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Location, Degree, and Direction of DNA Bending Associated with the Hin Recombinational Enhancer Sequence and Fis-Enhancer Complex

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The Fis protein of *Escherichia coli* and *Salmonella typhimurium* stimulates several site-specific DNA recombination reactions, as well as transcription of a number of genes. Fis binds to a 15-bp core recognition sequence and induces DNA bending. Mutations in Fis which alter its ability to bend DNA have been shown to reduce the stimulatory activity of Fis in both site-specific recombination and transcription systems. To examine the role of DNA bending in the activity of the Fis-recombinational enhancer complex in Hin-mediated site-specific DNA inversion, we have determined the locations, degrees, and directions of DNA bends associated with the recombinational enhancer and the Fis-enhancer complex. Circular-permutation assays demonstrated that a sequence-directed DNA bend is associated with the Fis binding sites in the proximal and distal domains of the recombinational enhancer. Binding of Fis to its core recognition sequence significantly increases the degree of DNA bending associated with the proximal and distal domains. The degree of DNA bending induced by Fis binding depended on the DNA sequences flanking the core Fis binding site, with angles ranging from 42 to 69°. Phasing analyses indicate that both the sequence-directed and the Fis-induced DNA bends associated with the proximal and distal domains face the minor groove of the DNA helix at the center of the Fis binding site. The positions and directions of DNA bends associated with the Fis-recombinational complex support a direct role for Fis-induced DNA bending in assembly of the active invertasome.

The basic molecular processes involved in many cellular activities including transcription, DNA replication, and DNA recombination require the formation of complex nucleoprotein structures. DNA bending often plays a key role in the formation of these nucleoprotein structures, as well as in the specific interaction of proteins with their DNA sites. The Fis (factor for inversion stimulation) protein of *Escherichia coli* and *Salmonella typhimurium* appears to utilize DNA bending in order to interact with its DNA binding site and to stimulate processes involving the formation of nucleoprotein complexes (9, 23, 26, 35). Fis was originally described as an accessory factor in the site-specific DNA inversion systems of *S. typhimurium* (Hin system; Fig. 1A) and bacteriophage Mu (Gin system), in which it dramatically stimulates recombination upon binding the recombinational enhancer sequence (for a review, see reference 8). The Fis protein binds as a homodimer to two sites of dissimilar sequence, called the proximal and distal domains, within the enhancer sequence (Fig. 1B). Mutational analysis of the Hin enhancer sequence and comparison of Fis binding sites in homologous DNA inversion systems and in transcription regulatory sites indicate that Fis has a degenerate 15-bp consensus core DNA binding sequence, GNNC/TA/GNNT/ANNT/CG/ANNC, where N is any base (5, 14). Based on the X-ray crystal structure of Fis, DNA bending may be required for the recognition helices of the Fis dimer to interact with its DNA sites (23, 35). Methylation interference assays showed that the Fis dimer must contact bases in two adjacent major grooves (3); the spacing between these contacts is approximately 34 Å on B-form DNA, while the recognition helices of the dimer are only 25 Å apart, indicating the need for flexibility either in the protein or the DNA binding site. Gel electrophoresis and chemical nuclease footprinting assays for protein-induced DNA bending indicate that Fis induces DNA bending upon binding DNA (e.g., see references 1, 6, 9, 28, and 33).

While it is apparent that DNA bending is associated with the Fis-enhancer complex, it is not known what role DNA bending at the recombinational enhancer plays in formation of the nucleoprotein synaptic structure (invertasome). It has been previously shown that the spatial arrangement of the Fis binding sites is important for directing the assembly of an active invertasome on supercoiled DNA (17) and that the functional recombinational enhancer has the Fis binding sites on different faces of the DNA helix (3) (Fig. 1). The importance of the positioning of the Fis binding sites within the recombinational enhancer suggests that the role of DNA bending associated with the Fis-enhancer complex would depend on the location, degree, and direction of sequence-directed and Fis-induced DNA bends. To understand the role of DNA bending in the activity of the Fis-enhancer complex in the site-specific recombination system of *S. typhimurium*, we have defined the DNA structure of the Fis-recombinational enhancer complex using circular permutation and phasing analyses.

MATERIALS AND METHODS

Fis protein. The Fis protein used in the circular permutation analyses was prepared as described by Johnson et al. (16). The Fis protein used in degree of DNA bending and phasing assays was a generous gift from Reid Johnson.

Plasmid constructions. Table 1 gives the 5′-to-3′ top-strand sequences corresponding to the oligonucleotides used in the construction of the plasmids listed therein. For plasmids pAG423, pAG424, and pAG542, single-stranded oligonucleotides were cloned into the *Bgl*II and *Sac*I sites of pCY4 as described by Prentki et al. (30). With the same cloning sites of pCY4, pAG437 was generated with annealed complementary oligonucleotides (Table 1) which had 3′- and

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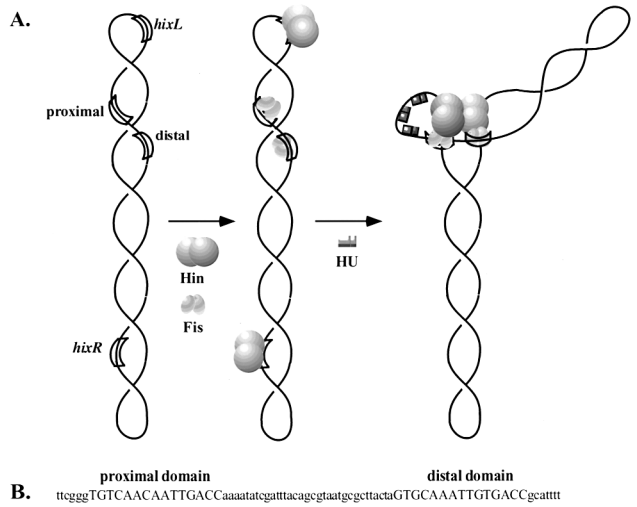

FIG. 1. Assembly of the invertasome for the Hin site-specific DNA inversion system. (A) The recombinase Hin binds as a dimer to the recombination sites *hixL* and *hixR* (7), and Fis binds independently as a dimer to the proximal and distal domains of the recombinational enhancer (3). The Fis-enhancer complex directs the assembly of a looped synaptic complex, trapping two interdomainal negative nodes (12, 13). HU binding facilitates the looping of the DNA when the distance between the recombinational enhancer and a recombination sites is less than approximately 100 bp (11). Once the invertasome is assembled, the Hin dimers are competent for carrying out DNA strand cleavage and exchange to specifically result in inversion of the DNA segment between the recombination sites (10). (B) Uppercase letters, sequence of the recombinational enhancer, with the 15-bp core Fis binding sites within the proximal and distal domains; lowercase letters, the upstream, downstream, and intervening sequences.

5′-protruding ends corresponding to the *Sac*I- and *Bgl*II-cleaved restriction sites. pAG426, pAG430, and pAG436 were created by ligating blunt-end double-stranded oligonucleotides corresponding to the indicated enhancer sequences (Table 1) into the *Sma*I cloning site of the polylinker in pCY4; pAG530, pAG531, and pAG533 were generated with double-stranded oligonucleotides (Table 1) with 5′-protruding ends complementary to the 5′ single-stranded ends of the vector after cleavage by *Hin*dIII in the multicloning site of pSL6 (9). The ligations were performed as described by Sambrook et al. (32).

The plasmids for phasing of the wild-type distal Fis binding sequence with the four-A-tract kinetoplast sequence (KIN4) were constructed as follows. Oligonucleotides corresponding to kinetoplast sequence containing four A tracts (34) (5′-AATTCTGCGCAAAAATGTCAAAAAATAGGCAAAAAATGCCAAAAATGGAATTCCGGCTAGAGTCGACCCA-3′ and 5′-AGCTTGGGTCGACTCTAGCCGGAATTCCATTTTTGGCATTTTTGCCTATTTTTTGACATTTTGCGCAG-3′) were annealed and ligated with pSL6, which had been digested with *Eco*RI and *Hin*dIII. The resulting plasmid, pAG532, was cut with *Hin*dIII, and the annealed oligonucleotides 5′-AGCTTACTAGTGCAAATTGTGACCGCATTTTT-3′ and 5′-AGCTAAAAATGCGGTCACAATTTGCACTAGTA-3′ were ligated with the cleaved vector, creating pAG534. pAG535, pAG536, and pAG537 were created similarly by using oligonucleotides with the same sequences as those used for pAG534, with the exceptions that 1 (T), 3 (GTT), and 5 (GGCTT) additional bp, respectively, were added to the sequence immediately adjacent to the 5′-*Hin*dIII sequence (AGCTT). Cleavage of pAG535 and pAG536 with *Hin*dIII, fill-in of the 4-bp overhangs with Klenow fragment (NEB) and all four deoxynucleotide triphosphates (dNTPs; Pharmacia), and religation of the plasmid by the methods described by Sambrook et al. (32) resulted in pAG538 and pAG539, respectively.

The plasmids for assaying the direction of DNA bending for a symmetric distal Fis binding sequence relative to three-phase A tracts were made as follows. Oligonucleotides encoding the symmetric distal Fis binding sequence (5′-AGCTTACTAGTGCAAATTGTGACCTAGTAAG-3′ and 5′-AGCTCTTACTAGGTCACAATTTGCACTAGTA-3′) were annealed and ligated into the *Hin*dIII site of pAG532 to give pAG541. The four-A-tract kinetoplast sequence was deleted from pAG541 by cutting with *Eco*RI and *Hin*dIII, and the annealed oligonucleotides encoding the three-phase A tracts (SYN3) (5′-AATTCGGTACCAAAAAAACGGCAAAAAAACGGGCAAAAAAACGA-3′ and 5′-AGCTTCGTTTTTTGCCCGTTTTTTGCCGTTTTTTGGTACCG-3′) were ligated into the *Eco*RI and *Hin*dIII sites to generate pAG545. The spacing between this A-tract sequence and the symmetric distal Fis binding sites was then varied by cleaving at the *Hin*dIII site followed by full or partial fill-in of the 3′-recessed ends with the Klenow fragment and all dNTPs or dATP, dGTP, and dCTP or dATP, dGTP, or dATP alone. The partial fill-in reactions were followed by digestion with mung bean nuclease (NEB) to remove the single-stranded ends, and all of the fill-in reaction mixtures were then ligated under the reaction conditions suggested by the manufacturer (NEB) for blunt-end ligation. The *Hin*dIII-cut pAG545 was also treated with mung bean nuclease and was blunt-end ligated. The resulting plasmids from the treatment with Klenow and/or mung bean nuclease were pAG546 (full fill-in; +4 bp), pAG548 (+2 bp), pAG551 (−3 bp), pAG547 (−4 bp), pAG549 (−5 bp), pAG550 (−6 bp), and pAG552 (−8 bp).

All plasmid DNAs were prepared from a *recA* strain, DH5α, by using the alkaline lysis and cesium chloride gradient protocols described by Sambrook et al. (32), and the expected sequence was confirmed by Sanger sequencing with the Sequenase kit (Pharmacia).

Circular-permutation analysis. DNA fragments with the same molecular weights containing the enhancer sequence circularly permuted in its position on the fragments were generated from the plasmids listed in Table 1. The restriction sites utilized for the pCY4-derivative plasmids included *Eco*RI, *Cla*I, *Bst*I, *Eco*RV, *Nhe*I, and *Bam*HI; for pSL6 derivatives, the restriction sites used were *Mlu*I, *Nhe*I, *Xho*I, *Eco*RV, *Pvu*II, *Ssp*I, and *Bam*HI. DNA fragments from the pCY4-derivative plasmids were end labeled with T4 polynucleotide kinase (NEB) and [γ-^{32}P]dATP (New England Nuclear) by the protocols described by

TABLE 1. Sequences of oligonucleotides used for construction of plasmids

Domain and vector	Plasmid	Enhancer sequence[a]	Angle (°)[b]
Proximal domain			
pSL6	pAG530	caAGCTTCGGGTGTCAACAATTGACCagcttgggtc	ND
pSL6	pAG533	caAGCTTCGGGTGTCAACAATTGACCAAAATATagc	ND
pCY4	pAG430	gaccCCTCGAGTCAACAATTGACCgggagctcgg	42
pCY4	pAG426	gaccCCTCGAGTCAACAATTGACCAAAATATCGA	55
pCY4	pAG542	GATCTCGAGATTCGGGTGTCAACAATTGACCAAAATATGAGCT	58
pCY4	pAG437	tcccgagctcGTCAACAATTGACCAAAATATCGATTTA CAGCGTAATGCGCTTACTAGTCCCGGGTCTA	44
Intervening sequence			
pCY4	pAG436	atctagacccATCGATTTACAGCGTAATGCGCTTACTAGTGggagctcgg	−
Distal domain			
pSL6	pAG531	aAGCTTACTAGTGCAAATTGTGACCGCATTTTagc	ND
pCY4	pAG423	aGATCTACTAGTGCAAATTGTGACCAAGCTTGAGCT	48
pCY4	pAG424	CGATCTACTAGTGCAAATTGTGACCGCATTTTTAAGCTTGAGCT	69

[a] 5′-to-3′ sequence of the oligonucleotides inserted into the indicated circular permutation vector is given in uppercase letters. The enhancer sequence is shown in large uppercase letters, and the core Fis binding sequence is underlined. At least 10 bp 5′ and 3′ to the core Fis binding site is given, including vector sequence in lowercase letters.

[b] Degree of Fis-induced DNA bending associated with the indicated enhancer sequence. The degree of bending for the Fis-enhancer complex was not determined for the pSL6-derived plasmids (ND), and Fis does not bind the isolated intervening sequence (−).

Sambrook et al. (32). DNA fragments from pSL6 derivatives were not radiolabeled. The binding of Fis to DNA fragments was performed as described by Bruist et al. (3). Electrophoresis of the Fis-bound or -unbound DNA fragments was performed at 4°C in 1× Tris-borate-EDTA buffer (32) on 8% nondenaturing polyacrylamide vertical gels (0.1 by 24.5 cm; 30:1 acrylamide-bisacrylamide) which were prerun at 200 V for 1 h and then run at 200 V after loading for approximately 18 h. DNA standards (pBR322 DNA-*Hin*fI digest kinased with [γ-^{32}P]dATP, ϕX174 DNA-*Hae*III digest, NEB, or pGEM DNA markers; Promega) were also run on each gel. Gels in which the DNA fragments had been radiolabeled were exposed to Kodak XAR film, and the gels containing unlabeled DNA were stained with ethidium bromide and photographed on a UV light box. The relative mobilities of the unbound DNA fragments and the Fis-enhancer complexes were calculated as the ratio of the observed gel mobility in millimeters (μ_{obs}) versus the maximum mobility (μ_{max} [theoretical mobility of unbent linear fragment extrapolated from a plot of the DNA standard fragment mobilities versus their molecular weights]). The μ_{obs}/μ_{max} values versus the distance of the center of the Fis core binding sequence from one end of the DNA fragment were plotted. The mobilities were also fit to a circular-permutation function described by Kerpola and Curran (19), which was derived from the relationship of the relative mobility of the circular-permutation fragments to the degree of DNA bending described by Thompson and Landy (33) as follows: $\mu_M/\mu_E = \cos \alpha/2$, where μ_M is the mobility of the fragment with the DNA bend located in the middle, μ_E is the mobility of the DNA fragment with the DNA bend at the end of the fragment, and α is the degree of DNA bending. Extrapolations of both the simple plot and the plot of the fitted data gave the same position for the center of the DNA bend for each fragment examined.

Empirical determination of DNA bend angles. The degree of DNA bending for the enhancer sequences was determined essentially as described by Thompson and Landy (33). DNA fragments with enhancer sequences at the end of the fragment were generated from the pCY4-derivative plasmids by digestion with *Eco*RI; digestion with *Eco*RV gave fragments with enhancer sequences in the middle. *Nhe*I and *Bam*HI separate digests of pJT170-2, pJT170-3, pJT170-4, pJT170-5, pJT170-6, and pJT170-7 (which were a gift from A. Landy) gave DNA fragments with the A-tract positions at the middle and the end, respectively. These digests were mixed and electrophoresed on 8% polyacrylamide gels beside the enhancer-containing fragments, which were bound by Fis. Fis-DNA binding, electrophoresis, and visualization were performed as described above for the circular-permutation analysis. The relative mobilities of the middle versus end DNA fragments were calculated from the distances that the DNA fragments ran on the vertical gel (μ_M/μ_E). The DNA bending angles for the enhancer sequences and Fis-enhancer complexes were extrapolated from a plot of the degrees of DNA bending versus the relative mobilities for the A-tract standard fragments. Determination of DNA bending angle by this empirical method required using DNA fragments with a linear length close to that of the A-tract standards, since the alteration in gel electrophoretic mobility of a DNA fragment with a DNA bend in the middle of the fragment versus one at the end is proportional to the change in the fragment end-to-end distance. The pCY4-derived DNA fragments (375 to 435 bp) were in the same size range as the A-tract standard fragments generated from the pJT170 plasmids (352 to 422 bp).

DNA bend direction phasing analysis. The plasmids in which the wild-type distal domain Fis binding site was phased with four A tracts (pAG534, pAG535, pAG536, pAG537, pAG538, and pAG539) and the symmetric distal Fis binding site was phased with three A-tracts (pAG546, pAG548, pAG545, pAG551, pAG547, pAG549, pAG550, and pAG552) were digested with *Eco*RV and then bound by Fis, electrophoresed, and visualized as described for the circular-permutation assays. The mobilities of the Fis-enhancer complexes from the phasing constructs were fitted to the phasing function described by Kerpola and Curran (19), and the distances from the center of the Fis binding site to the center of the A-tract bend were plotted versus the relative mobilities, which were defined as the ratios of the gel mobilities of the complexes (μ) versus the average mobilities of the phased complexes (μ_{avg}).

RESULTS

Location of DNA bending in the Hin recombinational enhancer. To determine the location of sequence-directed and Fis-induced DNA bending within the enhancer complex, we used circular-permutation assays (34). The basis of these assays is that the electrophoretic mobility of a DNA fragment in a polyacrylamide gel is directly related to the fragment end-to-end distance; therefore, when a DNA bend is located near the center of a DNA fragment, it has an altered mobility relative to the fragment of the same size with the DNA bend near the terminus (25). The position of a DNA bend within an enhancer sequence can be determined from the gel electrophoretic mobilities of DNA fragments in which the enhancer sequence is circularly permuted in its position on the DNA fragments. Recombinational enhancer DNA sequences (Table 1) were inserted into the circular-permutation vector pCY4 (30). DNA fragments with the enhancer sequences circularly permuted in location were generated with restriction endonucleases with cleavage sites in directly repeated sequences flanking the cloning site of the vector (Fig. 2A). These DNA fragments were electrophoresed on nondenaturing polyacrylamide gels along with molecular weight markers (Fig. 2B), and the relative mobility of each fragment was determined (see Materials and Methods). Extrapolation from plots of the position of the enhancer sequence on the DNA fragments versus relative mobilities (Fig. 2B) indicated that the Fis-induced DNA bend was centered in the core Fis binding site in both the proximal and distal domains. Additionally, a sequence-directed bend was associated with the distal domain when the wild-type enhancer sequence containing a T-tract 3′ of the core binding site was included. The proximal domain also exhibited slight sequence-directed DNA bending when the AT-rich wild-type enhancer sequence 3′ of the core binding site was present. No DNA bending associated with the isolated intervening sequence between the proximal and distal domains which contained no Fis recognition sequence was observed. However, Fis was found to bind a sequence in the duplicated region of the pCY4 vector near the *Eco*RV site with an affinity slightly lower than that for the proximal domain; this is the source of additional complexes observed in our bending assays with the pCY4 vector (Fig. 2 and 3). Since this Fis binding site in the vector DNA may have affected the bending assays for the enhancer sequences, we inserted the distal and proximal domain binding sites with the wild-type flanking sequences (pAG531 and pAG533, respectively) and the proximal domain core Fis binding sequence (pAG530) into a different circular permutation vector, pSL6, which differs from pCY4 in the directly repeated DNA sequence flanking the cloning site (9). No Fis binding was observed for pSL6 vector DNA fragments at the Fis concentrations used for all our bending assays (data not shown). The results of circular permutation assays with pAG530, pAG531, and pAG533 confirmed that Fis induces DNA bending at the core Fis binding sequence at the proximal and distal domains (data not shown).

Degree of DNA bending associated with Fis binding sites. The degrees of bending associated with each enhancer domain core sequence and the various flanking sequences (Table 1) were examined by the empirical method described by Thompson and Landy (33). The gel mobility of Fis-DNA complexes in which the Fis-binding site was at the end of the fragment or in the middle of the fragment was determined on 8% acrylamide gels, along with bending standards which contained helically phased A tracts (A_6), ranging from two to seven A tracts, at the end and middle of the DNA fragment (Fig. 3). Each A tract was associated with a DNA bend of approximately 18° facing the minor groove of the DNA helix at the center of the A_6 sequence (22). The predicted bending angles of the phased A-tract standards were plotted versus the relative mobilities (μ_M/μ_E) of the bending standard fragments with the A tracts positioned at the middle and at the end of the fragment. The estimated bending angle for each of the enhancer sequences assayed was interpolated from the standard curve. The angles calculated from the protein-DNA complex mobilities for all of the enhancer sequences analyzed are given in Table 1. Comparison of the DNA bending angles associated with the Fis proximal and distal domain sequences suggests that the degree of DNA bending induced by Fis binding depends on the DNA sequences flanking the core Fis binding site.

The relative mobilities of the unbound fragments for each of the enhancer sequences analyzed were less than that of the bending standard containing two A tracts which had a pre-

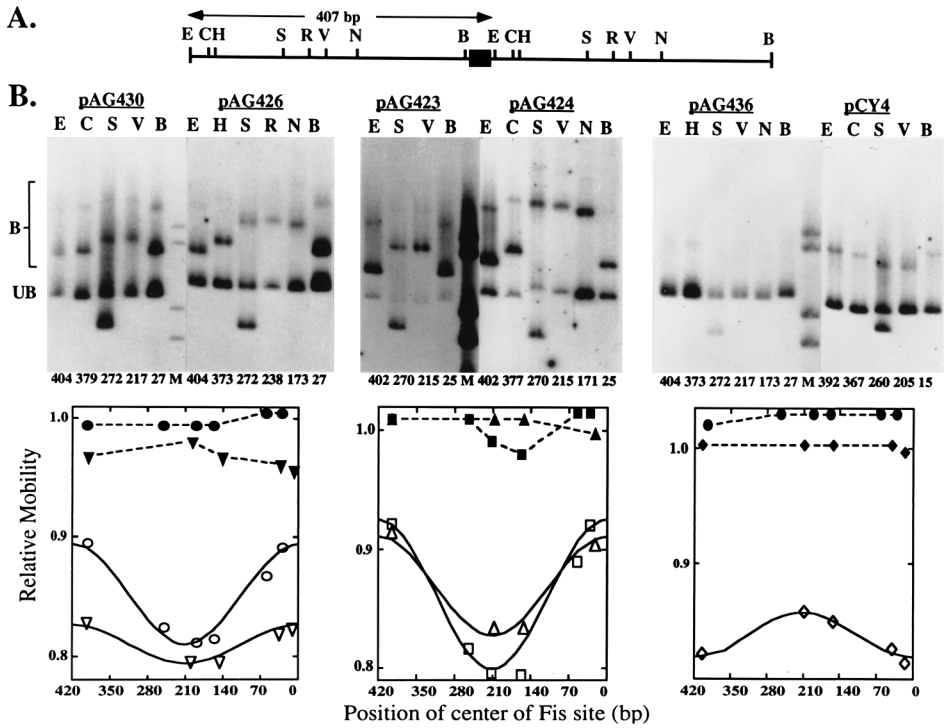

FIG. 2. Circular-permutation assays for proximal, distal, and intervening sequences of the Hin recombinational enhancer. (A) Generalized map of the duplicated region of the circular-permutation vector, pCY4, showing the polylinker region where the enhancer sequences were inserted (solid box). The indicated restriction endonuclease sites were used to generate the DNA fragments in which the enhancer sequence was circularly permuted in its position. The letter designations correspond to restriction endonucleases as follows: E, *Eco*RI; C, *Cla*I; H, *Hin*dIII; S, *Bst*NI; V, *Eco*RV; N, *Nhe*I; and B, *Bam*HI. (B) ^{32}P-end-labeled DNA fragments from pAG430 (proximal core sequence), pAG426 (proximal core with 3′ A · T-rich enhancer sequence), pAG423 (distal core sequence), pAG424 (distal core with 3′ enhancer sequence containing a T tract), pAG436 (intervening sequence), and pCY4 (parent circular-permutation vector) were incubated with purified Fis protein and electrophoresed on 8% polyacrylamide gels along with a DNA molecular weight standard (pBR322 *Hin*fI-digest [lane M]). Above each lane, the restriction endonucleases used to generate the DNA fragments for the Fis binding assay are indicated; below each lane, the positions (in base pairs) relative to one end of the fragment for the center of the core Fis binding sequence (for pAG430, pAG426, pAG423, and pAG424), the center of the intervening sequence (for pAG436), or the center of the polylinker (for pCY4) are given. The approximate positions of the unbound DNA fragments (UB) and Fis-bound (B) fragments are indicated. The additional low-molecular-weight band (lanes S) resulted from *Bst*NI cleavage sites in the pCY4 vector which did not affect the circular-permutation fragment. The relative mobilities of each fragment when bound by Fis (open symbols) and unbound (solid symbols) for pAG430 (▽ and ▼), pAG426 (○ and ●), pAG423 (△ and ▲), pAG424 (□ and ■), pAG436 (●), and pCY4 (◇ and ◆) are shown as a function of the position of the center of the enhancer sequence relative to the end of the fragment. The curves are a fit to a cosine function of the data from three independent circular-permutation assays for each sequence (see Materials and Methods).

dicted bending angle of 36°. DNA bending angles corresponding to this region of the standard plot cannot be predicted with any accuracy; therefore, sequence-directed DNA bends associated with the enhancer sequences could not be quantitated.

Determination of direction of sequence-directed and Fis-induced bending. To further probe the structure of the Fis-enhancer complex, we determined the directions of the bends along the DNA helix by using a phasing analysis as first described by Zinkel and Crothers (36) and Salvo and Grindley (31). In the phasing assay, changing the relative orientation of two bends on a DNA fragment shifts its gel electrophoretic mobility, i.e., fragments with the bends facing opposite directions have a faster mobility than fragments with the bends facing the same direction. To determine the direction of sequence-directed and Fis-induced bends at one of the Fis binding sites in the enhancer sequence, the distal domain sequence was phased with two characterized bent DNA sequences, i.e., the kinetoplast DNA sequence containing four phased A_{5-6} tracts (KIN4) (36) (Fig. 4A) and a synthetic sequence of three phased A_{5-6} tracts (SYN3) (31) (Fig. 4B). DNA containing the phased poly(A_{5-6}) tracts is known to bend toward the minor groove at the center of each A tract (21), and, therefore, the center of the overall bend associated with KIN4 is toward the major groove and the center of the bend for SYN3 is toward the minor groove. The distal sequences used were the wild-type distal Fis binding site (including 7 bp of 5′ and 3′ flanking sequence) and a symmetric distal site in which the 5′ 7 bp flanking sequence had been inversely repeated 3′ of the core site. The spacings between the A-tract sites and the Fis binding sites were varied by specific insertions or deletions (see Materials and Methods), such that the Fis binding site was rotated along the DNA helix relative to the center of the A-tract bend. When the direction of the Fis-induced bend was the same as the A-tract bend, a spacer length that separated the Fis core binding sequence from the A-tract sequence by an even number of DNA helical turns would position the Fis-induced bend in phase (*cis* isomer) with the A-tract bend, giving the shortest end-to-end distance and minimum gel electrophoretic mobility for the DNA fragment. A spacer length that gave a nonintegral number of helical turns between the center of the Fis binding site and the center of the A-tract sequence would move the DNA bends to more opposing positions (*trans* isomer), thus increasing the end-to-end distance and the gel mobility of the DNA fragment (Fig. 4B). DNA fragments of nearly equivalent lengths containing the wild-type Fis-enhancer complex helically phased with KIN4 and the symmetric distal site helically phased with SYN3 were electrophoresed on nondenaturing polyacrylamide gels; the relative electrophoretic mobilities of

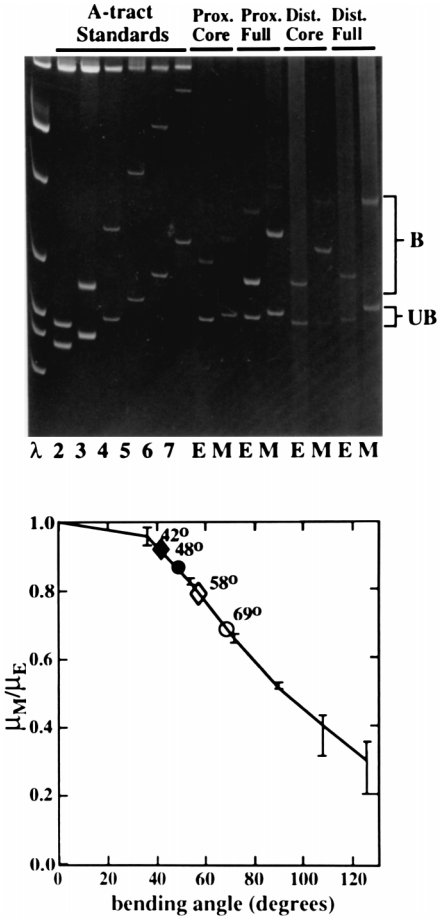

FIG. 3. Empirical determination of the degree of DNA bending associated with Fis-enhancer complexes. *Eco*RI and *Eco*RV digests (generating DNA fragments with the enhancer sequence at the end [E] and middle [M], respectively) of pAG430 (Prox. Core), pAG542 (Prox. Full), pAG423 (Dist. Core), and pAG424 (Dist. Full) were incubated with Fis to give approximately 50% Fis-complexed DNA fragments. The bound (B)/unbound (UB) DNA fragments were electrophoresed on an 8% nondenaturing polyacrylamide gel, along with DNA bending standards containing two to seven phased A tracts positioned at the middles and termini of the DNA fragments (lanes 2 through 7) and λ DNA *Hin*dIII digest (λ). The relative gel mobilities (μ_M/μ_E) of the A-tract DNA bending standards are shown as a function of the predicted bending angles (data from six independent experiments were used to generate the plot and error bars). Based on the relative mobilities of the bound DNA fragments for pAG430 (◆), pAG542 (◇), pAG423 (●), and pAG424 (○), the interpolated degrees of DNA bending are indicated on the standard plot for the enhancer sequences analyzed in the assay shown here.

the fragments were determined and plotted as a function of the spacer length (Fig. 4A and B, respectively). The results in Fig. 4A show that the maximum gel mobility for the fragments containing the wild-type distal site was seen when the center of the Fis binding site was approximately six full turns of the DNA helix from the center of the KIN4 sequence (62- and 64-bp spacer lengths), indicating that Fis induces a bend in the direction opposite to that of the four-A-tract DNA bend, i.e., toward the minor groove at the center of the binding site. In Fig. 4B, the fragments containing the symmetric Fis binding site exhibited minimum gel mobility when the center of the Fis site was close to three helical turns from the center of the SYN3 bend (31-bp spacer length). This result demonstrates that the Fis-induced bend was in the same direction as that of the three-A-tract bend, again indicating that the bend was toward the minor groove. It can be noted that the unbound DNAs in these assays have shifts in mobility similar to those of the bound DNAs and, therefore, that the DNA curvature associated with the distal sites used also was directed toward the minor groove at the center of the core binding sequence. The overall direction of the sequence-directed bend associated with the proximal domain is predicted also to be toward the minor groove, based on the relative mobilities of constructs in which the proximal and distal domains are helically phased in their relative positions on the DNA helix (reference 17 and data not shown).

The bend induced by Fis binding to the proximal domain is expected to have the same directionality as that of the Fis-distal domain complex. The Fis-DNA complex has essentially the same structure at the proximal and distal core sites, based on the results of methylation interference and DNase I protection studies (3). In addition, flipping the orientation of the core Fis binding sequence relative to that of the kinetoplast A-tract sequence gave the same results in phasing analysis as seen for the phasing constructs with the distal domain sequences in the same 5′-to-3′ orientation as that of the A tracts (data not shown); this result is consistent with the dyad symmetry of the Fis-DNA complex (18, 23, 35).

DISCUSSION

Fis plays a stimulatory role in many processes in the bacterial cell, including transcription, DNA replication, and bacteriophage λ Int-mediated site-specific recombination, as well as in site-specific DNA inversion. Fis-induced DNA bending is involved in the activity of Fis in all of these systems (2, 4, 6, 26, 27). There are two roles that DNA bending appears to have in the functioning of Fis. First, Fis interactions with its DNA binding sites are probably facilitated by DNA bending. The crystal structure of Fis indicates that the helix-turn-helix binding domains of the Fis dimer are spaced too closely to make the appropriate interactions with the adjacent major grooves of its recognition site (23, 35). Molecular modeling of Fis-DNA interactions based on nuclease protection and chemical interference data predicted an overall DNA bend of approximately 60° centered in the median minor groove of the core Fis binding site (35). We have shown by circular-permutation and phasing analyses that Fis does indeed induce a DNA bend, averaging approximately 56°, which is directed toward the central minor groove of its core recognition sequence. Our results indicate that the degrees of DNA bending induced at the proximal and distal Fis binding sites of the recombinational enhancer varied with the DNA sequences of the flanking regions. Analyses of Fis binding to specific sites in several systems and to nonspecific sites have shown that Fis induces DNA bends of various degrees upon interacting with DNA (1, 9, 28, 33). Pan et al. (29) have recently systematically analyzed the contributions of the core binding sequence and the flanking DNA sequences to the degree of DNA bending induced upon Fis binding. They found that DNA bending at the core sequences appears similar at different DNA binding sites and that the flanking sequences determine any variance in induced DNA bending.

Second, Fis-induced DNA bending may play a more-direct role in Fis-dependent stimulation of site-specific recombination or transcription. Bending of the DNA at the proximal and distal domains of the recombinational enhancer may be required to give the proper spatial relationship of the Fis molecules to direct the topology of supercoiled DNA domains in the synaptic intermediate structure (Fig. 1). It has been demonstrated that sequence-directed bends associated with kinetoplast DNA can determine the topology of branched super-

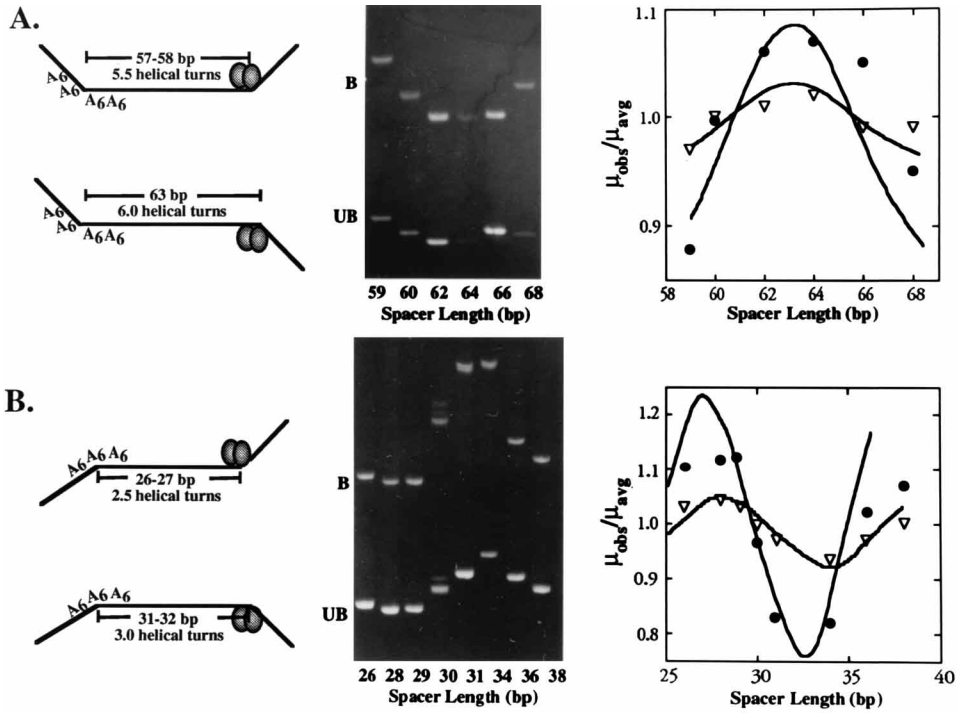

FIG. 4. Helical phasing analyses for determination of DNA bend direction associated with the distal domain of the recombinational enhancer. (A) Helical phasing of the Fis-bound wild-type distal site (Fis dimer shown as two ovals) relative to the KIN4 (four phased A tracts) sequence is schematically depicted for the spacer lengths which theoretically should place the center of the Fis binding site 5.5 and 6.0 helical turns from the center of KIN4. The overall curvature of the four-A-tract bend is toward the major groove; the Fis-induced DNA bend is depicted toward the minor groove at the center of the binding site, as determined in the phasing analyses. Phasing plasmids pAG534, pAG535, pAG536, pAG537, pAG538, and pAG539 were digested with *Eco*RV to yield DNA fragments containing the wild-type distal domain and the KIN4 sequence separated by 59, 60, 62, 64, 66, and 68 bp, respectively, as measured from the center of the core Fis binding sequence to the center of the four-A-tract sequence. These DNA fragments were bound with Fis and electrophoresed on an 8% nondenaturing polyacrylamide gel. The gel electrophoretic mobilities of the bound (B and ●) and unbound (UB and ▽) DNA fragments were used to calculate μ_{obs}/μ_{avg} values, which were then plotted as a function of spacer length; these data were used in a phasing function to generate the curves for each plot (see Materials and Methods). (B) Phasing analysis of the symmetric distal Fis binding site with the SYN3 sequence (three phased A tracts) as described for panel A. The direction of the SYN3 DNA bend is toward the minor groove at the center of the three-A-tract sequence, and, again, that of the Fis-induced bend is toward the minor groove at the center of the binding site. The spacer lengths that theoretically place these DNA bends in opposing directions and in phase are illustrated. Phasing plasmids pAG552, pAG550, pAG549, pAG547, pAG551, pAG545, pAG548, and pAG546 were digested with *Eco*RV to generate DNA fragments encoding the symmetric distal-domain Fis binding site and the SYN3 sequence spaced by 26, 28, 29, 30, 31, 34, 36, and 38 bp, respectively. These fragments were utilized as described for panel A.

coiled DNA (24). Fis-induced DNA bending at the core Fis binding sites within the proximal and distal domains could direct the formation of two branched domains on the supercoiled DNA substrate. DNA bending directed toward the bound Fis protein at the proximal and distal sites, which are spatially arranged on nearly opposite sides of the DNA helix, would set up negative nodes at each branch point (Fig. 1). This topological arrangement of the DNA strands in the invertasome facilitates the precise interactions between the Hin-bound recombination sites and the Fis-bound recombinational enhancer that result in DNA strand exchange and inversion of the DNA between the recombination sites (13, 18). Our results for Fis-induced DNA bending at the proximal and distal domains indicate that the Fis-enhancer complex has the appropriate structure to play this role in assembly of an active synaptic structure, i.e., the bends induced by Fis at the proximal and distal domains are directed toward the bound protein (toward the minor groove at the center of the core binding site) and no additional DNA bending is associated with the wild-type intervening DNA sequence. Since the centers of Fis-induced bends at the proximal and distal core sites are spaced approximately 4.6 turns of the DNA helix apart, these nearly opposing DNA bends would set up the two negative interdomainal nodes of the invertasome (Fig. 1). The small difference in the DNA bending angle associated with each site may be a fine adjustment to bring the Fis dimers into a more directly opposing position on the DNA helix, positioning the dimers for precise interactions with the Hin tetramer complex. Such a defined structure for the active Fis-enhancer complex predicts that minor alterations in the relative spatial arrangement of the Fis-bound proximal and distal domains could improve or impair enhancer function; this prediction is supported by mutational analyses of the intervening sequences of the recombinational enhancers from the Cin DNA inversion system (14) and from the Hin DNA inversion system (17). Specific single- or multiple-nucleotide changes in the sequence of the intervening region of an active enhancer resulted in increased or decreased enhancer function. For the Hin recombinational enhancer mutants, these changes in the intervening sequence created slight sequence-directed DNA bending in the intervening region that altered the phasing of the DNA bends associated with the proximal and distal domains (8a).

It is an attractive model that Fis-induced DNA bending within the recombinational enhancer spatially sets up the protein-protein interactions involved in the functioning of the Fis-enhancer complex in Hin-mediated DNA inversion. There is evidence from mutational analyses of Fis that both Fis-induced DNA bending and Fis-protein interactions are involved in Fis-mediated stimulation of site-specific DNA inversion and transcription. Osuna et al. (27) showed that amino

acid changes in Fis which affected DNA bending reduced recombinational enhancer activity by approximately 50-fold, as measured by the rate of DNA inversion. Mutations in the amino-terminal region of Fis, which did not affect DNA binding or bending, resulted in a >100-fold reduction in enhancer activity, suggesting an essential function for Fis-protein interactions in Hin-mediated DNA inversion. These results fit our model for the role of Fis-induced DNA bending at the recombinational enhancer sequence, since the structure of the Fis-enhancer complex would primarily facilitate the appropriate contacts between Fis and the Hin dimers, while the interaction between these proteins is probably required for DNA strand exchange to occur (10, 15, 20). In the Fis-stimulated transcription systems, Fis-RNA polymerase interactions are required for Fis-dependent activation of *rrnB* P1 transcription and a Fis mutant defective in DNA bending is less efficient in activating transcription (9). A role for Fis-induced DNA bending at the three Fis binding sites upstream of the *rrn* promoter may be to form a DNA loop with the Fis proteins facing the downstream RNA polymerase (2); evidence for such a nucleoprotein structure formed by RNA polymerase and Fis bound to three sites upstream of the *tyrT* promoter has been reported by Muskhelishvili et al. (26). These observations suggest that in different systems, Fis-induced DNA bending may set up the structure of specific nucleoprotein complexes in which Fis is then positioned to interact with other proteins to stimulate their function.

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