THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 267, No. 16, Issue of June 5, pp. 11183–11190, 1992
Printed in U.S.A.

The Effects of Symmetrical Recombination Site *hixC* on Hin Recombinase Function*

(Received for publication, October 29, 1991)

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An artificial recombination site *hixC* composed of two identical half-sites that bind the Hin recombinase served as a better operator *in vivo* than the wild type site *hixL* (Hughes, K. T., Youderian, P., and Simon, M. I (1988) *Genes & Dev.* 2, 937–948). *In vitro* binding assays such as gel retardation assay and methylation protection assay demonstrated that Hin binds to *hixC* as tightly as it binds to *hixL*, even when the sites are located in negatively supercoiled plasmids. However, *hixC* served as a poor recombination site when it was subjected to the standard inversion assay *in vitro*. *hixC* showed a 16-fold slower inversion rate than the wild type.

A series of biochemical assays designed to probe different stages of the Hin-mediated inversion reaction, demonstrated that Hin dimers bound to *hixC* have difficulty in forming paired *hix* site intermediates. $KMnO_4$ and S1 nuclease assays detected an anomalous structure of the center of *hixC* only when the site was in negatively supercoiled plasmids. Mutational analysis in the central region of *hixC* and assays of paired *hix* site formation with topoisomers of the *hixC* substrate plasmid suggested that Hin is not able to pair *hixC* sites because of the presence of the anomalous structure in the center of the site. The structure does not behave like a DNA "cruciform" since Hin dimers still bind efficiently to the site. It is thought to consist of a short denatured "bubble" encompassing 2 base pairs.

During the study of mutations in the center of *hixC*, it was found that Hin is not able to cleave DNA if a guanine residue is one of the two central nucleotides close to the cleavage site. Furthermore, Hin acts in a concerted fashion and cannot cleave any DNA strand if one of the four strands in the inversion intermediate is not cleavable.

The Hin recombinase is involved in gene rearrangement which leads to the alternative expression of the H1 and H2 flagellin genes in *Salmonella typhimurium* known as phase variation (Zieg *et al.*, 1977; Zieg and Simon, 1980). Hin inverts a 993-bp[1] chromosomal segment (H region) which is flanked by the two 26-bp recombination sites *hixL* and *hixR* (Johnson and Simon, 1985). Hin (22 kDa as a monomer) exists as a dimer in solution and binds to the two recombination sites as a dimer (Glasgow *et al.*, 1989). *In vitro*, two other proteins, Fis and HU, are required for inversion to occur efficiently (Johnson *et al.*, 1986). Fis binds to a DNA sequence called the enhancer located inside the H region. Two Fis dimers bind two domains of the enhancer (Bruist *et al.*, 1987). HU binds to DNA nonspecifically and promotes inversion by bending the DNA between the enhancer and *hixL* (Johnson *et al.*, 1986).

The first step in the inversion process is the binding of the necessary proteins to DNA. Binding of Hin, Fis, and HU occurs independently (Glasgow *et al.*, 1989). The next step appears to involve the random collision between bound Hin dimers to bring the two *hix* sites close together. This interaction is independent of subsequent steps in the inversion process (Lim and Simon, 1992). Negative supercoiling is required for formation of a productive intermediate, the invertasome. It consists of a structure formed by interaction between the Hin and Fis bound at the enhancer (Heichman and Johnson, 1990). It was proposed that the role of supercoiling in the formation of the invertasome is to promote physical contact between the Hin protomers and Fis molecules (Lim and Simon, 1992). The invertasome structure containing Hin and Fis molecules has been visualized by electron microscopy (Heichman and Johnson, 1990). Following the formation of the invertasome structure, concerted DNA strand cleavage occurs at the center of each of the recombination sites. Hin makes 2-bp staggered cuts producing protruding 3′-hydroxyl and recessed 5′-phosphate ends (Johnson and Bruist, 1989). Serine 10 of Hin is believed to make a transient phosphoester bond with the recessed 5′-P end. Strand rotation into the configuration necessary for inversion was proposed to occur through the exchange of subunits between Hin dimers (Heichman *et al.*, 1991). The subunit exchange hypothesis was proposed for the Gin invertase (Kanaar *et al.*, 1990) and for the Tn3 resolvase (Stark *et al.*, 1989). Finally, Hin religates four DNA strands and, presumably, the complex then dissociates.

The wild type *hixL* site is composed of two imperfect 13-bp inverted repeats. An *hix* sequence with two perfect 13-bp inverted repeat (*hixC*) was constructed using a half-site sequence that is identical in both *hixL* and *hixR*. This half-site has the highest binding affinity to Hin of any half-sites (Hughes *et al.*, 1988). This suggests that the Hin dimer, which possesses 2-fold symmetry, would bind best to a symmetric DNA-binding site assembled from the two highest affinity half-sites, *i.e.* the *hixC* site.

In this study, when a substrate plasmid with two *hixC* sites (pKH339) was subjected to the standard Hin-mediated inversion reaction *in vitro*, the rate of inversion was found to be extremely slow. A series of biochemical assays was performed to investigate the stage at which the inversion reaction is

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[1] The abbreviations used are: bp, base pairs; kb, kilobase(s).

blocked. The difference in secondary structure between the *hixL* and *hixC* sites was probed by $KMnO_4$ and S1 nuclease. We found that the central AT base pairs can form an anomalous DNA structure in *hixC*. Furthermore, under certain conditions Hin bound to *hixC* cannot bring two *hixC* sites close together because of the structure in the center of the DNA-binding site. *In vivo hixC* also shows extremely slow kinetics in inversion[2] (Moskowitz *et al.*, 1991).

MATERIALS AND METHODS

Proteins and DNA—Hin was purified using the methods described in Johnson and Simon (1985) from *Escherichia coli* strain DH1 (Hanahan, 1983) harboring pHL104 (Lim and Simon, 1992) which has the wild type *hin* gene under the control of the *tac* promoter. Fis and HU were prepared using previously described methods (Johnson *et al.*, 1986). Protein quantitation was performed by the method of Bradford (Bradford, 1976). Hin preparation used in this study was free from Fis and was 50% pure estimated by scanning SDS-polyacrylamide gel with a LKB Ultroscan XL laser densitometer. HU and Fis were purified to homogeneity. Calf thymus topoisomerase I and S1 nuclease were purchased from Bethesda Research Laboratories (BRL). Oligonucleotides were synthesized with the last trityl group retained to facilitate the purification of only the final products using Nensorb™ column (Du Pont). pKH173 was constructed as follows. The wild type *hin* gene in pES201 (Bruist and Simon, 1984) was inactivated by filling in the *Ase*I restriction site using the large fragment of *E. coli* DNA polymerase I (Klenow fragment from BRL) to make pKH165. The *hixL* site of pKH165 was removed and replaced with an oligonucleotide that has a *Sma*I restriction site by a oligonucleotide mutagenesis method (Sambrook *et al.*, 1989) to create pKH167. An oligonucleotide with a *Hpa*I site in the middle was ligated to the *Hin*dIII site of pKH167 to make pKH173.

A series of substrate plasmid for Hin-mediated *in vitro* reactions was constructed as follows. Two complementary oligonucleotides (26 bp) were hybridized and ligated to the *Sma*I and *Hpa*I sites of pKH173 separately. To insure only one insert was ligated, a linker-tailing method was used (Lathe *et al.*, 1984). After the sequence and orientation of inserts were confirmed, *Stu*I-*Eco*RV fragments between two plasmids were switched to make a substrate plasmid with two recombination sites cloned in *Sma*I and *Hpa*I sites (see Fig. 1*a*). Double-stranded DNA sequencing was performed as described in Lim and Pene (1989) with a Sequenase™ kit from United States Biochemical (USB).

Probing the Secondary Structure of hix Sites by $KMnO_4$ and S1 Nuclease—$KMnO_4$ assay was performed as described by Sasse-Dwight and Gralla (1989). The reaction was initiated by incubation of 0.5 μg of supercoiled substrate plasmid with 300 ng of Hin in a total volume of 17.5 μl of inversion buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, and 100 μg/ml polycytidylic acid (Pharmacia LKB Biotechnology Inc.)) at 37 °C for 20 min. When Hin was not needed, water was added instead of Hin to the reaction. $KMnO_4$ (2.5 μl) was added to a final concentration of 10 mM, and incubation continued for 2 min. To quench $KMnO_4$, 1.5 μl of β-mercaptoethanol was added to the reaction followed by a phenol/chloroform (1:1) extraction. DNA was loaded onto Sephadex G-25 spin column (400 μl). Fifteen μl of water was added to DNA eluted from the spin column to make the volume 35 μl in total. The DNA was used immediately for the primer extension reaction.

For the S1 nuclease assay, 1 μg of plasmid DNA was incubated with 50 units of S1 nuclease in a total volume of 50 μl of S1 nuclease buffer (30 mM sodium acetate, pH 5.3, 50 mM NaCl, 1 mM $ZnSO_4$, 5% (v/v)) glycerol at 37 °C for 1 h. Digested DNAs were resolved by electrophoresis in a 1% agarose gel in TBE buffer (89 mM Tris-borate, pH 8.0, 89 mM boric acid, and 2 mM EDTA) (Sambrook *et al.*, 1989).

Primer Extension—The basic principle of the primer extension reaction was followed as described in Borowiec *et al.* (1987). To 35 μl of DNA template, 1 μl of ^{32}P-end-labeled primer (0.1 pmol, 25,000 cpm), and 4 μl of 0.01 M NaOH were added. DNA samples were heated to 80 °C for 2 min and transferred to ice. Five μl of 10 × TMD buffer (0.5 M Tris-HCl, pH 7.2, 0.1 M $MgSO_4$, and 2 mM dithiothreitol) was added to samples, and the hybridization was performed at 50 °C for 3 min. Samples were returned to ice immediately, and 5 μl of dNTP (5 mM each) and 1 μl of Klenow fragment (1 unit) was added. DNA polymerization was performed at 50 °C for 10 min. For the $KMnO_4$ assay, DNA was precipitated by adding 17 μl of 4 M ammonium acetate and 167 μl of 95% ethanol. The DNA pellet was washed with 70% ethanol, dried in vacuum, and resuspended in 4 μl of dye mix (7 M urea, 1 mM NaOH, 0.02 mM EDTA, 0.01% xylene cyanol/bromphenol blue in deionized formamide). All 4 μl were loaded on a 6% polyacrylamide sequencing gel. For the Hin-mediated cleavage assay, 50 μl of dye mix was added to samples after the DNA polymerization step. Samples were boiled for 2 min, and 4 μl were loaded on 6% polyacrylamide DNA sequencing gel.

Assays of Reaction Intermediates—Pairing of *hix* sites, invertasome formation, and inversion were assayed by the methods described in the preceding paper (Lim and Simon, 1992). Hin-mediated strand cleavage was probed by two different methods. Concerted strand cleavage on both recombination sites after the cleavage reaction was detected by resolving DNA fragments on agarose gel electrophoresis (Johnson and Bruist, 1989). DNA cleavage on each strand of the *hixL* located at the *Sma*I site of substrate plasmids was analyzed by the primer extension method with two different primers hybridized to the top and bottom strands. For the primer extension reaction, DNA was extracted by phenol/chloroform (1:1) after the cleavage reaction, followed by a Sephadex G-25 spin column. Water was added to bring the final volume to 35 μl.

DNA Binding Assays—The gel retardation method (Fried and Crothers, 1981) was used to measure the Hin binding activity to linearized DNA fragments containing *hixL* and *hixC*. *Eco*RV-*Stu*I DNA fragments (300 bp) from pKH336 (*hixL*) and pKH339 (*hixC*) were dephosphorylated with calf intestinal phosphatase (BRL). End-labeling at the 5′ end of the dephosphorylated DNA fragments with T4 DNA kinase (New England BioLab) was performed according to standard procedures (Sambrook *et al.*, 1989). Conditions for binding and polyacrylamide gel electrophoresis were followed as described previously (Glasgow *et al.*, 1989). The apparent binding constant was measured as the concentration of Hin (monomer) at which 50% of DNA-binding sites are occupied at equilibrium.

Binding of Hin to *hixL* and *hixC* in supercoiled plasmid was performed as follows. The binding reaction was initiated by adding 300 ng of Hin with 12 units of *Nco*I (New England Biolab) to 0.5 μg of substrate plasmids in 50 μl of N buffer (10 mM Tri-HCl pH 7.9, 10 mM $MgCl_2$ 150 mM NaCl, and 10 μg/ml bovine serum albumin) at 37 °C. Incubation continued for 2 h. DNA fragments were analyzed by 1% agarose gel electrophoresis in TBE buffer.

RESULTS

Construction of Substrate Plasmids and hix Sequences—In order to facilitate the construction of a series of substrate plasmids prepared with different sequences and combinations of recombination sites, a plasmid pKH173 was constructed (Fig. 1*a*). This plasmid contains *Sma*I and *Hpa*I sites into which synthetic oligonucleotides (26 bp) could be inserted. The two restriction sites are separated by 0.9 kb. The recombinational enhancer is located in the same relative position as it is in the wild type chromosome of *S. typhimurium*. The different *hix* sequences which were used in this study are listed in Table I. The position of each base pair is indicated above the *hixL* sequence. The *hix* sequence used as the positive control is a modified *hixL* that has the sequence AT in its center, whereas the wild type *hixL* has the central sequence AA. *hixL* with the AT center will be referred to as *hixL* throughout this study. No differences in recombination efficiency between it and the wild type *hixL* have been observed (data not shown).

Hin-mediated Inversion Reaction on hixC Is Very Slow—The rate of the Hin-mediated inversion reaction was measured on pKH339 that has perfectly inverted repeat recombination sites (*hixC*) at the *Sma*I and *Hpa*I site of pKH173 and compared to that of pKH336 containing *hixL*. The rate of inversion at the *hixC* recombination site was so slow that no inversion was detected in 30 min. About 1.8% inversion was detected with the *hixC* substrates in 1 h whereas 30% inversion was observed with the comparable *hixL* substrates (Fig. 1*b*).

Hin Binds to hixC as Well as to hixL—Hughes *et al.* (1988)

[2] K. T. Hughes and M. I. Simon, unpublished data.

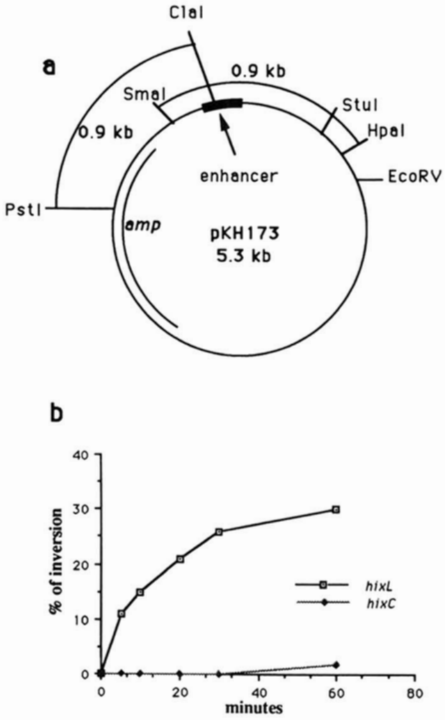

FIG. 1. **pKH173 and time course inversion reactions.** *a*, pKH173. Double-stranded synthetic oligonucleotides were inserted to *Sma*I and *Hpa*I site to create a series of substrate plasmids for the Hin-mediated reactions. *b*, measurement of the rate of inversion on *hixL* (pKH336) and *hixC* (pKH339) containing plasmid substrates.

TABLE I

Nucleotide sequences of hix recombination sites

The abbreviation used is: WT, wild type.

hixL(WT)		TTCTTGAAAACCAAGGTTTTTGATAA
		−12−10 −8 −6 −4 −2 2 4 6 8 10 12
hixL	:	TT**C**T**TG**AAAACCATGGTTTTTGATAA
hixC	:	TT**A**T**CA**AAAACCATGGTTTTTGATAA
hix1	:	- - - - - - - - - - - - TA - - - - - - - - - -
hix2	:	- - - - - - - - - - - - GC - - - - - - - - - -
hix3	:	- - - - - - - - - - - - CG - - - - - - - - - -
hix4	:	- - - - - - - - - - - A - - T - - - - - - - - - -
hix5	:	- - - - - - - - - - - T - - A - - - - - - - - - -
hix6	:	- - - - - - - - - - - G - - C - - - - - - - - - -
hix7	:	- - - - - - - - - - T - - - - A - - - - - - - - -
hix8	:	- - - - - - - - - - G - - - - C - - - - - - - - -
hix9	:	- - - - - - - - - - A - - - - T - - - - - - - - -
hix10	:	- - - - - - - - - - - - CC - - - - - - - - - - -
hix11	:	- - - - - - - - - - - - GG - - - - - - - - - - -
hix14	:	TTAT**T**AAAAACCATGGTTTTTGATAA
hix15	:	TTATC**G**AAAACCATGGTTTTTGATAA

showed that Hin binds to *hixC* slightly better than to *hixL in vivo* by the challenge phage assay. In order to further explore the affinity, a gel retardation assay was performed on *hixC* and *hixL in vitro*. Three-hundred-bp *Stu*I-*Eco*RV DNA fragments from pKH336 and pKH339 containing *hixL* and *hixC* sequences, respectively, were used. The concentration of Hin needed to bind 50% of *hixC* was 10 nM and that of *hixL* was 13 nM (Fig. 2*a*). This result is consistent with that of the challenge phage assay. This small difference in affinity of Hin in binding to the two sequences cannot account for the fact that *hixC* does not support the Hin-mediated inversion reaction *in vitro*.

The Hin-mediated inversion reaction occurs on negatively supercoiled DNA (Johnson *et al.*, 1984), and negative supercoiling promotes some unusual secondary structures (Lilley *et al.*, 1988) which could subsequently inhibit binding of Hin

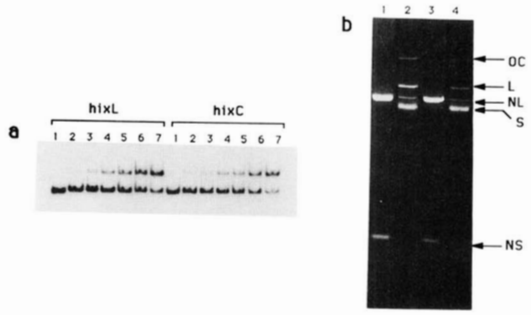

FIG. 2. **Hin binding assays.** *a*, Hin binding assay on a 300-bp *Eco*RV-*Stu*I fragment of pKH336 (*hixL*) and pKH339 (*hixC*). The concentration of Hin in each lane is: *1*, 0 nM; *2*, 0.71 nM; *3*, 1.4 nM; *4*, 2.8 nM; *5*, 5.6 nM; *6*, 9.3 nM; *7*, 23.25 nM. Fifty-two pM DNA is present in each binding reaction. *b*, Hin binding assay on *hixL* and *hixC* as they are in supercoiled plasmid. *Lanes*: *1*, *Nco*I-digested pKH336; *2*, *Nco*I-digested pKH336 in the presence of 300 ng of Hin; *3*, *Nco*I-digested pKH339; *4*, *Nco*I-digested pKH339 in the presence of 300 ng of Hin. *OC*, open circular plasmid. *L*, linearized plasmid. *NL*, *Nco*I-digested large fragment. *S*, supercoiled plasmid. *NS*, *Nco*I-digested small fragment.

to *hixC*. Therefore, we devised a binding assay that would determine the affinity of Hin toward *hixL* and *hixC* when these sequences are in negatively supercoiled plasmids. Both sequences have *Nco*I restriction sites in their centers. Therefore, the ability of *Nco*I to cut supercoiled substrate plasmids in the presence of Hin was examined in a buffer that supports both Hin binding and *Nco*I activity (see "Materials and Methods"). *Nco*I cleaves both pKH336 (*hixL*) and pKH339 (*hixC*) into two DNA fragments when there is no Hin present (Fig. 2*b lanes 1* and *3*, respectively). When Hin binds to the *hix* site, it prevents *Nco*I cleavage. The amount of residual cleavage (*i.e.* fragment labeled *NL* in Fig. 2*b*) found with pKH339 is almost the same as that found with pKH336 in the presence of Hin suggesting that both *hixC* and *hixL* are equally protected by Hin binding (Fig. 2*b*). The binding affinity of Hin to supercoiled *hixL* and *hixC* was further investigated by the methylation protection assay (Richet *et al.*, 1986; Glasgow *et al.*, 1990) in which supercoiled pKH336 and pKH339 were incubated with Hin for 20 min followed by dimethyl sulfate-mediated DNA methylation. This assay also showed that there is no difference in Hin binding to *hixL* and *hixC* (data not shown). These results clearly demonstrate that Hin binds to *hixC* as well as to *hixL* even when the binding sites are in negatively supercoiled plasmids.

Hin Bound on hixC Cannot Bring Two hixC Sites Close Together—The next step after binding of proteins to their DNA-binding sites, *i.e.* Hin to *hix* sites and Fis to the enhancer, is to bring two recombination sites close together by interaction between Hin molecules, followed by productive synapsis (invertasome formation) promoted by the enhancer complex. To determine which stage of the inversion reaction is blocked in the *hixC* recombination site, we performed a series of biochemical assays that were designed to probe the steps in the Hin-mediated inversion reaction. The pairing of *hix* sites assay was used to determine if there is interaction between dimers bound on different *hixC* sites. The formation of productive synapsis was probed by the invertasome formation assay (Lim and Simon, 1992). Pairing of *hix* sites followed by invertasome formation was found when *hixL* plasmids were used (Fig. 3*a*, *lanes 2* and *3*, respectively). However, neither formation of paired *hix* sites nor invertasome formation was detected with *hixC* (Fig. 3*a*, *lanes 4* and *5*). To test whether Hin was still bound to *hixC* sites, the same reactions (as shown in *lanes 2* and *4* in Fig. 3*a*) were set

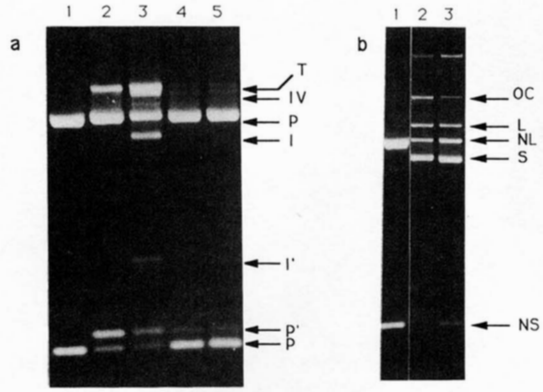

FIG. 3. **Pairing of *hix* sites and invertasome formation assays.** *a*, pairing of *hix* sites and invertasome formation assays on *hixL* and *hixC* containing plasmids. *Lanes*: *1*, *Pst*I-*Cla*I-digested pKH336; *2*, pairing of *hix* sites assay on pKH336; *3*, invertasome formation assay on pKH336; *4*, pairing of *hix* sites assay on pKH339; *5*, invertasome formation assay on pKH339. *T*, paired *hix* and Hin complex. *IV*, invertasome complex. *P*, *Pst*I-*Cla*I-digested DNA fragments. *I* and *I'*, DNA fragments generated by the low frequency inversion during the invertasome formation assay. *P'*, retarded small P band due to the bound Hin to the recombination site. The formation of P' band in the pairing *hix* sites assay on *hixC* (*lane 4*) was less than that on *hixL* (*lane 2*). If the pairing *hix* sites assay was performed without using glutaraldehyde, the amount of the P' band was the same between *hixL* and *hixC* (data not shown) which raises the possibility that the presence of glutaraldehyde in the pairing of *hix* sites assay could strip off only the *hixC*-bound Hin molecules from DNA. However, *Nco*I restriction digestion of glutaraldehyde-treated Hin and substrate plasmid rules out this possibility (*b*). Hin is still bound to *hixL* as well as to *hixC* even after the glutaraldehyde treatment (*b*, *lanes 2* and *3*, respectively). It is likely that some fraction of glutaraldehyde-treated Hin bound to *hixC* dissociates from the DNA during the initial stage of agarose gel electrophoresis. *b*, *Nco*I restriction digestion of substrate plasmids after the dialysis step of the standard pairing of *hix* sites assay. Each lane is *Nco*I digestion of *1*, pKH339 (*hixC*) absence of Hin in the reaction; *2*, pKH336 (*hixL*) in the presence of Hin; *3*, pKH339 (*hixL*) in the presence of Hin. *OC*, open circular plasmid. *L*, linearized plasmid. *NL*, *Nco*I-digested large fragment. *S*, supercoiled plasmid. *NS*, *Nco*I-digested small fragment.

up, and the *hix* sites were probed with *Nco*I restriction enzyme which cuts at *hix* sites when Hin is not bound. Fig. 3*b* shows that both the *hixC* and *hixL* reaction resulted in protection of the *Nco*I site at the center of the *hix* sequences. The *faint upper band* is presumably the paired *hix* and Hin complex formed on nicked pKH339 which corresponds to less than 5% of the plasmid preparation. This suggests that pairing of *hixC* sites might occur if pKH339 is fully relaxed (see below).

When Hin-mediated DNA cleavage was probed by the cleavage assay, (Johnson and Bruist, 1989) more than 50% of DNA substrate was cleaved at *hixL* sites; however, only background level cleavage was detected in our results with *hixC* substrates (data not shown) again indicating the failure of Hin bound to *hixC* to bring two sites together or form invertasomes.

hixC Has Unusual Secondary Structure—The possibility that there might be differences in secondary structure between *hixL* and *hixC* was probed by $KMnO_4$, which oxidizes pyrimidine bases if they are not engaged in hydrogen bonding (Hayatsu and Ukita, 1967). The oxidized bases can be detected by a primer extension reaction with the Klenow fragment of *E. coli* DNA polymerase I, which stops polymerization after the addition of a nucleotide complementary to oxidized T or C residues (Sasse-Dwight and Gralla, 1989). Fig. 4 shows the results of a $KMnO_4$ assay on *hixL* and *hixC* in negatively supercoiled plasmids in the presence and absence of Hin. DNA polymerization of $KMnO_4$-treated pKH339 stopped

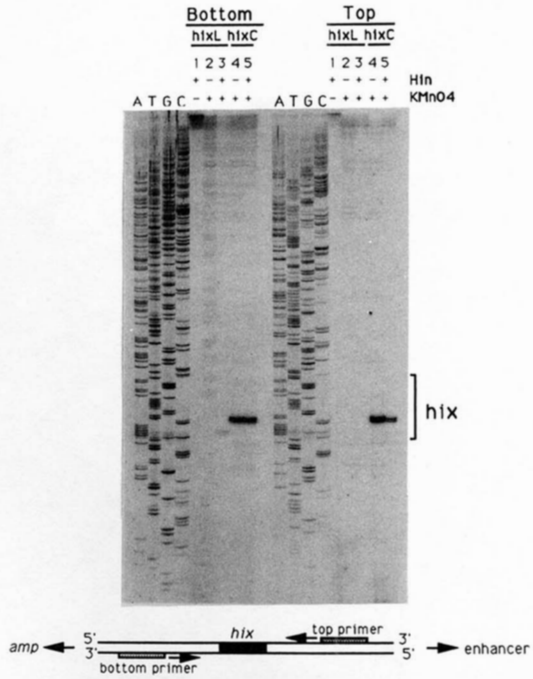

FIG. 4. **$KMnO_4$ assay on *hixL* and *hixC* containing plasmids.** Structural differences between *hixL* and *hixC* at the *Sma*I site of substrate plasmids is probed by $KMnO_4$ followed by primer extension with Klenow fragment. Each primer hybridizes to a DNA sequence that is 70 bp away from the *hix* sequence. The orientation of each primer is indicated below. ATGC are the dideoxy sequencing ladders.

after incorporation of A to complement the 1T at position 1 of *hixC* (see Table I) or the incorporation of T at position −1A in the reaction with the top primer (see Fig. 4). The same result was observed on the complementary strand with the bottom primer. Although A residues are not oxidized by $KMnO_4$, the Klenow fragment of *E. coil* DNA polymerase I tends to stop polymerization at an A residue if it is located adjacent to an oxidized T residue (Sasse-Dwight and Gralla, 1989). These results demonstrate that the central two bases (AT) in *hixC* do not form stable hydrogen bonds, whereas there is no such anomalous structure detected in the center of *hixL*. The presence of Hin induces small amounts of melting on the A residue at position −7 of *hixL* but does not seem to disturb the global structure of *hixL*.

The anomalous structure in *hixC* was also detected by S1 nuclease which was able to cut out a 0.9-kb DNA fragment between two *hixC* sites from negatively supercoiled pKH339. The 0.9-kb fragment was not observed when pKH339 was linearized, suggesting that the formation of the partially denatured structure on *hixC* forms with negative supercoiling (Fig. 5). S1 nuclease-sensitive sites were not detected on *hixL* whether the plasmid (pKH336) was linearized or negatively supercoiled.

Inversion on hixC Can be Restored by Central Base Changes—By the challenge phage assay, Hughes *et al.* (1991) showed that the central 6 base pairs (5'-CCATGG-3') of *hixC* are not involved in binding of Hin (except when −3C and 3G were changed to A and T, respectively). Therefore, the effect of base changes in the center and surrounding sequences of *hixC* on any biochemical assays can be tested without the complication of altered Hin binding to mutant *hixC* sites. The pairing *hix* sites and inversion assays were used for functional tests of base pair substitutions in the *hixC* sequences, whereas structural changes resulting from the substitutions were probed by $KMnO_4$ and S1 nuclease assays. Each of the 6 bases was changed to 3 other bases symmetrically so that all

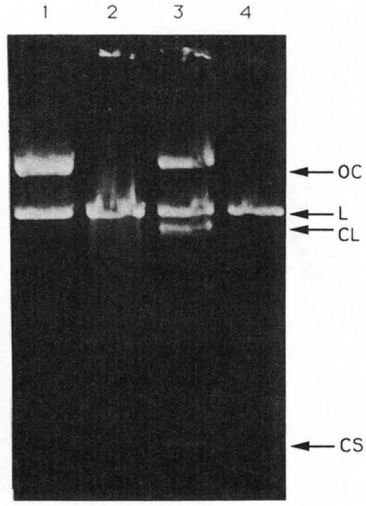

FIG. 5. **S1 nuclease assay on *hixL* and *hixC* containing plasmids.** *Lanes*: *1*, supercoiled pKH336; *2*, linearized pKH336; *3*, supercoiled pKH339; *4*, linearized pKH339. *OC*, open circular plasmid. *L*, linearized plasmid. *CL*, S1 nuclease-cleaved large DNA fragment (4.4 kb). *CS*, S1 nuclease-cleaved small DNA fragment (0.9 kb).

TABLE II

Results of assays on different recombination sites

Substrate plasmid	Structure *Sma-Hpa*	Pairing of *hix* assay[a]	Inversion assay[a]	KMnO4 assay[b]	S1 nuclease assay[b]
pKH336	hixL-hixL	100	100	N	N
pKH339	hixC-hixC	0	0	Y	Y
pKH328	hix1-hix1	0	0	Y	Y
pKH333	hix2-hix2	100	90	N	N
pKH334	hix3-hix3	100	0	N	N
pKH329	hix4-hix4	0	0	Y	Y
pKH330	hix5-hix5	0	0	Y	Y
pKH332	hix6-hix6	20	5	Y	Y
pKH340	hix7-hix7	0	0	Y	Y
pKH331	hix8-hix8	20	5	Y	Y
pKH335	hix9-hix9	10	5	Y	Y
pHL140	hixL-hixC	20	20	ND[c]	N
pHL152	hix2-hix3	70	0	ND	ND
pHL162	hix10-hix11	50	0	ND	ND
pHL163	hix10-hix10	50	0	ND	ND
pHL172	hix14-hix14	ND	5	ND	ND
pHL173	hix15-hix15	ND	5	ND	ND

[a] Numbers are % of the positive control value.
[b] Y denotes detection of anomalous structure; N denotes structure was not detected.
[c] Not determined.

9 possible *hixC* symmetric doubles were created (from *hix1* to *hix9* in Table I). All these substitutions maintain the perfect symmetry of the sites. A series of nine substrate plasmids was constructed by cloning each *hixC* symmetric double mutant into the *Sma*I and *Hpa*I sites of pKH173 (Fig. 1*a*). Results of functional and structural assays on these substrate plasmids are listed in Table II.

The most drastic changes occur in substitutions at the two central bases. Either G or C substitutions at −1A (pKH333, *hix2* and pKH334, *hix3*) restored pairing *hix* sites efficiency almost to that of the positive control (pKH336, *hixL*). Neither $KMnO_4$ nor S1 nuclease detected unpaired nucleotides in the center of these *hix* sites. The T substitution at −1A (pKH328, *hix1*) has no effect on function or structure of *hixC* (Table II). These results and the fact that *hixC*, but not *hixL*, has an anomalous structure suggest that there is a strong relationship between the formation and stability of this structure and the ability to form the paired *hix* and Hin complex. No inversion was detected on pKH334, even though this mutant restores pairing *hix* sites efficiency to that of the positive control, suggesting that a step in the inversion reaction after pairing of *hix* sites is blocked in pKH334 (see below). The effects of substitutions were less drastic as they moved to positions distant from the center. G substitutions at −2C and −3C restored pairing of *hix* sites to 20% and inversion to 5% of the positive control values. $KMnO_4$ and S1 nuclease, however, were able to detect the melted structure in these two mutants suggesting that only a small portion of the population of substrate plasmids lost the denatured structure at the center of the site, and only those were able to support pairing of *hix* sites and inversion. pHL140 which has a combination of two sites, *hixL* in the *Sma*I site and *hixC* in the *Hpa*I site of pKH173, showed 20% of pairing of *hix* sites and inversion suggesting that the *hixC* site can assume an effective conformation at a low frequency and be complemented by the more stable *hixL* site.

There are three base differences between *hixL* and *hixC* (Table I). *hix14* and *hix15* each incorporate one of the *hixL*-specific bases into *hixC*. Both break the perfect symmetry of *hixC*. In addition, one of the five consecutive A residues is lacking in *hix15*. The five consecutive A sequence has been shown to have intrinsic bending capability (Koo *et al.*, 1986). Those substrate plasmids with *hix14* and *hix15* (pHL172 and 173, respectively) restored inversion to 5% of the positive control value, suggesting that it is not a specific base change but the sum of all three base changes that leads to loss of function in *hixC*.

Inversion on hixC Can Also be Restored by Lowering Superhelical Density—The formation of an anomalous structure at the center of *hixC* only occurs when the plasmid is negatively supercoiled (Fig. 5). This raises the possibility that by lowering the superhelical density of the *hixC* substrate plasmid pKH339 pairing of *hix* sites and inversion could be restored. Therefore, we have generated a series of topoisomers of pKH339 with defined superhelical densities from 0 to −0.1 using different amounts of ethidium bromide with topoisomerase I (see "Materials and Methods"). The physiological superhelical density (σ) of pKH339 in this study is −0.075.

Lowering the superhelical density restores the pairing of *hix* sites and inversion on *hixC*. The pairing of *hix* sites reaches maximum at a σ value of −0.030 and decreases as σ approaches the physiological density (Fig. 6*a*). We have previously shown that pairing *hix* sites on *hixL* reaches maximum at a $\sigma = -0.025$ and remains constant even at greater than the physiological value of σ (Lim and Simon, 1992). Because the Hin-mediated inversion requires negative supercoiling of DNA, inversion on *hixC* starts to occur at a $\sigma = -0.030$, and increases slightly with supercoiling, but does not occur at all after the physiological superhelical density (Fig. 6*b*).

The efficiency of the *hixC* inversion reaction was calculated by the method described in Bruist and Simon (1984) and was compared with data obtained in similar experiments with *hixL* (Lim and Simon, 1992). Fig. 7 demonstrates that at lower superhelical densities (from −0.025 to −0.05), inversion on *hixC* occurs as well as on *hixL*. After a σ value of −0.05, however, inversion on *hixC* starts to drop and disappears completely whereas that on *hixL* continues to increase and remains elevated even after the physiological σ value (−0.072), suggesting that the sufficient negative supercoiling to drive the transition from a normal B-DNA conformation to the melted structure corresponds to a linking deficit of 23 in pKH339 ($\sigma = -0.046$).

Strand Cleavage Does Not Occur on hix3 Recombination Site—As shown earlier, G or C substitutions in place of −1A of *hixC* restored pairing of *hix* sites to the level of the positive

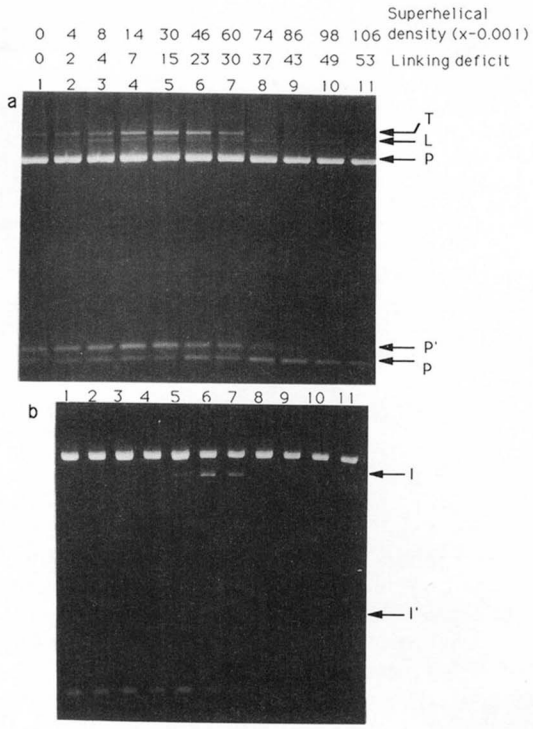

FIG. 6. **The pairing of *hix* sites (*a*) and inversion (*b*) assays on topoisomers of pKH339 (*hixC*).** The average superhelical density of each topoisomer and the corresponding linking deficit are the same between *a* and *b*. *T*, paired *hix* and Hin complex. *L*, linearized plasmid. *P*, *Pst*I-*Cla*I-digested DNA fragments. *P'*, retarded P band. *I* and *I'*, *Pst*I-*Cla*I-digested DNA fragments due to inversion.

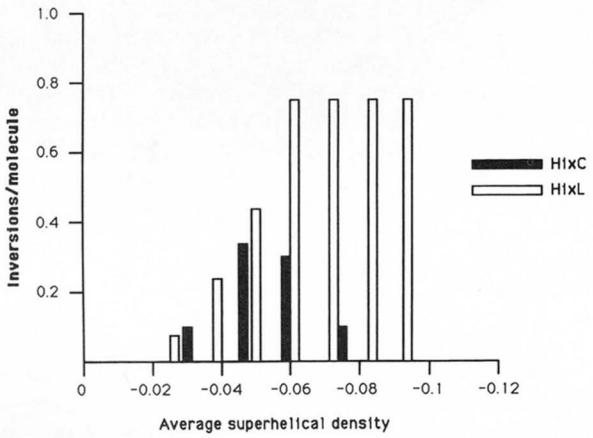

FIG. 7. **Dependence of the Hin-mediated inversion reaction on the average superhelical density of pKH336 (*hixL*) and pKH339 (*hixC*).** The negative from a photograph of the inversion assays gel (Fig. 5*b*) was scanned with a laser densitometer and a previously published method (Bruist and Simon, 1984) was used to quantitate the inversion efficiency as inversions/molecule.

control, but only the G substitution gives rise to inversion (Table II). Since the invertasome formation assays show that productive synaptic complexes form with both the G and C substitutions (*hix2* and *hix3*, respectively, data not shown), a cleavage assay was performed on *hix2* and *hix3*. An intermediate which has passed the strand cleavage stage accumulates in the presence of ethylene glycol and no Mg^{2+} in inversion buffer (Johnson and Bruist, 1989). Cleavage on both strands can be detected by either proteinase K digestion followed by agarose gel electrophoresis of cleaved DNA fragments or extension of two different labeled primers hybridized to the top and bottom strands. Hin makes a concerted two-base staggered cut at the center of the recombination site, producing a 3′ overhang. Fig. 8 shows the results of cleavage assays detected by the primer extension method, performed on various *hix* sequences. Efficient cleavage by Hin was detected on pKH336 (*hixL*), and the major cleavage site is consistent with the results of cleavage assay of the wild type *hixL* substrate plasmid (Johnson and Bruist, 1989). No cleavage was detected on either strand of *hix3*, whereas 50% of control cleavage of *hixL* was detected in *hix2* (*lanes 4* and *3*, respectively).

These results demonstrate that if a G is on the 3′ side of the cleavage site, then Hin is not able to cleave the phosphodiester bond between the G and the next base to the 3′ side. A similar result was observed in the $\gamma\delta$ resolvase system. $\gamma\delta$ resolvase could not cut the DNA when G is one of the two central bases close to the cleavage site (Falvey *et al.*, 1988). The same result was observed when cleaved DNA fragments were subjected to agarose gel electrophoresis (data not shown). pKH333 (*hix2*) showed 80% of the cleavage observed with the positive control. No cleavage was detected on pKH334 (*hix3*) suggesting that cleavage does not occur at either *hix3* sites in pKH334.

To investigate further the effects of central dinucleotide in the Hin-mediated cleavage reaction, a few combinations of G

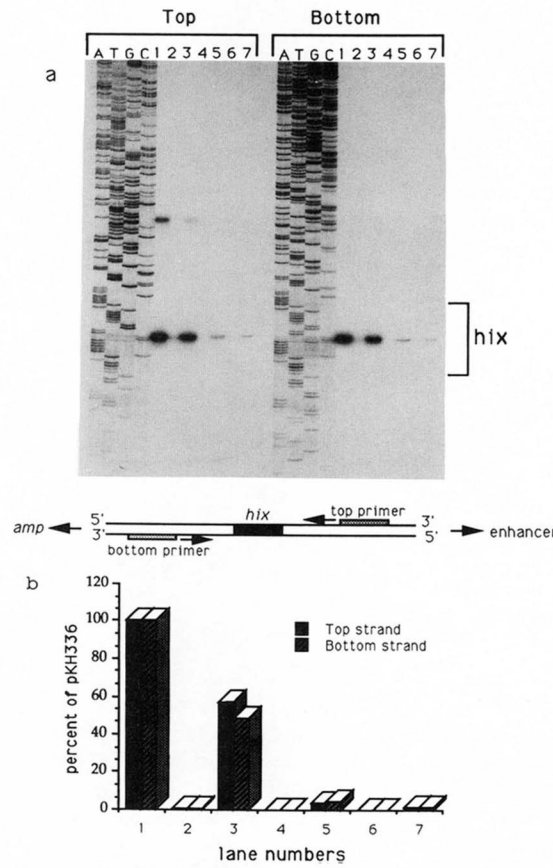

FIG. 8. **The Hin-mediated DNA cleavage reaction.** *a*, the cleavage assay was performed on a series of substrate plasmids and DNA cleavage at the *hix* site located in the *Sma*I site of the substrate plasmid was probed by the primer extension with the same primer used in the $KMnO_4$ assay. It is not clear why another weak cleavage was detected in the vector 58 bp away from the major cleavage site. The minor cleavage site maps at 4324 in pBR322 DNA sequence (Sutcliffe, 1979). The DNA sequence around the minor cleavage site has no similarity to *hix*. *b*, quantitation of the DNA cleavage of each lane was performed by the Phosphoimager (Molecular Dynamics) with ImageQuant™ software (V 3.0) system. *Lanes*: *1*, pKH336 (*hixL*); *2*, pKH339 (*hixC*); *3*, pKH333 (*hix2*); *4*, pKH334 (*hix3*); *5*, pHL152 (*hix2* × *hix3*); *6*, pHL162 (*hix10* × *hix11*); *7*, pHL163 (*hix10*).

and C substitutions in the central dinucleotide were made (Table II). pHL152 contains *hix2* (cleavable on both strands) at the *Sma*I site and *hix3* (uncleavable on either strand) at the *Hpa*I site. pHL162 and 163 were designed so that at least one strand is cleavable at both *hix* sites (Table II). When the Hin-mediated cleavage of these plasmid was probed by primer extension, none of them showed any significant cleavage at both strands (Fig. 8). However, these plasmids all showed significant levels of pairing *hix* sites (70, 50, and 50% of the positive control, respectively; Table II). These data demonstrate that Hin will not cleave any DNA strands if at least one out of four strands is not cleavable.

DISCUSSION

Why Cannot Hin Bring Two hixC Sites Together?—It was a surprising result that there is a 16-fold difference in the inversion rate between plasmids containing *hixL* and those with the perfectly symmetric recombination site *hixC* (Fig. 1*b*). In the related Gin-mediated inversion system, the perfect palindromic *gix* recombination site (psym2) showed 4-fold less inversion than the wild type *gix* site (Mertens *et al.*, 1988). The challenge phage assay (Hughes *et al.*, 1988), the gel retardation assay (Fig. 2*a*), *Nco*I cleavage assay on Hin-bound supercoiled plasmid substrates (Fig. 2*b*), and the methylation protection assay on supercoiled substrate plasmids, all demonstrate that Hin binds to *hixC* as well as to *hixL*, even when the sites are located in the negatively supercoiled plasmid. Therefore, it is evident that the Hin-mediated inversion reaction on *hixC* is blocked after the binding of Hin to DNA. Hin-mediated cleavage on *hixC* substrate plasmid (pKH339) probed by the primer extension method showed less than 1% of the positive control value (Fig. 8*a*), supporting the idea that the inversion reaction is blocked before the cleavage stage. The pairing of *hix* sites assay demonstrated that Hin bound to *hixC* has difficulty in bringing two *hixC* sites together to form the presynapsis complex which has been suggested to be the stage before invertasome formation (Heichman and Johnson, 1990).

Interaction between Hin dimers to form a presynaptic complex seems to occur only when they are bound to DNA. It was initially thought that Hin bound to *hixC* does not acquire the proper conformational change that is necessary for the pairing of *hix* sites. However, the observation that G and C substitutions in the center of *hixC* restored the formation of paired *hix* and Hin complex strongly argues against this possibility. Since the central four bases of *hixC* are not involved in Hin binding (Hughes *et al.*, 1991), it is hard to conceive of how base substitutions in the center of *hixC*, where actual contact with Hin is not made, could result in conformational changes in Hin. The $KMnO_4$ assay showed that there is a structural difference between *hixL* and *hixC*. The central AT base pairs are not engaged in hydrogen bonding in *hixC* (Fig. 4). The S1 nuclease treatment demonstrated that the anomalous structure at the center of *hixC* can be cleaved by S1 nuclease, and, furthermore, the structure only occurs when the plasmid is negatively supercoiled. Negative supercoiling promotes unusual DNA structures such as cruciforms (Lilley *et al.*, 1988) or melting in AT-rich regions (Kowalski *et al.*, 1988). *hixC* can adopt a cruciform structure because of its perfectly inverted symmetrical nature. However, the following data argue against this possibility. First, Hin would not be expected to bind to the cruciform-structured *hixC* site, since the center of the binding site is now at the tip of the cruciform. In fact, Hin was shown to be incapable of binding to a hairpin-structured *hixC* which formed spontaneously during preparation of the DNA in a gel retardation assay with *hixC* sequences (Hughes *et al.*, 1991; it was also shown that Hin binds well to the same sequence if it is prevented from forming a hairpin structure (Hughes *et al.*, 1991)). Second, our Hin binding assay and methylation protection assay showed that Hin binds to *hixC* as well as to *hixL* even when they are in supercoiled plasmid form and that the structure exists even when Hin is bound. Thus, the anomalous structure in the center of *hixC* cannot be a cruciform structure. We think that the anomalous structure is formed because DNA is underwound, driven by negative supercoiling. The center of *hixC* may resemble the "central bubble" structure in the proposed mechanism for S-type cruciform extrusion (Murchie and Lilley, 1987) where the major anomaly is in the center of sequences.

The symmetrical G or C substitution at −1A position of *hixC* (*hix2* and *hix3*, respectively) can both restore pairing of *hix* sites on pKH333 and pKH334 and at the same time remove the central "melted" structure from *hix2* and *hix3*. These results suggest that Hin cannot bring two *hixC* sites together due to the presence of this structure. This notion is further substantiated by the pairing of *hix* sites assay of topoisomers of pKH339 (*hixC*), which showed that two *hixC* sites can come together at a lower superhelical density when the melted structure does not exist. However, as more supercoiling is introduced the formation of paired *hix* sites drops and does not occur at all when the superhelical density reaches the value at which the stable melted structure is found.

Because *hixC* has an anomalous structure at the center and the negative supercoiling promotes the formation of this structure, we propose the following possible explanations as to why Hin dimers cannot bring two *hixC* sites together (these possibilities are not necessarily mutually exclusive). First, the two subunits of Hin dimer may not reside on the same face of DNA at *hixC*, due to the underwound structure of *hixC* driven by negative supercoiling. It has been suggested that the two subunits bind to the same side of DNA at *hixL* (Glasgow *et al.*, 1989). However, with reduced affinity, Hin is still able to bind to a mutant *hix* sequence which has one additional base pair in the center (Glasgow *et al.*, 1989). Addition of an extra base pair in the center of *hix* sequence twists two half binding sites by 36°. This mutant *hix* site cannot support inversion (Johnson and Simon, 1985). These data suggest that the Hin dimer is flexible enough to bind to the twisted half-sites but without function. Because Hin binds to *hixC* with the same affinity as to *hixL*, we propose that the relative twist angle between two subunits bound to *hixC* is more than 0° and less than 36°, Second, Hin may not be able to induce the appropriate bending at the center of *hixC* due to the formation of the melted structure. In the $\gamma\delta$ resolvase and Gin systems, protein binding to site I and *gix* site, respectively, induces DNA bending at the center of the binding sites, and the protein-induced DNA bending is required for the systems to work (Hatfull *et al.*, 1987; Mertens *et al.*, 1988). Thus, a specific bent configuration may be required in the Hin-Hin interactions that bring two recombination sites close together. The existence of the denatured structure at the center of the *hixC* site could eliminate this "critical" step.

DNA Cleavage Activity of Hin Is Coordinated—The anticipated result of the cleavage assay with pHL152 (*hix2* × *hix3*) was a linearized plasmid product cut at the *hix2* site, but no cleavage was observed at either *hix* sites (data not shown). Furthermore, no significant cleavage was observed on any strands of pHL152 when probed by primer extension (Fig. 8). A nick only on the cleavable strand of pHL162 and 163 might have been expected, but no nick was observed on either of the substrates. These results suggest that in the invertasome if

any of four strands is not cleavable, no cleavage by Hin occurs at all. Generation of DNA ends that would not be complementary for ligation in the inverted configuration, however, does not prevent cleavage by Hin. Thus, for example, a substrate plasmid containing one *hix* site with the sequence AA in its center and with AT in the center of the other *hix* site was cleaved efficiently by Hin, but no inversion was detected because the two cleaved ends were not compatible for efficient ligation (Johnson and Simon, 1985; Johnson and Bruist, 1989). $\gamma\delta$ resolvase, however, does not show coordinated cleavage activity on a mutant binding site I where one strand is cleavable and the other strand is not. It is able to make a single nick on the cleavable strand. As a result, an extensive relaxation of the mutant substrate plasmid through topoisomerase I-like activity of $\gamma\delta$ resolvase was detected (Falvey *et al.*, 1988). No such relaxation of any mutant substrate was observed by Hin (data not shown). This suggests that in the Hin-mediated inversion reaction, the cleavage and strand rotation reactions are highly concerted and occur in a tightly coordinated fashion.

Hin recombinase belongs to a family of minor groove DNA-binding proteins that include the homeodomain proteins in eukaryotic cells (Affolter *et al.*, 1991). Our present studies suggest that the ability of these proteins to interact with the other DNA-binding proteins to mediate recombination and perhaps to initiate transcription involves not only the base pairs that contact the protein directly but also the context of adjacent sequences. A simple base pair change can markedly influence the dependence upon superhelical density and the function of the proteins. Thus, while a large variety of proteins may recognize and bind efficiently to similar DNA sequences, their precise function can be sensitively modulated by adjacent sequence and structure.

Acknowledgments—We thank Robert Bourret and Jacques J. Pene for help in preparing this manuscript.

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