The recombinase of the Salmonella inversion system, Hin, mediates site-specific recombination between two 26 base pairs (bp) inverted repeat sequences (hixL and hixR) which flank a 993-bp DNA segment. We have investigated Hin recognition of, and association with, the hix recombination sites. Nuclease and chemical protection studies with linear and supercoiled DNA substrates demonstrate that Hin initially binds hixL and hixR independently of binding of the other protein components of the inversion system, Fis and HU. DNA-binding assays with mutant recombination sites and methylation interference experiments indicate that the critical bases for Hin recognition of its DNA-binding site are within an 8-bp sequence covering adjacent major and minor grooves of the DNA helix in each of the 12-bp half-sites of the hix recombination sites. The nature of the Hin-hix complexes in these binding studies and the results of gel filtration assays with purified Hin suggests that Hin binds the recombination sites as a dimer. The implications of the nature of the interactions of Hin with its recombination sites on the mechanism of the recombination reaction and on the novel features of DNA recognition by Hin are discussed.

Inversion of a 993-bp DNA segment controls the alternate expression of the flagellin genes of Salmonella typhimurium (see Fig. 1A; Ref 1). The recombinase, Hin, mediates site-specific recombination between two 26-bp inverted repeat sequences (hixL and hixR) resulting in inversion of the DNA segment between these two sequences (2). Hin has been purified to near homogeneity and has been shown to promote very low levels of recombination in vitro. Inversion of a wild-type DNA substrate is stimulated greater than 150-fold by a host factor called Fis and approximately 10-fold by a histone-like host protein, HU (3). The inversion reaction requires that the DNA substrate be supercoiled and contain a 60-bp sequence, the recombinational enhancer, in addition to the two recombination sites in inverted orientation (see Fig. 1A; Ref. 4).

Characterization of the inversion reaction has involved defining the essential sequences of the recombination sites and the recombinational enhancer, and determining the nature of Hin, Fis, and HU interactions with the DNA substrate. Comparison of the crossover regions of the Hin-related inversion systems, in which the recombinases Gin (bacteriophage Mu), Cin (bacteriophage P1), and Pin (e14 element of Escherichia coli) share 60 to 70% amino acid sequence identity with Hin, reveals a conserved 26-bp sequence exhibiting dyad symmetry at the recombination sites (5-7). This consensus sequence consists of two imperfect 12-bp inverted repeats separated by a 2-bp core (Fig. 1B). Nuclease and chemical protection studies with Hin, Gin, and Cin have shown that the recombinase binding site overlaps this 26-bp consensus sequence in the recombination sites (hix, gix, and cix) (8-12). Recent results demonstrate that Hin and Gin mediate strand cleavage at the 2-bp core of their respective recombination sites, creating a 2-bp 3' overhang (Hin) or nicks on either strand within the core (Gin) with the recombinase covalently attached to the 5' phosphoryl end (13).2

It has been demonstrated that the host factor Fis specifically interacts with the recombinational enhancer sequences of the Hin, Gin, and Cin inversion systems (10, 11, 14). Fis binds independently to two domains within the Hin recombinational enhancer sequence (14). The spatial orientation of these Fis-binding sites, which are separated by approximately 4.6 turns of the DNA helix, is critical for enhancer function (15). The properties of the Fis-enhancer interactions have been incorporated into a model for the role of the recombinational enhancer in Hin-mediated site-specific inversion (14-16). In this "looping-synapsis" model the Fis-bound enhancer functions as a "topological filter" directing Hin-bound recombination sites into the proper alignment and facilitating strand exchange.

In this paper we further define the structure of the Hin-hix complexes in order to gain an understanding of the interactions involved in the formation of a productive synaptic complex in the Hin inversion reaction. We present results of nuclease and chemical protection/interference experiments and gel electrophoresis-DNA binding assays which were used to investigate Hin recognition of, and association with, the hix recombination sites.

MATERIALS AND METHODS

Plasmid Construction—Plasmids pMS551, pMS570, pMS674, pMS612, and pMS614 have been described elsewhere (4). pMS649 (a gift from R. Johnson, UCLA School of Medicine) was derived from pMS614 by cleavage at the Ncol site (created by the A → T transversion at the 2-bp core sequence of hixL), filling in with T4 DNA polymerase and dNTPs, and blunt end ligation with T4 DNA ligase (Bethesda Research Laboratory); sequence analysis (17) revealed that 1 bp was lost, resulting in a 3-bp insertion at the center of hid. Plasmids pAG410 and pAG415 were constructed as follows. pBR322 was cleaved with Saff, the protuding ends filled in with the large fragment of E. coli DNA polymerase I (Klenow; Bethesda Research Laboratory) and dNTPs, and ligated in the presence of a 500-fold

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3 The abbreviations used are: bp, base pairs; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-2-piperazineethanesulfonic acid.

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DNA-binding Properties of the Hin Recombinase

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10072
molar excess of the oligonucleotides TTTCTCTCTTTAGAA (hixL; pAG410) or TTGGTTTTCAAGAA (nonconsensus half-site of hixL; pAG414) (hybridized with the complementary strand). Plasmids pAG400 and pAG403 resulted from cloning of the double-stranded oligonucleotide TCAGCTTTCTCTTTTGAAGCCTCG, and TCAGCTTTCTTTAGATAAGCCTCG, respectively, into the SalI site of pBR322. These oligonucleotides, which correspond to the hixR nonconsensus half-site and 10-mer half-site sequence, respectively, were synthesized with SalI protruding ends to facilitate cloning. The sequence of the pAG plasmids was confirmed by chemical cleavage methods (18a, 19).

Plasmids pMFB27 and pMFB36 were constructed as follows. The hixR-containing TaqI DNA fragment from the wild-type inversion region (base pairs +940 to +997 from the center of hin) was ligated into the Clal site of pBR322 creating pMFB16. The hixR-containing HindIII/HincII fragment of pMFB16 was then ligated with pMS551, which had been cleaved with HindIII and PvuII, giving pMFB25. The protruding end of the hixL, hixR-containing EcoRV/EcoRI fragment from pMFB25 was filled in with Klenow and dNTPs, and ligated into the filled in BamHI site of a pUC12-derivative which had the HindIII site filled in and blunt end ligated (pMFB24). This produced pMFB27 which has a unique HindIII site between the recombination sites. The 104-bp HaeIII fragment of pBR322, which contains a unique BamHI site, was ligated into the filled in HindIII site of pMFB27, creating pMFB32. This plasmid was restricted with BamHI, digested with Bal31, and religated. One of the deleted derivatives, pMFB35, retained 60 bp of the HaeIII fragment, as determined by DNA sequencing (18). pMFB36 has 229 bp between the two recombination sites.

Proteins—Purified Hin was prepared as previously described (3). The partially purified Hin used in one of the DMS protection experiments (see below) was prepared as reported (4); these preparations also contained some Fis activity. Molar concentration of Hin is given for the monomer. Fis and HU were purified as detailed in Ref. 3.

DNase I and DMS Protection Assays—The nucleoscale and chemical protection studies with purified Hin on linear substrates were performed as described (14). The adenine- (19) and guanine- (18) specific DNA sequence reactions for each DNA substrate were used as markers.

Methylation protection of the supercoiled or linear DNA substrate (pMFB36) by partially purified Hin extracts was assayed by the indirect end-labeling method of Church and Gilbert (20) as modified by Richet et al. (21). The indicated concentrations of Hin and HU (see "Results") were added to reaction mixtures containing 20 mM sodium cacodylate, pH 8.0, 5 mM MgCl2, 150 mM NaCl, 120 μg/ml bovine serum albumin, 5 μg of polycytidilic acid, and 0.8 μg of supercoiled or EcoRI-linearized pMFB36 plasmid DNA and incubated for 10 min to allow inversion to occur, before treatment with DMS. The methylated DNA substrate was restricted with EcoRV and then cleaved at the base-labile methylated residues. The methylation-specific cleavage products were electrophoresed on DNA sequencing gels, electrotransferred onto GeneScreen membranes (Du Pont), and detected by hybridization with the end-labeled oligonucleotide ATCGTCACCGCTCTGCGATT or its complement.
The DNA-binding properties of the Hin recombinase

The DNA-binding properties of the Hin recombinase were characterized in vitro. The Hin recombinase was purified from an E. coli strain and used in binding assays to determine the specificities and affinities of DNA binding. The experiments involved the use of DNA substrates containing the hixL and hixR sites, which are essential for DNA inversion reactions. The DNA substrates were labeled with radioactive isotopes and incubated with purified Hin recombinase to study the binding kinetics and stoichiometry.

**RESULTS**

**Binding of Hin, Fis, and HU to Supercoiled and Linear DNA Substrates**

It was of interest to determine whether protein-protein interactions occurred among the protein components of the Hin inversion system, including the recombinase Hin, the Fis protein, and the HU protein. The interactions were studied using DNase I cleavage protection assays, which allow for the detection of DNA-binding proteins and their binding sites.

- **Supercoiled DNA Substrates**: Binding of purified Hin recombinase to supercoiled DNA was analyzed. The results showed that Hin specifically binds to the hixL site, protecting approximately 36 bp which span the 26-bp inversion target. Purified Fis protects two regions of the supercoiled DNA, with one region at 50% occupancy and another at 70% occupancy. The addition of HU resulted in an enhancement of DNA binding by Fis, likely due to changes in DNA structure.

- **Linear DNA Substrates**: A linear substrate was used to study the binding of proteins in the presence of HU. The results indicated that HU enhances the DNA-binding properties of Fis by approximately 2- to 3-fold, possibly due to a microprecipitation of the Hin protein. Electrophoresis DNA binding assays were performed to confirm the binding patterns.

**DMS Methylation of the N-7 Position of Guanine**

The DNA substrates were also analyzed using DMS methylation of the N-7 position of guanine, which faces the major groove of the DNA helix. This analysis was performed to determine the DNA-binding specificity of the recombinase Hin and its interaction with the Fis protein.

- **Supercoiled DNA Substrates**: DMS methylation of the N-7 position of guanine was used as a marker for DNA binding sites. The results showed that Hin specifically binds to the hixL site, protecting approximately 36 bp which span the 26-bp inversion target.

- **Linear DNA Substrates**: DMS methylation was used to study the DNA-binding specificity of the recombinase Hin in the presence of Fis and HU. The results indicated that HU enhances the DNA-binding properties of Fis by approximately 2- to 3-fold, possibly due to a microprecipitation of the Hin protein.

**Conclusions**

The DNA-binding properties of the Hin recombinase were characterized using in vitro binding assays. The results showed that the recombinase specifically binds to the hixL site, protecting approximately 36 bp which span the 26-bp inversion target. Purified Fis protects two regions of the supercoiled DNA, with one region at 50% occupancy and another at 70% occupancy. The addition of HU resulted in an enhancement of DNA binding by Fis, likely due to changes in DNA structure. DMS methylation of the N-7 position of guanine was used as a marker for DNA binding sites. The results showed that Hin specifically binds to the hixL site, protecting approximately 36 bp which span the 26-bp inversion target. Purified Fis protects two regions of the supercoiled DNA, with one region at 50% occupancy and another at 70% occupancy. The addition of HU resulted in an enhancement of DNA binding by Fis, likely due to changes in DNA structure.
Fig. 2. Binding of Hin, Fis, and HU to a linear DNA substrate containing both recombination sites and recombinational enhancer. The DNase I cleavage protection patterns given by purified Hin (130 ng), Fis (45 ng), and/or HU (150 ng), as indicated above each lane, is shown for the EcoRI-XbaI fragment from pMFB36 which was 3'-end-labeled with [a-32P]dATP at the XbaI site (lanes 1-6) or the EcoRI site (lanes 7-12). This DNA substrate has hixL and the recombinational enhancer in their wild-type configuration (lanes 1-6), and hixR (lanes 7-12) is 299 bp from hixL. The DNase I reactions were performed as described under “Materials and Methods.” Fis protections and enhancements are indicated by vertical and horizontal bars, respectively; the two domains of the recombinational enhancer sequence are bracketed. Cross-hatched boxes indicate the location of hixL and hixR.

Fig. 3. Methylation protection assays with supercoiled and linear DNA substrates. Methylation-specific cleavage products from DMS protection assays (21) performed with linear (left panel) and supercoiled (right panel) pMFB36 are shown for the noncoding strand of hixL. The amounts of partially purified Hin, which contains some Fis activity, and purified HU in the binding reaction are indicated above each lane. For the last lane, 0.5% SDS and 0.3 M NaOAc was added to the binding reaction prior to incubation with DMS; to the right of this lane asterisks indicate the new bands resulting from DNA inversion during incubation with Hin and HU. The solid and open boxes indicate the consensus and nonconsensus half-sites, respectively, of hixL.

sequences, respectively, using direct end-labeling assays (see “Materials and Methods”; data not shown). In addition, the same levels of methylation protection and enhancement were observed at hixL or the enhancer in titrations with purified Hin or Fis, whether one or both proteins were present (data not shown). These results not only show that supercoiling does not alter the Hin-hix or Fis-enhancer contacts in a manner detectable by DMS but also indicate that the binding of Hin or Fis to their respective DNA-binding sites on linear or supercoiled substrates is independent of the binding of the other protein.

The last two lanes of Fig. 3 show the methylation pattern under recombination conditions with a supercoiled DNA substrate and all three protein components (Hin, Fis, and HU) present. After 10 min of the inversion reaction, SDS and sodium acetate were added to half of the reaction mixture (last lane) in order to denature and displace the proteins from the DNA prior to incubation with DMS. Therefore, the difference between the methylation patterns in these last two lanes indicates...
lans reflects the binding of Hin, Fis, and HU. The extra bands above hixL in these plus/minus SDS lanes (marked by asterisks in Fig. 3) arose from DNA inversion that occurred during the incubation, before DMS addition. The protections and enhancements seen at hixL are equivalent to those seen for Hin alone on linear or supercoiled DNA (lanes 2 and 6, respectively). These results demonstrate that under inversion conditions no alterations in the Hin-hixL contacts, as compared with those made in the absence of HU, are detectable by DMS. In addition, the presence of HU does not appear to affect the contacts made by purified Fis with the recombinational enhancer on pMS551 under conditions that give inversion (data not shown). However, since titrations with HU were not performed in these assays, the effect of HU on the binding affinity of Hin and Fis for their respective DNA sites could not be accurately measured.

Binding of Hin to hixL, hixR, and Their Isolated Half-sites—Previous studies (4) have shown that the minimum hixL sequence that functions efficiently as a recombination site corresponds to the 26-bp consensus sequence (see Fig. 1B). This 2-fold symmetric sequence is conserved to some extent in both hix recombination sites. The inner 12-bp half-sites of hixL and hixR exactly match the consensus sequence, and the outer half-site of hixL has only a single mismatch. However, the outer half-site of hixR deviates significantly from the consensus half-site with five mismatches in nine highly conserved bases (see Fig. 1B). DNase I cleavage and methylation protection assays indicate that the consensus and nonconsensus half-sites of hixL and hixR fill simultaneously with increasing concentrations of Hin (Fig. 3; data not shown).

Gel electrophoresis-DNA binding assays (31, 32) were used to further address the question of how Hin recognizes and binds the 2-fold symmetric 26-bp hix sequences. The formation of specific protein-DNA complexes was measured as a function of Hin concentration using DNA fragments containing hixL, hixR, or their isolated half-sites (Fig. 4; data not shown). Hin binding to the 269-bp hixL- or 253-bp hixR-containing DNA fragment gave a single protein-DNA complex migrating more slowly than the unbound DNA (Fig. 4A); this complex had both half-sites of the hix sequence occupied by Hin, as determined from methylation interference assays (see below). No intermediate complexes with only one half-site bound were observed. The 253-bp DNA fragment containing the isolated inner half-site of hixL and hixR (the consensus half-site) was specifically bound by Hin. The protein-DNA complex for the consensus half-site migrated with approximately the same mobility as the Hin-hixL or Hin-hixR complexes (Fig. 4B) suggesting that the same multimeric state of Hin was bound to the isolated consensus half-site. Unlike the Hin-hix complexes, the half-site complexes sometimes dissociated before electrophoresis (see Fig. 4B, 1.6 and 6.5 nM Hin), suggesting that the consensus half-site complexes are less stable than full site complexes.

A plot of the percent DNA bound versus the concentration of free Hin in these equilibrium binding reactions is shown in Fig. 4C for titrations with hixL and the isolated consensus half-site. The apparent binding constants for hixL and the consensus half-site, based on the monomer Hin concentration required for 50% occupancy of each site, are 10^8 M^-1 and 4 x 10^8 M^-1, respectively. These are minimum values for the binding constants since the fraction of the Hin concentration which is active for DNA binding was not determined in these assays.

Binding of Hin to the isolated outer half-site of hixL, which differs by only 1 bp from the consensus, was equivalent to binding to the consensus half-site (data not shown). Thus, the concerted filling of the hixL half-sites could simply be explained by the similar affinities of each half-site. However, very little binding was detected for the isolated nonconsensus half-site of hixR at the Hin concentrations represented in Fig. 4C. Less than 5% of the DNA was bound at 15.5 nM Hin. Since only complexes with both half-site bound were seen in titrations of the 26-bp hixR site, the low affinity of Hin for the isolated hixR nonconsensus half-site could be accounted for either by highly cooperative interactions between Hin monomers, facilitating binding to each half-site, or by Hin binding to the hix sequences as a multimer. The binding affinity of Hin for hixR is only slightly higher than that for the isolated consensus half-site (data not shown), possibly suggesting that most of the energy for Hin binding to hixR comes from interactions with the consensus half-site. In the case of multimer binding, a few critical contacts made in the nonconsensus half-site of hixR could stabilize the interactions of a Hin multimer with hixR as compared with Hin binding to the isolated consensus half-site (see “Discussion”).

FIG. 4. Titrations of Hin binding to hixL and isolated hix half-sites. The indicated concentrations of Hin were incubated with end-labeled NarI-HaeII fragments from pMS551 (panel A) and pMS570 (panel B) and the complexed DNA separated from unbound DNA on nondenaturing polyacrylamide gels as described under “Materials and Methods.” The autoradiograms show that a single protein-DNA complex was detected for both hixL- and consensus half-site-containing DNA fragments. No specific binding was detected in these assays to DNA fragments containing hix sequences (data not shown). Panel C shows a plot of the percent DNA bound versus the free Hin concentration for two independent titration experiments (open and closed symbols) with DNA fragments containing hixL (squares), consensus half-site (triangles), and the nonconsensus half-site of hixR (pAG400) (circles). A time course for Hin binding demonstrated that equilibrium had been reached in these binding assays (data not shown).
DNA Sequence Requirements for Hin Binding—To determine the extent, as well as structural features, of the 26-bp hix sequence required for Hin binding, a series of mutant hix sites, derived from synthetic oligonucleotides, were cloned into the Sall site of pBR322 and used in Hin binding assays. The DNase I cleavage protection pattern for wild-type and mutant hix sites are shown, and the results summarized, in Fig. 5. The binding of Hin to these hix sites was also assayed by gel electrophoresis-DNA binding assays (Fig. 4; data not shown). The DNase I protection pattern for Hin binding to the 26-bp hixL (pMS551) and hixR (pAG410) sequences indicates complete protection of the 2-bp core (base pairs +1, −1) and the 12-bp half-sites, including 2 to 3 neighboring base pairs. Due to the nature of the DNA cleavage properties of DNase I (33, 34) the precise boundaries of Hin DNA protection are uncertain. However, MPE Fe (methidiumpropyl-EDTA-Fe) cleavage protection studies with partially purified Hin extracts (8, 9) indicate that only the 26-bp consensus sequence is covered by Hin.

DNA fragments containing the consensus half-site of hixL and hixR (pMS570) or the outer half-site of hixL (pAG415) are good substrates for Hin binding (Fig. 4; data not shown). The DNase I cleavage pattern for each of these half-sites shows full protection of most of the hix sequence and enhancements at the 2-bp core (Fig. 5). In addition, partial protection from DNase I cleavage is seen at Hin concentrations as high as 103 nM (data not shown).

The effect of the spacing between half-sites on Hin binding was examined using mutant hixL sites with an additional one (27-mer; pMS612) or 3 (29-mer; pMS649) base pairs at the center of dyad symmetry. Titration of Hin with the 27-mer and 29-mer in gel binding assays (data not shown) indicated that the affinity of Hin for these sites was approximately 2-fold lower than for the wild-type hixL sequence. These mutant hix sites gave Hin-hix complexes of the same mobility as hixL. Hin protected 33 and 35 bp of the hix sequence (data not shown). The protection data for pAG415 were examined using mutant hixL sites with an additional one (27-mer; pMS612) or 3 (29-mer; pMS649) base pairs at the center of dyad symmetry. Titration of Hin with the 27-mer and 29-mer in gel binding assays (data not shown) indicated that the affinity of Hin for these sites was approximately 2-fold lower than for the wild-type hixL sequence. These mutant hix sites gave Hin-hix complexes of the same mobility as hixL. Hin protected 33 and 35 bp of the hix sequence. These mutant hix sites gave Hin-hix complexes of the same mobility as hixL. Hin protected 33 and 35 bp of the hix sequence.
observed, perhaps due to straining of a Hin multimer to contact both half-sites in this altered configuration.

To determine if the protected outer two base pairs of the consensus sequence are important contacts in Hin binding, the interaction of Hin with a mutant hix site which is deleted for the outer two base pairs of both half-sites of hixL (22-mer; pMS574) was assayed. Symmetric deletions facilitated measurement of the mutant phenotype since the affinity of Hin for each of the hixL half-sites is equivalent. The gel binding assays indicated that the affinity of Hin for the 22-mer site is at least 10-fold lower than for hixL (data not shown). This is consistent with the greatly reduced rate of inversion observed with pMS574 (4). The DNase I cleavage pattern (at a high Hin concentration) shows that the extent of protection of the 22-mer sequence by Hin (approximately 34 bp) is similar to the full hixL sequence. However, cleavage enhancements are seen within the protected region at the two base pairs immediately to the right of the 22-mer sequence, which might indicate the interaction of Hin with this site is altered (Fig. 5). These results indicate that the outer 2 bp of the hixL sequence are probably involved in the interaction of Hin with the recombination site.

Although the 2 core base pairs of the hix sequence are protected in both MPE-Fe and DNase I protection assays, the core sequence does not appear to be involved in Hin recognition of the hix site. The protection pattern for a mutant site containing a transversion mutant at the +1 position (+1:A→T; pMS614) is the same as for hixL (Fig. 5). Gel binding assays showed that the affinity of Hin for this mutant site is close to that of hixL (data not shown). These binding results and the normal inversion activity of this mutant site when recombined with another +1:A→T mutant site (4) suggest that the core 2 base pairs are not essential contacts in Hin binding to the recombination sites. In order to determine the inner boundary of specific Hin interactions with the hix half-sites, a consensus half-site deleted for the inner 2 base pairs (+2, +3), as well as the 2-bp core, (10-mer; pAG403) was used in the Hin binding assays. The 10-mer behaved like a consensus half-site (12-bp + 2-bp core) in gel binding assays (data not shown) and DNase I cleavage protection experiments (Fig. 5), indicating base pairs 2 and 3 from the center of hixL are not essential for Hin recognition of the consensus half-site sequence.

Hin-DNA Contacts at the Recombination Sites and the Secondary Hin-binding Site—To determine the critical DNA contacts made by Hin in binding each recombination site, methylation interference of binding studies (35, 36) were utilized. In these assays, modified residues which interfere with Hin binding to partially methylated hix-containing DNA substrates are identified as critical DNA contacts. As in the methylation protection assays (above), guanines or adenines are methylated with DMS in the major or minor grooves of the DNA helix, respectively. After binding of Hin to the modified DNA, the bound DNA is separated from unbound DNA in nondenaturing polyacrylamide gels as seen in Fig. 4. The methylation-specific cleavage products from uncomplexed (lane 1) and complexed (lane 2) DNA for the noncoding and coding strands of hixL and hixR are shown in Fig. 6. The modified residues that interfered with the formation of the Hin-hix complex (identified as uncleaved residues from the isolated complex DNA) are located in both half-sites of hixL and hixR. This confirms that contacts made by Hin in both half-sites of hixL or hixR are required for the formation of the single protein-DNA complex seen in the gel binding assay.
These contacts, which are summarized in Fig. 1B, are fairly symmetrical in hixL; contacts are made in the minor groove with adenines at positions (±)4 through 6 and in the major groove with a guanine at position +9. In hixR the contacted residues in the consensus half-site are the same as in hixL with the addition of the adenine at position +10. These contacted residues coincide with the purines protected from methylation by DMS when Hin is prebound to the DNA (see Fig. 1). In the nonconsensus half-site of hixR, methylation of a single adenine at position −6 from the center of hixR interferes with binding in the minor groove.

When a DNA fragment containing the wild-type hixL region is used in gel binding assays, a second protein-DNA complex appears as the Hin concentration is increased (data not shown). Methylation interference assays indicate that this slower-migrating complex has Hin bound to both hixL and a secondary Hin-binding site located immediately upstream of the hin translational start site (+75 bp). The interference data (summarized in Fig. 1) shows that Hin contacts purines on the coding and noncoding strands from base pair +38 to +72. The region from +44 to +71 exhibits limited homology with hixR. However, the half-sites are separated by 3 base pairs instead of a 2-bp core. Many more residues interfere with Hin binding to the secondary site than identified with hixR or hixL, including residues outside of the 27 bp “consensus sequence.” The additional contacts may be interpreted as reflecting that cumulative poor interactions can become important in stabilizing a weakly associated protein-DNA complex. It is also possible that the association of Hin with the secondary site requires additional contacts not involved in binding with the recombination sites.

The significance of the secondary Hin-binding site is uncertain. It can be deleted with no affect on the inversion activity of the DNA substrate (4). The proximity of secondary site to the translational start of hin suggests a possible regulatory role. However such a role has not been demonstrated. The affinity of Hin for the secondary site, as determined in gel binding assays, is close to that for an isolated hix half-site, and the Hin-secondary site complexes exhibit the same instability as the Hin-half-site complexes. Summarized in Fig. 1 is the methylation and DNase I cleavage protections for the secondary binding site obtained at high Hin concentrations (data not shown). The protection patterns are very similar to those for Hin binding to the isolated hix half-sites, suggesting that base pairs +59 through +70 are recognized by Hin as a good hix half-site.

**Hin Binds As a Dimer to the Recombination Sites**—The results of the DNA binding assays described above suggest that Hin binds the recombination sites as a multimer. To determine the molecular weight of the active DNA-binding species of Hin, purified Hin (90 μg/ml) was passed through a Superose 12 gel filtration column and fractions assayed for DNA binding and inversion activity. Western blot analysis of the fractions revealed a single peak at approximately 44,000 Mₙ (Fig. 7A), as determined by comparison to molecular weight standards (Fig. 7D). The molecular weight of the Hin polypeptide is approximately 21,000, as determined on denaturing SDS-polyacrylamide gels (3) and from the gene DNA sequence (2). Therefore, this peak most likely corresponds to a Hin dimer. Similar results were obtained with 25 μg/ml ¹⁴C-

![Fig. 7. Gel filtration of Hin.](image)

Hin (18 μg) was loaded on a Superose 12 column in HSB buffer containing 0.1% Triton and 0.6 M NaCl and 0.25-ml fractions were collected. Fraction numbers are indicated in panels A and C. Western blot analysis (panel A) of 30 μl of each fraction indicates a peak at approximately 44,000 Mₙ. The positions where monomer (21,000 Mₙ) and dimer (42,000 Mₙ) forms of Hin should elute are indicated above the blot. Panel B shows the results of in vitro inversion assays (3); the inversion products obtained after incubation with 8 μl of the fractions and subsequent cleavage of the DNA substrate with Clal are indicated to the left of the middle panel. The autoradiogram from gel electrophoresis DNA binding assays (see “Materials and Methods”) using 3 μl of the fractions and end-labeled hixL-containing DNA fragment (Narl-HaelII fragment of pM8501) is shown in panel C; the Hin-specific complexes and unbound DNA are indicated. It should be noted that a much lower concentration of Hin is required for saturation binding conditions (Fig. 7C) as compared to that required for detectable recombination levels in the inversion assay (Fig. 7B). A plot of molecular weight (Mₙ) versus the ratio of elution volume (Vₑ) to void volume (V₀) is shown in panel D for various standards: blue dextran (2,000,000 Mₙ), β-amylase (200,000 Mₙ), alcohol dehydrogenase (150,000 Mₙ), bovine serum albumin (66,000 Mₙ), carbonic anhydrase (29,000 Mₙ), and cytochrome c (12,400 Mₙ). The Hin peak is indicated by a solid circle and the dashed circle represents the expected position of a Hin monomer peak.
labeled purified Hin (data not shown), indicating that the multimeric state of Hin eluted is not concentration-dependent under these gel filtration conditions within the range of protein concentrations used (see "Materials and Methods"). In addition, the concentration of C-labeled Hin utilized is functionally relevant in that it is close to the concentration of purified Hin that gives inversion in the in vitro inversion assays (10 µg/ml; Ref. 3). No larger Hin multimers, which would have elution volumes (Vₑ) distinct from the dimer form, were detected by Western blot or DNA binding assays.

In vitro inversion assays (Fig. 7B) and gel electrophoresis DNA-binding assays with hixL (Fig. 7C) and the consensus half-site (data not shown) demonstrate that the dimer is active for recombination and DNA binding. There is some trailing of the main peak seen in the Western blots which gives both low level inversion and specific DNA binding activity. This activity may result from a small monomer peak which is indistinguishable from the trailing dimer peak. Although the predominant active species of Hin is apparently a dimer, the possibility that the monomer form can also bind has not been excluded.

**DISCUSSION**

The inversion system of *S. typhimurium* utilizes three proteins, Hin, Fis, and HU, and a supercoiled DNA substrate, containing two recombination sites, hixL and hixR, and the recombinational enhancer. A previous study (14) characterized the binding of Fis protein to the recombinational enhancer. Here we have characterized the binding of Hin, the recombinase, to the recombination sites and related sequences using DNase I and DMS protection assays, methylation interference assays, and gel electrophoresis DNA binding studies.

DNase I and methylation protection studies performed with linear and supercoiled DNA substrates (see Figs. 2 and 3) indicate that the interactions of Hin and Fis with their respective DNA-binding sites are unaltered by DNA supercoiling or by the presence of the other protein or HU. The apparent lack of cooperative interactions facilitating binding of Hin and Fis to the inversion substrate suggests that in the inversion reaction the hix sites and recombinational enhancer are initially occupied individually. Subsequent interactions between Hin and Fis in a synaptic intermediate, as suggested in the looping synapsis model for the role of Fis and the recombinational enhancer in the inversion reaction (14, 15), may be too transient, or may not change the DMS reactivity of the DNA, and therefore would not be detectable in these assays. Johnson and Bruini² report that two Hin-hix complexes may interact when they are on a single linear DNA fragment as indicated by limited double-stranded cleavage of the DNA at the hix sites. In the absence of Fis, this cleavage is affected only slightly by DNA supercoiling. These results are consistent with the notion that the individual proteins can make all the DNA contacts necessary to form the active synaptic complex on supercoiled DNA. This contrasts with the behavior of the Int-promoted site-specific recombination system of bacteriophage λ. In the λ system the DNase I cleavage pattern changes dramatically when all necessary proteins are present (40), and cooperative interactions for binding various sites have been indicated (41).

Previous work with a 52-amino acid synthetic peptide that corresponds to the carboxyl terminus (DNA-binding domain) of the Hin protein indicated that each half-site of the recombination sites should be occupied by a Hin monomer (42). The present work confirms this conclusion and indicates that Hin is a dimer in solution and while bound to a hix site. The 52-mer peptide binds the half-sites of hixL and hixR with different affinities (42). In contrast, titrations of hixL and hixR with Hin give a single protein-DNA complex in gel binding assays (see Fig. 4) and equal protection of both half-sites for each hix site in chemical and nuclease protection assays (see Fig. 3), indicating that the full site is bound as a unit. However, Hin does exhibit different binding affinities for the isolated hix half-sites, binding the nonconsensus half-site of hixR very poorly and binding the other three half-sites of hixL, and hixR with approximately 2.5-fold reduced affinities relative to the full hixL site. The DNase I protection patterns for the isolated hix half-sites indicate that Hin covers the nonspecific DNA at the missing half-site, but the interactions are altered compared to full site Hin-hix interactions (see Fig. 5). This is consistent with the fact that the isolated half-sites are not functional recombination sites (4). If Hin recognizes and binds the hix sites as a multimer, the high binding affinity of Hin for the isolated half-sites relative to hixL may simply be due to the contributions to binding energy of favorable nonspecific interactions between multimeric Hin and the DNA sequences adjacent to the isolated half-site (see Fig. 5). The binding energy for Hin association with hixR, which has an apparent binding constant close to the trailing dimer peak of the isolated consensus half-site, may be primarily from contacts within the consensus half-site, with a few specific contacts in the nonconsensus half-site of hixR stabilizing the interactions of a Hin multimer with the full site.

The nature of the Hin-hix complexes formed in this study suggests that Hin does bind the recombination sites as a multimer. Gel filtrations of purified Hin indicated that the predominant species of Hin in solution is a dimer. This dimer form of Hin is active for binding hixL and the consensus half-site and gives full inversion activity (see Fig. 7). It is simplest to imagine that Hin is also a dimer on the hix sites. These results differ from those obtained for the homologous recombinase Gin of the bacteriophage Mu inversion system. Gin exists in solution predominantly as a monomer (43) and binds differentially to the unequal half-sites of the gix recombinase sites (12). These differences in the recombinase-recombination site interactions may be relevant to the differences seen in the DNA cleavage properties of Hin and Gin; Hin mediates concerted double-stranded cleavage at the 2-bp core site of the hix sites while Gin apparently produces single-stranded breaks within the core of the gix sequences (13). The cleavage properties of Hin suggest a recombination mechanism involving concerted cleavage of the DNA at both recombination sites followed by strand rotation leading to the recombinant product. The nicking of one strand at each recombination site by Gin could suggest that recombination entails sequential strand exchange involving a Holliday intermediate such as that proposed for the Int-mediated site-specific recombination of bacteriophage λ (13; for review see Ref. 44). However, it would be surprising if the inversion reactions mediated by these homologous recombinases were not mechanistically the same. This discrepancy may be explained by the DNA binding properties of Gin and Hin in that the unequal binding affinity of Gin monomer for the gix half-sites may result in detection of primarily nicked intermediates, and the simultaneous binding of hix half-sites by the Hin dimer leads to the observed concerted double-stranded cleavage.

The binding of Hin to mutants altered in the spacing between half-sites indicates that there may be some flexibility in the dimeric Hin molecule, as the dimer binds both half-sites of hix sequences containing 2-(wild-type), 3-, or 5-bp

² A. C. Glasgow and M. I. Simon, unpublished data.
cores. However, the interactions of Hin with these spacing mutants are not productive since plasmid substrates containing these mutant sites, combined with a wild-type site, do not show inversion activity (4). Resolvase of the γδ resolution system, which exhibits 35% amino acid sequence identity with Hin (45), also appears to exhibit this flexibility, binding three res subites, each with a different size core (7, 10, and 16 bp). However, there are probably essential contributions to DNA structure made by these core sequences which are needed for resolvase binding and activity (46).

Changing the sequence of the 2-bp core of hixL from AA to AT altered neither the binding of Hin to the hix site, nor the recombination activity when this mutant site is combined with the same site in the inversion reaction (4). However, it is possible that other mutations in (or near) the core sequence may result in a change in Hin binding and inversion activity if these bases contribute to altered DNA structures in the hix sites. Preliminary results of circular permutation assays, which detect altered DNA structure (47), suggest that the binding of Hin induces a bend in the hix sequence as has been seen for the interactions of Gin with the gix recombination sites (12). We are presently studying the role of such altered DNA structures in the recombination reaction.

Hin protects approximately 36 bp of DNA at each hix site from cleavage by DNase I. The DNA binding surface of DNase I extends 4 bp 5′ and 6 bp 3′ from the cleavage site (34, 37). Thus, Hin blocks only the 26-bp consensus sequence at hixL and hixR (Fig. 1) from cleavage. This agrees with earlier results using the low molecular weight DNA cleaving probe methidiumpropyl-EDTA-Fe(II) and a crude preparation of Hin (8, 9). The extent of the consensus sequence required for recombinase recognition and binding was tested using mutant recombination sites in nucleoside protection studies and gel electrophoresis-DNA binding assays. The essential bases for Hin recognition of the recombination sites appear to be within the sequence between base pairs ±4 through ±11 from the center of dyad symmetry (see Fig. 1). This conclusion is based on the facts that Hin still recognizes sites with the outer two base pairs of each half-site deleted, sites with insertions and alterations at the core, and a half-site comprised of only base pairs +4 through +13 of hixL. In addition, methylation interference assays indicate that the critical contacts for Hin binding to the hix sites are in this region (see Fig. 1). The same critical bases were revealed in mutagenesis studies with the hix sequences in vivo (38).

The disposition of the purine contacts made by Hin with the hix sequences, as revealed by methylation interference (Fig. 1B), suggest that the Hin molecule binds primarily to one face of the DNA helix in adjacent major and minor grooves of the hix half-sites. The partial interference by methylation of the +10 adenine may indicate that the COOH-terminal tail of Hin reaches around the DNA helix (39) and contacts this adenine in the minor groove, or that alkylation of this residue alters the DNA in such a way that the major groove contacts at this sequence are affected.

The importance of the minor groove contacts made by Hin with the three adenines near the center of dyad symmetry is supported by work with the 52-mer peptide and various deleted derivatives. The position of the amino terminus of the 52-mer when bound to hixL or the secondary site was determined by attaching EDTA to the NH2-terminal end of the 52-mer and initiating cleavage of the DNA by addition of Fe(II) and a reducing agent. The cleavage pattern indicates that the NH2 terminus of the 52-mer is bound to the minor groove near the symmetry axis of hixL (base pairs ±4 to ±6) (39). In a deletion analysis of the 52-mer peptide, removal of the amino-terminal residue (glycine) resulted in a significant reduction in binding affinity for hixL. Deletion of the next amino-terminal residue (arginine) resulted in a 50-mer peptide which could no longer bind hixL but still could bind the secondary Hin-binding site. These results indicate that the contacts made by the NH2-terminal two amino acids of the 52-mer are critical for recognition of the hixL sequence but not for sequence-specific binding. In vivo mutagenesis studies with Hin have also shown these two residues (Gly-139 and Arg-140) to be essential for Hin recognition and binding to the hix sequence. Within the Hin carboxyl-terminal domain is an amino acid sequence indicative of the helix-turn-helix motif (42) which has been shown to bind the major groove of DNA (48). The methylation interference data presented here for Hin binding and the results described for the synthetic DNA-binding peptides indicate that Hin binds the outer major groove of the hix sites using this the putative helix-turn-helix motif, but, in addition, utilizes another structure which recognizes features within the adjacent minor groove.

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DNA-binding Properties of the Hin Recombinase

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