is a temperate phage that is related to phage $\lambda$. Both share an identical mechanism of integration and excision. However, the two phages integrate at different $attB$ sites on the $E.coli$ chromosome (10). The Int proteins of the two phages are 67% homologous but they are highly specific, i.e. one Int cannot substitute for the other (65). The 55 N-terminal amino acids of both $\lambda$ and HK022 integrases are identical (Fig. 3). This part of the protein comprises most of the arm binding domain. Thus, the reason for the specificity lies within the 33% heterologous amino acids scattered throughout the C-proximal region. On the DNA level, it has been shown that the C and B' Int binding sites of the $att$ core carry the major DNA determinants responsible for the difference in specificity (37). By constructing a series of chimeric HK022-$\lambda$ Int proteins as well as missense mutants, Dorgai et al. (11) and Yagil et al. (66) have shown that a five amino acids substitution in Int-$\lambda$ with the corresponding HK022 residues (arrows in Fig. 3) confers nearly exclusive HK022 recombination specificity. These 5 amino acids reside within the core-binding domain and the catalytic domain of Int. However, it is not yet clear how the specificity is determined, is it due to the binding properties of Int to the core or due to a subsequent topoisomeric function of Int, i.e. DNA unwinding, DNA cleavage, strands transfer and ligation.
Figure 3. Alignment of the λ and HK022 Int proteins. The different domains are indicated in the scheme. Bold red letters represent conserved amino acids within the Int family. Arrows indicate amino acids of λ that were substituted for HK022 amino acids and rendered specific HK022 activity (66).

Xis of HK022 is composed of 72 amino acids (Fig. 4). It differs from Xis-λ by only one amino acid (Ser59 in HK022 corresponds to Gly59 in λ, Fig. 4) and the proteins are interchangeable. Hence, Xis cannot be responsible for the specificity difference between the two phages. Xis is not only required for excisive recombination, it also inhibits integrative recombination and a possible interaction between Int and Xis proteins has been suggested (2). Xis has at least two functional
domains, an amino-terminal domain that binds to its sites on the DNA and a carboxy-terminal domain that is required for interaction with Int (41).

![Amino acids sequence of Xis protein. Ser59 in HK022 (purple) corresponds to Gly59 in λ (green). Notice the single Cys residue at position 28 (Black).](image)

IHF is a heterodimeric protein composed of two subunits IhfA and IhfB. It is a histone-like protein that binds to a specific DNA sequence and induces a bend (15). IHF participates in the control of many processes in E.coli and other gram-negative bacteria. In addition to its role in the site specific recombination of lambdoid phages it is involved in plasmid replication, DNA transposition, DNA inversion and gene expression. IHF is also part of the SOS regulon (reviewed in 9;14;34). Though its crystal-structure has been solved (46) the role of IHF in many of these processes is not yet known.

**GOALS OF RESEARCH**

The purpose of this research was to determine the molecular basis of the specificity differences between the two site specific recombination reaction systems of λ and of HK022 and to elucidate how Xis is involved in the structure-function relationship of the intasome complex.
RESULTS

A. CLONING, EXPRESSION, PURIFICATION AND CRYSTALLIZATION OF XIS

To investigate the nature of the specificity differences between the two Int proteins of \(\lambda\) and of HK022 I chose to construct an \(attR\) intasome in vitro, because its core carries the most important elements that determine specificity, B’ and C (see next chapter) (37). To accomplish that, I needed all the components that assemble an \(attR\) intasome in a purified form (Fig. 2B). The \(attR\) substrates were obtained by a polymerase chain reaction (PCR) and the proteins Int and IHF were available in our laboratory, but the Xis protein was not. Though the \(xis\) gene of \(\lambda\) (\(xis-\lambda\)) was cloned and its protein was previously purified by Abremski and Gottesman (2), its expression was not efficient enough, probably because of the inappropriate distance between the promoter and the initiation codon. It was therefore necessary to construct a better expression and purification systems of Xis. Since Xis-\(\lambda\) and Xis-HK022 are exchangeable (65) I chose to purify Xis of HK022 and to develop the proper assay systems to determine its activity.

Cloning and expression of Xis

A PCR product of the \(xis\) open reading frame was cloned in the expression vector pETI1 downstream of its ATG initiation codon; this expression vector carries the T7 promoter. The plasmid containing the \(xis\) gene, designated pPG1 was transformed into strain BL21(DE3) that contains a chromosomal copy of the gene for T7 polymerase, under the control of the \(lac\) promoter (58). Overexpression of the native Xis protein with the expected molecular weight of 8.6kD was obtained in an Isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) induced culture (Fig. 5 lane E).
In vivo assay for Xis activity

To test if plasmid pPG1, cloned with the *xis* gene, produces an active protein, I transformed a lysogenic strain that carries an HK022 prophage (strain EY1529) either with pPG1 or with the vector pETI1 as a control and measured the titer of the released phage. This assay indicates if the excess of Xis, encoded by the plasmid pPG1, can enhance the excision of the prophage. Logarithmic cultures of the transformed strains were grown for 150 minutes, in the presence of IPTG or without it and at time intervals samples were drawn to measure cell density (Fig. 6) and to assay the formation of viable phage particles that were released to the medium (Fig. 7). The growth of the strain containing plasmid pPG1 was inhibited in the presence of IPTG (Fig. 6A) whereas in the control it was unaffected (Fig. 6B). This inhibition could have been the result either of cell lysis caused by an increased release of the phage or by an inhibitory effect caused by the overexpression of the protein. The strain containing plasmid pPG1 released phage particles by over 4 orders of magnitudes more than the control (Fig. 7), indicating the presence of an active Xis protein. However there was no difference in the level of phage released between the induced and the uninduced cultures that carried plasmid pPG1 showing that the basal
uninduced level of the cloned Xis was sufficient to excise that much phage. Thus, the
growth of the IPTG induced culture was probably inhibited by a toxic effect of the
overexpressed protein and was not due to cell lysis.

Figure 6. Growth curves of strains containing the *xis* clone (A) and strains containing the
cloning vector as a control (B). Full squares – uninduced cultures; Empty squares – induced
cultures.

Figure 7. Viable phage titer (phage/ml) in the medium of an HK022 lysogen transformed
with plasmid pPG1 (blue) and the vector pETI1 (purple), induced (+) and uninduced (-) with
IPTG. The results are an average of three experiments.
**In vitro assay for Xis activity**

Figure 8a shows a scheme of the plasmid (pPG18) that was constructed as a substrate for an in vitro excision assay of Xis. It contains the sites of the HK022 excision recombination, *attL* and *attR*, in tandem, separated by a Kanamycin resistance (Km<sup>R</sup>) cassette and a single EcoRI restriction site. In the presence of Int, IHF and Xis, recombination between the two *att* sites is expected to result in two smaller plasmids, one that contains *attB* and the other that contains *attP* (Fig. 8b). To monitor the recombination I cut the reaction products with the restriction enzyme EcoRI, end-labeled the cut sites, separated them on a gel and observed them by autoradiography. If recombination took place a labeled product of 2.7kb should appear in addition to the labeled substrate of 4.6kb (Fig. 8c). Figure 9 shows the results obtained in this excision assay. Lane a is the untreated substrate, lanes b and c, in which no product is observed, are incomplete reactions missing either Xis or Int, respectively. Lanes d, e and f are the complete reactions, containing Int IHF and Xis (in a later chapter I will show that the reaction is also dependent on IHF, Fig. 23A, page 35). Lane d includes purified Xis-λ as a positive control (kindly provided by H. Nash) and lanes e and f include crude extracts of cells transformed with plasmid pPG1 in increasing dilutions (1/20 and 1/40). Both Xis-λ and the cell extract of the overexpressing Xis-HK022 strain yielded the expected product of 2.7kb. Cell extracts missing the pPG1 plasmid were also tested by the in vitro assay and found to be inactive (data not shown), thus the activity of the cell extracts transformed with pPG1 plasmid is the result of the presence of an active Xis protein.
**Figure 8.** Substrate and products in the in vitro assay of Xis.

**Figure 9.** In vitro excision reaction, a – no protein was added, b,c – incomplete reactions, missing Xis and Int, respectively. d,e,f – complete reactions with Xis-λ (d) and with 1/20 (e) and 1/40 (f) diluted extract of an Xis overexpressing strain. S and P indicate the position of the 4.6kb substrate and the 2.7kb product, respectively.
Purification of Xis protein

In order to use the one-step Nickel-column chromatography method to purify Xis, I constructed two plasmids that have overexpressed two different His-tagged variants of Xis. One, designated pPG14, has a relatively long (2kD) tail, containing 6 successive His residues and a thrombin cleavage site upstream to the native N-terminus of the protein (in total 20 extra amino acids). This tail comprises about 20% of the fused protein but can be removed after purification, by proteolysis with thrombin. The other His-tagged variant, designated pPG15 contains a Met residue and the 6 His residues, just upstream to N-terminus of the native protein such that 79 amino acids are translated instead of 72 of the wild type. Should the N-terminal tails of the proteins show no negative effect on Xis activity, the latter variant (pPG15) will be preferable for further use. Otherwise, the tail of the protein encoded by pPG14 can be removed by thrombin to yield a restored native protein. Figure 10, shows extracts of IPTG induced and uninduced cultures each cloned with one of the three plasmids, pPG1 that encodes for the native protein (lanes A,B), pPG14 encoding the long-tailed Xis (lanes C,D) and pPG15 that encodes the short tailed Xis (lanes E,F). Both His-tagged-Xis extracts overexpressed Xis with the expected molecular weight of 9.2kD (pPG15) and 10.6kD (pPG14), running somewhat slower than the native Xis (8.6kD).

To test if the His tails affect Xis activity, I repeated both the in vivo and the in vitro activity tests with the two His-tagged constructs (pPG14 and pPG15). In the in vivo assay, I titered only uninduced overnight cultures for free (excised) phage of the HK022 lysogen (Fig. 11). Both strains transformed with pPG14 and pPG15 had the same activity as that of the strain transformed with the native Xis (pPG1). All three excised phage particles by over 3 orders more than the control. Results of an in vitro
test, shown in Figure 12, indicate that the cell extracts of both His-tagged-Xis variants are active (Fig. 12, lanes d,e).

Figure 10. 15% SDS PAGE of cell extracts, induced (+) and uninduced (-) with 1 mM IPTG. Lanes A, B, - native Xis expressing strain pPG1. Lanes C, D, E and F - His-tagged-Xis expressing strains (C, D – pPG14, and E, F - pPG15). Lane G - Molecular weight makers (kD). Arrow heads indicate the position of the different overexpressed Xis variants.

Figure 11. Viable phage excised from overnight cultures. The results are an average of three experiments.
Each of the His-tagged Xis proteins (clones pPG14 and pPG15) was purified using Nickel chromatography. The larger protein from clone pPG14 was eluted in fractions 10-13 (Fig. 13A) and the smaller one from clone pPG15 was eluted in fractions 8-15 (Fig. 13B). Extracts From 1 liter of overexpressed cultures of each of the variants that were loaded on the column yielded 7mg and 10mg of Xis of pPG14 and pPG15, respectively, both with a purity of about 95%. The purified protein in each fraction was then tested by the in vitro assay and found to be active (Fig. 14).

Figure 12. In vitro excision assay of the His tagged Xis proteins. a-MW markers (kb); b-control without Xis; c-l/20 diluted extracts transformed with pPG1 (the native protein), d,e-l/20 diluted cell extracts transformed with plasmids pPG14 and pPG15, respectively.

Figure 13. 15% SDS PAGE of fractions eluted from the Nickel column. A. Purified Xis protein of clone pPG14 eluted in fractions 10-13, X – sample of the over expressed extract loaded on the column. B. Purified Xis protein from clone pPG15, eluted in fractions 8-15, M – Molecular weight markers.
Figure 14. In vitro activity of the purified His-tagged Xis proteins. A. Activity of strain pPG14. Lane a, no Xis was added, Lanes b-e activity assay of fractions 10-13 shown in Fig. 11A. B. Activity of strain pPG15. Lane a, no Xis was added, Lane b, Activity assay using Xis from the extract loaded on the column. Lanes c-h activity assay of fractions 9-14 shown in Fig. 11B.

Though both His-tailed Xis proteins were active, I removed the tail of variant pPG14. The His-tail was cleaved by thrombin whose cleavage site is just upstream to the native N-terminus. The “restored” native protein retains its activity however, the yield of this procedure was relatively poor, only 20% of the protein were recovered (data not shown). Together with the facts that the initial amount of the protein produced by the pPG14 strain was lower then that of pPG15 (7mg vs. 10mg), and both proteins show a similar level of activity, I used pPG15 as the source of Xis protein for all the following experiments described in this work.
Crystallization of Xis

Preliminary crystallization experiments with concentrated Xis were carried out using the “crystal screen” kit (Hampton Research). After seven days, needle shaped crystals grew in hanging drops with 1mM of purified Xis protein from variant pPG15 (Fig. 15). The precipitant contained 0.1M Sodium Acetate, 0.2M Sodium Cacodylate and 30% Polyethylene glycol (PEG) 8000. The crystals reached a maximum length of 0.3mm within 7 days and were stable. However, at this stage, their dimensions were not large enough for diffraction experiments. Further attempts to receive larger crystals were not within the scope of this work and will be done later.

Fig. 15. Xis crystals. A. Magnitude x10. B. Magnitude x40.
B. CORE BINDING OF INT DETERMINES ITS SPECIFICITY

The site-specific recombination systems of bacteriophages λ and HK022 share the same mechanism and their two integrase proteins show a strong homology. Nevertheless the integrase protein of each phage can only catalyze recombination between its own att sites (24). Previous work has shown that the specificity determinants on the att sites are not within the arms, rather they are located within the sites in the core that bind the Int weakly. Moreover, it was shown that the B’ and C sites of the core carry the major specificity determinants (37). The B site carries weaker determinants whereas the C’ site is identical in both phages (Fig. 16). However, it has not been tested yet if these determinants are involved in the binding ability of Int to the core or in a later stage in the recombination reaction, e.g. in its topoisomerase activity (cutting properties, strands transfer or ligation).

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**Figure 16:** Alignment of the attB and attP core sequences of λ and HK022.

Because Int alone binds very weakly to its core sites, I tested the ability of each Int (of λ and of HK022) to form an intasome complex. For that purpose I used the gel retardation technique. As already described in the introduction, Int is a bivalent
protein that binds first tightly to the arms of the att sites and for the formation of the high-order intasome Int subsequently binds to the core (39). The intasome complex can be identified on a gel as the most retarded species (21;47). Using this approach I tested if the binding of Int-λ or Int-HK022, is restricted to its own core sites.

The B’ and C sites are the ones that carry the strongest specific determinants. Since both are located together on attR (Fig. 1, page 7), I first used attR that is an excision substrate to form an intasome complex. attR carries two strong arm binding sites (P1,P2) for the N-terminal domain of Int, two sites for IHF (H1,H2), two tandem binding sites for Xis (X1 and X2) and the two core sites, C and B’ to which the C-terminal domain of Int binds weakly. The affinity of IHF to H2 is higher then to H1 and it has been shown that excess IHF, when bound to both H sites, prevents the formation of the attR excision intasome complex (21). Hence, an accurate calibration of IHF binding to attR was required to ensure that IHF binds predominantly to the H2 site. A radioactively labeled attR fragment was challenged with increasing amount of IHF (Fig. 17). The occupation of H2 alone by IHF is seen only with the two lower concentrations (0.48µM and 0.96µM). At the highest concentration (4.6µM) both sites are occupied. Similar results were obtained with attR-HK022 (data not shown). In all shift assays described below, I have used 0.48µM of IHF that bind only to the H2 site.

Figure 17. Gel shift experiment of attR-λ bound with increasing concentrations of IHF (indicated in the figure). H2 represents the complex formed by IHF occupying H2 site only, H1+H2 represent the subsequent occupation of both sites.
As already mentioned, core binding of Int and the formation of the intasome complex is conditioned first by binding of Int, IHF and Xis to the arm sites. Core binding and the formation of an intasome becomes apparent only at higher concentrations of Int because the intasome can be seen on a gel as the most retarded species (21).

Gel shift assays were preformed using radioactively labeled DNA fragments that carry attR-λ (Fig. 18A) and attR-HK022 (Fig. 18B). Each was challenged with the proper amounts of Xis and IHF, and increasing amounts of Int-HK022 or Int-λ. Lane a in each panel shows the untreated attR. The retarded complexes formed with Xis alone are shown in lane b of each panel, and the ones formed with Xis+IHF are shown in lanes c. In both cases the attR fragments challenged with both proteins (Xis+IHF) migrated slower than with Xis alone. By adding low amounts of Int (up to 0.6µM, lanes 1-3), the binding of Int is restricted to the P2 arm site (Lanes 1-3 in each panel), and is shown by a further retarded migration of complexes (open arrow). So far, there are no differences in the retardation patterns between the cognate and noncognate Int. However, in the presence of higher amounts of Int (1.2µM and 2.5µM, lanes 4 and 5), each Int forms two stronger retarded complexes only with its cognate substrate (full arrows). These high-order complexes are, presumably, intasome complexes (Int monomers bound to its two core sites, see discussion). Suggesting that indeed the core binding is specific. It should be mentioned that attR has two arm binding sites of Int (P1 and P2), it has been shown that in the presence of Xis Int binds predominantly to the P2 site (6).

In order to further test this presumption, I carried out a similar experiment using the same substrates but whose core sequences were deleted (∆core, Fig.18C and 18D). Challenging these ∆core-attR substrates of λ and of HK022, each with its cognate and
Figure 18. Gel shift experiments of attR-λ (A), attR-HK022 (B), Δcore attR-λ (C) and Δcore attR-HK022 (D). Untreated substrates (lanes a), substrates supplemented with Xis (lanes b), with Xis+IHF (lanes c), and with increasing amounts of Int-λ or Int-HK022 (0.15, 0.3, 0.6, 1.2, 2.5 µM Int, lanes 1-5 respectively).
noncognate Int, showed that they were all identical in their arm-binding properties
(arrow in Fig. 18C and 18D). However, the formation of the two highest complexes
was abolished. This proves that the high order complexes observed in Figure 18A and
18B have resulted from specific core binding. These results confirm that specificity is
determined by core binding that forms the intasome complex.

Having demonstrated that the specificity difference between Int λ and Int HK022 is
caused by their ability to bind to the core sites B’ and C (of attR). I have also tested if
the third variable core site, B, is also important in binding-specificity. Similar
experiments were performed using attL substrates, these carry the B and C’ sites (Fig.
1B, page 7). The sequence of the C’ site is identical in both phages and therefore it is
not expected to carry any specificity determinant. However, the B sites differ in 3 out
of 6bp  (Fig. 16). attL contains three strong arm binding sites for the N-terminal
domain of Int (P’1, P’2, P’3), one binding site for IHF and no binding site for Xis
(Fig. 1B, page 7).

The results (Fig. 19A and 19B) show that IHF alone retarded both attL substrates
in a similar way (lanes b). Adding increasing amounts of Int has resulted in two types
of arm-bound complexes, one protomer bound to P’1 and a second subsequently
bound to P’2 (lanes 1-3, open arrows). The P’3 site is not involved in excisive
recombination (21) (Fig. 2B, page 10). Again, at the highest concentrations of Int,
high-order complexes are seen only with the cognate Int (lanes 4, 5, closed arrows).
These results show that also with attL binding of Int to the core is possible only with
its own att substrate. Figures 19C and 19D show, again, that when the attL substrates
lack their core sites (∆attL) no high-order complexes were formed, confirming that the
ones formed with the intact attL sites were indeed the result of core binding that
requires a prior binding of the arms.
Figure 19. Gel shifts of *attL-λ* (A), *attL-HK022* (B), coreless *attL-λ* (C) and coreless *attL-HK022* (D). DNA substrates IHF and were as in Fig. 18.
Taken together, the core binding assay used in this study shows that the specificity of Int is due to its binding ability to the core sites, both of \textit{attR} and of \textit{attL}. The results also confirm previous conclusions that arm binding by Int is not involved in the specificity difference between \(\lambda\) and HK022 (37). This part of the work has been published (17).
C. THE ROLE OF XIS AND ITS BINDING SITES

There are two adjacent binding sites for Xis (X1 and X2) on the P arm of the phage (Fig. 20A and the bold-faced bases in Fig. 20B line a). Each site contains the conserved tandem repeated sequence TATGT in its 5’ end (underlined in Fig. 20B line a). In order to test the role of these binding sites in the process of site specific recombination, I mutated them by site directed mutagenesis and tested the ability of

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<th>X2 site</th>
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<td>a. Wild type</td>
<td>TATGGTTCGTTTTGTCGCA TATGTAGTCTATCA</td>
<td></td>
</tr>
<tr>
<td>b. x1</td>
<td>AtcGaTGCGTTTTGTCGCA TATGTAGTCTATCA</td>
<td></td>
</tr>
<tr>
<td>c. x2</td>
<td>TATGGTTCGTTTTGTCGCA TtcGaAGTCTATCA</td>
<td></td>
</tr>
<tr>
<td>d. x1x2</td>
<td>AtcGaTGCGTTTTGTCGCA TtcGaAGTCTATCA</td>
<td></td>
</tr>
<tr>
<td>e. x2’</td>
<td>TATGGTTCGTTTTGTCGCA TgaGctcTCTATCA</td>
<td></td>
</tr>
<tr>
<td>f. x1x2’</td>
<td>AtcGaTGCGTTTTGTCGCA TgaGctcTCTATCA</td>
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Figure 20. A. Schematic presentation of the attR site. COB’ is the core, P indicates arm-binding sites for Int, X for Xis, H for IHF and F for Fis. B. The sequence of the two Xis binding sites X1 and X2 (line a, bold face) and that of the various mutants described in the text (lines b-f).
Xis to bind to the mutated substrates and to perform the site specific recombination reactions. The mutations were designed to replace the conserved TATGT sequences with restriction sites (Fig. 20B lines b-f). At first, three types of P arm site mutants were constructed, one mutated in the X1 site to form a ClaI restriction site (x1 in Fig. 20B line b), the second was mutated in the X2 site to form a SfuI site (x2 in Fig. 20B line c) and the third was the double mutant x1x2 containing both ClaI and SfuI sites (Fig. 18B line d). For binding experiments and to test the excision activity, the mutations were constructed on a plasmid that carries attR. To test the integration activity the mutations were constructed on a plasmid that carries attP. All constructs were confirmed by restriction and by DNA sequencing.

**Binding assays**

First, the ability of Xis to bind to the three mutant sites was tested by the gel retardation assay. A radioactively labeled wild type attR fragment of 450bp and three similar attR fragments that contained the mutant X-sites were challenged with increasing amounts of Xis. The results (Fig. 21) show that at the lower concentrations Xis and the wild type attR (Fig. 21a) form, as expected, two types of complexes. The less retarded complex (C1) represents Xis occupying a single site and the second, more retarded complex (C2) represents the occupation of both sites. At the highest concentration of Xis C2 is evident predominantly. The double mutant (Fig. 21d) is, as expected, completely incapable of binding Xis. The single mutants show partial binding to one site only (Fig. 21b and 21c), that is attributed to their single wild type site. The much stronger binding of Xis to the x2 mutant implies that Xis binds to X1 more efficiently and suggests a cooperative mode, i.e. binding of Xis to X2 depends on a prior binding to X1. This is also supported by the observation that at the smallest
concentrations of Xis the wild type attR was always bound to both sites (Fig. 21a). Even at the lowest concentration of Xis in which binding was evident C2 is the predominant complex (Fig. 22). Binding to a single site alone cannot be seen.

**Figure 21.** Gel shifts by Xis bound to a wild-type and to mutated attR sites. Triangles represent the increasing concentrations of Xis (none, 0.1µM, 0.2 µM, 0.4 µM and 0.8 µM). S, indicates the labeled substrates, C1 and C2 are Xis-DNA complexes.

![Gel shifts by Xis bound to a wild-type and to mutated attR sites.](image)

**Figure 22.** Binding of Xis to wild type attR in low concentrations. a-e increasing concentrations of Xis (none, 0.025µM, 0.05 µM, 0.1 µM and 0.4 µM, respectively) to the wild-type attR site.

![Binding of Xis to wild type attR in low concentrations.](image)

**Excision assays**

The effect of the site mutations was tested on the excision process both in vitro and in vivo. The in vitro assay was essentially as described in the previous chapter, (Fig. 8, page 18). The substrates were four circular plasmids (pPG123 and pPG125-pPG127), each cloned with a 1kb fragment that carried tandem attR-attL sites separated by a
sequence of 420bp that contained the *rrnB, t1* and *t2* transcription terminators (these terminators are only relevant for the in vivo assay, see below). Each substrate carried one of the three *attR* mutations described above and the control was a wild type *attR*. Here, the excision product is expected to be a labeled linear product of 3kb that is shorter than the labeled linearized substrate of 4kb.

The results, presented in Figure 23A, show that in the wild type the expected product (closed arrow) appears only in the presence of Int, IHF and Xis, confirming that this is indeed an excision reaction. The single mutant *x1* showed a weak activity and mutant *x2* was completely inactive. However, the double mutant (*x1x2*), unexpectedly, restored a significant level of activity. The density of the substrate and product in each lane was measured and a quantitative analysis has revealed that mutant *x1* showed 11 percent activity of the wild type and that the double mutant (*x1x2*) has restored 29 percent of the wild type activity (Fig. 23B). To confirm these in vitro observations I carried out an in vivo assay using the same *attL-t1t2-attR* fragments. In this assay these fragments were cloned into a reporter plasmid, between the *lacZ* gene and its promoter, preventing the expression of β-galactosidase (Fig. 24). Excisive recombination (*attL x attR*) removes this fragment and allows the synthesis of β-galactosidase (11). These four plasmids (pLD205 that carries the wild type *attR* and the three *x attR* mutants pPG128, pPG131 and pPG132) were transformed into a *lacZ* strain of *E.coli* (TAP114) and the transformants were infected with wild type HK022 phage that supplies Int and Xis in vivo. The level of β-galactosidase was measured 40 minutes after infection. The results, shown in Figure 23C, confirm the in vitro results, i.e. the *x1* mutant showed 3.4 percent activity, the *x2* was practically inactive and the *x1x2* double mutant showed 25 percent of the wild type activity.
Figure 23. Excision tests of attR sites mutated at their Xis binding sites X1 and X2. 

A. In vitro test. Open arrow point to the substrate and closed arrow to the product. 

B. In vitro activities of the mutants relative to the wild type (w.t.). 

C. In vivo activity measured as % of β- galactosidase activity of the w.t. The data are an average of 3 experiments. The in vivo activity of the wild type was measured to be 1047 Miller Units.
Figure 24. Substrate and products of the in vivo excision assay. Following recombination, the terminators are removed and the \textit{tac} promoter (red) becomes close to the \textit{lacZ} gene (yellow), allowing the expression of \(\beta\)-galactosidase (11).

Is the excision activity in the \(x1x2\) mutant due to a tandem repeat?

As mentioned above, the two wild type Xis binding sites consist of a tandem repeat of five base pairs (TATGT, Fig. 20B line a). It may be noticed that the \(x1x2\) double mutant carries a different tandem repeat of four base pairs (tcGa, Fig. 20 line d). To reconfirm the validity of the results and to test if the elevated activity of the double mutant is due to the newly-formed tandem repeat I have constructed a new mutation (\(x2'\)) in the X2 site (Fig. 20, line e) that forms a SacI restriction site. I also constructed the double mutant (\(x1x2'\)) that does not carry any tandem base-pair repeat (Fig. 20, line f). I repeated the binding experiment and the excision activity assay using the new constructed substrates. Binding of Xis to this second set of mutants (Fig. 25) was very similar to its first set, i.e. some binding to one site in \(x2'\) (Fig. 25b) and no binding in \(x1x2'\) (Fig. 25c). The in vitro test, presented in Figure 26 and quantified in Figure 27 shows again that the single mutant (\(x2'\)) was inactive and the double mutant (\(x1x2'\)) showed a significant activity that was measured to be 29 percent of the wild type. In the in vivo test the \(x2'\) showed neglected activity (0.3% of the wild type) and the \(x1x2'\)
had 26 percent activity of the wild type (Fig. 27). Thus, the data agree with the first set of mutants and the activity of the first double mutant (x1x2) was unrelated to the fact that it had a tandem sequence in its binding site.

Figure 25. Gel shift experiments of Xis to a wild-type and to the x2’ mutated attR sites. Triangles represent the increasing concentrations of Xis (none, 0.1 µM, 0.2 µM, 0.4 µM and 0.8 µM). S, indicates the labeled substrates, C1 and C2 are Xis-DNA complexes.

Figure 26. In vitro excision test of attR sites carrying the x2’ mutations. Upper arrow indicates the substrate. Lower arrow indicates the excised product.

Figure 27. A quantitative presentation of the in vitro assay results (dark blue) and of the in vivo results (light blue) of the x2’ containing substrates. Data are average of 3 experiments, the in vivo activity of the wild type was measured to be 1143 Miller Units.
Integration assay

Though it is known that Xis does not participate in the process of integration I tested if the site mutations on $attR$ had any effect on the integration process. For that purpose I inserted the three mutations of the first set ($x1$, $x2$, and $x1x2$) into an $attP$-carrying plasmid and performed an in vitro integration assay. Each circular $attP$ plasmid was reacted with a radioactively labeled linear wild type $attB$ fragment in the presence of Int and IHF (Fig. 28). Recombination between the circular $attP$ plasmid (3.3kb) and the linear $attB$ fragment (100bp, S in Fig. 28) is expected to create a linear 3.4kb-labeled fragment (P1 in Fig. 28). In addition, each reaction included an internal control.

Figure 28. Substrates and expected products of the in vitro integration assay. A. The internal control (blue) contains a wild type $attP$. The test molecules (pink) contain either a wild type $attP$ or mutated ones. The radioactively-labeled (red asterisk) $attB$ (yellow) is indicated by S. B. P2 is the linear product of the internal control. P1 is the linear product of the tested molecule. C. A scheme of the expected autoradiogram of an active test molecule (product P1) and the active internal control (product P2).
control i.e. a larger plasmid of 4kb carrying the wild type \textit{attP}. In this case the product of integration is a 4.1kb linear DNA fragment (P2 in Fig. 28). The results, presented in figure 29, show that none of the mutants inhibited the integration process. On the contrary, the strong P1 products in lanes F and G indicate that the \textit{x2} mutant and the \textit{x1x2} double mutant may have enhanced the integration. Perhaps the impairment of the sequences that are important for excision enhances the integration process. This part of the work is now in the press (16).

\textbf{Fig. 29.} In vitro integration test of \textit{attP} sites carrying the X mutations and their wild type parent. Their expected product is P1. An internal larger wild type \textit{attP} substrate that expects to give the larger product P2 was included. S – labeled \textit{attB} fragment. Lane a - no Int; land b - wild type test parent alone; lane c - wild type internal control alone; lane d - both wild type \textit{attP}’s together; lane e - mutant \textit{x1}; lane f – mutant \textit{x2}; lane g - mutant \textit{x1x2}. The reactions in lanes d - g also included the internal control.
D. Dimerisation of Xis and the Role of its Cys Residue

During the purification procedure it was noticed that the purified Xis protein tends to form dimers that have accumulated with time and whose formation could be resolved to monomers in the presence of β-mercaptoethanol. Figure 30 shows a gel electrophoresis of purified Xis that was run under denaturing conditions in the presence or absence of β-mercaptoethanol treatment. When run on the day of purification, dimers are hardly seen in the absence of β-mercaptoethanol treatment. However 14 days later at 4°C in the absence of β-mercaptoethanol treatment the dimers become very obvious (arrow in Fig. 30). In either case when treated with β-mercaptoethanol no dimers are seen. This indicates that the single Cysteine residue at position 28 of the protein (Fig. 4, page 13) is responsible for the formation of the dimers. Further investigations have indicated that these dimers are also formed in cell extracts of the Xis overproducing strain (data not shown).

<table>
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<th>Age of pure protein (days)</th>
<th>1</th>
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<tbody>
<tr>
<td>β-mercaptoethanol</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
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**Figure 30.** 15% SDS-PAGE of purified Xis protein. Xis-Dimers (indicated by the arrow) accumulated with time and reduced by β-mercaptoethanol.
Since the cellular milieu is of a reducing nature that does not favor disulfide bond formation (4) it is likely that the Cys-Cys bonds are spontaneously formed in the purified protein and accumulated with time.

However, in a different set of experiments, when the purified protein was separated on a gel filtration column, a western blot of each fraction has revealed two peaks of Xis (Fig. 31A). The protein was evident in fraction 17 whose molecular weight of 18kb corresponds to a dimer and in fractions 22-24 that corresponds to a monomer of 9kD. Selected fractions were tested for their activity (Fig. 31B). The peak fractions 17 and 23 were found to be active whereas fraction 19 that is between them and that according to the western blot did not react with the Xis antiserum, was inactive. This indicates that Xis was really separated into two peaks, a dimer and a monomer. The positive activity of the dimeric fraction does not necessarily mean that the dimeric form is active. Since the filtration is done under native conditions, the fraction may also include non-covalent dimers and below I show that Xis indeed forms such non-covalent dimers both in vivo and in vitro.

![Figure 31](image)

**Figure 31.** A. Western blot of the different fractions eluted from the column. B. Activity assays of fractions 17, 19 and 23 and of a sample taken from the load.
To further investigate the nature of the dimeric fraction I adopted the cross-linking technique, using Disuccinimidyl suberate (DSS) (Fig. 32) as a cross-linker. It is a bifunctional N-hydroxysuccinimide ester (NHS-ester) that reacts with side chain amines (especially with that of the Lysine) and therefore enables the fixation of native protein-protein interactions. DSS has the advantage that it can penetrate the bacterial cell thereby permitting in vivo cross-linking experiments (19).

Cross-linking experiments were done both in vivo and in vitro. In the in vivo experiments, intact cells expressing either the native Xis (plasmid pPG1) or the His-tagged Xis (plasmid pPG15) were treated or untreated for 30 minutes with DSS. The samples were washed and their extracts were treated or untreated with β-mercaptoethanol before being subjected to SDS-PAGE (Fig. 33A,B). A western blot analysis with Xis antiserum reveals that in the presence of the cross-linker Xis dimers become apparent in vivo in both strains and that the dimers are not affected by the treatment of β-mercaptoethanol (Fig. 33A,B, lanes d). The disulfide dimeric fraction, previously observed with purified Xis (Fig. 30), is not seen at all in vivo (Fig. 33A,B, lanes b), indicating that all dimers seen with DSS treatment, are originally non covalent ones. Because the native Xis (pPG1) has also formed cross-link dimers the

Figure 32. Structure of the cross-linker DSS.
presence of the His tail have no influence on the dimerisation. In the in vitro experiments, extracts of cells expressing the His-tailed Xis (plasmid pPG15), were treated or untreated with DSS. After 30 minutes the samples were treated or untreated with β-mercaptoethanol (Fig. 33C). Again, dimers are predominantly evident in the DSS treated extracts (lane d). A disulfide fraction of dimer is also faintly seen in extracts untreated with DSS and β-mercaptoethanol (Fig. 33C, lane b). Similar results were obtained with pPG1 extracts in vitro (data not shown). This shows again that spontaneous disulfide bonds are formed under oxidative conditions and confirms the existence of non-covalent Xis dimers. In all lanes in Figure 33 a slower migrating band is evident. This band appears also in the uninduced samples (lanes a) and is probably a non-specific protein.

In summary, these results have revealed the existence of dimers both in vivo and in vitro which are the result of non-covalent interaction between two monomers. In addition, in the cell extracts and in the purified protein disulfide dimers could spontaneously be formed as a result of an exposure to the oxidative environment.

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<th>In vitro</th>
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<tr>
<td></td>
<td>A. native Xis (pPG1)</td>
<td>B. His-tailed Xis (pPG15)</td>
</tr>
<tr>
<td>IPTG (1mM)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DSS (2mM)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-mercaptoethanol (2mM)</td>
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**Figure 33.** Western blot of induced and uninduced Xis-expressing strains. **A** and **B** are in vivo cross-linking experiments performed with strains pPG1 and pPG15, respectively. **C** is an in vitro cross-linking experiment performed with strain pPG15. The samples in the different lanes were treated as indicated in the above table.
To test if the single Cys residue plays any role in dimerisation, I have mutated the Cys codon into a Serine codon by site directed mutagenesis and constructed an overproducing strain of the mutant protein (plasmid pPG165). Similar cross-linking experiments done with this strain have shown the dimers even more extensively than in the wild type (Fig. 34). Hence the Cys residue has no role in the formation of the non covalent dimers. The existence of the dimeric form of the mutant protein was again verified by gel filtration chromatography (Fig. 35). The protein was eluted from the column in two peaks at fractions 17 and 23 that correspond to the dimeric and monomeric forms of the protein (Fig. 35A). As before (in the wild type protein) a western blot analysis of the different fractions has shown that both peaks (fractions 16-19 and 22-25) reacted with Xis antiserum, confirming the existence of the two forms of the protein (Fig. 35B).

<table>
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<td>A. His-tailed Xis (pPG15)</td>
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</tr>
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<td>DSS (2mM)</td>
<td>-</td>
</tr>
<tr>
<td>β-mercaptoethanol (2mM)</td>
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**Figure 34.** Western blot of induced and uninduced Xis-expressing strains. A and B are in vivo cross-linking experiments performed with strains pPG15 and the mutant pPG165, respectively. C is an in vitro cross-linking experiment performed with the mutant strain pPG165. The samples in the different lanes were treated as indicated in the above table.
In Figure 34, a higher band whose molecular weight fits the size of a tetramer (~40kD) is clearly apparent in the mutant (lane d in panels B and C). Traces of this putative tetramer might also present in the wild type (lane d in Fig 34A and Fig 33C). However under the native conditions of the gel filtration chromatography tetrameric fraction were not detected in the wild type (Fig. 31) or in the mutant (Fig. 35).

![Figure 35](image)

**Figure 35.** A. Spectrophotometric ($\lambda=280\text{nm}$) profile of purified Xis separated by gel filtration. B. Western blot of the different fractions eluted from the column.

Further characterization of the Cys-Ser mutant protein has shown that it has lost its activity in vivo (Fig. 36) and was no longer capable of binding to its sites on $attR$ (Fig. 37). This implies that the Cys residue has a yet unknown important role in the activity of Xis.
Figure 36. The effect of Xis expressing plasmids on phage excision, pET14m (control vector), pPG15 (wild type Xis) and pPG165 (mutant Xis). The results are an average of three experiments.

Figure 37. Gel-shifts of wild-type Xis and mutated Xis of an attR site. a. wild type Xis. b. Mutant Xis. Triangles represent the increasing concentrations of Xis (0.2 μM, 0.4 μM and 0.8 μM). S, indicates the labeled substrates, C1 and C2 are Xis-DNA complexes.
DISCUSSION

*The specificity of Int:* On the DNA level previous work has shown that the specificity differences between the Int proteins of λ and HK022 were located within the weak Int-binding sites of the core, predominantly in the B’ site (37). On the protein level, 5 amino acids of Int HK022 were determined as being responsible for the specificity (Fig. 3, page 12) (11;66). However, it was not clear if the difference resides in site binding or in a later step in the recombination process. The gel shift experiments presented in this work have demonstrated that the specificity difference between the two related recombinases, lies within their ability to bind to the core. They have also confirmed that binding to the arms plays no role in specificity. This became apparent not only in *attR*, that carries the B’ and C sites but also in *attL* that carries only one variable core-binding site (B site). Since all three variable core sites (B’, B and C) were found to possess specific binding properties, it is likely that *attP* that contains one variable core site (C) and more likely *attB* that contains two variable core sites (B and B’) will also carry specific core binding properties.

The finding that Int binding to the core is specific does not rule out the possibility that the specificity lies also within subsequent events of the site specific recombination process. DNA unwinding or the endonucleolytic activity of Int could also be involved in a specific interaction of Int with the core. Of the five amino acids of Int that have been previously assigned to be important specificity determinant (11), only the one at position 99, resides in the proposed core-binding domain (between residues 65-169). The other four residues are at positions between 287-319 that belong to the catalytic domain of Int (60). Perhaps the catalytic domain carries other specificity determinants that are not necessarily involved in core binding.
Alternatively, the so-called catalytic domain also carries determinants that are important for core binding. To seek for other functional specificity differences between the two systems it is possible to use “relaxed” Int proteins that can recombine att sites of HK022 as well as of λ (11;66). These proteins have acquired mutations that lost their specificity towards the phages. Mutations that would be found outside of the core binding domain, could be associated with the relevant stage in site specific recombination.

The high-order complexes between Int and attR that have shown specificity towards the cognate Int were of two types that differed in their migration pattern (closed arrows in Figs. 18A and 18B, page 27). This is consistent with previous work suggesting that in attR two Int protomers bind to the core by means of protein-protein interactions between them and a third protomer tightly bound to the P2 arm binding site (21). With regards to attL previous work has indicated that in attL one protomer of Int forms an intramolecular bridge between the P’1 arm site and the C’ core site (22;33). Another protomer binds to the P’2 arm site and a third one is recruited from solution and through protein-protein interactions binds to the weak B core site (Fig. 2b, page 10) (21;22). In the case of HK022, two different high-order complexes are seen. In λ, however, they are not so obvious (Fig. 19B, page 29). If the C’ site is identical in both phages, one might expect that a non-cognate Int tightly bound to P’1 site will also form a C’-bound nonspecific high-order complex. Formation of such a complex between arm-bound Int of HK022 and a COC’ core of λ was observed at high concentrations of Int by MacWilliams et al. (33). However, the results presented in this work, showed that this is not the case. This could be because in the present work I have used an attL with a native BOC’ core whereas in the analysis of MacWilliams et al. (33) they used a COC’ core. The weak binding to C’ may be
depended on a prior binding of the solution-recruited protomer to B, which itself represents a specific binding event. Alternatively, in the high-order attL complex the C’ site of each phage may acquire a difference in structure which is sequence-independent.

In summary, the results presented in this part of the work have demonstrated that at least one reason for the specificity difference is the ability of Int to bind to the core of the attachment sites.

The role of Xis and its binding sites in the excision process: The binding assays of Xis to its sites (X1, X2) have shown a strong cooperativity. Even at the lowest concentration of Xis that showed a gel shift, a major complex of Xis bound to a single site could not be observed (Fig. 22, page 33). These results confirmed previous observations of cooperative binding in phage λ (6). The binding studies to the single mutants, done in the present work have indicated that binding of Xis to X1 stimulates further binding of Xis to X2.

Mutations in X1 or in X2 abolish Xis binding as well as excision activity in the HK022 system, this is in agreement with results obtained in λ (40). The observation that in the double mutants Xis can restore a significant excision activity of the wild type (25 percent or over) though the protein is unable to bind to any of its sites, is a novel one. It shows that Xis is active in another way beside its ability to bend the DNA. Indeed, previous evidence has pointed on an essential protein-protein interaction between Xis and Int. It has been shown that the 15 C-terminal amino acids of Xis carry residues that are required to promote binding of Int to the P2 site. A site that is dispensable for integration but is required for excision (64). Furthermore,
Abremski and Gottesman (1), have shown that Xis protects Int from heat inactivation. Therefore the cooperative Xis-Int interaction is probably a DNA-independent protein-protein interaction. The results presented in this work support this notion. In the case of the single mutants, where little or no activity was evident, it is possible 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 \textit{attR} is evident, the partial excision activity becomes possible because the incorrect binding of Xis to the P arm is prevented and the available Xis molecules can still interact with Int and allow part of the excision reaction to take place even without arm binding.

The Fis protein, that is dispensable when Xis is abundant and capable of binding to its sites, can compensate for the unavailability of Xis by binding to its F site on \textit{attR} that overlaps with X2 (Fig. 1B, page 7). However, it is unlikely that Fis is involved in the partial restoration of excision activity in the double mutants because Fis promotes the excision by a cooperative interaction with Xis, when the latter is bound to its X1 site (59). The mutations in X2 site were not in the region where the binding sites of Xis (X2) and of Fis (F) overlap. In the in vivo tests, where Fis could have been available and complement the aborted binding of Xis to X2 it did not do so because the \textit{x2} mutations were not active in vivo. Furthermore, the in vitro experiments were done with purified proteins and did not include Fis.

The Int proteins of both phages, \textit{\lambda} and HK022, were expressed in mammalian cells and both found to enable site specific recombination reactions in these cells (23;31;51). Kolot et al. (23) have shown that the \textit{int} gene of phage HK022 can promote site-specific recombination in mouse cells in the absence of IHF and Xis. In addition in vitro experiments with phage \textit{\lambda} have shown that eukaryotic cells have DNA bending proteins, which can replace the bacterial IHF (52). Since the single
mutations in the X sites presented in this work had no influence on the integration process, but were practically inactive in excision, they may also eliminate the activity of the eukaryotic Xis-like protein (if it exists). Thus, they can be useful for an efficient unidirected site specific integration of genes, promoted by Int. Such a system can be advantageous over the Cre-lox site specific recombination system of phage P1 and the yeast FLP system, that have already been engineered to insert desired DNA sequences into specific sites of eukaryotic genomes (12;20;30;50). Because of the identity of their recombining sites and because both integration and excision are catalyzed by the recombinase alone, the insertions are less stable since they are subjected to the reverse excision. In contrast, Int integration and excision are not reversible reactions and if the excision process can be eliminated or reduced, Int may catalyze stable integrations.

**Xis dimers and the role of the Cys residue:** The disulfide dimers of Xis that have accumulated with time, must have resulted from the exposure of the protein to oxidative conditions and therefore have no biological significance. However gel filtration and the cross-linking experiments have indicated that a non-covalent Xis dimers does exist both in vivo and in cell extracts. The tandem arrangement of the two binding sites X1X2 on the P arm supports this notion. However, it is not yet clear if the dimerisation of Xis is essential and whether the protein forms dimers prior to its binding to its tandem sites or whether first two separate monomers bind their sites and subsequently interact to form dimers that may be necessary for the activity of the protein. Experiments are in progress to test this question. In any case, the results presented here show that the protein carries a self-recognition site. This site can be
identified by proteolysis of the cross-linked dimers and sequence analysis of the covalent peptides.

The moderate substitution of the single Cys residue in position 28 with Ser, abolishes the ability of the protein to bind to its sites. This is in agreement with previous work that determined the 53 N-terminal amino acids of Xis as the DNA-binding domain (41). The ability of the mutant Xis to form dimers was unaffected and therefore its single Cys residue does not play a role in the dimerisation process.

Finally, this work has demonstrated that the His tail that was engineered on Xis did not interfere with its activity. The overexpression and relatively easy purification of this soluble protein yielded 7-10 mg/liter. Preliminary attempts to crystallize the protein have yielded small needle-shaped crystals. This latter observation is encouraging to test if a higher concentrated protein and/or a gentle modulation of the precipitant composition, can improve the quality of the crystals. Work in this direction is now in progress, preferably the protein structure will be determined in the presence of its DNA binding sites. Moreover, being a relatively small protein (72 amino acids) and soluble in water, Xis is also most appropriate for NMR analyses. Such attempts were recently reported by the laboratory of Reid Johnson (personal communication). Determining the three-dimensional structure of Xis will be a step forward in elucidating its mode of interaction with its sites on the \textit{attR} and with Int, and will help understand the three-dimensional structure of the intasome complex.
MATERIALS AND METHODS

Strains, plasmids and oligomers

Bacteria, phage strains and plasmids used in this study are listed in Table 1. The way the plasmids that were constructed in this study, is described in Table 2. Oligomers used are listed in Table 3.

Table 1: Bacteria, Bacteriophage and plasmids

<table>
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<tr>
<th>Relevant Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>BL21(DE3) Host for T7 promoter plasmids.</td>
<td>(58)</td>
</tr>
<tr>
<td>EY1529 BL21(DE3)(HK022)</td>
<td>Lab collection</td>
</tr>
<tr>
<td>TAP114 Δ(lacZ)M15</td>
<td>(11)</td>
</tr>
<tr>
<td><strong>Phages</strong></td>
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</tr>
<tr>
<td>OR228 HK022 wild type</td>
<td>(65)</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
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<td>Stratagene</td>
</tr>
<tr>
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<td>(68)</td>
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<tr>
<td>pET14b ApR expression vector to clone N-terminal His-tag proteins under the T7 promoter.</td>
<td>Novagen</td>
</tr>
<tr>
<td>pET14m Derivative of pET14b</td>
<td>M. Kolot</td>
</tr>
<tr>
<td>pETI1 ApR, derivative of pET-22 (Novagen).</td>
<td>I. Morad</td>
</tr>
<tr>
<td>pEY1391 xis-HK022 cloned in pEMBL18.</td>
<td>(65)</td>
</tr>
<tr>
<td>pKY110 attP–HK022 cloned in pEMBL19</td>
<td>(24)</td>
</tr>
<tr>
<td>pKY113 attP-HK022 cloned in pUC18</td>
<td>M. Kolot</td>
</tr>
<tr>
<td>pLD205 attR-t1t2-attL clone in pLD46</td>
<td>(11)</td>
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<tr>
<td>pLD46</td>
<td>vector used to construct pLD205 derivatives</td>
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<tr>
<td>-------</td>
<td>---------------------------------------------</td>
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<tr>
<td>pMK24</td>
<td>attrR -HK022 cloned in pUC18</td>
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<tr>
<td>pMK25</td>
<td>attL -HK022 cloned in pUC18</td>
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<tr>
<td>pMK27</td>
<td>attR -λ cloned in pUC18</td>
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<tr>
<td>pMK28</td>
<td>attL -λ cloned in pUC18</td>
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<tr>
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<td>attP with x1 mutation in pUC18</td>
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<tr>
<td>pPG102</td>
<td>attP with x1x2 mutations in pUC18</td>
</tr>
<tr>
<td>pPG107</td>
<td>attR with x1 mutation in pUC18</td>
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<tr>
<td>pPG109</td>
<td>attR with x1x2 mutations in pUC18</td>
</tr>
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<td>attR with x2 mutation in pUC18</td>
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<td>pPG116</td>
<td>attP with x2 mutation in pUC18</td>
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<td>x1 mutation in pPG123</td>
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<td>pPG126</td>
<td>x2 mutation in pPG123</td>
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<td>pPG131</td>
<td>x1x2 mutations in pLD205</td>
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<tr>
<td>pPG143</td>
<td>x1x2’ mutations in pPG123</td>
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<td>pPG186</td>
<td><em>attR</em> with x2' mutation in pUC18</td>
</tr>
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<td>pPG187</td>
<td><em>attR</em> with x1x2' mutations in pUC18</td>
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<td>pPG68</td>
<td>Core-deleted <em>attR</em>-HK022 cloned in pUC18</td>
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Table 2: Plasmid construction

a. Plasmid constructed by PCR and ligation

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<th>Template</th>
<th>Oligomers</th>
<th>Restriction</th>
<th>Ligated in vector</th>
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<tr>
<td>pPG1</td>
<td>xis</td>
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<td>pPG165</td>
<td>f+oEY149</td>
<td>HindIII+Ndel</td>
<td>pET11.(HindIII+Ndel)</td>
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<td>pMK24</td>
<td>f+oEY148</td>
<td>HindIII</td>
<td>pUC18.(SmaI+HindIII)</td>
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<td>EcoRI</td>
<td>pUC18.(SmaI+EcoRI)</td>
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<td>x1</td>
<td>pMKY110</td>
<td>f+oEY137</td>
<td>EcoRI+ClaI</td>
<td>pUC18.(EcoRI HindIII)</td>
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<td>pP15</td>
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### b. Plasmid constructed by ligation only

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<tr>
<td>pET14m</td>
<td>His-tag</td>
<td>none</td>
<td>Annealing of oEY115 and oEY116</td>
<td>pET14b.(<em>NcoI</em>-<em>NdeI</em>)</td>
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<td><em>xis</em></td>
<td>none</td>
<td><em>NdeI</em>-<em>XhoI</em> <em>xis</em> fragment of pPG1</td>
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<td><em>x1</em></td>
<td><em>HindIII</em>-<em>Asel</em> of pPG101 and <em>Asel</em>-<em>EcoRI</em> of pMK24</td>
<td>pUC18.(<em>HindIII</em>-<em>EcoRI</em>)</td>
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<td><em>x2</em></td>
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<td><em>x2</em></td>
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<td><em>x2</em></td>
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<td><em>BamHI</em>-<em>PstI</em> fragment of pPG143</td>
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Table 3. List of oligonucleotides

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<tr>
<th>Oligonucleotide</th>
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<th>Restriction Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>oEY115</td>
<td>CATGGGCCCATCATCATCATCA</td>
<td></td>
</tr>
<tr>
<td>oEY116</td>
<td>TATGATGATGATGATGATGGCC</td>
<td></td>
</tr>
<tr>
<td>oEY137</td>
<td>GACTGG<strong>G</strong>atcGatGCGGCTTTTTGTCGCATTATG</td>
<td>(ClaI)</td>
</tr>
<tr>
<td>oEY138</td>
<td>GCGACAAAACGCA<strong>T</strong>catTCCAGTCACTATG</td>
<td>(ClaI)</td>
</tr>
<tr>
<td>oEY139</td>
<td>GTCGCATT<strong>t</strong>caGATCTATCATTTAACCACAG</td>
<td>(SfuI)</td>
</tr>
<tr>
<td>oEY140</td>
<td>GGTTAAATGATAGACTT<strong>c</strong>gaAATGCAGACAAAACG</td>
<td>(SfuI)</td>
</tr>
<tr>
<td>oEY141</td>
<td>GCCTTGAAAAAGATTCTCGTGATGGGTGCGGAATCCAGG</td>
<td></td>
</tr>
<tr>
<td>oEY145</td>
<td>TATATTTAAAAATCTCTTTAAATTATC</td>
<td></td>
</tr>
<tr>
<td>oEY146</td>
<td>GCATTATAAAAAGCATGATTCTTC</td>
<td></td>
</tr>
<tr>
<td>oEY147</td>
<td>AACGAGAAACGTAAAATGATAG</td>
<td></td>
</tr>
<tr>
<td>oEY148</td>
<td>GACAAAAATACATTAATCAC</td>
<td></td>
</tr>
<tr>
<td>oEY149</td>
<td>GCGGAGACTTTTCATATGTAC</td>
<td></td>
</tr>
<tr>
<td>oEY150</td>
<td>GTCGCATT<strong>g</strong>aGctcTCTATCATTTAACC</td>
<td>(SacI)</td>
</tr>
<tr>
<td>oEY151</td>
<td>GGTTAAATGATAGAG<strong>c</strong>ctAATGCGAC</td>
<td>(SacI)</td>
</tr>
</tbody>
</table>

Newly created restriction sites (in parentheses) are underlined and the mutations are in lower case.

Proteins

Protein concentration was measured using the method of Bradford (5) using bovine serum albumin as a standard. Proteins were separated on polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (26). If not otherwise indicated the gels were stained with Comassie Brilliant Blue.
DNA

All DNA manipulations were according to Sambrook et al. (49). Antibiotics were added when necessary.

Growth Media

Luria broth (LB) rich medium as described in (49)

Cloning of xis-HK022 and its different variants

A 370bp PCR fragment was generated using plasmid pEY1391 as template and oligomers oEY149 and the universal primer of the vector pEMBL18. The PCR product was cut with \textit{NdeI} and \textit{HindIII} and cloned into plasmid pETI1, obtaining plasmid pPG1. The \textit{NdeI-HolI xis} fragment of 355bp of pPG1 was then subcloned into the same sites of the His-tagged expression vectors pET14b (Novagen) and pET14m obtaining plasmids pPG14 and pPG15, respectively.

Plasmid pET14m is a derivative of pET14b containing only the 6 His codons instead of the \textit{NcoI-NdeI} fragment of pET14b. To construct it pET14b was cut with \textit{NcoI-NdeI}, the vector was purified, annealed with oligomers oEY115 and oEY116 and ligated.

The \textit{xis} mutant with the substitution of the single Cys residue to Ser was constructed by a PCR reactions using oligomer oEY141 that carries the mutation as well as a native XmnI restriction site and the T7 terminator oligomer (Novagen) of the vector pET14b. Plasmid pPG15 was used as a template. The PCR product and plasmid pPG15 were then restricted with the enzymes XmnI and XhoI and the PCR product was cloned into pPG15 replacing the corresponding wild type sequence. The
new plasmid was designated pPG165. Table 3 summarizes the construction of the above plasmids. The presence of the mutation was confirmed by DNA sequencing.

**Protein purification**

The three different Xis variants and IHF were purified from strain BL21(DE3) transformed with plasmids pPG14, pPG15, pPG165 or pEE2003, respectively. The His-tailed proteins were induced at the logarithmic growth phase with 1mM IPTG for 2 hours. The supernatant of a sonicated extract was used to purify Xis by a one step chromatography on a nickel resin column according to the Novagen pET system manual.

Int-λ and Int-HK022 were purified according to Nash (38) from IPTG-induced cultures of strain BL21(DE3) transformed with plasmid pNR169 or pNR69, respectively.

**Construction of the mutants in the Xis-binding sites**

Plasmids with mutations in the X sites were each constructed by two PCR reactions each including one oligomer with a mutated sequence and a second universal oligomer (forward or reverse) of the vector pUC18 as specified in Table 2A. The oligomers used for a single mutation were complementary. The PCR products and the vector were then restricted with the enzymes indicated in Table 2A 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 onto other cut vectors as specified in Table 2B.
In vitro excision assay

The reaction mixture (10 µl) contained 50nM KCl, 50mM tris-HCl pH 7.5, 5mM spermidine, 1mM Na2EDTA, 1mg/ml BSA, 0.2µM Xis, 0.1µM IHF, 0.1µM Int and 0.6nM of the appropriate plasmid as a substrate. The reaction was incubated at room temperature for 10 min. Reaction products were cut by the restriction enzymes EcoRI or XhoI. Restriction was inactivated for 10 min at 75°C and the restriction products were labeled by filling-in with αP32dCTP (in the case of XhoI) or αP32dATP (in the case of EcoRI) and Klenow polymerase. The products were separated on 1% agarose gel electrophoresis followed by autoradiography.

In vivo excision assay measured by β-galactosidase activity

Strain TAP114 transformed with either plasmid pPG123 or its mutant derivatives in the X sites, were each infected with phage HK022 at the logarithmic phase at a m.o.i.=10. After 40 min the cells were sonicated and assayed for β-galactosidase activity (35;66).

In vivo excision assay measured by phage release

Strain EY1529 transformed with a plasmid that carries xis or a control plasmid, was grown in LB+Ap to a cell density of A540=0.2. Each culture was divided into two subcultures, one of which was induced with 1mM IPTG. At time intervals, samples were taken to measure growth (A540) and the titer of free phage particles in the supernatant. In some cases, indicated in the text, the titer of free phage particles was measured after the cells were grown overnight.
In vitro integration assay

The integration assay was done as previously described (24), using 6nM of 100bp linear $^{32}$P-labeled $attB$ and 0.6nM of $attP$ carrying plasmid.

Quantitative analyses

The autoradiograms were exposed to a BAS1000 FUJI phosphorimager screen and the relative intensities of the bands were calculated using the TINA program package (version 2) from Rayset.

Substrates for gel retardation assay

Plasmids pMK27 and pMK28 were the source of wild type $attR$-$\lambda$ and $attL$-$\lambda$, respectively. pMK24 and pMK25 were used as the source of wild type $attR$-HK022 and $attL$-HK022, respectively. pPG107, pPG109, pPG113, pPG186 and pPG187, were used as the source of the various $attR$-HK022 mutants ($x1$, $x2$, $x1x2$, $x2'$ and $x1x2'$, respectively).

Core-deleted $attR$-$\lambda$ (195bp) fragment was obtained as a PCR fragment using plasmid pMK27 as template with oligo oEY147 and the reverse primer of pUC18 as primers. The PCR product was cut with EcoRI and cloned between the SmaI-EcoRI sites of pUC18. The plasmid obtained (pPG69) was used as the source of $\Delta attR$-$\lambda$.

Core-deleted $attR$-HK022 (415bp) fragment was obtained as a PCR fragment using plasmid pMK24 as template with oligo oEY148 and the forward primer of pUC18 as primers. The PCR product was cut with HindIII and cloned between the SmaI-HindIII sites of pUC18. The plasmid obtained (pPG68) was used as the source of $\Delta attR$-HK022.
The X-site mutations were inserted to the *attR* as described above in "Construction of the mutants in the Xis binding sites" and specified in Table 2 using pMK24 as template for PCR.

All *att* sites mentioned above were obtained by cutting the fragments out from their pUC18 vector with *Eco*RI and *Hind*III and the staggered *Hind*III ends were labeled by filling-in using the Klenow polymerase and $^{32}$P-labelled dCTP.

Core-deleted *attL-λ* and *attL-HK022* fragments (225pb and 165bp, respectively) were obtained as PCR fragments using plasmids pMK28 and pMK25, respectively, as substrates. In both reactions one primer was the reverse primer of pUC18 and the second primer was oligo oEY146 for the λ site, and oEY145 for the HK022 site. The PCR products were cut with *Eco*RI (the site derived from the polylinker of the vector) and the staggered *Eco*RI ends were labeled by filling-in using the Klenow polymerase and $^{32}$P-labelled dATP.

**Gel-retardation assays** (53)

Reactions were performed in 10 µl and contained 4 nM $^{32}$P-labeled *att* DNA, 3 µg sonicated salmon sperm DNA, 44 mM Tris-Cl (pH 8), 60 mM KCl, 5 µg BSA, 11 mM tris borate (pH 8.9), 1 mM spermidine, and 8.5% glycerol. In Figures 18 and 19 the final concentrations of the proteins were 0.48 µM IHF, 1.2 µM Xis and 0.15-2.5 µM of Int. In the rest of the shift assays the concentration of Xis was 0.1-0.8 µM and that of the IHF 0.5-8 µM, as described in text. Reactions were incubated for 10 minutes at room temperature, separated by electrophoresis on a 5% native polyacrylamide gel at 100 Volts in 0.5 TBE buffer. Subsequently, the gels were dried and autoradiographed using intensifying screens.
In vivo cross-linking experiments

1ml cultures of strain BL21(DE3), transformed with plasmid pPG1, pPG15 or pPG165, were induced at the logarithmic growth stage with 1mM of IPTG and pelleted after 2 hours. The pellet was resuspended in 20µl of a phosphate buffer saline (PBS) containing 2mM Disuccinimidyl suberate (DSS) at room temperature. The controls were resuspended in PBS without DSS. After 30 minutes the cells were centrifuged and resuspended in 30 µl containing 1M tris (which inhibits the cross-linker) 5% Glycerol, 4% SDS and 5% Bromphenol blue (loading buffer). β-mercaptoethanol was added to the relevant samples to a final concentration of 2mM and the samples were boiled for 3 minutes. Aliquots were subjected to 15% SDS PAGE and the protein was detected by a western blot.

In vitro cross-linking experiments

Induced cultures as above, were sonicated in PBS and centrifuged. 20µl sample of the supernatant were treated with 2mM DSS for 30 minutes at room temperature. The controls were incubated under the same conditions but without DSS. Loading buffer was added to a final concentrations as above and the samples were subjected to 15% SDS PAGE and a western blot.

Western-blot analyses

Gels were transblotted to a nitrocellulose membrane. Membranes were blocked overnight at 4°C in blocking solution (3% long life milk), treated for 60 min with primary polyclonal anti-Xis antibody (1:1000), and then incubated with peroxidase conjugated goat anti-rabbit IgG (1:50,000) for 45 minutes. The reaction was detected
using enhanced chemiluminescence (ECL). The Super Signal Chemiluminescence Substrate Kit (Pierce) was used according to the manufacturer’s instructions.

**Gel filtration chromatography**

2mg of purified Xis Protein (either native or mutant) were loaded on a Superdex 75 PC column (Pharmacia Biothech Inc.) that was equilibrated in 50 mM Tris buffer containing 200mM NaCl and calibrated with Ovalbumin (45kD), Cytochrome C (12.4kD) and Bacitracin (1.45kD). 1ml fractions were collected. Samples of 30µl of each fraction were separated on 15% SDS-PAGE and the protein was detected by a western blot.

**Crystallography**

Crystals were grown in hanging drops using the “Crystal screen” kit of Hampton Research. Each drop contained 3µl of the precipitant solution (100mM Sodium Cacodylate pH 6.5, 50 mM of Sodium acetate trihydrate and 15% w/v PEG 8000) and 3-6µl of 1mM concentrated sample of Xis. Crystals were obtained after 7 days.
REFERENCES


ייחוזיות הקניון במכנוזים והקודמים שלהם של הבקרטריאופאנים

ו- חפצים של התוכן Xis וה食べるית HK022 במっぴית.

תֶזֶר לְשָׁמֶךָ חֵזֶר ו־"דוקְטֵר ל'פִילוֹסְפְּניִית"'

מאת:

פִינְגַה גוֹטפֶרִיד

הוֹנֶשׁ לְסַנְאָט אוֹנוֹבְסֵייט תַלֶאָבִיב

יֵלֵי 2001
עבודה ועשתה בחדירות פורפ' עזרה גל
The bacteriophage HK022-1 λ Integartion host factor (Int) HK022-
, (att sites) DNA helicases, and (Xis) Excisionase. The
cofacilitated magnesium enters and leaves the helicase
which is essential for the mechanism and can be
between the catalytic site and the integrase. α)
The proteins λ and HK022 are
(X1) and Integartion host factor (Int). The
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