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The *Escherichia coli* Fis Protein Stimulates Bacteriophage λ Integrative Recombination In Vitro

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The *Escherichia coli* nucleoid-associated protein Fis was previously shown to be involved in bacteriophage lambda site-specific recombination in vivo, enhancing the levels of both integrative recombination and excisive recombination. While purified Fis protein was shown to stimulate in vitro excision, Fis appeared to have no effect on in vitro integration reactions even though a 15-fold drop in lysogenization frequency had previously been observed in *fis* mutants. We demonstrate here that *E. coli* Fis protein does stimulate integrative lambda recombination in vitro but only under specific conditions which likely mimic natural in vivo recombination more closely than the standard conditions used in vitro. In the presence of suboptimal concentrations of Int protein, Fis stimulates the rate of integrative recombination significantly. In addition, Fis enhances the recombination of substrates with nonstandard topologies which may be more relevant to the process of in vivo phage lambda recombination. These data support the hypothesis that Fis may play an essential role in lambda recombination in the host cell.

Fis is a 98-amino-acid homodimeric protein found in *Escherichia coli* and numerous other prokaryotes. Fis is a member of a group of proteins known as the nucleoid-associated proteins, which are often isolated as part of the mass of protein-DNA which forms the *E. coli* nucleoid (13). Members of this family are often involved in DNA interactions involving bending, looping, or condensation of DNA substrates. Fis was first identified for its role in regulating recombination reactions carried out by the DNA invertase, Hin (8, 9). Other activities for Fis were later identified, including transcriptional activation of a wide number of promoters (11, 15, 17), repression of another set of promoters (4, 10, 18), involvement as a cofactor for DNA replication (7) and cell division and chromosome separation (14), and participation in site-specific recombination of bacteriophage lambda (2, 3, 16). Cellular levels of Fis vary dramatically during the *E. coli* cell cycle depending on the growth stage and the availability of nutrients (4, 16). During log phase growth, Fis is present at concentrations up to 50 μM (1), or enough Fis to bind every 500 bp along the chromosome (4). However, as cells enter stationary phase or are deprived of nutrients, levels of Fis drop to almost undetectable amounts (1, 4).

Fis is capable of nonspecific binding to DNA in vitro, but it has a 50-fold higher affinity for a series of sites with a degenerate 15-bp consensus sequence which loosely resembles an inverted repeat (5, 6, 13). DNA footprinting shows clear contacts between the protein and the DNA in these 15-bp Fis binding sites; however, the DNA sequence alone appears to be a poor predictor of Fis binding affinity, and local DNA structure may influence the activity of a given Fis binding site (12). The role of Fis in lambda site-specific recombination was first identified through observation of a 20-fold stimulation of lambda excision in vitro by Fis in the presence of suboptimal levels of the lambda Xis protein (16). Genetic evidence presented by Ball and Johnson (2, 3) demonstrated not only that Fis could stimulate excision of phage lambda but also that lysogeny was enhanced by the presence of the *fis* gene. These experiments, which were carried out in vivo using phage mutated in the F site and/or *E. coli* lacking Fis, showed a 15-fold drop in lysogenization frequency when the *fis* gene was deleted (3). A portion of this decrease was shown to be due to the loss of Fis as a regulator in non-recombination-related events. However, a mutation of the F site which eliminates Fis binding without affecting Xis binding still leads to a loss in lysogenization frequency of two- to threefold, suggesting that Fis plays a role in integration as well as excision. Previous experiments carried out in vitro with Fis to examine integration did not identify any effect of Fis on the reaction (16).

We demonstrate here that Fis does affect in vitro integrative recombination, but it does so only under nonstandard conditions which have not previously been examined. Fis stimulates recombination at levels of Int which are considered suboptimal for the in vitro reaction. In addition, Fis plays a role in enhancing recombination between substrates in topological states different from those of the normal in vitro substrates. The level of enhancement in these reactions agrees well with that observed in experiments carried out in vivo. These data strongly support the proposal first made by Ball and Johnson that Fis may play an important or even essential role in lambda integrative recombination.

MATERIALS AND METHODS

Oligonucleotides. The synthetic oligonucleotides (Invitrogen) used in this study were as follows: DE09, 5′-GGGGGCTGCAGGCAAGAAGACAAAAATCACCTTGCGC; DE10, 5′-GGGGGCCCGGGCAGAGGCAGGGAGTGGGACAAAATTG; DE46 (Fis start), 5′-GGAGGGAATTCAGGAGGTATAAATTAATGTTCGAACAACGCGTAAATTCTG; DE49 (Fis stop), 5′-GGAGGGGATCCTTATTAGTTCATGCCGTA; and DE162, 5′-GGAAGGAGATCTTCCGATCGACCGCTATTACATAATACTGTAAAACACAAC.

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Cloning of *E. coli fis*. The *fis* gene was amplified by using PCR on *E. coli* DH10B chromosomal DNA with Platinum *Taq* Hi Fidelity (Invitrogen) and primers (DE46 and DE49, respectively) corresponding to the 5′ and 3′ ends of the gene. The 5′ primer was constructed to provide a strong Shine-Dalgarno initiation sequence prior to the start of the *fis* gene. The PCR product was digested and cloned into pRAD19, a high-copy-number expression vector carrying the lambda P_L promoter under the control of the heat-inducible lambda *c*I857 gene. The final clone (pLDE15) was sequence verified to ensure that no mutations were present and was introduced into *E. coli* BL21 for expression.

Purification of *E. coli* Fis protein. A 5-ml overnight culture of pLDE15 was diluted into 1 liter of Luria broth and 0.1 mg of ampicillin/ml in a 2.8-liter Fernbach flask and was grown at 30°C to an optical density at 600 nm of 0.7. At that point, production of Fis was induced by shifting to 42°C for 2 h, and cells were subsequently collected by centrifugation; 7.5 g of wet cells was obtained and was frozen at −80°C. Cells were thawed and resuspended in 15 ml of buffer containing 50 mM Tris-Cl (pH 8.0), 5 mM EDTA, 10% glycerol, 1 M NaCl, and 1 mM dithiothreitol (DTT). The cell solution was sonicated four times for 45 s, and the sonicated extract was incubated at 65°C for 15 min, after which the denatured proteins and cell debris were removed by centrifugation at 30,000 × *g* for 40 min. Clarified extract was stored at −80°C. The extract (15 ml) was diluted with 35 ml of buffer A (20 mM Tris-Cl [pH 8.0], 1 mM EDTA, 10% glycerol, 1 mM DTT) and applied to a Pharmacia HiTrap heparin column (1 ml) at a flow rate of 0.25 ml/min. The column was washed with 400 mM NaCl in buffer A for 10 column volumes (CV) and eluted with a 15-CV gradient from 400 to 800 mM NaCl in buffer A. A broad peak of Fis was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fractions containing Fis were pooled and dialyzed against buffer A with 200 mM NaCl. This sample was applied to a Pharmacia HiTrap MonoS column (1 ml) equilibrated in the same buffer. The column was washed with 15 CV of 200 mM NaCl in buffer A and eluted with a 20-CV gradient of 200 mM to 1 M NaCl in buffer A. Two peaks were observed from the column, with the second sharp peak representing most of the Fis protein. The cleanest fractions were pooled to give a sample containing >95% Fis by Coomassie staining. Purified Fis was obtained at a concentration of 1 mg/ml after dialysis into Fis storage buffer containing 20 mM Tris-Cl (pH 8.0), 1 mM EDTA, 50% glycerol, 1 mM DTT, and 0.5 M NaCl and was stored at −20°C.

Fis binding assay. A gel retardation assay was developed to quantitate Fis activity. A PCR product containing the lambda *attP* sequence was amplified using primers DE9 and DE10. The 400-bp product was cut with *Ava*I and labeled at the ends with [^{32}P]dCTP by using the Klenow fragment of *E. coli* DNA polymerase I. Reactions (20-μl volume) were carried out in 20 mM Tris-Cl (pH 8.0), 5% glycerol, 25 mM NaCl, 200 μg of salmon testis DNA/ml, and 1.17 ng (10,000 dpm/fmol) of PCR product. Fis protein was added, binding was carried out for 10 min at room temperature, and samples were loaded on a Novex 6% gel retardation gel running in 0.5× Tris-borate-EDTA buffer for 60 min at 100 V. Gels were dried and visualized on a Typhoon phosphorimager (Molecular Dynamics) after a 60-min exposure. In the presence of competitor DNA, a single discrete shift was observed, which allowed the calculation of an apparent K_d value by measurement of the concentration of Fis required to shift 50% of the substrate.

Recombination assays. pATTP2 contains the bacteriophage lambda *attP* site cloned into the *Hin*dIII and *Eco*RI sites of pUC19. pATTB2 contains the *E. coli* *attB* site cloned into the *Bam*HI site of pUC19. Linear radiolabeled substrates for recombination assays were made by restriction enzyme digestion followed by fill-in synthesis using Klenow fragment of DNA polymerase I. Substrates (1 μg) were incubated with 0.5 U of Klenow fragment, 1 mM dATP, 1 mM dGTP, 1 mM dTTP, and 30 μCi of [^{32}P]dCTP for 15 min, and the labeled DNA was purified using Concert PCR purification columns (Life Technologies) and eluted in 50 μl of Tris-EDTA. The average specific activity of labeled substrates was 500 dpm/fmol. Recombination reaction mixtures (20 μl) consisted of 25 mM Tris-Cl (pH 8.0), 1 mM EDTA, 6 mM spermidine, 15% glycerol, and 75 mM NaCl (unless indicated otherwise), 100 fmol of each substrate, and approximately 30,000 cpm of ^{32}P-labeled linear substrate. Standard integration reactions contained 80 ng of *E. coli* integration host factor and 150 ng of λ Int. Reactions were incubated for 60 min at 25°C, stopped by the addition of 50 μg of proteinase K/ml, heated for 15 min at 65°C, and electrophoresed on a 0.7% agarose gel. Gels were dried and visualized on a Typhoon phosphorimager. Recombination levels were determined by quantitation of substrate and product bands by using ImageQuant software (Molecular Dynamics).

Mutation of Fis binding sites. The lambda F site was mutated by first introducing a single A-to-G base change in the *attP* region, which created a *Bgl*II restriction site. This change, located 3 bases 3′ of the end of the F site, did not affect recombination efficiency. The new left arm of the *attP* site was amplified by PCR using an oligonucleotide (DE162) containing the mutated Fis binding site flanked by a *Bgl*II site. This mutated left arm fragment was then used to replace the wild-type arm in the *attP* construct by using the *Bgl*II restriction site. The final construct was sequence verified to ensure that no unintended mutations had been introduced.

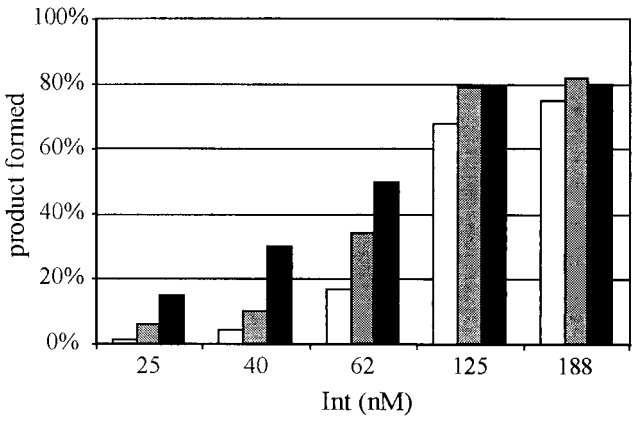

FIG. 1. Fis stimulates integrative recombination at low Int concentrations. Integration reactions (20-μl volume) were performed as described in Materials and Methods by using 100 fmol of supercoiled pATTP2, 100 fmol of linear pATTB2, and the indicated amounts of Int and Fis. The percentages of recombination product observed at given Int concentrations are plotted for three different Fis concentrations: no Fis (white bars), 150 nM Fis (gray bars), and 500 nM Fis (black bars).

RESULTS

Fis stimulates integration at suboptimal levels of Int. Previous experiments designed to examine the effect of Fis on integrative recombination were carried out with optimal in vitro levels of Int protein. We have observed effects on lambda integration by other host factors in which stimulation was seen only at suboptimal levels of Int (E. Flynn, D. Esposito, and G. F. Gerard, unpublished data). The role of Fis in the in vitro integration reaction was examined at various levels of Int (Fig. 1). At optimal levels of Int (120 to 180 nM), Fis showed little effect on the amount of product produced. However, at suboptimal levels of Int, where reaction efficiency was dramatically decreased, Fis increased levels of product formation by as much as sevenfold. A titration of Fis in a reaction mixture containing suboptimal levels of Int (Fig. 2) showed that there is a broad optimal concentration of Fis once a threshold amount is reached. Concentrations between 0.3 and 3 μM produced similar levels of stimulation. At lower Int concentrations, the same range of Fis concentrations produced optimal stimulation, suggesting that Fis/Int ratios are not relevant to the level of stimulation. This in turn indicates that Fis did not affect the reaction by interacting directly with Int but rather likely acted through its DNA binding activity.

Fis stimulates reactions with substrates of varied topologies. Lambda integration reactions in vitro are most efficient when the *attP* substrate is supercoiled. The likely reason for this is that supercoiling energy is used to assist in the formation of the larger protein-DNA complexes called intasomes, which are required for proper control of recombination. In the absence of *attP* supercoiling, reaction efficiencies decrease nearly 10-fold. In addition, these linear *attP* reactions only occur at

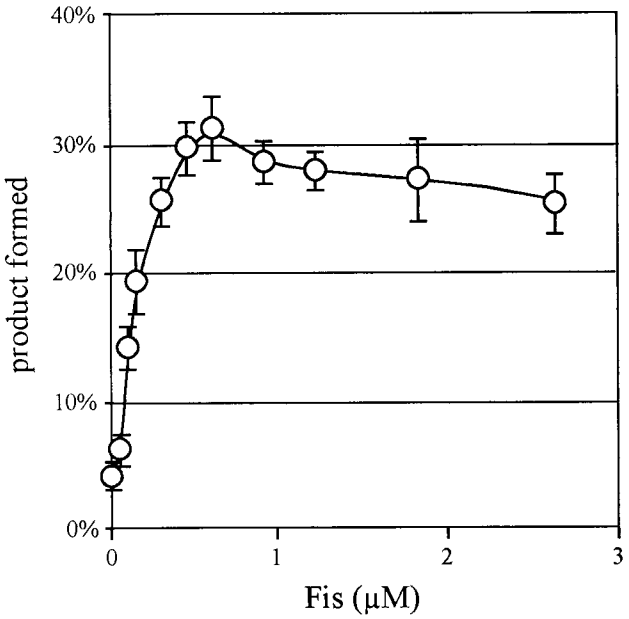

FIG. 2. Fis stimulates low Int recombination over a broad range of concentrations. Integration reactions (20-μl volume) were performed as described in Materials and Methods by using 100 fmol of supercoiled pATTP2, 100 fmol of linear pATTB2, 40 nM Int, and the indicated amounts of Fis. The percentages of recombination product observed at the given Fis concentrations are plotted. Data shown are averages of three experiments, with standard deviations shown with error bars.

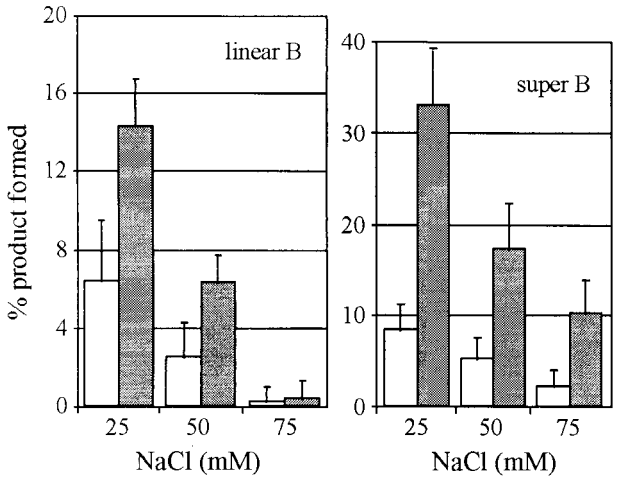

FIG. 3. Fis stimulates integrative recombination when substrates with nonstandard topologies are used. Reactions (20-μl volume) were performed as described in Materials and Methods by using 100 fmol of linear pATTP2, 100 fmol of linear (left panel) or supercoiled (right panel) pATTB2, and 150 nM Int. The percentages of recombination product observed at the given NaCl concentrations in the absence of Fis (white bars) or in the presence of 500 nM Fis (gray bars) are plotted. Data shown are averages of three separate experiments, with standard deviations shown with error bars.

very low ionic strength (<25 mM NaCl), whereas standard supercoiled *attP* integration reactions are optimal at a much higher level (75 mM). The most inefficient reaction occurs when both *attP* and *attB* are linear; when Fis is added to these reactions (Fig. 3, left panel), product formation is stimulated two- to threefold. In the case of reactions containing supercoiled *attB* and linear *attP* (Fig. 3, right panel), the addition of Fis stimulates product formation to even higher levels. At low salt concentrations, a threefold effect is observed, while at higher ionic strength, Fis stimulation increases to nearly fivefold. This effect requires the same concentration of Fis as does standard integration reactions at reduced Int concentrations (data not shown).

Elimination of the F site abolishes Fis stimulation. To demonstrate more clearly that the stimulation by Fis is due to the interaction of Fis with its specific DNA binding site, we created mutant substrates which eliminated the Fis binding motifs. Thirteen of the 15 bases of the F site were mutated to bases observed least at those positions in all known Fis binding sites. All six strongly conserved residues in the binding site were removed. The new sequence, 5′-ATAGCGGTCGATCGG showed no similarity to the consensus Fis binding site 5′-Gnt YAnWWWnTRanC and did not score as a Fis binding site by computer algorithm. A gel retardation assay (Fig. 4) demonstrated that Fis was no longer capable of binding the mutant F site even at levels of Fis which completely bound the wild-type site. The binding affinity of Fis for the mutated site was at least 2 orders of magnitude lower than for the F site and was equivalent to the normal nonspecific DNA binding affinity of Fis for random sequence DNA. Integration reactions were carried out using the mutated F-site substrates in various topologies. The standard supercoiled *attP*-linear *attB* reaction was performed with suboptimal levels of Int, while the other reactions involving linear *attP* were carried out with optimal levels of Int. The results (Fig. 5) demonstrate that elimination of the F site abolishes all of the stimulatory effects of Fis. In each case, levels of recombination with the F-site mutant substrates in the presence of Fis were indistinguishable from levels with wild-type substrates in the absence of Fis, indicating strongly that Fis requires binding to the wild-type F site in order to stimulate integrative recombination.

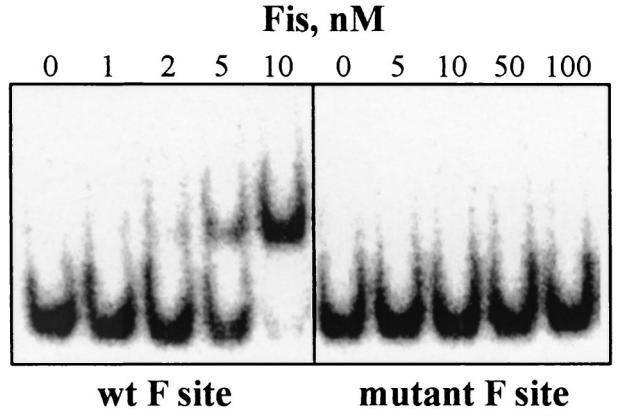

FIG. 4. Fis is incapable of specific binding to the mutated F site. A gel retardation assay with Fis was performed on DNA fragments containing the wild-type or mutated F site. The left lane of each set contains no Fis; the next four lanes contain various concentrations of Fis as indicated. Competitor DNA (salmon sperm DNA) was present at a concentration of 0.2 mg/ml in all samples.

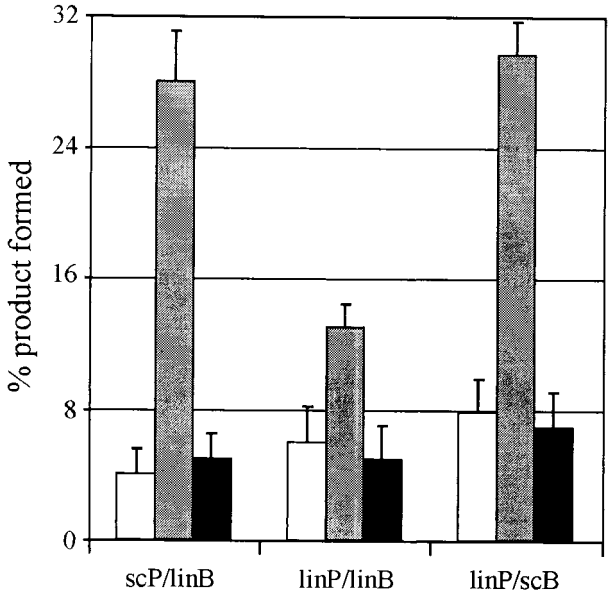

FIG. 5. Fis stimulation is abolished by mutation of the F site. Reactions (20-μl volume) were performed using 100 fmol of pATTP2 (wild-type or mutant F site) and 100 fmol of pATTB in the topological forms indicated. Reactions with supercoiled *attP* contained 40 nM Int, while reactions with linear *attP* contained 150 nM Int. Substrates containing wild-type F sites contained no Fis (white bars) or 500 nM Fis (gray bars). Substrates containing the mutated F site contained 500 nM Fis (black bars). The percentages of recombination product observed are plotted; data shown are averages of three separate experiments, with standard deviations shown with error bars.

Kinetics of Fis stimulation. To better understand the role of Fis stimulation of integration, we examined the early kinetics of the reaction in the absence or presence of Fis. In the standard supercoiled *attP*-linear *attB* reaction at suboptimal levels of Int, Fis stimulates the initial rate of recombination (based on time points from 2 to 15 min) fourfold, achieving a rate of 1.6 fmol of product produced per min in the presence of Fis, compared with 0.4 fmol/min in the absence of Fis. This rate of recombination is still only half of that seen in the presence of optimal levels of Int (3.0 fmol/min). Likewise, in reactions in which linear *attP* and supercoiled *attB* were used, the initial rate of recombination was again stimulated nearly fourfold by the presence of Fis (1.2 fmol/min, compared with 0.3 fmol/min in the absence of Fis). These data suggest that the effect of Fis is on a relatively early step in the pathway, as the stimulation is observed not just in the total amount of product formed but also in the early rate of recombination.

DISCUSSION

When examining the role of Fis in lambda site-specific recombination, it is important to consider that effects observed in vitro under optimal conditions may be highly artificial for a system whose main function is to carry out a single recombination event to introduce or excise one molecule of phage DNA, not to catalyze recombination of large amounts of highly supercoiled plasmid substrates. The in vivo data strongly suggested an essential role for Fis in both integrative recombination and excisive recombination of phage lambda, as demonstrated by the dramatic 50-fold drop in phage lysis rates and the 15-fold drop in lysogenization frequency in the absence of Fis. While the role of Fis in excision is mirrored in results seen with in vitro experiments, explanations for the role of Fis in lysogeny have been considerably more elusive.

The results of this study identified a likely source of the stimulation observed in vivo during integrative recombination. A two- to threefold effect, similar in magnitude to the effect seen in vivo by Ball and Johnson, is clearly observed in vitro when *attP* substrates are not supercoiled. It has long been known that, in vitro, supercoiling energy appears to be essential for proper establishment of the protein-DNA structures known as intasomes which must be formed prior to the onset of recombination. This argument has been used to explain the much lower recombination efficiency observed with nonsupercoiled *attP* substrates in vitro. However, it has been demonstrated that DNA inside the cell is not supercoiled to the high levels of superhelicity seen in isolated plasmid DNA. Ball and Johnson first proposed the notion that Fis may be used in the cell to enhance integration under conditions where such high superhelicity is not present (3). Given the fact that many nucleoid-associated proteins appear to be involved in DNA compaction of the nucleoid, it is reasonable to assume that the ability of Fis to bind and bend DNA may well mimic the compaction of DNA by supercoiling, and such an event may allow proper intasome formation even in the absence of high superhelicity.

A similar explanation may account for the stimulation by Fis of the standard topology reaction at suboptimal Int concentrations. In the cell, where Int levels are presumed to be much lower than the concentrations used in reactions in vitro, Fis may be necessary for any recombination reaction to proceed. It is plausible that Fis binding to the DNA promotes the formation of a structure which allows for tighter binding of Int to its binding sites, thus reducing the levels of Int necessary for the intasome to form. A similar argument has been made for another small DNA binding protein which stimulates lambda recombination, the ribosomal protein S20. Like Fis, S20 is a potent stimulator of integration when levels of Int are low (Flynn, Esposito, and Gerard, unpublished). Though S20 is not a specific DNA binding protein, it is capable of strong nonspecific binding which may create a general compaction of the DNA, which allows tighter Int binding. This could also explain the role of Fis binding; it is notable that the optimal Fis concentrations in these reactions were quite high (500 nM), given the strong specific binding of Fis to its sites (K_d = 5 nM). It may be that Fis also coated the DNA substrates and affected the compaction of the sites, assisting in formation of the intasomes. However, the mutation data clearly indicate that the specific Fis binding site must be present in order for any of its stimulatory effects to occur. One possibility is that Fis must bind to that site first, prior to additional Fis binding along the neighboring DNA; however, given the strong nonspecific binding of Fis to DNA, this seems less likely to be the case. A better understanding of where Fis interacts with the intasome DNA and what effect that has on other protein binding may help to elucidate this mechanism.

Finally, the fact that Fis stimulation is manifest in the early rate of recombination argues for a possible role for Fis in

intasome formation, which must occur early in the reaction prior to any catalytic steps. In the in vitro experiments using linear *attP*, intasome formation is presumably the rate-limiting step, as proper protein-DNA complex formation is likely to be dependent in part on the energy of supercoiling derived from the *attP* substrate. In this case, the action of Fis on the linear *attP* apparently assists protein binding enough to overcome the lack of superhelicity. A better understanding of the role of Fis in these early steps in recombination will require a more detailed analysis of the kinetics of the reaction, in both the presence and absence of Fis, and will also require a more detailed look at the very early events of the reaction which lead to the formation of the active intasome complexes.

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