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Fis targets assembly of the Xis nucleoprotein filament to promote excisive recombination by phage lambda

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SUMMARY

The phage-encoded Xis protein is the major determinant controlling the direction of recombination in phage lambda. Xis is a winged-helix DNA binding protein that cooperatively binds to the *attR* recombination site to generate a curved microfilament, which promotes assembly of the excisive intasome but inhibits formation of an integrative intasome. We find that lambda synthesizes surprisingly high levels of Xis immediately upon prophage induction when excision rates are maximal. However, because of its low sequence-specific binding activity, exemplified by a 1.9 Å co-crystal structure of a nonspecifically bound DNA complex, Xis is relatively ineffective at promoting excision in vivo in the absence of the host Fis protein. Fis binds to a segment in *attR* that almost entirely overlaps one of the Xis binding sites. Instead of sterically excluding Xis binding from this site, as has been previously believed, we show that Fis enhances binding of all three Xis protomers to generate the microfilament. A specific Fis-Xis interface is supported by the effects of mutations within each protein, and relaxed, but not completely sequence-neutral, binding by the central Xis protomer is supported by the effects of DNA mutations. We present a structural model for the 50 bp curved Fis-Xis cooperative complex that is assembled between the arm and Holliday junction Int binding sites whose trajectory places constraints on models for the excisive intasome structure.

Keywords

site-specific DNA recombination; DNA architectural proteins; DNA bending; nonspecific DNA binding; X-ray crystallography

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INTRODUCTION

Integration and excision of phage lambda DNA into and out of the *E. coli* chromosome transpire via elaborate and carefully regulated site-specific DNA recombination reactions. Integration occurs between the 240 bp *attP* and 20 bp *attB* sites, resulting in the prophage DNA flanked by the recombinant products *attL* and *attR* (Fig. 1A,B) ^{1; 2}. The phage-encoded Integrase (Int) protein, a member of the tyrosine recombinase family, and the host-encoded accessory protein Integration Host Factor (IHF) are required for integration, while the host protein Factor for Inversion Stimulation (Fis) moderately stimulates the reaction ^{3; 4}. The excision reaction, in which *attL* and *attR* recombine to regenerate *attP* and *attB*, requires an additional phage protein, Xis, and is strongly stimulated by Fis ^{5; 6; 7; 8}. Other tyrosine recombinases, such as the well studied Cre and FLP enzymes, promote unregulated reactions using similar chemical steps and much simpler DNA substrates. By contrast, phage λ has recruited the three different host and phage accessory factors to enable it to exquisitely control the timing and direction (integration vs. excision) of recombination with respect to the physiological state of the cell.

Xis is a 72 amino acid residue, highly basic, monomeric DNA-binding protein that functions as the key directionality factor in lambda site-specific recombination. Xis binds cooperatively to *attR* through its N-terminal winged-helix domain, where it bends DNA to promote excisive intasome assembly ^{9; 10; 11; 12; 13; 14}. Xis also prevents phage reintegration by discouraging formation of an integrative intasome structure at *attP* ^{8; 9; 15}. Recent biochemical data and X-ray crystallography have demonstrated that three Xis monomers form a curved micronucleoprotein filament over the Xis binding region (Fig. 1C, D) ¹⁶. Xis binding to a site called X1.5, located between the related X1 and X2 sequence elements, stabilizes the filament by forming related intermolecular contacts with the upstream and downstream protomers. The Xis protomer bound at X1 also cooperatively recruits Int to the arm binding sites, which activates the Int C-terminal domains to bind to the core *att* sites leading to synapsis and initiation of DNA exchange ^{9; 17; 18; 19; 20; 21; 22}.

The Fis protein is a general host nucleoid-associated DNA bending factor that was originally identified because of its critical role in promoting site-specific recombination by DNA invertases ^{23; 24}. Fis also has been shown to regulate transcription and replication reactions, and it likely plays a role in chromosome condensation ^{25; 26}. The Fis homodimer is modeled to bind successive major grooves of DNA by its C-terminal helix-turn-helix motifs, consistent with the protection of guanines from dimethylsulfate attack over the Fis-bound F site at *attR* ^{7; 27; 28} (this paper). Thompson et al. ⁷ first demonstrated that Fis binds cooperatively with Xis at *attR* and increases excision *in vitro* up to 20-fold when Xis is limiting. *In vivo*, the absence of Fis decreased *attP* formation from an induced lysogen to undetectable levels by Southern blotting, with phage yields reduced 100–1000 fold ^{6; 11}. Moreover, both Fis and Xis binding to the *attR* region was shown to be essential for transcriptional repression in an *in vivo* P22 challenge phage system ²⁹. Because the F site overlaps much of the X2 site (Fig. 1C,D), the higher affinity binding by Fis has been thought to occlude Xis from binding to X2.

We show in this work that Xis is an abundant DNA binding protein at the time of phage excision and that it binds DNA in a largely indiscriminant manner. An X-ray structure of Xis in complex with a DNA sequence unrelated to the λ Xis binding sites was determined, which provides an example of how Xis associates with diverse sequences. We posit that this property is important for assembly of the microfilament at *attR*, particularly with respect to binding of the central Xis protomer at site X1.5. On the other hand, the relatively high affinity of Xis to competing random sequence DNA inhibits selective binding to the prophage *attR* site, necessitating the presence of Fis. We investigate how Fis recruits multiple Xis protomers to specifically bind to the *attR* arm to promote assembly of the excisive intasome. Earlier models postulated that the Fis-Xis cooperative complex consisted of Xis bound at X1 and Fis bound at F ⁷. However,

these models would place the Fis and Xis proteins over 30 Å apart at their closest approach, even with a large amount of bending modeled into each DNA-protein interface. Surprisingly, we find that three Xis proteins are present when Fis is bound to *attR*, and we delineate the sequence and spatial determinants for Fis-Xis binding cooperativity leading to assembly of the Xis nucleoprotein-microfilament. A molecular model of the 50 bp Fis-Xis-*attR* nucleoprotein complex is presented that places new constraints on the structure of the fully assembled excisive intasome. Recent studies from Landy and co-workers also have concluded that three Xis monomers together with a Fis dimer are bound to the P-arm of the excisive intasome³⁰.

RESULTS

In vivo levels of Xis and kinetics of excision during prophage induction

Levels of Xis produced at various times after synchronous induction of a temperature-sensitive (*cI857*) prophage were determined by quantitative immunoblotting (Fig. 1E). Prophage excision was also followed by measuring the formation of the *attP* and *attB* products by quantitative PCR. Xis levels rise sharply and peak at over 15,000 copies per cell within 10 minutes after induction. Xis is known to be unstable in the cell due to the activities of the Lon and FtsH proteases³¹, and we find that cellular Xis levels decrease rapidly after reaching peak levels. The *attP* and *attB* excision products increase at least 10⁴-fold within the first 5 min, and another 100- to 1000-fold over the next 15–20 min (Fig. 1E and data not shown). Levels of *attB* are 50- to 75-fold and *attP* are 75- to over 200-fold lower in isogenic *fis* mutant cells throughout the time course, consistent with the 10²- to 10³-fold reduction in viable phage yields from *fis* mutant strains⁶. We conclude that phage excision occurs rapidly upon inactivation of the *cI* repressor, which controls the transcription of *xis* via the P_L promoter, and that large amounts of Xis are present in cells at the time of excision. Therefore, although Xis can function efficiently without Fis under certain in vitro conditions^{7; 8}, an abundance of Xis appears to be insufficient to promote efficient excision in the absence of Fis in vivo. We also note that the in vivo kinetics of excision are much faster than reported for integration³¹, which may be due to a burst of P_L-driven Xis synthesis immediately after infection that temporarily inhibits integration.

Sequence-independent binding of Xis

To evaluate sequence discrimination of Xis binding to DNA, we tested Xis binding to a 266 bp ³²P-labeled fragment of yeast DNA using an electrophoretic mobility shift assay (EMSA). In the absence of competitor DNA, Xis-DNA complexes aggregate, preventing them from migrating into the gel. Upon addition of competitor DNA, a ladder of nonspecific Xis-DNA complexes is generated on the random sequence DNA probe as a function of increasing Xis concentrations (Fig. 2A). We next assessed the consequence of introducing a single Xis binding site into a DNA fragment. Even when the 13 bp X1 site was cloned into a 250 bp fragment derived from pUC18, Xis produced a ladder of DNA complexes that was remarkably similar from the ladder formed on the vector fragment (Fig. 2B, compare lanes 7–10 to lanes 2–5, respectively). Together, these data indicate that Xis binds DNA in a largely sequence-neutral manner.

Crystal structure of Xis bound nonspecifically to DNA

An example of a nonspecifically bound Xis-DNA complex was revealed from a 1.9 Å crystal structure of Xis and an 18 bp DNA segment containing the X2 binding site. As in previous co-crystal structures with Xis, we used a truncated version (^{Δ55}Xis) missing the C-terminal 17 amino acid residues that are disordered in solution¹¹. In the present crystal, ^{Δ55}Xis bound to the X2 site in an essentially identical fashion as observed in a previously determined 1.7 Å Xis-X2 structure containing a 15 bp sequence¹³ and with only minor differences in the Xis-X2 segment of the 2.6 Å X1/X1.5/X2 crystal structure¹⁶. The DNA molecules within

asymmetric units in the Xis-X2(18mer) crystal aligned to form near perfect pseudocontinuous double helices with an additional Xis monomer bound to the sequence spanning the junction (X_{ns}) (Fig. 2C). The DNA sequence at X_{ns} is unrelated to sites X1, X1.5, or X2 (Fig. 2D). Nevertheless, Xis bound to X_{ns} in a similar overall manner as to the *attR* sites, burying 1360 Å² of solvent exposed surface area as compared to values ranging from 1345 – 1486 Å² from Xis complexes with *attR* sites in isolation or in the filament. Almost all the DNA backbone contacts are at the same relative positions, and the wing is inserted into the minor groove with the side chain of Arg39 engaged in hydrogen bonding and extensive van der Waals contacts with the floor and sides of the groove. At the *attR* sites, the side chains of Glu19, Arg22, Arg23, and Arg26 from helix 2 protrude into the major groove, though only Glu19 and sometimes Arg23 are directly hydrogen bonded to bases. The side chains of Arg22 and Arg26 at X_{ns} are positioned nearly identically as in each of the *attR* sites, and Arg23 at X_{ns} contacts the backbone in a nearly identical manner as at X1.5 and one of the two conformations at X2 in the *attR* filament (Fig. 2D). Glu19 is the only residue contacting a base in the major groove in the X_{ns} complex, but its side chain is oriented to hydrogen bond to the C at position 3 rather than to the C at position 18 (two nucleotides shifted on the opposite strand) that is present at each of the *attR* sites. The conserved C at position 18 of the *attR* sites is replaced by a T at X_{ns} , which would not support direct hydrogen bonding with the glutamic acid.

In summary, Xis can bind to a variety of different DNA sequences by forming a conserved set of contacts to the phosphodiester backbone. These contacts anchor the protein to the duplex in a similar manner as seen in the specific complexes at X1 and X2, but the presence of different nucleotide sequences are accommodated by repositioning of long interfacial side chains. As described below, this flexibility of DNA recognition is important for formation of the Xis microfilament complex at *attR*, particularly with respect to Xis binding to the intervening X1.5 site where protein-protein interactions with adjacent Xis protomers further stabilize binding.

Binding of Xis and Fis to wild-type and mutant *attR* substrates

Unlike Xis binding to random sequence DNA, Xis cooperatively binds to the Xis regulatory region in *attR* to form a distinct Xis-DNA complex on a 263 bp fragment that displays low electrophoretic mobility (Fig. 3A, lanes 4–6)⁹. This complex contains three protomers of Xis bound to X1, X1.5, and X2¹⁶. Additional Xis protomers are recruited into the complex at slightly higher concentrations to extend the Xis filament on the P2 side (C.V.P. and R.C.J., unpublished data). When Fis is added, a single, slower migrating complex appears at lower Xis concentrations, corresponding to the Fis-Xis cooperative complex (Fig. 3A, lanes 7–10)⁷.

We evaluated a series of DNA changes within the Xis binding region for their effects on Xis and Xis-Fis cooperative binding. Insertion of 5 bp of DNA within the X1.5 binding site between X1 and X2/F disrupts Xis cooperativity, resulting in a binding pattern that resembles the nonspecific DNA substrates (*attR*+5; Fig. 3B, lane 3–6). Addition of Fis resulted in a weak diffuse low mobility complex at very high concentrations of Xis (Fig. 3B, lane 10). Insertion of 10 bp at the same location places X1 and X2/F on the same side of the DNA in the *attR* +10 substrate. Formation of an Xis cooperative complex was also abrogated in this mutant (Fig. 3C, lanes 3–6), but a distinct Fis-Xis complex was generated (lanes 9–10). The mobility of the Fis-Xis complex on *attR*+10 was markedly slower than that of the wild-type complex, suggesting the presence of an additional Xis protomer bound within the expanded intervening segment. Xis binding to the +5 and +10 substrates are consistent with a role for the DNA sequence over the Xis binding region and a requirement for protein-protein interactions for targeted binding.

The *attR*-10 substrate shown in Fig. 3D deletes 10 bp between the X1 and X2/F sites, including X1.5, which positions the X1 site adjacent to most of the X2/F site. In the absence of Fis,

multiple Xis complexes were formed on *attR-10* (lanes 3–6). We interpret the faster migrating complex to contain a single Xis and the most prominent slower complex in lanes 5 and 6 to contain Xis monomers bound to X1 and X2; additional Xis protomers bound at high Xis concentrations (lane 6). Significantly, a robust Fis-Xis complex was formed in the presence of Fis on *attR-10* at an Xis concentration which is nearly identical to that required to form the Fis-Xis cooperative complex on the wild-type *attR* substrate. However, this complex migrated faster than the wild-type Fis-Xis complex, consistent with the presence of only two Xis monomers bound at X1 and X2 with Fis. Because the X1.5 sequence is not present in this substrate, the 1.5 site must not be essential for assembly of a Fis-Xis complex.

We next addressed the importance of the DNA sequence between X1 and X2 for Fis-Xis cooperativity under conditions where the native spacing is maintained. Two substrates, *attR sub1* and *attR sub2*, contain different 5 bp mutations between X1 and X2. As shown in Figs. 3E and F, the 5 bp substitutions in *attR sub1* are well tolerated for Xis and Fis-Xis cooperative binding, whereas the substitutions in *attR sub2* strongly disrupt formation of both complexes. The 5 bp substitutions of both mutant substrates reside within the major groove of the Xis 1.5 binding site. Two of the changes within the *attR sub2* substrate, at positions –82 and –81 (Fig. 1C), have been previously shown to disrupt repression by Xis binding in an in vivo challenge phage assay²⁹. The effects of these mutations indicate that Xis binding at X1.5 exhibits some sequence specificity, although it still can bind to a site (*attR sub1*) where all 5 bp within its major groove interface have been changed. Taken together, the binding data on *attR* mutated substrates imply that multiple Xis protomers bind within the Fis-Xis complex and that cooperativity involves interactions between Xis protomers that bind with a degenerate sequence specificity.

Binding of Xis and Fis to short *attR* fragments suggests three Xis protomers are present in the Fis-Xis complex

Cooperativity between Xis monomers becomes increasingly reduced on substrates smaller than 100 bp¹⁶. The effect of substrate length on Xis binding is illustrated in Fig. 4A (lanes 3–7) with a 54 bp *attR* fragment, as compared with the 263 bp fragment in Fig. 3A (lanes 3–6). The reduced cooperativity results in three distinct Xis-DNA complexes over a broad range of Xis concentrations, representing binding of the three Xis protomers in the *attR* complex. Fis plus Xis binding on the 54 bp *attR* fragment also exhibits reduced cooperativity, revealing the presence of two novel species migrating between the Fis and the Fis+Xis cooperative complex bands (compare Fig. 4A lanes 8–12 to Fig. 3A lanes 7–10). This result suggests that three Xis monomers are also present in the Xis-Fis complex.

We next tested Fis + Xis binding to a set of oligonucleotide substrates that contain an intact Fis binding site but are truncated in the Xis binding region. The longest, *attR* –87 to –54, begins at the left boundary of the minimal X1.5 site. Nevertheless, only one Xis protomer was able to weakly bind to this substrate (Fig. 4B, lanes 3–6). In the presence of Fis, a single, prominent, supershifted complex was formed with increasing Xis on *attR* –87 to –54, demonstrating cooperative binding between Fis and one Xis protomer. No Xis binding was observed on the *attR* –81 to –54 substrate, which only contains an intact X2 site, but a weak Fis-Xis complex still formed (Fig. 4C). These results highlight the cooperativity between Fis and a single Xis bound at the overlapping X2 site.

Stoichiometry of Xis and Fis within the Xis-Fis cooperative complex

The number of Xis proteins in the Fis-Xis cooperative complex was quantified using a differential labeling approach. Fis-Xis complexes were assembled on fluorescein-labeled *attR* (–104 to –54) substrates using $\Delta^{55}\text{Xis}^{\text{HMK}}$ that was ^{32}P -labeled at a predetermined specific activity. $\Delta^{55}\text{Xis}^{\text{HMK}}$ contains the disordered C-terminal 17 residues replaced with the

recognition sequence for heart muscle kinase. Previous studies have shown that $\Delta^{55}\text{Xis}$ cooperatively binds with itself and with Fis on the 263 bp *attR* fragment in a similar manner as full-length Xis¹¹, and we find here that $\Delta^{55}\text{Xis}$ binds to short DNA substrates more cooperatively than full-length Xis (e.g., Fig. 5 below). The gel displaying the complexes formed with ^{32}P -labeled $\Delta^{55}\text{Xis}^{\text{HMK}}$ was fluoroimaged together with fluorescein-labeled DNA standards to determine the numbers of *attR* molecules, and the radioactivity within the cooperative complex was measured to determine the number of Xis molecules. The ratio of Xis monomers to *attR* DNA molecules from nine independent measurements was 3.3 ± 0.6 .

A similar strategy was used to confirm that one Fis dimer is present in the complex, except a derivative of Fis was used that was ^{32}P -labeled via a kinase tag on its C-terminus. As predicted, the ratio of Fis^{HMK} dimers to *attR* in the complex was determined to be 1.2 ± 0.1 from three independent experiments.

Crosslinking three Xis protomers in the Xis-Xis and Fis-Xis cooperative complexes

A chemical crosslinking approach, employing a variety of succinimidyl crosslinkers to probe the proximity of primary amines between the multiple proteins, was used to investigate the nature of the Xis and Xis-Fis cooperative complexes. Binding reactions containing fluorescein-labeled *attR* (−104 to −54) substrates and ^{32}P -labeled $\Delta^{55}\text{Xis}^{\text{HMK}}$ were subjected to crosslinking in solution in the presence and absence of Fis using the crosslinkers bis (sulfosuccinimidyl)suberate (BS3, 11 Å spacer), disuccinimidyl glutarate (DSG, 8 Å spacer) and tris-succinimidyl aminotriacetate (TSAT, 4 Å spacer). The crosslinked reactions were electrophoresed through a native polyacrylamide gel to separate complexes from free DNA, and subjected to fluoroimaging and autoradiography to visualize the $\Delta^{55}\text{Xis}^{\text{HMK}}$ complexes (Fig. 5A). The crosslinked protein complexes were then extracted from the gel and applied to a SDS polyacrylamide gel to identify the crosslinked species (Fig. 5B). As shown previously, Xis-*attR* complexes treated with BS3 and DSG generate two new bands whose migration is consistent with a crosslinked dimer and trimer of Xis (Fig. 5B, lanes 2 and 3)¹⁶. Crosslinking of the Fis-Xis complexes reveals the same Xis dimer and trimer bands, strongly suggesting that the Fis-Xis cooperative complex is comprised of three Xis monomers (lanes 6–8). In addition, several new species are obtained, particularly with the 11 Å crosslinker. The migrations of the two most prominent Fis-dependent bands are consistent with a Fis subunit linked to one or two Xis protomers. The identities of the Fis-Xis crosslinked products were confirmed by performing identical experiments with disulfide-linked Fis dimers, Fis S18C or Fis S30C³². In both cases, bands corresponding to those containing a Fis monomer were absent and higher molecular weight bands were enriched (data not shown).

Fis-Xis crosslinking reactions were also performed using a ^{32}P -labeled kinase tagged version of full-length Xis (Xis^{HMK}) and BS3 as the crosslinker. Xis^{HMK} forms similar intermediates on the fluorescein-labeled *attR* (−104 to −54) substrates as does wild-type Xis (data not shown; refer to Fig. 4A for a representative gel). The Xis^{HMK} intermediate that is predicted to contain two Xis protomers from the native gel generates a single product that corresponds to two linked Xis molecules (Fig. 5C, lane 2), whereas the slower migrating complex contains a high proportion of three linked Xis monomers (lane 3)¹⁶. Crosslinking efficiency of Xis^{HMK} is enhanced compared to $\Delta^{55}\text{Xis}^{\text{HMK}}$ crosslinking, presumably because of the presence of the lysine rich C-terminus on full-length Xis, which is unstructured in the absence of Int and consequently highly accessible for crosslinking. In the presence of Fis, the intermediate Fis-Xis complex containing two Xis protomers generates the crosslinks expected between two Xis monomers and one or two Fis subunits, and the cooperative Fis-Xis complex generates additional bands corresponding to crosslinked products containing three Xis protomers (lanes 4 and 5, respectively).

We assessed the region on Fis responsible for Fis-Xis crosslinking by using the Fis mutant $\Delta(2-26)$ that eliminates the unstructured N-terminal ten amino acid residues and the β -hairpin arms³³. This region contains residues that are responsible for the activation of the Hin recombinase but have no effect on phage excision *in vivo*³⁴. Although Fis $\Delta(2-26)$ exhibits wild-type Fis-Xis cooperativity *in vitro* (data not shown), BS³ crosslinking between Fis and Δ^{55} Xis^{HMK} is eliminated (Fig. 5D, lane 4). Amine crosslinking from the N-terminal region of Fis could involve residue 25, the only lysine in the region, or the amino-terminus. To distinguish between these possibilities, we performed BS³ crosslinking on Fis K25C. Fis K25C produced the same level of Fis- Δ^{55} Xis^{HMK} crosslinked products, suggesting the N-terminus of Fis is responsible for the observed crosslinks (Fig. 5D, lane 6).

DNA footprinting localizes Fis and Xis molecules within the Fis-Xis complex

Guanine contacts made by Xis and Fis within the DNA major groove were examined using DMS methylation protection experiments. The experiment shown in Fig. 6A probed the top strand of the 263 bp *attR* fragment in the presence of wild-type Xis and/or Fis. Xis alone completely protects the guanine at positions -96 in X1 and partially protects the guanine at -76 in X2 from methylation (lane 2, denoted by arrows) (see also Yin et al.¹⁰). The crystal structure of the X1/X1.5/X2 complex shows that Arg23 contacts G -96 and alternates between contacting G -76 and the phosphate backbone, consistent with the DMS protection results¹⁶. Only a single position, G -73, that is located within the F site is protected by Fis alone (lane 1). As shown in lane 3, when Xis and Fis are bound together, the Fis protection at -73 and the Xis protection at -96 remain unchanged, but Xis protection of G -76 is lost, and in some experiments, modification-cleavage of G -76 is enhanced. Thus, G -76 in X2 switches from being partially protected by Xis to being moderately hyperactive to DMS attack when Fis is present.

Protections from hydroxyl radical cleavage were used to identify Xis and Fis interactions within the minor groove³⁵. As shown in lane 4 of Fig. 6B and the corresponding scan in Fig. 6C, Xis protects the following regions of *attR* from hydroxyl radical cleavage: -91 to -86, -80 to -76, and to a lesser extent, the DNA between -69 to -67. These positions correlate with the insertion of the wing motif of Xis into the minor grooves at X1, X1.5, and X2, respectively¹⁶. Fis binding to *attR* (Fig. 6B, lane 3 and Fig. 6C) reveals three regions of protection on this strand, -76 to -73, -65 to -62, and -57 to -55 (not shown in the figure). Fis protections between positions -65 to -62 are located in the minor groove positioned within the center of the Fis binding site, whereas protections at -76 to -73 and -57 to -55, which flank the 15 bp core Fis site, are associated with the sides of the Fis dimer due to DNA wrapping. When Fis and Xis are bound together (Fig. 6B, lane 5 and Fig. 6C), the hydroxyl radical footprinting pattern reflects the combination of the individual Xis and Fis complexes. These results indicate that the positions of the wings of the three Xis protomers are not detectably altered by the presence of Fis.

A model of the Fis-Xis complex: Fis-Xis intermolecular interactions

The 2.6 Å X-ray structure of the Δ^{55} Xis-X1/X1.5/X2 microfilament¹⁶ (Fig. 1D) was combined with a model of the Fis dimer docked to DNA³⁶ in a manner consistent with the DMS and hydroxyl radical footprinting data to construct a structural model of the Fis-Xis cooperative complex (Fig. 7A). Fis and Xis bound at X2 are located adjacent to each other such that their DNA recognition helices form a continuous solvent-excluded protein surface in the major groove. Presumably, this shifts the Arg 23 side chain of the Xis bound at X2 to exclusively contact the phosphate backbone, which occurs with Xis bound at X1.5 and at X_{ns}. Residues 1-10 are normally disordered in Fis crystal structures, but if the residues 1-10 that are resolved in the structure of Fis R71L³⁷ are added onto the Fis structure shown in Fig. 7A, the NH₂-terminus would be close to Xis at X2, consistent with the Fis-Xis crosslinks observed.

The location of Fis in the model creates a plausible interface between Xis bound at X2 (colored gold) and residues in helix C of Fis (colored cyan) (Fig. 7B). We tested whether substitutions of Fis and Xis residues within the putative interface region compromise assembly of Fis-Xis cooperative complexes. Of the Fis mutants tested, T75A had the greatest effect on cooperative binding with Xis (Fig. 7C). Fis N73S also exhibited a defect in recruitment of Xis to X2. The Asn73 side chain is not predicted to be oriented towards Xis, but rather to be bending DNA through a backbone contact^{28; 36}. Thus, DNA structural effects may also be influencing Fis-Xis cooperativity. Other Fis mutants containing changes in the vicinity of the interface (R76A, R76E, L79E, and N84C) had no measurable effect on Xis binding. Of the Xis mutants evaluated, Q6E and L18D had the strongest inhibitory effects (Fig. 7D). Interestingly, an L18R substitution improved nonspecific DNA binding (not shown) as well as Xis-Xis, and Xis-Fis cooperative binding (Fig. 7D). The general increase in DNA affinity is not unexpected, since an arginine side chain at residue 18 could readily contact DNA.

DISCUSSION

Xis can be considered to be an essential accessory factor for the phage λ excision reaction. Without Xis, excision between the *attR* and *attL* recombination sites is virtually undetectable under standard in vitro conditions and numbers of viable phage generated from an induced lysogen are reduced by 10^6 in vivo^{5; 11}. Over 15,000 Xis molecules per cell ($>30 \mu\text{M}$) are synthesized upon prophage induction, and Xis exhibits surprisingly weak specificity for its binding site in *attR*, with binding to a DNA fragment containing a single Xis binding site no better than to random sequence DNA. The abundance, low sequence specificity, and DNA bending properties of Xis resemble properties of bacterial nucleoid proteins. Specific binding by Xis to *attR* involves the cooperative formation of a micronucleoprotein filament over the X1-X1.5-X2 region. However, the Xis cooperative complex exhibits only modest affinity for the *attR* region in vitro, and this appears to be insufficient in vivo to promote efficient excision without Fis. We show here by qPCR methods that the in vivo rate of phage excision is reduced about 100-fold when Fis is absent.

Fis binds to the *attR* site in vitro with about 100-fold greater affinity than Xis alone. We propose that Fis binding at F plays a crucial in vivo role in targeting Xis to *attR* by first recruiting Xis to X2. Thus, even though Fis and Xis bind over a common F/X2 DNA segment, the two proteins bind cooperatively rather than competitively. Xis bound at X2 is proposed to then recruit an Xis protomer to X1.5, followed by an Xis protomer to X1. Cooperative assembly of the Xis filament requires only the first 50 residues of Xis, which fold into a minimal winged-helix motif^{11; 29}. The C-terminal 20 residues of Xis bound at X1 recruit Int to bind to an arm site by specifically interacting with its N-terminal DNA binding domain^{16; 21; 22}.

The Xis filament assembled over the 33 bp X1/X1.5/X2 region in the crystal induces about 75° of curvature into the DNA¹⁶, and bending introduced by Fis is predicted to be similarly phased with the curvature of the Xis microfilament^{36; 38}. This bending is believed to be critical to enable the bivalent Int protomers to simultaneously bind to the P2 arm and core binding sites within the excisive intasome.

Xis-DNA binding and filament formation

There is considerable flexibility in the nucleotide sequences that allow Xis binding. This property is best illustrated by the *attR sub1* mutant, which supports filament assembly yet has five contiguous base pairs changed over the major groove binding region of the X1.5 site. Three X-ray structures showing six Xis-DNA complexes have been determined, and these show that Xis is positioned on DNA in a nearly identical manner, regardless of the sequence, and that Glu19 is the only residue that always contacts a base within the major groove (this paper)^{13; 16}. The X-ray structures and mutant binding properties suggest that a hydrogen bonding donor,

which can be satisfied by the cytosine N4 or adenine N6 (e.g., at the mutant *attR sub1*) groups, must be available at positions 18 or 3 (numbering of Fig. 2D) to interact with Glu19. The *attR sub2* mutant does not satisfy this requirement over X1.5, and thereby disrupts filament formation. The orientation of the Glu19 side chain present at each of the *attR* sites may be preferred because the negatively charged carboxylate in this configuration is neutralized by salt bridges with Arg22 and Arg26. Arg23 is flexible: at X1 or on an isolated X2 site, Arg23 directly contacts 2–3 bases within the major groove, whereas at X_{ns}, X1.5, and apparently at X2 in the presence of Fis, Arg23 exclusively contacts the phosphate backbone. Xis appears to be able to accommodate many sequences over the minor groove region because of the flexibility of hydrogen bonding by the guanidinium group of Arg39 at the tip of the wing.

Xis-like recombinational directionality proteins (RDFs) in other systems often cooperatively bind over extended segments of diverse sequence³⁹. In many cases, these proteins have been demonstrated or inferred to fold into winged-helix structures and are likely to form filaments along DNA. An ability to bind DNA in a largely sequence neutral manner, as described here for phage λ Xis, would also enable these RDFs to oligomerize on DNA. Localized nucleoprotein filaments comprised of structurally diverse proteins have been implicated in the control of DNA replication, transcription and chromosome condensation and are the platform in which DNA pairing and exchange occur in homologous recombination.

Targeting of the λ Xis microfilament by Fis to *attR*

The Fis-Xis complex contains three distinct protein interfaces: Fis/F-Xis/X2, Xis/X2-Xis/X1.5, and Xis/X1.5-Xis/X1. The two Xis protein interfaces within the filament involve mostly common residues but differ somewhat in their molecular interactions¹⁶. The proposed interface between Fis and Xis bound at X2 involves a small number of interprotein contacts that include Thr75 on Fis and Gln6 and Leu18 on Xis. Recruitment of Xis to X2 by Fis is most clearly shown in Fig. 4B, where a robust Fis-Xis complex is formed on a substrate missing the X1 and an intact X1.5 binding site despite poor binding by Xis in the absence of Fis. Fis and Xis can also cooperatively bind to a mutant *attR* substrate where the 10 bp region containing X1.5 is absent (*attR-10*, Fig. 3D). Moreover, a Fis-Xis cooperative complex forms, albeit with reduced affinity, when an additional 10 bp separates X1 from X2/F (*attR+10*, Fig. 3C). As the DNA binding interface by Xis is about 10 bp, the *attR+10* complex presumably contains a filament consisting of four Xis molecules in addition to Fis.

Protein interfaces involving only a small number of residues within each DNA bound partner are not uncommon. For example, alanine substitutions of only two CRP residues within the carboxyl-terminal domain of the RNA polymerase α subunit decrease transcriptional activation greater than 2-fold^{40; 41}. In the phage λ cI-RNA polymerase sigma subunit ternary complex, only two residues within the helix-turn-helix of cI contact sigma⁴². Interestingly, one of the residues on cI is in the same relative location on the helix prior to the DNA recognition helix as is Thr75 in Fis. And likewise, recent evidence indicates that Thr75 contacts sigma at the *proP* (P2) promoter, where Fis is bound adjacent to the –35 promoter element⁴³. By contrast, the Fis T75A mutant has no effect on activation of the *rrnB* P1 promoter, where Fis-mediated activation is mediated only by an interaction with the C-terminal domain of the RNA polymerase alpha subunit, or on Fis-activated DNA inversion by Hin (R.C.J., unpublished).

Protein-induced DNA distortions that favor mutual binding may also contribute to cooperative binding between Fis and Xis. In the model of the Fis-Xis complex, the DNA recognition helices of Xis bound at X2 and the proximal Fis subunit are positioned adjacent to each other, nearly forming a continuous protein surface within the major groove. Both Xis (by X-ray structure) and Fis (by modeling) compress the major groove over their recognition helices as well as the minor groove, where the Xis wing inserts between the two Fis subunits. Experimental support for protein-induced DNA conformational changes contributing to Fis-Xis cooperativity is

provided by the Fis mutants N73S (Fig. 7C) or N73A (data not shown), which are defective for Fis-Xis cooperativity and exhibit altered DNA bending^{26; 36}.

Biological implications

Fis has been proposed to act as a sensor of host physiology for controlling lambda excision⁴⁴. Cellular Fis levels are directly proportional to growth rates and rapidly increase upon nutrient upshifts^{45; 46}. Robust growth conditions, where Fis levels are high, are optimal for producing high phage yields upon excision. In contrast, the *attR* F site is not occupied in quiescent or stationary phase cells where prophage excision would be expected to result in failed phage production⁴⁴. We have shown here that Fis enhances the excision reaction by promoting Xis binding and subsequent assembly of a microfilament at *attR*, which otherwise occurs inefficiently due to the competing nonspecific DNA binding activity. Fis activity on lambda is not restricted solely to the excision reaction. Integration is more efficient when Fis is present, and Fis directly or indirectly stabilizes the lysogenic state^{3; 4}. However, no effect of Fis on phage development during lytic growth has been observed⁶.

MATERIALS AND METHODS

Xis levels and qPCR analysis of λ excision in vivo

RJ5606 (MG1655 Δ lacX74 λ cI857 monolysogen) and RJ5607 (RJ5606 *fis::kan-767*) were grown in LB at 30° to an OD₆₀₀ = 0.3, shifted to 42° for 15 min, and then incubated at 37°. Aliquots were taken at various times immediately before and after the shift to higher temperature. Quantitative immunoblotting was performed on whole cell extracts using anti-Xis antibody (gift of H. Nash, NIH). The blots were developed with ECL-Plus (Amersham) and fluorimaged using a Typhoon (Amersham). Cell number (colony forming units) was determined immediately before induction. DNA for qPCR was prepared using the Wizard Genomic DNA Purification Kit (Promega). The *attP* and *attB* PCR products were quantitated relative to a standard curve of λ DNA and *E. coli* chromosomal DNA, respectively, using 0.075x SYBR Green in the PCR reaction mix. Minor relative adjustments to the product yield from each DNA sample were made based on a control chromosomal amplicon internal to the *recA* gene. Primer sequences were: *attP*, TTCTCTGGAGTGCGACAGGTT, GCAGATAAGCGATAAGTTTGCT; *attB*, CCAGACGGGAACTGAAAT, GGTCGGGTTTAACGTTTCATT; *recA*, GTTATCGTCGTTGACTCCGT, GGGTTACCGAACATCACACC.

In vitro DNA-binding assays

Binding reactions were performed as follows: 50–100 fmol labeled DNA probe was incubated at room temperature with varying amounts of Xis and Fis, as appropriate, in 20 μ l binding buffer containing 20 mM Hepes (pH 7.5), 100 mM NaCl, 5% (v/v) glycerol, 100 μ g/ml BSA, 2 mM DTT, 1 mM EDTA, and 50 μ g/ml sonicated herring testes DNA (Promega), unless otherwise stated. For EMSAs, samples were subjected to PAGE in 0.5X TBE. Wild-type and Xis mutants have been described elsewhere^{11; 16}, or were constructed by the QuikChange protocol (Stratagene). Δ^{55} Xis derivatives have cysteine 28 substituted with alanine. Fis mutants used in this work were from the laboratory collection or constructed by the QuikChange protocol. Xis¹¹ and Fis³⁶ proteins were purified essentially as described, with chromatography through an FPLC Superdex 75 column usually added as a final step. ³²P-5'-end-labeled DNA probes were typically generated by PCR and were purified by native PAGE. The 263 bp wild-type *attR* probe (nucleotides –220 to +43) contains 122 bp and 102 bp from the left and right ends of X1-X2/F region, respectively. The *attR* +5, *attR* –10, *attR* +10 mutants and *attR* 5 bp substitutions were introduced into the same template as wild-type *attR* by the QuikChange method as follows: CGGCC and CATGGATCCG were inserted after *attR* bp –83 to create *attR* +5 and *attR* +10, respectively, while the *attR* –10 substrate removed the sequence

from *attR* -83 to -74. The 54 bp *attR* fragment, *attR* -104 to -51, was amplified such that the X1-X2/F region was 5 bp and 8 bp from each end, respectively. Nonspecific DNA probes were a 266 bp segment of the *S. cerevisiae* *MET14* gene and a 250 bp region surrounding the multiple cloning site region of pUC18. The pUC18 segment was also amplified from pRJ2273, which has the X1 binding site sequence (TATGTTGTGTTTT) cloned between the *Bam*H1 and *Sac*I sites.

Xis and Fis crosslinking reactions and stoichiometry measurements

$\Delta^{55}\text{Xis}^{\text{HMK}}$, Xis^{HMK} , and Fis^{HMK} (5–10 μg) were labeled with ^{32}P in a reaction containing 20 mM Hepes (pH 7.5), 10 mM MgCl_2 , 50 mM NaCl, 10% (v/v) glycerol, 40 mM DTT, 1 mCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (7000 Ci/mmol; ICN), and ten units of heart muscle kinase (Sigma) at 4°C for 5 min and quenched with 12.5 mM EDTA. Binding reactions were performed on 6-FAM-labeled duplex oligonucleotides (-104 to -54) using 0.5 – 0.7 μM labeled Xis and 17.6 nM Fis, incubated at 23°C or 37°C with various crosslinkers (3.4 mM) for 15 to 60 min, quenched with the addition of 20 mM glycine, and applied to 8% polyacrylamide gels. The gels were exposed to film and complexes of interest were excised from the gel. Complexes were eluted overnight at 37°C overnight in 200–300 μl 10 mM Hepes (pH 7.5), 1 mM EDTA, and 1% SDS. The eluate was recovered by centrifugation, and equal ^{32}P counts were applied to a 15% SDS gel. For crosslinking reactions performed with disulfide-linked Fis S18C and Fis S30C, reactions were quenched with 20 mM glycine and 0.4 mM diamide, and 10 mM oxidized glutathione was added to the gel slice incubation buffer and SDS loading dye. The succinimidyl crosslinkers (Pierce) used were as follows: BS³, [Bis(sulfosuccinimidyl) suberate] (11.4 Å); DSG, Disuccinimidyl glutarate (7.72 Å); TSAT, (Tris-succinimidyl aminotriacetate) (4.4 Å), and dissolved at 10 mg/ml in DMSO. Stoichiometry experiments employing ^{32}P -labeled $\Delta^{55}\text{Xis}^{\text{HMK}}$ or Fis^{HMK} and the 6-FAM 51 bp oligonucleotides were performed as described in ¹⁶.

Dimethylsulfate (DMS) and hydroxyl radical DNA footprinting

DNA binding reactions for DMS protection assays were performed using the ^{32}P -end-labeled 263 bp *attR* fragments and 0.5 μM Xis and 44 nM Fis. Reactions were incubated with 10 mM DMS for 2 min at 23°C , and DNA cleavage was performed as described ⁴⁷. Hydroxyl radical footprinting was achieved essentially using the procedure described by Tullius ³⁵. Xis (0.5 μM) and Fis (44 nM) were bound to 5'- ^{32}P -end-labeled 203 bp *attR* fragments (-160 to +43) in reactions lacking glycerol and then incubated with 3 mM sodium ascorbate, 0.09% hydrogen peroxide, and 0.3 mM Fe-EDTA for 3 min at room temperature and quenched with 26 mM thiourea. Scans were generated using ImageQuant software (Amersham).

The footprinting, as well as stoichiometry and crosslinking, experiments were performed using linear DNA substrates without Int or IHF in the reaction. The Xis-dependent excision reaction occurs efficiently on linear substrates ⁴⁸, justifying their use in our binding studies. Although complex cooperative or antagonistic effects occur amongst each of the components of the λ recombination reaction on a global scale, previous studies have not detected localized effects of IHF on Xis or Fis binding (summarized in reference 2). Hydroxyl radical footprint experiments indicate that additional Xis protomer(s) can assemble onto the Xis microfilament on the P2 side of the Xis binding region (C.V.P. and R.C.J., data not shown). Int bound at P2 does cooperatively bind with the Xis+Fis complex and thus would be expected to prevent additional polymerization of Xis, but this has not been directly tested.

Crystallization and structure determination of the Xis-X2(18 bp) complex

$\Delta^{55}\text{Xis}$ (residues 1–55 with a Cys to Ser mutation at position 28) and an 18-mer DNA duplex containing the X2 binding site plus 6 additional base pairs on the left side (5'-gtattaTGTAGTCTGTT/5'-aAACAGACTACAataac) were used to form the Xis-DNA

complex as previously described¹³. Crystals that diffracted to a 1.9 Å resolution limit were grown using the vapor-diffusion hanging-drop method in 30% polyethylene glycol monomethyl ether 2000, 0.2 M NH₄SO₄ and 0.1 M NaOAc (pH 4.6). Crystals were cryo-protected by the addition of 25% (v/v) glycerol to the mother liquor and cryo-cooled under a nitrogen stream. A complete native dataset was collected using a Rigaku FR-D generator. This dataset was integrated and scaled using the DENZO and SCALEPACK programs⁴⁹. The complex was solved by molecular replacement with EPMR⁵⁰ using the Δ^{55} Xis-X2(15 bp) structure (PDB: 1RH6) as the search model¹³. The model was refined using CCP4-REFMAC⁵¹, and the remainder of the structure was built using the program O⁵² and further refined by applying restrained and TLS parameters. An example of the final *2Fo-Fc* map is provided in supplemental Fig. 1. In the final model, no traceable density was observed for residues L52-P55 of the Xis molecule bound to the nonspecific site and residues N53-P55 of the Xis molecule bound to X2. Data and refinement statistics are given in the supplemental Table 1; the PDB code will be provided. The separation of bases at the junction between DNA duplexes in adjacent unit cells is 3.35 Å.

Structural model of the Fis-Xis DNA segment

Fis (PDB: 1F36)³³ docked to a 35 bp DNA containing an overall bend of about 50°³⁶ was superimposed onto the Δ^{55} Xis-33 bp microfilament X-ray structure (2IEF)¹⁶ over the DNA backbones surrounding *attR* G-73, which is protected from DMS methylation by Fis both in the absence and presence of Xis (Fig. 6A). Modeling and structure figures were generating using the Insight II software package (Accelrys) and PYMOL⁵³.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) The phage λ integration and excision reactions. The supercoiled phage genome inserts into the *E. coli* chromosome by recombination between the *attP* and *attB* sites on the phage and bacterium, respectively, and excises from the chromosome by recombination between the hybrid *attL* and *attR* sites. (B) Binding sites within *attL* and *attR* for the proteins involved in formation of the excisive intasome. Int binds to core-type sites (B, C', C, B'), where DNA strand exchange (O) takes place, and arm-type sites (P'1, P'2, P2) to promote excision. Efficient excision also requires IHF bound to sites H' and H2. Xis monomers bind to X1, X1.5, X2 within *attR*, while the Fis dimer binds to site F, which overlaps the X2 site. Additional binding sites that are used during the integration reaction are denoted with parentheses. (C) Sequence of the

Xis-Fis binding region within *attR*; the coordinates are numbered relative to the center of the core site COB'. Solid lines designate the minimal DNA interfaces based on X-ray structures of Int- and Xis-DNA complexes; dashed lines indicate flanking DNA backbone contacts^{16; 54}. Demarcation of the Fis segment is derived from oligonucleotide binding, footprinting, and structural modeling²⁵. (D) X-ray structure of Δ^{55} Xis bound to the X1/X1.5/X2 binding sites¹⁶. The DNA segment within X2 that overlaps the Fis core binding site (solid line) and minimal flanking region (dashed line) is denoted. (E) Xis protein levels upon induction of a λ prophage and kinetics of excision. λ cI857 lysogens were induced by temperature shift and aliquots were removed at various times. Xis levels were determined by quantitative immunoblotting (top panel and open circles in graph). Phage excision (*attP* formation) was measured using qPCR; *attP* molecules were determined from parallel cultures of *fis*⁺ (squares) and *fis* mutant (triangles) lysogens.

Figure 2.

Nonspecific DNA binding by Xis. (A) Xis binding to nonspecific DNA. Xis was incubated with a ^{32}P -end-labeled 266 bp fragment derived from the *S. cerevisiae* *MET14* coding region followed by electrophoresis in a native polyacrylamide gel. Competitor DNA (50 $\mu\text{g}/\text{ml}$ herring testes DNA) was included in these reactions to prevent aggregation at the top of the gel. (B) Titration of Xis on a 250 bp ^{32}P -end-labeled fragment from pUC18 (lanes 1–5) or the same fragment containing the X1 binding site (lanes 6–10). (C) X-ray crystal structure of an Xis monomer bound to X2 (blue protein) and X_{ns} (red protein). Shown are DNA segments plus the Xis bound to X2 from two asymmetric units plus an Xis from one unit cell bound to X_{ns} at the DNA junction. Selective side chains that are contacting DNA (see panel D) are shown for each protein. The right panel illustrates Xis bound at X1.5 within the Xis-*attR* microfilament complex. (D) Diagrams of the Xis-DNA contacts at the different binding sites. The contacts

between Xis and X2 in the 18mer co-crystal structure depicted here are virtually identical with the Xis-X2 structures reported from the 15 bp complex and the 33 bp filament complex except Arg23 is also bound to the 5' PO₄ of T1 about 75% of the time in the microfilament. Contacts with the X1 sequence in the context of the microfilament (not shown) are also nearly identical to the Xis-X2 contacts shown here. Contacts representing the Xis-X1.5 interface in the microfilament are given for comparison.

Figure 3.

Binding properties of Xis with and without Fis on wild-type and mutant *attR* DNA substrates. (A) The 263 bp wild-type *attR* fragment; (B) *attR* +5; (C) *attR* +10; (D) *attR* -10; (E) *attR* sub1; (F) *attR* sub2. Concentrations of Xis added are in nM. 2.2 nM Fis is added as indicated, except in panel B where 4.5 nM Fis was added, which results in a slower-migrating secondary Fis-DNA complex. Schematic drawings of each substrate are shown below the individual panels with the top strand sequence of part of the segment between X1 and X2 shown. The triangles in the *attR* +5 and *attR* +10 panels indicate insertions of either 5 or 10 bp, respectively, between the G and T. The *attR* -10 substrate removes 10 bp of *attR* sequence between -83 to -74 (see Fig. 1C). The 5 bp substitutions present in *attR* sub1 and *attR* sub2 are written below

the wild-type sequence in panel E and F, respectively. The locations of relevant complexes are indicated: free designates the labeled *attR* DNA probe without protein, F represents Fis bound to site F, X represents the cooperative Xis complex, XF denotes the cooperative Fis-Xis complex.

Figure 4.

Binding properties of Fis and Xis on short *attR* fragments. (A) A titration of wild-type Xis was performed on a 54 bp fragment (-104 to -51) containing the X1 to F region of *attR* in the absence and presence of 2.2 nM Fis. (B and C) Binding of increasing concentrations of Xis with and without 4.5 nM Fis on *attR* oligonucleotides missing Xis binding sites. A small amount of contaminating Fis protein is evident in lanes 5 and 6 where large amounts of Xis were added. The sequence of the DNA used in panels B and C is written below the panels with the endpoints of the substrates denoted. The core (solid lines) and flanking (dashed lines) sequences representing the Xis/X2 and Fis/F binding sites are denoted. A shorter substrate (-78 to -54

denoted on the sequence) gave only a trace of a Fis/Xis complex (not shown). Free designates unbound substrate; X denotes an Xis protomer; F denotes a Fis dimer.

Figure 5.

Fis and Xis protein crosslinking. (A) Native PAGE performed with fluorescein-labeled DNA substrates containing the Xis-Fis binding region (-104 to -54) and 32 P-labeled Δ^{55} Xis^{HMK} plus Fis. A fluorimetry image is shown in lanes 1–5, and an autoradiogram is shown in lanes 6–9. Xlink indicates reactions were incubated with the crosslinking reagent BS³ prior to electrophoresis. The dominant complexes containing multiple Δ^{55} Xis^{HMK} (Δ^{55} Xis) and multiple Δ^{55} Xis^{HMK} + Fis (Δ^{55} Xis+Fis) proteins are labeled. Note that in the autoradiogram only complexes containing the radiolabeled Δ^{55} Xis^{HMK} are visible. (B) Autoradiograph of crosslinked Δ^{55} Xis^{HMK} plus Fis complexes after SDS-PAGE. Crosslinked radiolabeled complexes were eluted from native gels such as shown in panel A and subjected to SDS-PAGE. Lanes are labeled by the lengths of the various crosslinkers: (-) denotes no crosslinker added, 11 Å (BS³), 8 Å (DSG), 4 Å (TSAT). (C) Autoradiograph of the SDS gel displaying complexes assembled using full-length 32 P-labeled Xis^{HMK} in the absence or presence of Fis and crosslinked with BS³, as denoted. Lane 1 is uncrosslinked Xis, lane 2 is crosslinked Xis that was gel-purified from the complex predicted to contain 2 Xis protomers (e.g., XX in Fig. 4A lane 7); lane 3 is crosslinked Xis that was gel-purified from the complex predicted to contain

3 Xis protomers (e.g., XXX in Fig. 4A lane 7); lane 4 is crosslinked Fis+Xis that was gel-purified from the complex predicted to contain Fis + 2 Xis protomers (e.g., XXF in Fig. 4A lane 12); lane 5 is crosslinked Xis that was gel-purified from the complex predicted to contain Fis + 3 Xis protomers (e.g., XXXF in Fig. 4A lane 12). (D) Autoradiograph of the SDS gel of Fis-Xis complexes formed with ^{32}P -labeled $\Delta^{55}\text{Xis}^{\text{HMK}}$ and wild-type Fis, Fis $\Delta(2-26)$, or Fis K25C, and crosslinked with BS³ as indicated. The composition of the crosslinked bands are designated on the sides of the panels: X denotes an Xis monomer and F denotes a Fis subunit. For example, the band labeled XXX in panels B–D contains 3 crosslinked Xis monomers, and the band labeled XXF contains 2 Xis and one Fis monomer crosslinked together. The identities of the crosslinked bands were deduced by their migrations relative to protein molecular weight markers and by the components added in the particular reactions, including the use of disulfide-linked Fis dimeric proteins (not shown). Band identities in parentheses are tentative.

Figure 6.

DNA footprinting of the Xis-Fis binding region of *attR*. (A) Dimethylsulfate (DMS) footprinting of the 5'-labeled top strand of *attR* in the presence of Fis and/or Xis as denoted. Arrows to the left of the panel, along with the sequence coordinates, designate the locations of relevant protections by Fis and Xis. The sequence of the X1-X2/F region is shown to the right of the panel; the binding sites for Xis and Fis are colored as follows: X1 and X2 (blue), X1.5 (black), F (red), X2/F overlap region (green). The guanines protected from DMS modification by Xis and Fis are circled. (B) Hydroxyl radical footprinting of the 5'-labeled top strand of *attR* in the presence of Fis and/or Xis as denoted. In lane 1, a G chemical sequencing reaction on *attR* is shown. The *attR* sequence is shown to the right of the panel and colored as in (A).

Filled circles denoting hydroxyl radical protections are placed over the relevant bases; smaller circles represent weaker protections. The circle over G-76 is colored green because both Xis and Fis individually protect this base. (C) Hydroxyl radical footprint scans of panel (B) where the black solid line represents the (–) protein control and the colored lines represent the presence of Xis alone (blue), Fis alone (red) or Fis + Xis (green). The sequence coordinates and protected regions are indicated directly above each individual graph. The *attR* sequence over the region, reading 3' to 5', is given at the top of panel (C) and color coded as described above.

Figure 7.

(A) Model of the Fis-Xis cooperative complex. The X-ray crystal structure of three Δ^{55} Xis monomers bound to the X1 (magenta), X1.5 (blue), and X2 (gold) binding sites was superimposed onto the model of the Fis K36E X-ray structure docked to DNA representing the F site. Fis subunits are colored cyan and yellow. (B) A detailed view of the potential interface between Fis and Xis, highlighting side chains of selected residues within the region. (C) Gel mobility shift assays of binding to an X2-F DNA probe (-87 to -54) by Xis and Fis wild-type (4.4 nM), Fis T75A (6.6 nM), FisR76A (4.4 nM), and N73S (11 nM) proteins. Lanes 2–5, 6–9, 10–13, 14–17, and 18–21 contain 29, 115, 460, 1840 nM Xis, respectively. (D) Binding of mutant Xis proteins in the absence or presence of Fis. Lanes 1, 6, 11, 16, and 21

contain 1840 nM Xis without Fis; lanes 2–5, 7–10, 12–15, 17–20, and 22–25 contain 29, 115, 460, and 1840 nM Xis plus 4.4 nM wild-type Fis, respectively.