A Genetic Analysis of Xis and FIS Interactions with Their Binding Sites in Bacteriophage Lambda

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The bacteriophage P22-based challenge-phage system was used to study the binding of Xis and FIS to their sites in attP of bacteriophage lambda. Challenge phages were constructed that contained the X1, X2, and F sites within the P22 Pmar promoter, which is required for expression of antirepressor. If Xis and FIS bind to these sites in vivo, they repress transcription from Pmar allowing lysogenization to occur. Challenge phages carrying the X1X2F region in either orientation exhibited lysogenization dependent on both Xis and FIS. Neither Xis nor FIS was capable of functioning by itself as an efficient repressor in this system. This was the first time challenge phage have been constructed that require two different proteins bind simultaneously to act as a repressor. Mutations in the X1, X2, and F sites that inhibit Xis and FIS from binding were isolated by selecting mutant phages that still expressed antirepressor synthesis in the presence of Xis and FIS. DNA sequence analysis of the mutants revealed 38 unique mutations, including single-base-pair substitutions, multiple-base-pair changes, deletions, and insertions throughout the entire X1, X2, and F regions. Some of the mutations verified the importance of certain bases within the proposed consensus sequences for Xis and FIS, while others provided evidence that the DNA sequence outside of the proposed binding sites may affect the binding of the individual proteins or the cooperativity between them.

During lysogenic development, bacteriophage λ integrates into the Escherichia coli chromosome via site-specific recombination (for a review, see reference 21). Integration occurs by recombination between specific sites in the λ and E. coli chromosomes called attP and attB, respectively, and generates the two hybrid prophage sites, attL and attR. The lysogenization reaction requires the λ-encoded integrase (Int) protein and the E. coli-encoded integration host factor (IHF) protein. Upon induction of a λ lysogen, the prophage excises its DNA from the E. coli chromosome and commences lytic growth. Excisive recombination occurs between attR and attP and requires Int, IHF, and a second bacteriophage-encoded protein excisionase (Xis). In addition, the E. coli protein factor for inversion stimulation (FIS) enhances excision (32, 40).

Each of these proteins binds to specific targets within the att sites. In footprinting experiments using nuclease protection with attR, Xis protected a 40-bp region of DNA that contained two imperfect 13-bp repeats, designated the X1 and X2 sites (Fig. 1 [42]). FIS was similarly shown to protect a region of DNA called the F site that lies adjacent to the X1 site and overlaps the X2 site (Fig. 1 [40]). Early experiments showed that Xis occupancy of the X1 and X2 sites promoted excision (6). Later work indicated that Xis bound to X1 and FIS to F perform the same role (40). Thus, the intracellular concentration of FIS may influence excision. For example, because the concentration of FIS is higher in cells growing exponentially than in those in stationary phase, excision will be favored in exponentially growing cells (40).

Nuclease protection experiments showed that Xis binds the DNA cooperatively at the X1 and X2 sites (7) and also binds X1 cooperatively with FIS at the F site (40). Xis binds DNA as a monomer (7), whereas FIS binds as a dimer (17), and the DNA is bent when they have bound to their respective sites (39). Thus, the X1, X2, and F sites may play a structural role in excision by making or stabilizing a bend required for recombination. To date, only nuclease protection and deletion experiments have been used to delineate the X1 and X2 sites. No substitution mutants in the sites have been isolated. Many FIS binding sites on the bacterial chromosome have been identified, but a comparison of the sequences from 15 sites binding-site mutants that decreased FIS-dependent DNA inversion reveals a degenerate, symmetric 15-bp consensus sequence, with eight positions left completely unspecified (14).

The goal of this study was to determine more thoroughly the DNA sequence requirements for efficient binding by Xis and FIS. We have performed a mutational analysis of the DNA sequences required for Xis and FIS to bind their respective sites, using the P22-based challenge-phage system (4). In this study, we show that neither Xis nor FIS acts individually as an effective genetic repressor in the challenge-phage system, but that the concomitant binding of Xis to X1 and FIS to F is required for effective repression. We have isolated 38 different variants containing mutations that abolished repressor activity by disrupting the binding of Xis or FIS. Our analyses show that bases both within and external to the previously reported Xis and FIS sites are important for binding.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains are listed in Table 1. Salmonella typhimurium JG1126 and JG1127 contain Tn10 insertions in the dnaQ and mutS genes of MS1883, respectively. The dnaQ and mutS insertions were transduced into MS1883 by P22-mediated generalized transduction from strains RM822 (20) and MS1933 (36), respectively. S. typhimurium JG1160 contains a chloramphenicol acetyltransferase gene (cat) inserted into the fis gene of MS1868 and was made as follows. Plasmid pTNI143 (described below)

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contains the cat gene cloned into the fis gene from S. typhimurium. The plasmid was moved from MS1868 to the polA strain MS1571 by P22-mediated generalized transduction. Plasmids with ColEl origins of replication, such as pTN143, cannot replicate in MS1571 because it contains the polA2 allele (12). Therefore, chloramphenicol-resistant transductants could arise only by homologous recombination between pTN143 and the chromosome. The cat insertion was moved from MS1571 into MS1868 by P22-mediated generalized transduction and named JG1160. To demonstrate that the fis gene was mutated in JG1160 because of a cat insertion, the absence of FIS was tested by transducing the cat marker into the TH714 Lac− strain in which FIS activity could be assayed. Strain TH714 contains a MudJ- lacZ operon fusion to the H2 flagellin structural gene, which is controlled by Hin and FIS (10). Therefore, lactose utilization in TH714 is directed by Hin and FIS and can be turned on or off in a manner similar to phase-variation control. However, in a TH714 FIS− strain, lactose utilization is stabilized in either a Lac− or Lac+ phenotype. All of the TH714 chloramphenicol-resistant transductants remained Lac+. Thus, the chloramphenicol resistance in JG1160 is linked to the FIS− phenotype, confirming that JG1160 is itself FIS−.

Media, chemicals, and enzymes. The media and buffers used have been described previously (23). All antibiotics were purchased from Sigma and added to the media to the following concentrations: ampicillin and kanamycin, 50 μg/ml; tetracycline, 15 μg/ml; and chloramphenicol, 30 μg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) were obtained from Sigma and supplemented as indicated.

T4 DNA ligase, T4 polynucleotide kinase, and most restriction endonucleases were obtained from Bethesda Research Laboratories. Mung bean nuclease and SmaI endonuclease were obtained from New England Biolabs. Sequenase and Taq polymerase were obtained from U.S. Biochemical and Promega, respectively. IFF and Int were purified from strains HN356 and HN695, respectively, as described previously (30, 31). Xis production by pPS2-3ARS was tested in E. coli and S. typhimurium strains by making crude extracts as described previously (1) and measuring the Xis activity in an in vitro excision assay (32). Buffers for Int, IFF, and Xis were made as described previously (32).

Transformation, transduction, DNA isolation, and sequencing. Plasmid DNA was isolated by a rapid alkaline lysis method (35). Transformations were performed either by making cells competent by the CaCl2 method (35) or by electroporation at 2.5 kV, 25 μF, and 200 Ω in a Bio-Rad Gene Pulser apparatus (28). P22-generalized transduction was performed as described previously (25) except that phage lysates were exposed to UV light (14 ergs/mm2) for 5 s prior to infection.

P22 DNA was prepared for sequencing and cloning by a method described for λ DNA isolation (37). Two different methods were used to determine the sequence of Punc and the X1X2F insert. The Taq Tag system from Promega was used to determine the sequence of Punc and X1X2F by sequencing the P22 DNA directly, following the manufacturer's protocol for sequencing λ DNA. Otherwise, the Punc X1X2F region was sequenced after cloning it into the EcoRI site of pUC18. An EcoRI digest of the P22 challenge phages produces a DNA fragment containing both the gene for kanamycin resistance (kan') and the Punc operon region, providing a strong selection for the correct clones. The template DNA for the pUC18 clones was prepared as described previously (19). Three different oligodeoxynucleotides were used as primers. The O1 primer contained the sequence 5'-d(GCGCATTTTGCTCATTCC)-3', which is complementary to the 5' end of arc and was used to sequence the X1X2F insert and the promoter region in phase DNA to position −35. The anti-O1 primer contained the sequence 5'-d(GATCATCTCTAGACCATGC)-3', which is complementary to the 5' end of mnt and thus primes DNA synthesis towards Punc and the insert. The New England Biolabs 1212 primer was used to sequence the insert and Punc to position −35 in the pUC18-X1X2F clones.

Plasmid constructions. Plasmids are listed in Table 1. Plasmid pPS2-3ARS contains the xis gene under control of the Punc promoter. It was made by first cloning the EcoRI-BamHI fragment containing xis from pRK5 (2) into the EcoRI-BglII sites of pCRK101. Next, 190 bp of DNA between xis and Punc were removed by digesting the DNA with SmaI and EcoKI, rendering the ends blunt with the Klenow fragment of DNA polymerase, and religating them. This cloning procedure placed the xis gene downstream of the Punc promoter on pCRK101 and under control of the lacF gene on the same plasmid.

Plasmids pTN130 and pTN131 were constructed to place the X1, X2, and F sites at the P22 ant operator locus of pPY190, a plasmid that contains the P22 DNA surrounding the Omnt site. However, the Omnt site itself was deleted in pPY190 and replaced with a SmaI site (15). Complementary oligodeoxynucleotides (oligonucleotides 1 and 2 in Fig. 1), 47 bases in length, were synthesized that contained the X1, X2, and F binding sites from λ. The oligodeoxynucleo-
cleotides were annealed and inserted into the Smal site of pPY190 as described previously (15, 22). Plasmids pTN130 and pTN131 contain inserts with the orientations of the X1 site and F site nearest the p_rac promoter, respectively.

Plasmid pRJ807 contains the fis gene from E. coli cloned downstream of a p_rac promoter on a plasmid expressing the lacI* repressor, ampicillin resistance, and kanamycin resistance (33). To use this plasmid in the challenge-phae assay, we inactivated the Kan' gene. The DNA encoding the gene for Kan' contains three PvuII sites. Thus, pRJ807 DNA was digested with PvuII and religated. The resulting plasmid was named pRJ807B.

Plasmid pRJ898 contains 5 kb of chromosomal S. typhimurium DNA cloned into the EcoRI site of pUC9. The fis gene lies within the 5-kb insert and contains two BsrEI sites at positions 62 and 213 of the 247-bp fragment that comprise it. Plasmids pTN143 and pTN144 were made by inserting the cat gene for chloramphenicol resistance between the two BsrEI sites. The cat gene was derived from Tn9 and isolated on a 1.8-kb PsI fragment from plasmid pTN129. Plasmid DNAs for pRJ898 and pTN129 were cut with BsrEI and PsI, respectively. The desired DNA fragments were separated by agarose-gel electrophoresis and isolated by the DEAE-paper method (9). The DNA fragments were made blunt ended with mung bean nuclease as described previously (13) and ligated with T4 DNA ligase (35). The mixture was drop dialyzed over Tris-EDTA, electroporated into E. coli, and spread onto LB plates containing chloramphenicol. Plasmids pTN143 and pTN144 contain the cat gene transcribed in the same direction and the opposite direction of the fis gene, respectively.

**Challenge-phae construction.** Challenge phages containing the X1, X2, and F binding sites were made by homologous recombination between pTN130 or pTN131 (described above) and phage P22-1000. Phage P22-1000 is a P22 derivative containing the gene for Kan' inserted into the mnt gene and an amber mutation in the arc gene (4). The recombinant phages were selected by plating the phage on S. typhimurium MS1582, a strain that contains an mnt* P22 prophage that prevents P22-1000 from growing lytically (23). Therefore, only recombinants of O_mnt mutants of P22-1000 form plaques on MS1582. The recombinant phages were made as described previously (23), and the presence of the desired insertions was verified by DNA sequencing. Challenge phages containing the insert were named P22xis2B and P22xis2D for the orientations P_mnt-X1-X2-F and P_mnt-F-X2-X1, respectively.

**Challenge-phae assay.** Unless stated otherwise, strain MS1868 carrying plasmid pPS2-3ARS was used to measure the frequency of lysogeny for each challenge phage. The assay was carried out as described previously (23). Xis production (or FIS production for strains containing pRJ807B) was induced prior to each infection with IFI3.

Challenge phages and induced cells were combined at a multiplicity of infection for 20. Dilutions of each infection were spread onto LB plates supplemented with ampicillin, kanamycin, and IPTG at a concentration identical to that used to induce the cells. The percent lysogeny was calculated as 100 times the number of Kan' lysogens divided by the number of cells infected.

**Mutagenesis of the challenge phages and selection of binding-site mutants.** Mutations in the X1, X2, and F binding sites of the challenge phages were induced by four different methods. In one method, lysates of each challenge phage were exposed to UV light (14 ergs/mm²) for 30 s and then used to infect strain MS1868 carrying pGW1700. Plasmid pGW1700 contains the mucAB mutator genes that promote UV-induced mutagenesis (34). Mutated-phae lysates were also produced by growing the phage on either JGI1126 and JGI1127, which contain Tn10 insertions in the dnaQ and mutS genes, respectively. The dnaQ gene encodes the proofreading activity of DNA polymerase III (20). The mutS gene is required for the methyl-directed mismatch repair system (29). Thus, deficiencies in either of these genes increases the frequency of spontaneous mutagenesis in replicating DNA. Finally, the challenge phages were also mutated by hydroxyamide, which causes GC-AT base pair transitions (8) as described previously (25).

Challenge phages containing mutations that disrupted Xis and FIS binding were selected by using the mutated-P22 lysates to infect THS64 carrying plasmid pPS2-3ARS, which had been induced with IPTG at a concentration of 1 mM. Plaques resulting from mutant phages were purified, and the mutational changes were determined by sequencing the DNA.

**Construction of ant-lacZ fusions.** Challenge phages containing ant-lacZ fusions were made by homologous recombination between the phages and plasmid pMS580 as described previously (11). Lysogens of the ant-lacZ phages were grown to mid-log phase (optical density at 600 nm of 0.3 to 0.7) and analyzed for β-galactosidase activity as described previously (25), with each assay being performed in triplicate. The units of β-galactosidase activity were calculated as described by Miller (27).

**RESULTS**

Xis and FIS binding. To analyze the binding of Xis and FIS to their attP binding sites in λ, we used the bacteriophage P22-based challenge-phae system (4). This system has been used to study protein-DNA interactions in vivo by providing a method for isolating mutant binding sites (3, 5, 15, 23, 24). The challenge-phae assay takes advantage of the two immunity regions (immC and immI) of bacteriophage P22 that determine whether the phage undergoes lytic or lysogenic growth during infection of S. typhimurium (for a review, see reference 38). The immC region encodes the c2 repressor protein, which is analogous to the c repressor of λ. When c2
is present and active, it binds to the two operators O₅ and O₆, thus preventing transcription of the genes required for lytic growth. The imml region contains the ant and mnt genes that encode the antirepressor (Ant) and the maintenance (Mnt) proteins, respectively. Ant inactivates the c2 protein, thereby promoting lytic development of the phage. Transcription of the ant gene initiates at its promoter Pₐnt and is negatively regulated by the Mnt protein when bound to its operator site (O_mnt), located immediately downstream of Pₐnt. Hence, repression of Pₐnt is required for lysogenization to occur.

A challenge phage contains the recognition sequence of a DNA-binding protein substituted for O_mnt. The protein is usually plasmid encoded, and its concentration in the cell can be controlled by cloning its structural gene downstream of a regulatable promoter (e.g., P_tac). The intracellular level and binding affinity of the protein control ant expression and thus determine the lysis-lysogeny alternative upon infection by a challenge phage. In addition, the challenge phage contains a gene encoding Kan' inserted into the mnt gene (4), so that the efficiency of lysogeny can be measured as the percent of Kan' survivors following a challenge-phage infection.

We constructed two challenge phages that contained the X1, X2, and F binding sites of bacteriophage λ substituted for O_mnt. Details of the constructions are described in Materials and Methods. The challenge phages P22xis2B and P22xis2D carry the inserts in the Pₐnt-X1-X2-F-ant and Pₐnt-F-X2-X1-ant orientations, respectively. Plasmid pPS2-3ARS (see Materials and Methods) was made to supply a regulatable source of Xis for the challenge-phage assays. It contains the xis gene cloned downstream of the P_tac promoter on a plasmid carrying the lacFᵢ gene. Xis production by pPS2-3ARS was tested in E. coli and S. typhimurium by growing cells in the presence of various concentrations of IPTG, making crude extracts, and measuring the Xis activity in an in vitro recombination assay (32). The expression of Xis was regulated by IPTG and was produced in comparable amounts in S. typhimurium and E. coli (data not shown). FIS protein was supplied endogenously by the S. typhimurium host itself.

The challenge-phage assays were performed with S. typhimurium MS1868 containing pPS2-3ARS as an inducible source of Xis. Prior to each infection, the cells were grown either without IPTG or with various IPTG concentrations, ranging from 50 μM to 1 mM. The results of a typical challenge-phage assay with phages P22xis2B and P22xis2D are shown in Fig. 2 and are presented as the percentage of viable cells lysogenized as a function of the IPTG concentration in the media. Both P22xis2B and P22xis2D formed lysogens on this strain, and their frequencies of lysogenization increased from 3 to 5 orders of magnitude as the IPTG was increased to a concentration of 1 mM (Fig. 2). Phage P22xis2B was unable to produce lysogens in the absence of IPTG, whereas P22xis2D exhibited a low level of lysogeny (≤10⁻³%) on cells grown in the absence of IPTG. At an IPTG concentration of 1 mM, the two phages formed lysogens at equal frequencies (approximately 2 to 4%). Above 1 mM IPTG, the frequency of lysogenization decreased gradually for both phages (data not shown). These results demonstrate that lysogeny by these two phages depends on the induction of Xis.

Analysis of the effects of FIS on Xis binding. In phage P22xis2D, the orientation of the X1, X2, and F binding-sites positions the F site adjacent to Pₐnt. In the absence of IPTG, P22xis2D lysogenized MS1868 cells carrying pPS2-3ARS more efficiently than did P22xis2B, in which the orientation of the binding sites is reversed. This finding raised the possibility that chromosomally encoded FIS had bound to the F site on the challenge phage, and because the F site is adjacent to Pₐnt in P22xis2D, this binding would partially repress ant expression. To test this possibility, strain MS1868 containing pCKR101, the parent plasmid without the xis gene, was used in the challenge-phage assay. Neither P22xis2B nor P22xis2D formed lysogens on MS1868 containing pCKR101, thereby eliminating the possibility that FIS was the sole agent responsible for the P22xis2D lysogeny observed with pPS2-3ARS in the absence of IPTG. As an additional test, the challenge phage-assay was repeated with MS1868 containing plasmid pRJ807B, in which the fis gene is cloned downstream of the P_ac promoter, so that FIS expression could be regulated by the concentration of IPTG. Phage P22xis2B was unable to lysogenize MS1868 containing pRJ807B, whereas P22xis2D exhibited a 3 × 10⁻⁴% lysogenency at all concentrations of IPTG. Therefore, endogenous FIS encoded by the chromosomal fis gene was not produced in a concentration high enough to act by itself as an efficient repressor on P22xis2B or P22xis2D. However, when the intracellular FIS concentration was increased by a multicopy plasmid (pRJ807B), FIS acted as a weak repressor, partially blocking transcription from Pₐnt in P22xis2D. It is possible that the F site is fully saturated at the FIS levels obtained in cells grown under these conditions (see Discussion).

Another way to test the requirement for FIS in the challenge-phage assay was to perform the assays in a host that is deficient in FIS production. Therefore, a FIS-deficient mutant was made in S. typhimurium MS1868 by inserting a gene encoding chloramphenicol resistance into fis. The presence of the fis mutation in MS1868 was confirmed by demonstrating that the strain transduced with the fis::cat insertion was unable to support flagellar phase variation, a process known to require FIS (see Materials and Methods). When the strain MS1868 fis::cat containing the Xis-encoding plasmid pPS2-3ARS was used in the challenge-phage assay with P22xis2B and P22xis2D, no lysogens were produced. Thus, under our conditions, neither Xis nor FIS individually functions as an efficient repressor in the challenge-phage assay. However, when both proteins are expressed simultaneously in sufficient amounts, they act as efficient repressors to inhibit ant expression. These results demonstrate that lysogeny by these two phages depends on the induction of Xis, that FIS and Xis may be binding cooperatively, and that under identical conditions, the construction with the FIS site adjacent to the promoter is more easily repressed. The direct requirement for the FIS binding site was confirmed by the finding that several of the mutants isolated that prevented repression contained mutations in the FIS binding site (see below).

Isolation of binding-site mutants. Mutations that disrupted Xis and FIS binding of P22xis2B and P22xis2D were generated by subjecting the phages to mutagenesis. Four different mutagenic treatments were used to increase the spectrum of possible mutations. In one method, the phages were exposed to UV light, followed by infection of strain MS1883 carrying a plasmid encoding the mucAB mutator genes (see Materials and Methods). Challenge phages were subjected to mutagenesis by growing them on strain MS1883 containing either a mutS or mutD (dnaQ) mutation. Finally, the challenge phages were also subjected to hydroxylamine mutagenesis.

Challenge phages containing mutations that disrupted Xis and FIS binding were selected by isolating phages that formed plaques on S. typhimurium TH564 carrying plasmid
FIG. 2. Challenge-phage assay. The frequency of lysogeny (Materials and Methods) is shown for P22xis2B (●) and P22xis2D (■) when used to infect cells induced at different IPTG concentrations. The points on the graph represent the averages of three assays.

pPS2-3ARS, under conditions such that lysogeny would be maximal (i.e., at 1 mM IPTG). This strain contains a defective P22 prophage that produces c2 protein (23). Thus, a wild-type challenge phage is unable to form plaques on this strain, because Xis and FIS bind to the X1 and F sites and prevent ant expression from the phage. However, phages containing mutations that interfere with Xis or FIS binding produce the antirepressor protein and form plaques.

We isolated and determined the DNA sequence changes of 86 mutants from P22xis2B and 24 mutants from P22xis2D. Although all the mutants were isolated independently, some were isolated repeatedly. Altogether, 38 unique mutations were generated in the binding sites (Fig. 3). P22xis2B mutants contained changes affecting each of the proposed binding sites (X1, X2, and F), and in addition, some mutations occurred outside of these sites. All of the changes in the P22xis2D mutants disrupted the proposed F site, and two of them were also in the overlapping portion of the X2 site. Although mutants containing changes in the X1 site were not isolated from P22xis2D, the importance of the X1 site was
confirmed by constructing challenge phages containing only the X2-F sites in both orientations relative to P_22x2s2B. The X2-F challenge phages were constructed from oligodeoxyribonucleotides 3 and 4 shown in Fig. 1. The P_22x2sis2B construct was isogenic to P22xis2D with respect to the P_22x2sis2B and F fusion sequence. Both classes of challenge phages were unable to significantly lysogenize MS1868 carrying pPS2-3ARS, even when cells were grown at 1 mM IPTG (data not shown).

The mutations comprise 16 single-base-pair substitutions, 6 single-base-pair deletions, 3 single-base-pair insertions, and 13 multiple-base-pair changes. Among the four methods of mutagenesis, the mutS method worked best for inducing single-base-pair changes. It produced as many single-base-pair substitutions as single-base-pair deletions and insertions and produced no multiple-base-pair changes (Table 2). UV mutagenesis with the plasmid-encoded mucAB genes generated more deletions and insertions than single-base-pair substitutions and also produced many multiple-base-pair changes. Mutagenesis with the mutD strain yielded only single-base-pair deletions and insertions. Hydroxylation of mutagenesis generated almost all single-base-pair substitutions, but unfortunately, most of the substitutions were identical. The mutS and mucAB methods of mutagenesis complemented each other well, as each generated a distinct subset of mutations. For example, of the 15 unique single-base-pair substitutions produced, only two were isolated in common as a result of both methods of mutagenesis.

The 38 mutants were tested in the challenge-phage assay to determine the severity of the effects of the mutations on

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\begin{array}{cccc}
P_{ant} & X1 & X2 & F \\
\hline
1 & 2 & 3 & 4 \\
\hline
\end{array}
\]

FIG. 3. Binding-site mutations and frequency of lysogenization observed upon infection of cells grown at an IPTG concentration of 1 mM. The proposed X1 and F sites (14, 42) are overlined in the sequences and represented by hyphens in the mutants. The designation of each mutant precedes the sequence showing the mutation. A Δ in the sequence signifies that that particular base was deleted. Mutants B5, B18, B27, and D8 contain insertions designated by a + followed by the base inserted. Following each sequence is the percent lysogenization (see Materials and Methods) for that mutant at 1 mM IPTG. These values are given as percentages and represent the averages of three assays. The underlined bases represent the transcription start sites.
The only optimal binding site in the category.

Expressed values in parentheses represent the number of unique mutations within each category.

We used the P22-based challenge-phage system to further define the interactions between Xis and FIS and their respective binding sites in the P arm of λ. These sites have previously been identified only by nuclease protection experiments and by deletions in the DNA that eliminated them. We constructed two challenge phages with the region encompassing X1, X2, and F acting as an operator for the P_{ant} promoter. The two phages were identical except for the orientations of the sites; phages P22xis2B and P22xis2D carried the orientations P_{ant}-X1-X2-F-ant and P_{ant}-F-X2-X1-ant, respectively. In addition, we constructed a plasmid pPS2-3ARS that contains the xis gene cloned downstream of the F_tac promoter so that the intracellular level of Xis could be varied by changing the concentration of IPTG in the medium.

When the phages were used to infect an S. typhimurium strain carrying pPS2-3ARS, the frequencies of lysogenization depended on the IPTG concentration in the medium (Fig. 2). Although the frequencies of lysogenization at 1 mM IPTG were the same for the two phages, P22xis2B grew lytically in the absence of IPTG, whereas P22xis2D lysogenized at a very low level (<10^{-3}%). These observations suggested that endogenous FIS was binding to the F site and that when this occupied site was immediately adjacent to P_{ant} (P22xis2D), it was partially repressing ant expression. However, when the challenge phages were used to infect a strain lacking the plasmid producing Xis, lysogens did not form. Thus, FIS alone cannot account for the P22xis2D lysogenization of strain MS1868 carrying pPS2-3ARS in the absence of IPTG. We conclude that lysogeny in both P22xis2B and P22xis2D depends on the induction of Xis.

To determine whether Xis binding required FIS binding to F for repression of P_{ant}, we constructed a S. typhimurium strain deficient in FIS production. When the challenge phages were used to infect the fis strain carrying the Xis-producing plasmid, no lysogens were formed even after induction with 1 mM IPTG. Thus, Xis requires FIS to act as a repressor. These two experiments indicate that neither Xis nor FIS can independently bind their sites effectively enough to inhibit transcription from P_{ant}. However, when both FIS and Xis are bound, ant expression is inhibited.

The differences in lysogeny observed between P22xis2B and P22xis2D in the absence of IPTG may be explained in several ways. For example, FIS may disrupt the RNA polymerase-promoter interaction better than Xis. FIS binds as a homodimer (M_r = 24,000 [16]), making the bound form almost three times more massive than Xis and thus possibly better at interfering with transcription initiation through steric exclusion. Alternatively, slight differences in the context of the P22xis2B and P22xis2D binding sites or the relative phasing of the sites with respect to P_{ant} may have affected the ability of Xis and FIS to act as repressors.

We have demonstrated that both Xis and FIS are required for maximal repression of P_{ant}. Previous work showed that Xis and FIS bind cooperatively in vitro (40), and thus the requirement for the presence of both proteins for repression suggests that Xis and FIS may also bind cooperatively in vivo. However, another possibility is that FIS normally occupies the F site on the challenge phages but cannot function by itself as an efficient repressor. This possibility is supported by the observation that the F site on a pBR322-based plasmid is bound by FIS in vivo during exponential growth (40). Consequently, effective repression may simply require both Xis and FIS bound to their sites or the formation of a higher-order FIS-Xis-DNA structure.

The three phages λ, 434, and HK022 use the same general molecular mechanism for integrative and excisive recombination. Not only are their attachment sites highly homologous, but their Xis proteins are identical except for one amino acid, making the proteins functionally interchange-
able (26, 41). Alignment of the X1, X2, and F binding sites in the three bacteriophages reveals a consensus sequence for Xis binding (Fig. 4). The 13-bp consensus sequence, TATGT-GT-T-TT, contains the indicated bases completely conserved in five of six Xis binding sites of these three phages and is the same as the consensus published for λ (42). Figure 4 also shows the FIS consensus sequence as determined by Hubner and Arber (14).

To find which bases within the X1X2F region were important for Xis and FIS binding, we mutagenized the challenge phages and selected variants that were able to form plaques on S. typhimurium TH564 carrying an inducible xis gene (Materials and Methods). We selected and determined the DNA sequences of 110 independent isolates, of which 38 were unique (Fig. 3). The mutations occurred in the X1, X2, and F binding sites and most completely disrupted Xis or FIS binding, as determined by the challenge-plague assay (Fig. 3). The isolation of mutants with changes in the F site supports the earlier cooperativity argument in that disruption of FIS binding affects Xis binding.

We isolated three mutants, B4, B24, and B29, that contained single-base-pair substitutions in the X1 site. Mutations in B24 and B29 were at positions conserved in all six sites (positions 4 and 7) of the Xis consensus sequence (Fig. 4). The failure of these mutants to bind Xis verifies these positions as critical for Xis recognition. Mutant B4 contained a substitution that changed the T in position 5 of the consensus to C. Position 5 is conserved in five of six Xis sites, differing only in the X2 site of bacteriophage 434, where it is a C. Thus, one might have expected mutant B4 to be tolerated since the change appears in nature in the aforementioned site. That it was not suggests that FIS binding to the adjacent site compensates for the weaker X2 binding site created by the sequence change. Alternatively, the T in the consensus sequence is context dependent, with Xis binding the X2 site differently than the X1 site in phage 434. In either case, the mutant confirms that a T at position 5 is required for Xis recognition of the X1 site.

Six other mutants contained deletions within the X1 site. These variants may have either eliminated important direct contacts for Xis or altered the spacing or angular relationships of important contact sites in the target sequence. In either case, the mutants support the conclusion that FIS is not an efficient repressor without Xis.

We isolated 10 mutants (B11, B12, B16, B19, B20, B23, B26, B28, D5, and D7) that contained single-base-pair substitutions in the F site, eight of which also occurred in the overlapping X2 site (Fig. 3 and 4). Xis fails to bind the X2 site in vitro when FIS is bound to the F site (40). Because FIS is required for lysogenization of the challenge phages, and 11 of the mutants isolated (B3, B5, B14, B15, B28, D1, D2, D3, D4, D5, and D8) contained mutations solely in the F site, it is likely that FIS binds the F site, thereby preventing Xis from binding at the overlapping X2 site. Thus, the disruptive effects of mutations that occur in both the F and X2 sites in the challenge-plague assays are likely on FIS binding to F, not Xis binding to X2. Four of these mutations (B11, B20, B28, and D5) changed conserved bases in the postulated FIS consensus sequence described by Hubner and Arber (Fig. 4 (14)). These mutations confirm the importance of certain bases in the consensus sequence, by showing that changes within these positions disrupt FIS binding. However, five mutations that prevented FIS binding either changed bases unspecified by the postulated FIS consensus sequence (B12, B16, B19, and B23) or even allowed by it (B26). For example, although the first position of the Hubner and Arber consensus site (Fig. 4) is specified as either A (as in the λ F site) or T, mutant B26 demonstrates that a T at this position in the λ site prevents FIS from binding to it. The FIS binding site is an imperfect palindromic sequence in which each monomer of the FIS dimer binds half the site (14). The nature of this binding is complex, since different FIS sites containing different bases at the same positions, resulting in ambiguities in the consensus sequence. However, as our four mutations show, particular changes in these unspecified bases disrupt FIS-DNA interaction. Perhaps significantly, all nine mutations in the F site are at positions completely conserved in phages λ, 434, and HK022 (Fig. 4).

Seven variants were isolated that changed the spacing between the X1 and F sites. For example, mutant B27 contained a single-base addition to a run of T residues in the X1 site. Thus, although the mutation failed to change any of the bases of the consensus sequence of Xis, it nonetheless disrupted Xis and/or FIS from binding effectively. Again, this mutant may disrupt the spacing or angular relationships between Xis and DNA contacts outside the proposed X1 consensus sequence. Additionally, since Xis and FIS bind cooperatively in vitro (40), the deletions and insertions in mutants B10, B17, B18, and B27 may have disrupted the cooperativity between Xis and FIS. Cooperativity of binding between these proteins could occur through protein-protein or protein-DNA interactions. Any protein-protein interactions between Xis and FIS that give rise to cooperativity may require strict spacing and phasing between the binding sites and could have been disrupted by the mutations. Alternatively, Xis and FIS may recognize structural features
of the DNA as well as the DNA sequence itself. Hence, the cooperativity between Xis and FIS could be due to their effects on the DNA and not necessarily to direct protein-protein interactions. Each protein binds the DNA when bound to its target sequence (39). When the DNA is bent by one protein (either by Xis at X1 or FIS at F), the second protein may bind the DNA better. The mutations between the X1 and F sites may have altered the intrinsic bendability of the DNA required for the second protein to bind effectively. For instance, in mutant B27 the additional T. A base pair added to a run of such base pairs may increase the curvature of the DNA at these sites (18), and such a change may affect Xis or FIS binding.

Evidence supporting an explanation depending on protein-to-DNA effects was reported recently, when the carboxyl and amino portions of the FIS protein were found responsible for binding the DNA and for cooperativity with Hin, respectively (33). Interestingly, mutations in FIS which disrupt protein-protein cooperativity between FIS and Hin have no effect on α excision (33), suggesting the fis mutants still bind cooperatively with Xis. Thus, this finding lends support to the model that FIS-Xis cooperativity may be effected through the DNA and not via direct protein-protein interactions, at least not with the domain of FIS used in the interaction with Hin.

Three other mutants (B9, B22, and B30) contain single-base-pair substitutions between the proposed X1 and F sites. Unless these mutations interfered with Xis-FIS cooperativity by changing the intrinsic structure of the DNA around the sites, their effects suggest that protein-DNA interactions occur outside the proposed Xis or FIS sites. In either case, these results show that single-base-pair changes at positions outside of the proposed consensus bases can disrupt Xis or FIS binding.

In summary, we have shown that the challenge-phage system is a valuable means of monitoring the interactions of Xis and FIS with their respective binding sites and possibly with each other. Neither Xis nor FIS separately functions efficiently as a repressor in this system, but together they inhibit transcription from Fam. This is consistent with the observation that Xis and FIS bind cooperatively in vitro (40). In addition, this is the first time that challenge phases have required two different proteins that are bound simultaneously to repress ant transcription. Thus, the challenge-phage system should be useful for studying other systems employing multiple-protein interactions with DNA. We have isolated mutations both within and flanking the postulated consensus sequences that disrupt protein binding. Some of these changes verify the importance of bases within each consensus sequence, whereas the others provide evidence that the DNA sequences outside of the proposed binding sites may also affect binding. The mutants isolated may provide a means for further understanding the protein-DNA interactions in this system by facilitating the isolation of suppressors that recognize an altered site. We are currently seeking Xis mutants that are deficient in excision but remain functional in DNA binding. Such experiments should help define the requirements for functional protein-DNA interactions as well as the exact roles of these proteins in λ recombination.

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