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Characterization of the bacteriophage lambda excisionase (Xis) protein: the C-terminus is required for Xis-integrase cooperativity but not for DNA binding

Thomas E.Numrych[1], Richard I.Gumport[2] and Jeffrey F.Gardner[1,3]

[1]Department of Microbiology, and [2]Department of Biochemistry and College of Medicine, University of Illinois, Urbana, IL 61801, USA)
[3]Corresponding author (Department of Microbiology, 131 Burrill Hall, 407 S.Goodwin, Urbana, IL 61801, USA)

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We have performed a mutational analysis of the *xis* gene of bacteriophage λ. The Xis protein is 72 amino acids in length and required for excisive recombination. Twenty-six mutants of Xis were isolated that were impaired or deficient in λ excision. Mutant proteins that contained amino acid substitutions in the N-terminal 49 amino acids of Xis were defective in excisive recombination and were unable to bind DNA. In contrast, one mutant protein containing a leucine to proline substitution at position 60 and two truncated proteins containing either the N-terminal 53 or 64 amino acids continued to bind λ DNA, interact cooperatively with FIS and promote excision. However, these three mutants were unable to bind DNA cooperatively with Int. Cooperativity between wild-type Xis and Int required the presence of FIS, but not the Int core-type binding sites. This study shows that Xis has at least two functional domains and also demonstrates the importance of the cooperativity in DNA binding of FIS, Xis and Int in λ excision.

Key words: cooperativity/DNA binding/mutagenesis/recombination

Introduction

Bacteriophage λ is a temperate phage that infects *Escherichia coli*. Upon infection, it may replicate and produce progeny phage or, alternatively, its chromosome may integrate into the bacterial DNA to form a prophage. If induced, the prophage excises from the chromosome and initiates lytic development. Both integration and excision occur by unique, reciprocal, site-specific recombination reactions that occur at specific regions of the interacting DNA molecules called attachment (*att*) sites (for a review, see Landy, 1989). The phage *att* site (*attP*) and the bacterial *att* site (*attB*) contain a common core sequence (O) of 15 bp, flanked by non-homologous DNA sequences called arms that are designated as P and P′ for *attP* or B and B′ for *attB*. Integration of λ into the bacterial genome forms two recombinant prophage sites called *attL* (BOP′) and *attR* (POB′) which themselves react to reform *attP* (POP′) and *attB* (BOB′) during excision (Weisberg and Landy, 1983).

Integrative recombination requires the λ-encoded protein integrase (Int) and the *E.coli*-encoded integration host factor (IHF) protein (Gingery and Echols, 1967; Miller and Friedman, 1977). Excisive recombination requires these two proteins and the λ-encoded protein excisionase (Xis) (Guerneros and Echols, 1970). In addition, the host protein factor for inversion stimulation (FIS) enhances excision (Thompson *et al.*, 1987; Numrych *et al.*, 1990; Ball and Johnson, 1991a). Footprinting experiments show that each of these proteins binds to different specific regions within the *att* sites. Int binds to five arm-type sites in the P and P′ arms (P1, P2, P′1, P′2 and P′3) and to two sites in each core [C and C′ in *attP* and B and B′ in *attB*; (Ross *et al.*, 1979; Hsu *et al.*, 1980; Mizuuchi *et al.*, 1981; Ross and Landy, 1982, 1983)]. IHF binds to two sites in the P arm (H1 and H2) and to one site in the P′ arm [H′ (Craig and Nash, 1984)]. Xis binds to the X1 and X2 sites in the P arm (Yin *et al.*, 1985) and FIS binds to the F site, which overlaps the X2 site (Thompson *et al.*, 1987).

The formation of a higher order protein-DNA complex in which the DNA is wrapped around a combination of the above four proteins bound to their respective sites is necessary for λ recombination. The different protein requirements for integration and excision indicate that excision is not simply the reversal of integration, and thus probably requires a different higher order DNA conformation from that used in integration. For example, the P2, X1, X2 and F sites in *attR* (Figure 1) are important for excision, but are not required for integration (Guarneros and Echols, 1970; Bushman *et al.*, 1985; Bauer *et al.*, 1986; Numrych *et al.*, 1990; Ball and Johnson, 1991b). Previous studies of these sites showed that Xis binds the DNA cooperatively at the X1 and X2 sites (Bushman *et al.*, 1984), and also binds the X1 site cooperatively with FIS at the F site (Thompson *et al.*, 1987). The DNA is bent when Xis or Xis-FIS bind to their respective sites (Thompson and Landy, 1988), and thus Xis and FIS may play structural roles in excision by forming or stabilizing a bend required for recombination. However, Xis may also play an additional role in excision by helping Int bind P2 through cooperativity since the presence of Xis reduces the amount of Int needed to bind the P2 site by 32-fold *in vitro* (Bushman *et al.*, 1984).

The goal of this study is to analyze the functions of the Xis protein in excision. We have mutagenized the *xis* gene and isolated 30 unique mutants. The mutant Xis proteins were examined for excision proficiency, DNA binding and their ability to bind DNA cooperatively with FIS and Int. We show that only the first 53 amino acids of Xis are required for binding the Xis sites in DNA and for Xis-FIS cooperativity of binding, whereas the carboxyl-portion of Xis is required for Xis-Int cooperativity in binding to the Xis and P2 sites, respectively. In addition, the cooperativity between Xis and Int required FIS.

Results

Mutagenesis and excision

In order to study the functions of Xis in excisive recombination, mutants were generated by altering the *xis* gene in several ways as described in Materials and methods. The mutants were made on plasmids pPS2-3ΔRS and

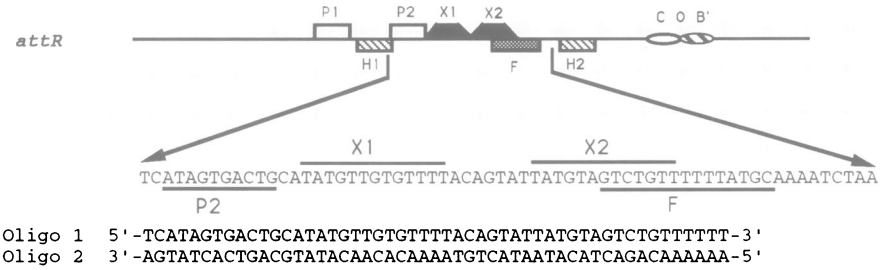

Fig. 1. The *attR* site of bacteriophage λ and the two oligodeoxyribonucleotides used to make the challenge phages. The *attR* sequence contains the binding sites for the proteins Int, IHF, Xis and FIS (Landy, 1989). Int binds to two arm-type sites, P1 and P2 (▭) and to the two core-type sites, C and B′ (⬭ and ⬭). IHF binds to the H1 and H2 sites (▨). Xis binds to the X1 and X2 sites (▬). FIS binds to the F site (◢◣) overlapping the X2 site. Oligos 1 and 2 are complementary and contain the P2, X1, X2 and F* sites.

Table I. Xis mutants and characteristics

Mutant[a]	Amino acid change	Type of mutagenesis[b]	Percent lysogeny at 1 mM IPTG[c]		Excision[d]
			P22xis2B	P22xis2D	
Xis-1I	M to I	HA	–	–	–
Xis-4I	T to I	HA	–	–	–
Xis-8	W to opal	HA	–	–	–
Xis-8	W to amber	HA	–	–	–
Xis-9S	N to S	$MnCl_2$	–	–	–
Xis-10V	A to V	HA	–	–	–
Xis-12	Q to amber	HA	–	–	–
Xis-13	R to opal	HA	–	–	–
Xis-19K	E to K	HA	–	–	–
Xis-22H	R to H	HA	–	–	–
Xis-23Q	R to Q	HA	–	–	–
Xis-24	W to amber	HA	–	–	–
Xis-26W	R to W	HA	–	–	–
Xis-28W	C to W	HA	–	6.7×10^{-4}	–
Xis-30T	I to T	$MnCl_2$	–	–	–
Xis-34S	P to S	HA	–	–	–
Xis-38R	G to R	HA	–	–	–
Xis-38E	G to E	HA	–	–	–
Xis-40K	E to K	HA	–	1.6	+
Xis-45	E to ochre	site-directed	–	–	–
Xis-46	S to amber	$MnCl_2$	–	–	–
Xis-47V	A to V	HA	–	–	–
Xis-49E	K to E	$MnCl_2$	–	–	–
Xis-54	R to opal	HA	8.5×10^{-4}	10.2	+
Xis-60P	L to P	$MnCl_2$	11.6	11.8	+
Xis-65	R to opal	site-directed	3.5	10.1	++
Wild-type			5.6	11.5	+++

[a]The nonsense mutants were named as 'Xis-' followed by the amino acid number containing the mutation. The missense mutants were named in the same manner, but the amino acid number was followed by the one-letter symbol representing the new amino acid at that location.
[b]See Materials and methods for a description of each type of mutagenesis.
[c]These values are given as percentages and represent the averages of three assays. A '–' value represents a lysogenization of $<10^{-6}$%, the lower limit of the assay.
[d]Excision was measured as described in Materials and methods and scored based on the length of time required for the colonies to change color on plates containing 1 mM IPTG. The level of excision was scored as '+++', '++' and '+' for colonies that turned red after 24, 48 and 72 h of incubation respectively. Colonies that remained white after 72 h of growth were scored as '–'.

pTN146, or moved from bacteriophage M13mp8xis into plasmid pTN160. The plasmids are all pCKR101 derivatives that placed the mutated *xis* gene downstream of a P_{tac} promoter on a plasmid that also contains $lacI^Q$. Therefore, the concentration of each Xis mutant inside a cell may be regulated by the concentration of IPTG in the growth medium. Overall, the various methods of mutagenesis procedure generated 30 different Xis mutants, consisting of 21 missense mutations and nine nonsense mutations. Twenty-six of the mutations reduced the ability of Xis to catalyze λ excision as measured by the red colony test (see Materials and methods) and are shown in Table I. Colonies of LE292/pIntB1 that contain a plasmid encoding the wild-type *xis* gene turned bright red on plates containing 1 mM IPTG after 24 h of incubation and red on plates lacking IPTG after 48 h of incubation. However, if the host strain contained a *fis::kan* mutation, colonies containing the wild-type *xis* gene formed red papillae on white colonies only after 48 h of growth on plates containing 1 mM IPTG (data not shown). The majority of Xis mutants were scored as excision

negative, because they were unable to form red colonies after 72 h of growth on MacConkey galactose plates containing 1 mM IPTG (Table I). However, LE292/pIntB1 transformants containing the Xis-40K, Xis-54, Xis-60P and Xis-65 mutants formed small red papillae on white colonies after 48–72 h of growth on plates containing 1 mM IPTG. These four mutants were leaky and thus for excision to take place, a higher concentration of Int and/or the mutant Xis protein was required. In addition, the behavior of the Xis-54 mutant (Table I) demonstrates that the N-terminal 53 amino acids of Xis were sufficient to promote λ excision.

Analysis of DNA binding in challenge phage assays

Each Xis mutant was tested for its ability to bind DNA using the bacteriophage P22-based challenge phage system (Benson *et al.*, 1986). In a previous study, we constructed two challenge phages that contained the X1, X2 and F binding sites of bacteriophage λ acting as an operator for P_{ant} (Numrych *et al.*, 1991). Phages P22xis2B and P22xis2D contain these sites in the P_{ant}-X1-X2-F-*ant* and P_{ant}-F-X2-X1-*ant* orientations respectively (Figure 2). The frequency of lysogeny of these phages varies with the intracellular Xis concentration and requires the presence of FIS. Thus, lysogenization by these challenge phages requires a repression complex of Xis at X1 and FIS at F. When P22xis2B and P22xis2D were used to infect *Salmonella typhimurium* MS1868 containing a plasmid encoding wild-type Xis, the highest frequency of lysogeny was reached at an IPTG concentration of 1 mM (Numrych *et al.*, 1991). Therefore, these two challenge phages were used to analyze the ability of each Xis mutant to bind with FIS to the X1 and F sites respectively, *in vivo*. The challenge phage assays were performed with strain MS1868 carrying plasmids containing the different *xis* mutants. Prior to each infection, the cells were induced with 1 mM IPTG. The results are presented in Table I.

Based on these results, the Xis mutants may be separated into two different groups, the most populous of which contains those mutants unable to prevent lytic growth of the challenge phages (i.e. the mutant proteins may not be able to bind the DNA or may be unstable). In contrast, the second group comprises those mutants that support lysogenic growth of both challenge phages, and includes the Xis-60P and Xis-65 mutants. Although the Xis-54 mutant only weakly supported lysogenic growth of P22xis2B, it was included in the second group because it supported lysogenic growth of P22xis2D (Table I). The reason for the difference between Xis-54 and the other two mutants is under investigation, and is possibly due to a decrease in the relative stability of the Xis-54 protein. In any case, the latter class of mutants bind the X1 site.

Analysis of Xis–Int cooperativity with the challenge phage assay

To investigate the cooperativity of binding of Xis and Int, we constructed two challenge phages containing the Int P2 site in addition to the X1, X2 and F sites used in a previous study (Numrych *et al.*, 1991). These phages were originally designed to contain only the P2, X1 and X2 binding sites. However, an altered F site (F*) is present and functional (see below) in both phages. The challenge phages P22xis4B and P22xis4D carry the inserts in the P_{ant}-P2-X1-X2-F*-*ant* and P_{ant}-F*-X2-X1-P2-*ant* orientations, respectively (Figures 1 and 2).

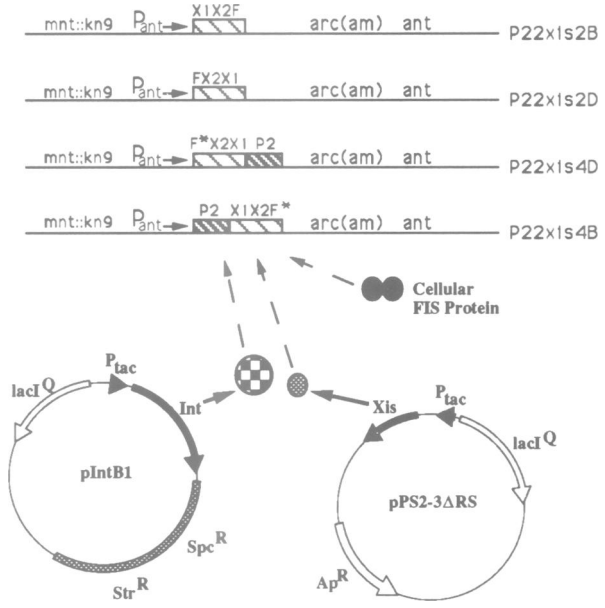

Fig. 2. A comparison of the challenge phages used in this study and a cartoon depicting the requirements for lysogenization by P22xis4B. See Materials and methods for a description of each phage, plasmid, and the challenge phage assay.

The challenge phage assays were performed with MS1868 containing either pPS2-3ΔRS as a source of Xis, pIntB1 as a source of Int, or both plasmids as sources of both proteins. Prior to each infection, the cells were grown either without IPTG, or with various IPTG concentrations ranging from 0.2 mM to 1 mM. The results of these challenge phage assays with P22xis4B and P22xis4D are shown in Figure 3, and are presented as the percent of viable cells lysogenized as a function of the IPTG concentration in the medium. P22xis4B and P22xis4D were unable to lysogenize MS1868 containing only pIntB1 (data not shown). Consequently, Int alone cannot bind the P2 site well enough to act as a repressor in the challenge phage assay.

The frequency of lysogenization of P22xis4D on MS1868/pPS2-3ΔRS increased by four orders of magnitude as the concentration of IPTG was increased to 1 mM (Figure 3), but dropped to zero when the assay was repeated in a *fis* host (data not shown). On closer inspection of the inserts in P22xis4B and P22xis4D, the F* site contains the first 11 bp of the original λ F site, followed by the nucleotides GGGA. The last four nucleotides of the Hübner and Arber (1989) FIS consensus sequence are RNNC (where R represents an A or a G, and N represents any nucleotide). Therefore, it is not surprising that FIS still binds the F* site in P22xis4B and P22xis4D, because these sites contain 14 out of 15 bases of the FIS consensus sequence. Thus, the frequency of lysogenization of P22xis4D varies with the intracellular Xis concentration, and requires the presence of FIS.

Unlike P22xis4D, the phage P22xis4B was unable to form lysogens on MS1868 carrying pPS2-3ΔRS. Although the phage P22xis4B (P_{ant}-P2-X1-X2-F*-*ant*) contains the same orientation of binding sites as P22xis2B (P_{ant}-X1-X2-F-*ant*), the additional P2 sequence probably causes Xis and FIS to bind too far downstream from the promoter in P22xis4B to act as efficient repressors.

When challenge phage assays were performed on MS1868 carrying pPS2-3ΔRS and pIntB1, both P22xis4B and P22xis4D were able to form lysogens, and their frequencies

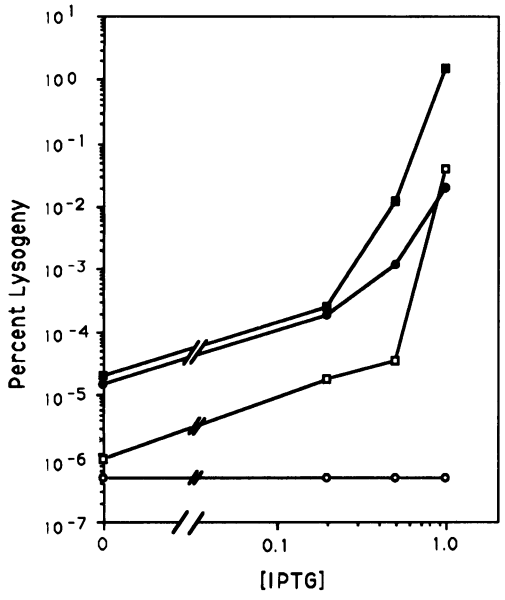

Fig. 3. The challenge phage assay. The frequency of lysogenization (Materials and methods) is shown for P22xis4B (circles) and P22xis4D (squares) when used to infect cells induced at different IPTG concentrations. The strain MS1868 containing pPS2-3ΔRS only is represented by open symbols; the strain MS1868 containing both pPS2-3ΔRS and pIntB1 (produces both Xis and Int) is represented by filled symbols.

of lysogenization increased by four to six orders of magnitude respectively, as the IPTG concentration in the medium was increased to 1 mM (Figure 3). Both phages exhibited the same frequency of lysogenization (10^{-5}%) on cells grown without IPTG, but P22xis4D displayed a 100-fold higher frequency of lysogenization than P22xis4B on cells that had been induced with 1 mM IPTG. In addition, P22xis4D exhibited a 100-fold higher frequency of lysogenization on cells containing both plasmids than on those containing only pPS2-3ΔRS, at 1 mM IPTG. In order to determine if FIS was still required for Int and Xis to act as repressors, the challenge phage assays were repeated in JG1160 (MS1868::*fis-3*) carrying pPS2-3ΔRS and pIntB1. P22xis4B and P22xis4D were unable to form lysogens in the absence of FIS. The frequency of lysogenization by P22-P'123 (a challenge phage containing the contiguous P'1, P'2 and P'3 sites) was unaffected by the presence or absence of FIS, demonstrating that FIS is not required for P22 to form lysogens (data not shown). Therefore, lysogenization by P22xis4D depends on Xis and FIS, and is enhanced by Int (Figure 3). In contrast, P22xis4B requires Xis, Int and FIS to form a repression complex with Int at P2, Xis at X1, and FIS at F* to form lysogens. Because P22xis4B requires both Int and Xis for lysogenization, and these proteins have been shown to bind cooperatively *in vitro* to λ DNA (Bushman *et al.*, 1984), it is likely they are also binding cooperatively *in vivo* to this challenge phage.

Analysis of the Xis mutants for Xis–Int cooperativity in vivo

Based on the results of the challenge phage assays with P22xis2B and P22xis2D, the Xis-54, Xis-60P and Xis-65 mutants bind DNA and interact with FIS like wild-type Xis (Table I; see below). However, these mutants were unable to catalyze λ excision efficiently, and thus must have been deficient in a critical function of wild-type Xis. To see if these mutants might be unable to promote Int binding to the P2 site, challenge phage assays were performed using P22xis4B (which requires Int, Xis and FIS for lysogenization) in MS1868 and MS1868/pIntB1 carrying plasmids encoding these Xis mutants. All cells were induced prior to infection with 1 mM IPTG. The results are presented in Table II. Neither the three mutants nor wild-type Xis repressed lytic growth of P22xis4B in the absence of Int (Table II). The addition of Int to MS1868/pPS2-3ΔRS via pIntB1 increased the frequency of lysogeny 20 000-fold (Table II). However, the addition of Int via pIntB1 to MS1868 carrying plasmids expressing the Xis-54, Xis-60P or Xis-65 mutants had little or no effect on lysogeny (Table II). Therefore, these mutants were unable to promote the binding of Int to P2. This finding shows that the C-terminal region of Xis is required for Xis–Int DNA binding cooperativity. The observation that these mutants are deficient in excisive recombination *in vivo* indicates that this cooperativity is important for excision.

Table II. Results of challenge phage assays analyzing Xis–Int cooperativity

Mutant	Percent lysogeny on P22xis4B[a]	
	−pIntB1	+pIntB1
Xis-54	$<10^{-6}$	$<10^{-6}$
Xis-60P	2.5×10^{-6}	1.5×10^{-6}
Xis-65	$<10^{-6}$	8.8×10^{-5}
Wild-type	$<10^{-6}$	2.0×10^{-2}

[a]The assays were performed with MS1868 (−pIntB1) or MS1868/pIntB1 (+pIntB1) containing the plasmid with the mutant shown. The assays were performed with cells induced with 1 mM IPTG prior to infection. These values are given as percentages and represent the averages of three assays.

Gel retardation assays

Requirements for Xis–Int cooperativity. A gel retardation assay was used to study the interactions between Xis, FIS and Int occurring upon DNA binding. The DNA fragments were each ^{32}P-labeled, and each contained the P2, X1, X2, F and H2 sites from bacteriophage λ and the Int core-type sites from either λ or the λ-like bacteriophage HKO22 (the fragments comprised essentially the P arm of *attR*). Similar cooperativity of binding between Xis and Int was also observed with a DNA substrate containing pUC19 DNA in place of the Int core-type sites (data not shown). This control experiment showed that all the DNA fragments used were potentially equal substrates in the gel retardation assay because cooperativity between Xis and Int did not depend upon the Int core-type binding sites. In addition, a crude extract isolated from MO (the parent strain lacking a plasmid expressing Xis) was unable to retard these fragments, and crude extracts containing Xis and Xis mutant proteins were unable to retard DNA fragments lacking Xis binding sites (data not shown). Therefore, the retarded protein–DNA complexes described below result from specific interactions between the respective proteins and their binding sites.

The different protein–DNA complexes migrated differently in the gel depending upon which proteins were bound to their target sites. Therefore, it was important to define the relative mobility of each type of protein–DNA complex. For example, a reaction containing Xis in an extract lacking FIS (isolated from MO *fis::kan*/pPS2-3ΔRS)

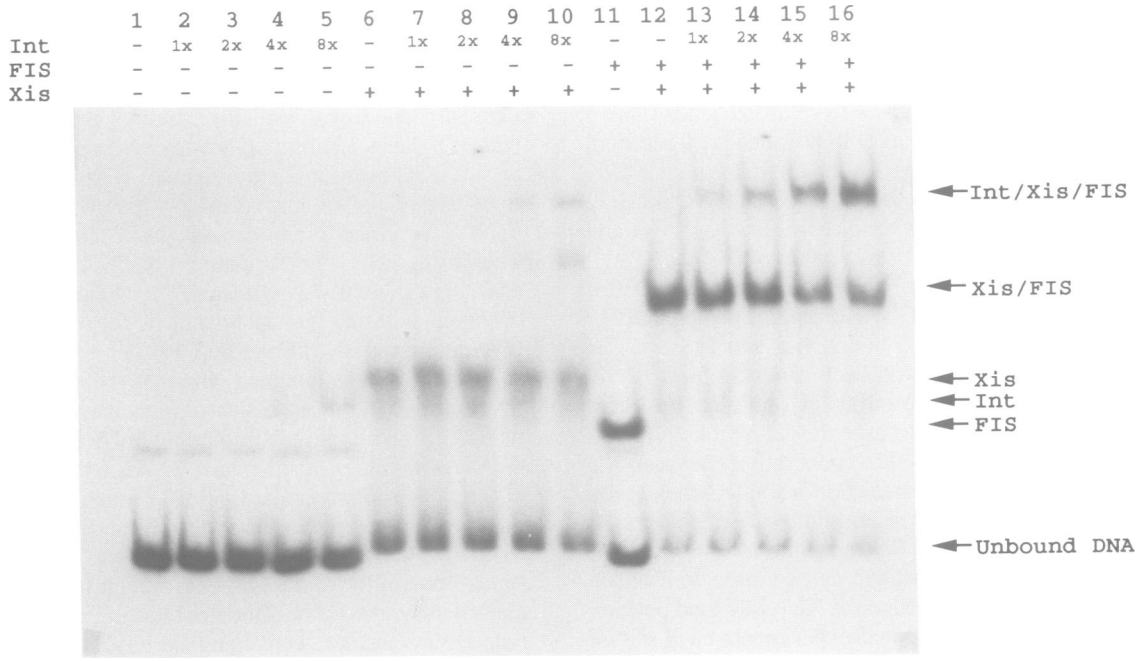

Fig. 4. Binding of Xis, FIS and Int proteins in a gel retardation assay. The assay was performed as described in Materials and methods. The ^{32}P-labeled DNA fragment was 150 bp long and derived from a *Bgl*II/*Dde*I digest of pNR141. It contains the λ P2, X1, X2, F, H2 and HKO22 C and C′ sites. A crude protein extract containing Xis was isolated from MO *fis::kan*/pPS2-3ΔRS, and 13 μg total protein of this extract was added where indicated. FIS was added where indicated to a final concentration of 17 nM. Int was added as indicated. An '8×' amount of Int contained 1.6 units of activity. Each retarded band contains the proteins indicated after the arrow. An extra band may be seen in lanes 1–5 and 11 from a contaminant DNA isolated during purification.

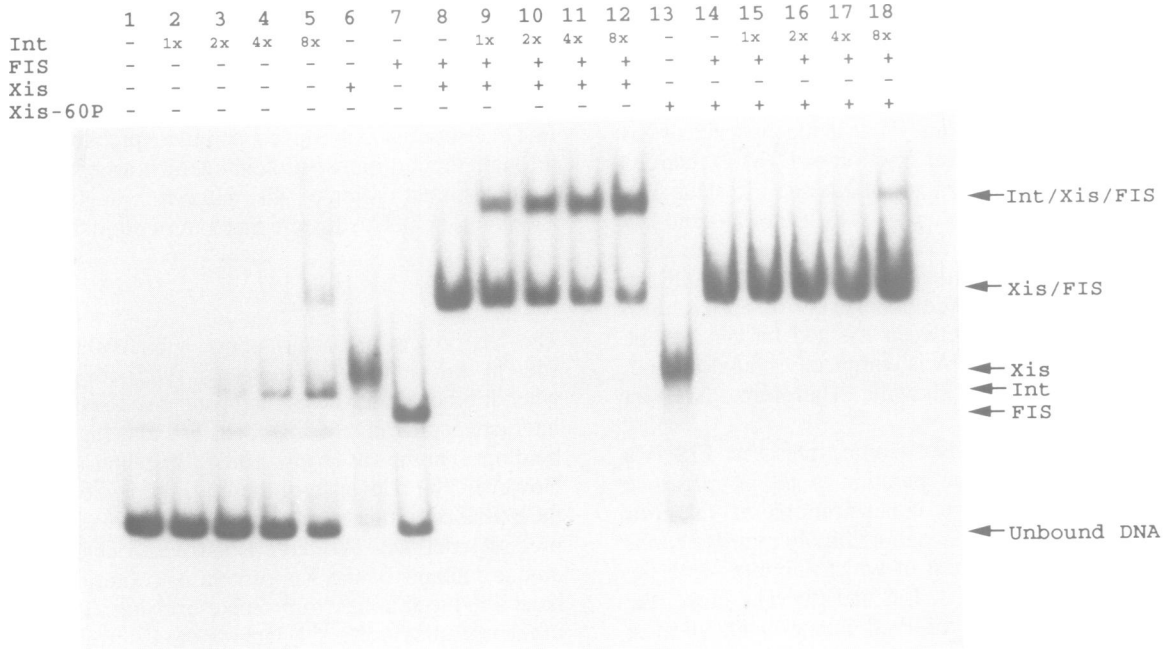

Fig. 5. Binding of Xis and Xis-60P proteins in a gel retardation assay. The assay was performed as described in Materials and methods. The ^{32}P-labeled DNA fragment was 150 bp long and derived from a *Bgl*II/DdeI digest of pNR141. It contains the λ P2, X1, X2, F, H2 and HKO22 C and C′ sites. The crude extracts containing Xis or Xis-60P were isolated from MO *fis::kan* carrying the respective plasmid. Total amounts of protein for Xis were 17 μg (lane 6) or 8.5 μg (lanes 8–12); those for Xis-60P were 12 μg (lane 13) or 6 μg (lanes 14–18). FIS was added where indicated to a final concentration of 17 nM. Int was added as indicated. An '8×' amount of Int contained 1.6 units of activity. Each retarded band contains the proteins indicated after the arrow. An extra band may be seen in lanes 1–5 from a contaminant DNA isolated during purification.

produces only one retarded band (lane 6 in Figure 4). We presume this band represents an Xis bound to both X1 and X2 in accordance with previous gel retardation assay results (Thompson *et al.*, 1987a). Because Xis binds the X1 and X2 sites cooperatively, if two bands are seen, the most intense of the retarded bands has Xis bound to both sites

(Thompson *et al.*, 1987). As can be seen in lane 11 of Figure 4, FIS alone retards the DNA in the gel to a point just below the band corresponding to the DNA with Xis bound to both sites, and Int alone retards the DNA to a point in between the Xis-retarded and FIS-retarded bands (lane 5 in Figure 4). When both Xis and FIS are added to a reaction, a single retarded band is observed that migrates more slowly than the band containing Xis bound to X1 and X2 (lane 12 in Figure 4). We believe this band represents Xis at X1 and FIS at F (Thompson *et al.*, 1987; Numrych *et al.*, 1991). Lastly, as can be seen in lanes 13–16 of Figure 4, Xis, FIS, and Int together retard the DNA to a point on the gel that is above the band containing bound Xis and FIS.

Cooperativity in binding has been reported for Xis and FIS (Thompson *et al.*, 1987) and for Xis and Int (Bushman *et al.*, 1984). Accordingly, we used the gel retardation assay to examine these interactions. The cooperativity between Xis and FIS was easily demonstrated. For example, when either Xis or FIS was present at a concentration sufficient to retard only a portion of the DNA (lanes 6 and 11 in Figure 4), the addition of the second protein caused almost all of the DNA to be completely retarded to the Xis–FIS band position (lane 12 in Figure 4). Additional experiments have shown that the presence of FIS reduces the concentration of Xis required to retard 100% of the DNA by 4-fold (data not shown), in agreement with what has been previously reported for Xis–FIS cooperativity (Thompson *et al.*, 1987).

Xis–Int cooperativity could not be demonstrated in the absence of FIS. For example, in Figure 4, lanes 2–5 contain reactions with increasing amounts of Int, lanes 7–10 contain reactions with the same Int dilution pattern in the presence of Xis (isolated from MO*fis::kan*/pPS2-3ΔRS), and lanes 13–16 contain reactions with the same Int dilution pattern in the presence of both Xis and FIS. As can be seen in lanes 5 and 10, only a small fraction of DNA is retarded with 1.6 units of Int, whether alone (lane 5) or in the presence of Xis (lane 10). However, a similar fraction of DNA is retarded by 0.2 units of Int in the presence of Xis and FIS (lane 13). Furthermore, 1.6 units of Int in the presence of Xis and FIS (lane 16) retards at least 8-fold more DNA than the same concentration of Int, whether alone (lane 5) or in the presence of Xis (lane 10). Additional experiments have also shown that cooperativity between Xis and Int can best be demonstrated when the DNA is completely bound by both Xis and FIS (data not shown). Therefore, Xis–Int cooperativity requires FIS.

Interestingly, Figure 4 also demonstrates that FIS is a contaminant in our Int preparation with its presence becoming apparent at high concentrations of Int. For example, there are actually two more highly retarded bands in lanes 9 and 10, the highest of which migrates with the band representing bound Xis, FIS and Int. Therefore, the lower protein–DNA band observed in lanes 9 and 10 most probably represents Xis bound to X1 and X2, and Int bound to P2. This suggests that our purified Int has traces of FIS. Furthermore, gel retardation experiments also demonstrated that FIS is present in crude Xis extracts isolated from MO (data not shown).

Analysis of the Xis mutants for Xis–Int cooperativity in vitro. The Xis-60P mutant repressed lytic growth by P22xis2B and P22xis2D, but not by P22xis4B. These results demonstrate that this mutant protein binds DNA as well as wild-type Xis, but fails to promote the binding of Int to P2 in P22xis4B. Therefore, we used the gel retardation assay as an additional test of whether the Xis-60P mutant was truly deficient in Xis–Int cooperativity. As seen in Figure 5, the Xis-60P mutant retards the DNA to the same degree as wild-type Xis (compare lane 6 with lane 13) and binds DNA cooperatively with FIS (lanes 13 and 14); however, more DNA is retarded by 0.2 units of Int in the presence of wild-type Xis and FIS (lane 9) than the amount retarded with 1.6 units of Int alone (lane 5) or in the presence of Xis-60P and FIS (lane 18). Therefore, the Xis-60P mutant is unable to promote binding of Int as efficiently as wild-type Xis. Similar results were observed with the Xis-54 mutant in gel retardation assays (data not shown). Therefore, the gel retardation assays support the results obtained with the challenge phage assay by also showing that these two mutants are defective with respect to wild-type Xis in binding cooperatively with Int.

DNA-binding proteins retard DNA fragments differently during electrophoresis in a gel as a function of the size of the protein and the degree to which the DNA is contorted by the protein binding (Wu and Crothers, 1984). In order to test the ability of the Xis-60P mutant to bend the DNA as well as wild-type Xis, the gel retardation assay was used with DNA substrates containing the P2, X1, X2 and F* sites either in the middle or at the end of the fragments. As can be seen in Figure 6, the Xis–FIS or Xis-60P–FIS complexes to sites in the middle of the fragment are less mobile than the complexes at the end of the fragment (compare lanes 4 and 6 with lanes 10 and 12). However, it is apparent that there is little or no difference in the relative mobilities of the Xis–FIS and Xis-60P–FIS complexes when they occur at the middle or at the end of the fragment. Because differences in bending manifest themselves more in alterations at the center than at the ends of fragments, this result suggests that both complexes bend the DNA to the same extent. Taken together, these results provide the first evidence that Xis–Int cooperativity probably takes place through direct protein–protein interactions between Int and the C-terminal region of Xis, and not through Xis-induced alterations in DNA structure that in turn promote Int binding.

Discussion

The Xis protein of bacteriophage λ binds the X1 and X2 sites in *attR* and *attP* (Yin *et al.*, 1985), bends the DNA when it binds these sites (Thompson and Landy, 1988) and interacts cooperatively with both FIS and Int during DNA binding (Thompson *et al.*, 1987; Bushman *et al.*, 1984). However, the relevance of these properties to excision and the existance of functional domains within Xis have not been precisely defined. We have isolated and characterized 26 unique mutants of the Xis protein of bacteriophage λ that have a reduced ability to promote excision. The properties of the mutant Xis (Xis-54) lacking the C-terminal 19 amino acids in the excision, challenge phage and gel retardation assays demonstrate that the N-terminal 53 amino acids of Xis are sufficient to promote λ excision, bind the DNA and interact cooperatively with FIS.

In addition, the Xis-54, Xis-60P and Xis-65 mutants comprise a special class of mutants that bind DNA in a manner similar to wild-type Xis, but are impaired in λ excision. These results indicated that the C-terminal portion of Xis is important in excision for something other than binding and bending the DNA. One possible function is cooperative DNA binding between Xis and Int. Therefore,

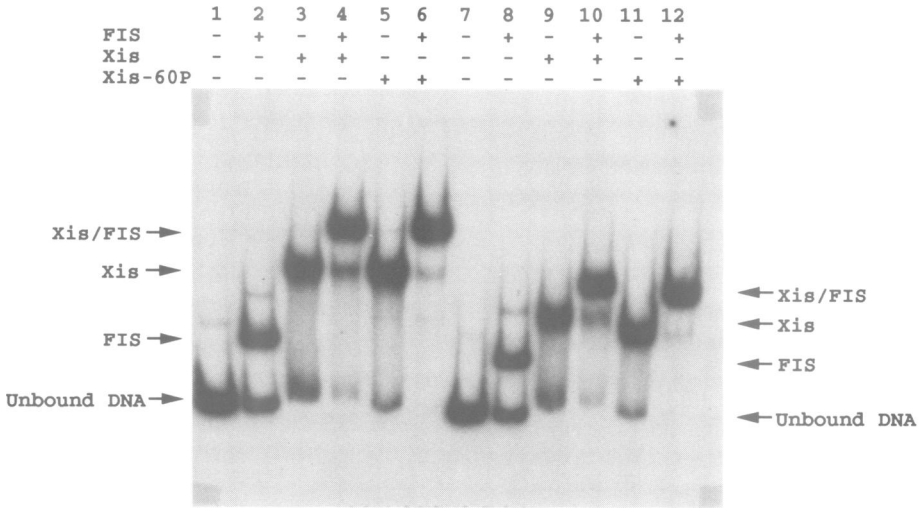

Fig. 6. Binding of Xis and Xis-60P proteins to *Eco*RV- and *Bam*HI-cleaved fragments of pTN165. The gel retardation assay was performed as described in Materials and methods. The ^{32}P-labeled DNA fragments were 172 bp long and derived from pTN165, a plasmid that contains the λ P2, X1, X2 and F* sites located between two identical multiple cloning sequences (see Materials and methods). The fragment in lanes 1–6 was derived from an *Eco*RV digest, thereby placing the P2, X1, X2 and F* sites in the middle of the fragment. The fragment in lanes 7–12 was derived from a *Bam*HI digest, and placed the P2, X1, X2 and F* sites at the end of the fragment. The crude extracts containing Xis or Xis-60P were isolated from MO *fis::kan* carrying the respective plasmid. Proteins were added from crude extracts where indicated for Xis (8.5 μg) and Xis-60P (6.0 μg), respectively. FIS was added where indicated to a final concentration of 170 nM.

we constructed the challenge phage P22xis4B that contained the region encompassing the P2, X1, X2 and F* sites act as an operator for P_{ant} in the P_{ant}-P2-X1-X2-F*-*ant* orientation of a challenge phage. Lysogenization by this challenge phage required the simultaneous binding of Int, Xis and FIS to the P2, X1 and F* sites respectively. We used P22xis4B to test the ability of the Xis-54, Xis-60P and Xis-65 mutants to bind along with Int and FIS to prevent lytic growth. These three mutants (in the presence of Int and FIS) were unable to effectively repress lytic growth by P22xis4B, revealing that they had lost the ability to interact with Int cooperatively. These findings demonstrate that the C-terminal portion of Xis is required for Xis–Int cooperativity. Furthermore, the reduced ability of these mutants to promote λ excision suggests that this cooperativity is important in excision. Even more dramatically, the results with the Xis-65 mutant show that removing as few as eight amino acids from the C-terminus of Xis reduces cooperativity with Int and thereby impairs excision.

By taking a genetic approach to analyze a multi-protein regulatory system, we have been able to screen and test Xis mutants to reveal that the protein has two functional domains; the N-terminal domain is required for the protein to bind DNA and the C-terminal tail is responsible for Xis–Int DNA-binding cooperativity. The results of this study (see below) suggest that the cooperative interactions between Xis and Int are mediated through protein–protein interactions. We are continuing our genetic study of this system by seeking additional missense mutants in Xis that are deficient in Xis–Int cooperativity. Such mutants may be used to isolate suppressors in Int, revealing the regions of Int that interact with Xis, and helping to define the requirements for the protein–protein cooperativity.

We used the gel retardation assay to examine the cooperativity between Xis and Int *in vitro*. In these assays, similar cooperativity between Xis and Int was observed with different DNA substrates, even when the Int core-type sites were replaced with the core-type sites from phage HKO22 (Figures 4 and 5) or replaced with pUC19 DNA (data not shown). These results show that cooperativity between Xis and Int does not involve the Int core-type sites. In addition, we were unable to demonstrate cooperativity between Xis and Int in the gel retardation assays in the absence of FIS (Figure 4). Thus, under our conditions, FIS is required for cooperativity between Xis and Int. This ternary interaction may also explain the results of the challenge phage assays, where Xis and Int were unable to act as repressors for P22xis4B in the absence of FIS. Furthermore, the level of excision observed in the red colony assay was reduced to the same extent when the bacterium contained Int, FIS and the mutant Xis-60P which cannot promote Xis–Int cooperativity, as when the bacterium contained Int and wild-type Xis but lacked FIS. Collectively, these results suggest that the role of FIS in excision is not only to promote Xis binding to the DNA, but also to help Xis promote Int binding to P2. Although Int–Xis cooperativity is probably mediated through protein–protein interactions, FIS may stabilize Xis at X1 or create a unique DNA conformation required for Xis–Int cooperativity. The requirement of FIS for cooperativity in DNA binding *in vitro* between Xis and Int has not previously been reported, possibly due to FIS contamination of Int or Xis preparations (see Results).

The results of the gel retardation assays also confirmed the challenge phage results by demonstrating that the Xis-54 and Xis-60P mutants can no longer facilitate Int binding to the P2 site. Because these two mutants retard the DNA to the same degree as wild-type Xis under all the conditions tested, and still bind cooperatively with FIS, Xis–Int cooperativity is probably mediated through protein–protein interactions between the C-terminal portion of Xis and an unknown domain of Int.

It is probable that the purpose of an Int arm-type site is to bind the N-terminal domain of Int and present its catalytic site to a core-type site (Moitoso de Vargas *et al.*, 1988; Richet *et al.*, 1988; Kim *et al.*, 1990). Thus, the requirement for different arm-type sites in integration versus excision may

be a result of the different DNA conformations occurring during these reactions. For example, the *attR* conformation would probably have the DNA bent around Xis and FIS bound at X1 and F, as well as around IHF bound to H2. The roles of Xis and FIS in excision would be to promote the binding of Int to the P2 site while bending the DNA into a conformation in which the Int at the P2 site can be presented to a core-type site.

The results of this study demonstrate the complexity of the nucleoprotein complex that is required for excisive site-specific recombination. The P22-based challenge phage system was useful for demonstrating the simultaneous requirement for as many as three proteins to interact with the DNA. The extreme power of the P22 challenge phage system is potentially useful for dissecting other complicated, multicomponent nucleoprotein systems. For example, eukaryotic enhancer systems may be amenable to analysis with the challenge phage system.

Materials and methods

Bacterial strains, media, chemicals and enzymes

Bacterial strains are listed in Table III. The media and buffers used have been described previously (Lee *et al.*, 1990). Luria–Bertani (LB) medium, Tris–EDTA (TE) buffer and Tris–borate–EDTA (TBE) buffer were made as described by Maniatis *et al.* (1982). All phage dilutions were made in λ-Ca buffer [10 mM Tris–Cl (pH 7.9), 10 mM $MgSO_4$ and 5 mM $CaCl_2$]. All antibiotics were purchased from Sigma and added to the media in the following concentrations: ampicillin and kanamycin, 50 μg/ml; spectinomycin, 100 μg/ml; chloramphenicol, 30 μg/ml; and tetracycline, 15 μg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Sigma.

T4 DNA ligase, T4 polynucleotide kinase, bacterial alkaline phosphatase (BAP) and all restriction endonucleases were obtained from Bethesda Research Laboratory. Sequenase and *Taq* DNA polymerase were obtained from United States Biochemical and Promega respectively.

DNA manipulations

Plasmid DNA was isolated by the rapid alkaline method (Maniatis *et al.*, 1982). Transformations were performed by electroporation at 2.5 kV, 25 mF and 200 Ω in a Bio-Rad Gene Pulser apparatus (Miller, 1988). Plasmid templates were prepared and sequenced by the dideoxy method using Sequenase as described by Kraft *et al.* (1988). Single-stranded M13 template DNA was prepared in *E.coli* KK2186 as described (Bauer *et al.*, 1985) and sequenced by the dideoxy method following the United States Biochemical protocol for sequencing single-stranded DNA with Sequenase. Two different oligodeoxyribonucleotides were used as primers to sequence the wild-type and mutant *xis* genes. The Int-8 and Xis-8 primers contain the sequences d(CCTGTAGCAGTAATATCC) and d(ATATAAAGGTTAGGGG) respectively, which are complementary to the DNA 3′ of *xis*, and thus prime DNA synthesis towards the *xis* gene. Other primers used for sequencing are described as indicated.

Plasmid and M13 constructions

The plasmid pPS2-3ΔRS contains the *xis* gene under control of the P_{tac} promoter, $lacI^Q$ and ampicillin resistance genes and the pBR322 origin of replication (Numrych *et al.*, 1991). The plasmid pTN146 is a derivative of pPS2-3ΔRS that contains an M13 intergenic (IG) region. The plasmid pIntB1 contains the *Int* gene under control of the P_{tac} promoter, $lacI^Q$, spectinomycin and streptomycin resistance genes and the pSC101 origin of replication.

The phage M13mp8xis was made by cloning the *Eco*RI–*Bam*HI *xis*-containing fragment from pRK5 (Abremski and Hoess, 1983) into the same sites in M13mp8 (Messing, 1983). The plasmid pTN160 was constructed to serve as a vector for cloning *xis* mutants generated in M13mp8xis. It contains an *Eco*RI site directly adjacent to the previous *Sna*BI site of pCKR101. Therefore, mutants generated in M13mp8xis could be cloned into the *Eco*RI–*Bgl*II sites of pTN160 and be almost isogenic to pPS2-3ΔRS. The only difference between plasmids containing *xis* mutants in pPS2-3ΔRS and those constructed by cloning the mutants into pTN160 is 3 bp in the 150 bp region between P_{tac} and the *xis* gene.

The plasmids pTN163 and pTN164 were constructed to place the P2, X1, X2 and F* sites adjacent to the P_{ant} locus of pPY190. Complementary oligodeoxyribonucleotides (Figure 1) were annealed and inserted into the *Sma*I site of pPY190, as described (Hughes *et al.*, 1988; Lathe *et al.*, 1984). The plasmids pTN163 and pTN164 contain inserts with orientations of F* and P2 nearest the P_{ant} promoter respectively, as determined by sequencing the plasmids with primers described previously (Numrych *et al.*, 1991). The plasmid pNR141 contains *attP* from λ, but with the Int core-type sites from phage HKO22 (Nagaraja and Weisberg, 1990). The plasmid pTN121 contains the λ *attR* site (Numrych *et al.*, 1990).

The plasmid pTN165 was constructed to place the P2, X1, X2 and F* sites in between the two identical multiple cloning sequences of pBend2 (Zweib *et al.*, 1989). This plasmid was constructed in two steps. First, the *Eco*RI–*Sca*I fragment of pBend2 was replaced with the *Eco*RI–*Sca*I fragment of pUC19, thereby removing the *Hin*cII site from the *bla* gene of pBend2. Then, complementary oligodeoxyribonucleotides (Figure 1) were annealed and inserted into the remaining *Hin*cII site as described (Hughes *et al.*, 1988; Lathe *et al.*, 1984). *Eco*RV digestion of pTN165 places the P2, X1, X2 and F* sites in the middle of the fragment (P2 is 71 bp from the left end and F* is 68 bp from the right end). *Bam*HI digestion places the sites at the left end of the fragment (P2 is 18 bp from the left end and F* is 121 bp from the right end).

Mutagenesis of the xis gene

One method used to generate mutations in *xis* was to treat the plasmid pPS2-3ΔRS with hydroxylamine (HA), which causes GC to AT base-pair transitions (Drake, 1970). The mutagenesis was performed as described by Maloy (1990). The strain LE292/pIntB1 (described below) was transformed by the HA-mutagenized pPS2-3ΔRS, plated onto MacConkey galactose–ampicillin–spectinomycin plates, and incubated at 37°C for 2 days. The pPS2-3ΔRS plasmids containing potential *xis* mutants were isolated from colonies that remained white, separated from pIntB1 and sequenced as described above.

Two nonsense mutations in *xis* were constructed at amino acid positions 45 and 65 by oligonucleotide-directed mutagenesis using strain M13mp8xis and *dut ung* selection (Kunkel *et al.*, 1987; Schena, 1988). The mutation at amino acid 45 was made by changing the codon from GAA to UAA using the oligodeoxyribonucleotide d(GCTGATTAGTGGAAC). The mutation at amino acid 65 was made by changing the codon from AGA to TGA using the oligodeoxyribonucleotide d(CCATTTCAGATCCTC). Phages containing the mutations were identified by colony hybridization (Bauer *et al.*, 1985), isolated by plaque purification and used to make templates for sequencing as described above. The mutations were moved from M13mp8xis to a suitable expression vector by cloning the *Eco*RI–*Bam*HI *xis*-containing fragment from M13mp8xis into the *Eco*RI–*Bgl*II sites of pTN160.

The frequency with which *Taq* DNA polymerase incorporates a wrong nucleotide during DNA synthesis may be increased by the addition of $MnCl_2$ to the reaction buffer and by increasing the ratio of one of the dNTPs relative to the other three (Leung *et al.*, 1989). Furthermore, when the initial concentrations of three of the four required dNTPs are suboptimal, mutations are limited to within 200 nucleotides of the primer (Liao and Wise, 1990). These methods were combined and used to synthesize the second strand of DNA from a single-stranded DNA template of pTN146 or M13mp8xis, which were prepared from a *dut ung* strain as described (Zagursky and Berman, 1984; Bauer *et al.*, 1985).

A typical reaction contained 1 μg uracil-containing template, 50 pmol phosphorylated Xis-8 primer, 2 μl 10× *Taq* buffer [500 mM Tris–Cl (pH 9) and 100 mM $MgCl_2$], 2 μl 20 μM dNTPs (dGTP, dATP, dTTP and dCTP), 2 μl 650 μM of either dGTP or dATP, 2 μl 5 mM $MnCl_2$, 1 μl *Taq* DNA polymerase (2.5 U/μl) and double distilled H_2O to a total volume of 20 μl. The mixture was covered with 25 μl mineral oil and heated to 90°C for 3 minutes, and then cooled to 55°C, where it was incubated for 1 h. At this time, 2 μl chase solution (1 mM dNTPs and 20 mM ATP) was added to the reaction, and it was incubated for one more hour at 55°C. The reaction was cooled to 37°C, 2 U of T4 DNA ligase were added and the mixture was incubated at 37°C for 1 h. Finally, the mixture was used to transfect KK2186 (M13mp8xis) or transform LE292/pIntB1 (pTN146) as described (Bauer *et al.*, 1985; Miller, 1988). Mutations in pTN146 were screened in an identical manner to the HA-induced mutations in pPS2-3ΔRS (described above). Mutations in M13mp8xis were characterized by sequencing single-stranded DNA from phages that formed plaques on KK2186, and then subcloning the *xis* mutants into pTN160 as described above.

In vivo excision assay

The ability of each *xis* mutant to promote λ excision was tested using *E.coli* LE292. This strain contains a defective λ prophage (Int^- Xis^-) inside its *galT* gene, making the cells Gal^- (Enquist and Weisberg, 1976). However, when Int and Xis are introduced into LE292 on compatible plasmids, they

Table III. Bacterial strains and phages

	Relevant characteristics	Source
Escherichia coli		
KK2186	Δ(*lac-pro*) *supE thi strA sbcB15 endA*/F' *traD36 proA B lacI Z M15*	J.Gardner
RJ1529	*fis::kn*	R.Johnson
LE292	HfrH *argEam rpoB galT*::(λΔ[*int-FII*])	L.Enquist
LE292*fis*	LE292 *fis::kn*	This study
MO	F^- derivative of HfrH	W.Reznikoff
MO*fis*	MO *fis::kn*	J.Gardner
RZ1032	HfrKL16PO/45[*lysA*(61−62)] *dut-1 ung-1 thi-1 relA1 zbd-279*::Tn*10 supE44*	J.Gardner
Salmonella typhimurium		
JG1160	MS1883*fis3*	J.Gardner
MS1582	*leuA414supE40*Fels$^-$ *ataA*::[P22 *sieA44 16-amH1455tpfr49*]	M.Susskind
MS1868	*leuA414 hsdSB* Fels$^-$	M.Susskind
MS1883	*leuA414 hsdSB supE40* Fels−	M.Susskind
M13 phages		
M13mp8		J.Gardner
M13mp8xis	M13mp8*xis*	This study
M13KO7	helper phage	Pharmacia LKB
P22 challenge phages		
P22-P'123	P_{ant}-*P'1P'2P'3-ant*	J.Gardner
P22xis2B	P_{ant}-*X1X2F-ant*	J.Gardner
P22xis2D	P_{ant}-*FX2X1-ant*	J.Gardner
P22xis4B	P_{ant}-*P2X1X2F*-ant*	This study
P22xis4D	P_{ant}-*F*X2X1P2-ant*	This study

catalyze excision of the cryptic phage, making the strain Gal^+. The excised phage is incapable of replication and is diluted out of the population during cell division.

The mutants made in M13mp8xis were not screened by the red colony test. Furthermore, the mutants isolated in pPS2-3ΔRS were screened in the absence of IPTG in the medium. Therefore, LE292 cells carrying pIntB1 were transformed with plasmids containing each *xis* mutant, and then spread onto two sets of MacConkey galactose-ampicillin-spectinomycin plates: one set contained 1 mM IPTG while the other set contained no IPTG. The plates were incubated at 37°C for 3 days. As discussed in the Results, by inducing protein expression with IPTG in the medium, a class of Xis mutants was identified that otherwise would have been classified as unable to promote λ excision.

Challenge phages: construction and assays

The challenge phage assay is used to study protein−DNA interactions *in vivo* (Benson *et al.*, 1986; Lee *et al.*, 1990, 1991; Numrych *et al.*, 1991). When bacteriophage P22 infects *S.typhimurium*, the presence of the antirepressor (Ant) protein helps determine whether the phage will grow lytically or form a lysogen (for a review, see Susskind and Botstein, 1978). The production of Ant from *ant* stimulates lytic growth of the phage, and thus repression of *ant* promotes lysogenic growth. A challenge phage contains the recognition site of a DNA-binding protein acting as a repressor for the promoter of *ant* (P_{ant}) and contains a gene conferring kanamycin resistance (Kan^R). Therefore, the intracellular concentration and binding affinity of the DNA-binding protein regulate *ant* expression, and thus determine whether an infecting challenge phage commences lytic or lysogenic growth.

Challenge phages containing the X1, X2 and F sites were constructed previously (Numrych *et al.*, 1991) and named P22xis2B and P22xis2D for the orientations of P_{ant}-X1-X2-F and P_{ant}-F-X2-X1, respectively (Figure 2). Challenge phages containing the P2, X1, X2 and F* sites were made by homologous recombination between pTN163 or pTN164 (described above) and the phage P22-1000 (*mnt::kn9 arc-am1605*; Benson *et al.*, 1986; Lee *et al.*, 1990). That the phage were recombinant was verified by sequencing the insert and P_{ant} region as described (Numrych *et al.*, 1991). Challenge phages containing the insert were named P22xis4B and P22xis4D for the orientations of P_{ant}-P2-X1-X2-F and P_{ant}-F-X2-X1-P2, respectively (Figure 2). The challenge phage P22-P'123 was described previously (Lee *et al.*, 1990) and contains the contiguous Int arm-type sites P'1, P'2 and P'3.

The challenge phage assay was performed as described previously (Lee *et al.*, 1990; Numrych *et al.*, 1991) with the strain MS1868 carrying either the wild-type Xis or mutant Xis-producing plasmids and/or pIntB1. Xis and/or Int production was induced prior to each infection with IPTG, while FIS was supplied by the host chromosomal gene. The percent lysogenization was calculated as 100 times the number of kanamycin resistant lysogens divided by the number of cells infected.

Gel retardation assay

Wild-type Xis, select Xis mutants, FIS and Int were analyzed for DNA-binding by gel retardation assays. The DNA fragments used in each assay are described in the figure legends and were ^{32}P-labeled and isolated as described (Maxam and Gilbert, 1980). The fragments were resuspended in TE to ~9000 c.p.m./μl.

Each gel retardation reaction (8 μl) initially contained 9000 c.p.m. of ^{32}P-labeled DNA and 500 ng competitor salmon sperm DNA in a buffer of 27.5 mM Tris−Cl (pH 8), 30 mM KCl, 25 mM NaCl, 0.25 mg/ml BSA and 3% glycerol. Next, 1 μl of each protein (FIS, Xis and Int) or its respective dilution buffer was added to each reaction. Proteins were added as indicated in the figure legends in the order FIS, Xis and Int for each reaction. The reactions were incubated for 15 min at room temperature, mixed with 2.5 μl XC dye solution [0.15% xylene cyanol, 33 mM Tris−Cl (pH 7.7), 120 mM KCl and 2% glycerol, and then loaded on a 5% polyacrylamide gel (29:1 acrylamide: *bis*-acrylamide). The reactions were initially subjected to electrophoresis at 200 V for 15 min, and then the voltage was reduced to 150 V for 2.5−4 h. Each gel was dried onto Whatmann 3 MM paper and exposed to film for autoradiography.

Recombination proteins

Cell extracts of Xis and select Xis mutants were made from strains MO and MO/*fis::kan* as follows. Cells containing the respective Xis or Xis mutant producing plasmids were grown in one liter of LB media supplemented with ampicillin. When the OD_{600nm} reached 0.6, IPTG was added to a final concentration of 1 mM and the cells were grown for one more hour. The cultures were transferred to an ice bath for 15 min and then centrifuged in a Sorvall GSA rotor at 4000 r.p.m. for 15 min. The supernatant was discarded and the cell pellet was resuspended in 3 ml of an ice-cold solution of 20 mM Tris−Cl (pH 7.4), 100 mM EDTA, 20 mM NaCl and 10% glycerol, and then frozen in liquid nitrogen. The cells were thawed on ice and then lysed by six rounds of sonication, each burst lasting for 15 s at 45 W, with a 30 s pause between bursts. The lysed cells were transferred to TI (Beckman Inc.) tubes and centrifuged for 1 h at 40 000 r.p.m. at 4°C in a Beckman TI70.1 rotor. The supernatant was placed in aliquots

in Eppendorf tubes and frozen in liquid nitrogen. Protein concentations were determined using the Bio-Rad protein assay (Bradford, 1976). Int was isolated and purified as described (Nash, 1983). FIS was a gift from Dr Reid Johnson (UCLA).

Xis was diluted in 50 mM Tris−Cl (pH 7.4), 250 mM NaCl, 1 mM EDTA, 2 mg/ml BSA and 5% glycerol. Int was diluted in 25 mM Tris−Cl (pH 7.4), 300 mM KCl and 5% glycerol. FIS was diluted in 20 mM Tris−Cl (pH 7.4), 1 M NaCl, 0.1 M EDTA and 50% glycerol.

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