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Intermediates in Hin-mediated DNA inversion: a role for Fis and the recombinational enhancer in the strand exchange reaction

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The site-specific inversion reaction controlling flagellin synthesis in *Salmonella* involves the function of three proteins: Hin, Fis and HU. The DNA substrate must be supercoiled and contain a recombinational enhancer sequence in addition to the two recombination sites. Using mutant substrates or modified reaction conditions, large amounts of complexes can be generated which are recognized by double-stranded breaks within both recombination sites upon quenching. The cleaved molecules contain 2-bp staggered cuts within the central dinucleotide of the recombination site. Hin is covalently associated with the 5′ end while the protruding 3′ end contains a free hydoxyl. We demonstrate that complexes generated in the presence of an active enhancer are intermediates that have advanced past the major rate limiting step(s) of the reaction. In the absence of a functional enhancer, Hin is also able to assemble and catalyze site-specific cleavages within the two recombination sites. However, these complexes are kinetically distinct from the complexes assembled with a functional enhancer and cannot generate inversion without an active enhancer. The results suggest that strand exchange leading to inversion is mediated by double-stranded cleavage of DNA at both recombination sites followed by the rotation of strands to position the DNA into the recombinant configuration. The role of the enhancer and DNA supercoiling in these reactions is discussed.

Key words: DNA cleavage/DNA supercoiling/nucleoprotein complexes/recombinational enhancer/site-specific DNA recombination

Introduction

Site-specific recombination reactions are found in a variety of genetic contexts and mediate the conservative joining or rearranging of DNA sequences in a highly precise manner. These reactions are distinguished in part from homologous recombination reactions in that extensive homology within the region of DNA strand exchange is not required. Specialized enzymes (recombinases) catalyze the rearrangement of DNA at specific loci. Several of these reactions can be studied *in vitro*. These systems vary as to their degree of complexity and almost certainly with respect to the mechanics of the reaction (for review see Sadowski, 1986; Craig, 1988).

Hin is a member of a family of recombinases which catalyze site-specific inversion. Hin regulates flagellin synthesis in *Salmonella* by switching the orientation of a promoter contained on a 1-kb DNA segment in the chromosome (Silverman and Simon, 1980; Zieg and Simon, 1980). Two homologous enzymes, Gin and Cin, control the host range of phage Mu and P1 respectively, by activating different tail fiber protein genes encoded in an invertible DNA segment (Kamp *et al.*, 1979; Iida *et al.*, 1982). These enzymes share 60–70% amino acid sequence identity and have been shown to function on each others' substrate (for review see Plasterk and van de Putte, 1984). One of the distinguishing features of the DNA invertases is their requirement for a recombinational enhancer sequence (Huber *et al.*,1985; Johnson and Simon, 1985; Kahmann *et al.*, 1985). This sequence can function at many positions on a plasmid substrate to stimulate inversion. Substrates lacking this sequence recombine at $<1\%$ of the wild-type rate.

DNA strand exchange occurs within a 26-bp sequence which has been defined in the Hin system as the minimal recombination site (Johnson and Simon, 1985). This sequence consists of two imperfect repeats separated by two nucleotides (see Figure 1). Hin binds to the recombination site as a dimer, interacting with nucleotides within each of the inverted repeat segments (Glasgow *et al.*, submitted). A spacing of two nucleotides between the inverted repeats is critical for site function. The sequence of these nucleotides can vary as long as it is identical in the two recombination sites. The DNA substrates containing recombination sites with non-identical base pairs within the central residues that have been tested do not support efficient inversion (Johnson and Simon, 1985). The biochemical consequences of sequence differences within the core nucleotides is explored further in this report.

In addition to the Hin protein, which is shown in this paper to peform the cleavages and ligations required for strand exchange, two other cellular proteins are required for high rates of inversion. These include Fis, a sequence-specific DNA binding protein, and HU, a non-specific DNA binding protein (Johnson *et al.*, 1986). Fis is a 98 amino acid protein which binds to two sites within the 60-bp recombinational enhancer sequence (Bruist *et al.*, 1987; Johnson *et al.*, 1988; Koch *et al.*, 1988). Mutations which prevent Fis binding at either of these two sites result in loss of enhancer function. The relative position of these binding sites on the DNA helix has been shown to be critical for activity (Johnson *et al.*, 1987). This finding, combined with topological considerations and the observation that the enhancer cannot function when located very close to one of the recombination sites, has lead to the proposal that Fis proteins bound at the enhancer domains are interacting with Hin proteins bound at the recombination sites to promote inversion (Bruist *et al.*, 1987; Johnson *et al.*, 1987; Johnson and Simon, 1987). HU is thought to facilitate the required bending of the DNA

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needed to achieve this interaction. The magnitude of HU stimulation of the reaction varies with respect to the location of the enhancer. Substrates containing large segments of intervening DNA between the enhancer and recombination sites are less dependent on HU than substrates where the enhancer is close to one of the recombination sites (Johnson *et al.*, 1986).

A related group of site-specific recombinases are the resolvases found associated with the Tn*3* family of transposons (Grindley and Reed, 1985). These enzymes share ~30% amino acid homology with the DNA invertases and, like the DNA invertases, also require a supercoiled DNA substrate. However, the resolvases catalyze deletions *in vitro* without the need for additional host factors. In addition, the resolvase recombination site is far more complex than the recombination site for inversion.

In all site-specific recombination reactions, proteins must assemble the recombination sites into a highly precise complex which aligns the DNA strands. Strand exchange can proceed by one of two fundamentally different pathways: (i) sequential single-stranded nicks and strand transfer reactions involving a Holliday intermediate or (ii) concerted cleavages in both strands generating a transient intermediate containing eight ends of DNA, followed by ligation in the recombinant configuration. The experiments reported here support the latter pathway for Hin-mediated inversion. These experiments also address the mechanism of action of the recombinational enhancer and the role of DNA supercoiling in the reaction.

Results

pMS551 (Figure 1) is used as the wild-type substrate plasmid. It is a derivative of pBR322 with a fragment containing *hixL* and the enhancer at its normal position substituted between the *Eco*RI and *Hin*dIII sites. A second recombination site (*hixL* sequence) in the appropriate orientation to generate inversion is located at the *Sal*I site. Incubation of pMS551 with Hin, Fis and HU under optimal conditions for recombination (with Mg^{2+}) results in the invertible segment being inverted to an equilibrium state where both orientations are equally represented (see Johnson *et al.*, 1986). If the products of the reaction are electrophoresed in an agarose gel without prior digestion with restriction enzymes, numerous topoisomers are observed (Figure 2, lane 2). These topoisomers are the consequence of many strand exchange events which cause a decrease in the linking number of the supercoiled plasmid DNA. This activity is dependent on the three proteins (Hin, Fis and HU) which are required for high rates of inversion (Figure 2, lanes 3–5). However, as has been observed with inversion, significant topoisomerase activity can be measured in the absence of HU when the enhancer is located >500 bp from either recombination site (data not shown).

Isolation of complex containing double-stranded cleavages within the recombination sites

Previous studies with resolvase have demonstrated that a protein–DNA adduct accumulates in reactions lacking Mg^{2+} (Reed and Grindley, 1981). The absence of Mg^{2+} from the Hin inversion reaction combined with the addition of ethylene glycol results in a reduced rate of inversion and the loss of topoisomerase activity. The product that is generated under these conditions is recognized by the formation of novel electrophoretic products after quenching the reaction and digestion with proteinase (Figure 2, lane 6). The use of proteinase is necessary to generate discrete bands upon electrophoresis since the Hin protein is covalently associated with one of the DNA ends (see below). One of the cleavage products co-migrates with linear pMS551. The

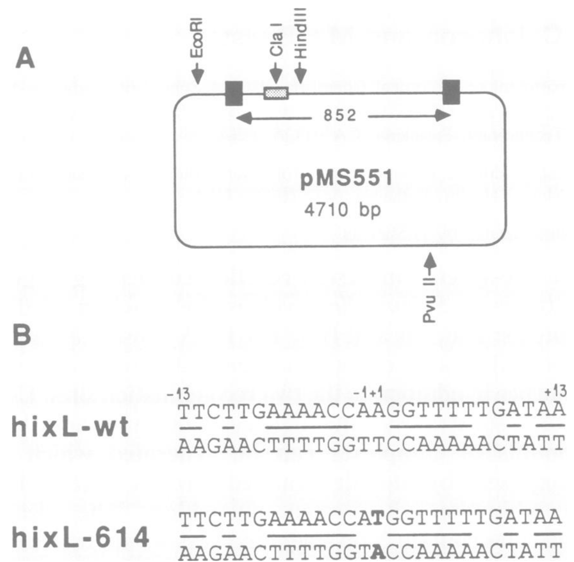

Fig. 1. Structure of pMS551 and sequence of the recombination sites. (**A**) pMS551 is a derivative of pBR322 and contains two *hixL* recombination sites (filled boxes) in inverted orientation with respect to each other. The stippled box denotes the recombinational enhancer segment. pMS614 has the same structure except that the recombination site distal to the enhancer contains a base substitution mutation. (**B**) The sequence of the wild-type *hixL* recombination site and the mutant site present on pMS614 is depicted. The bars indicate the nucleotides in an inverted repeat structure.

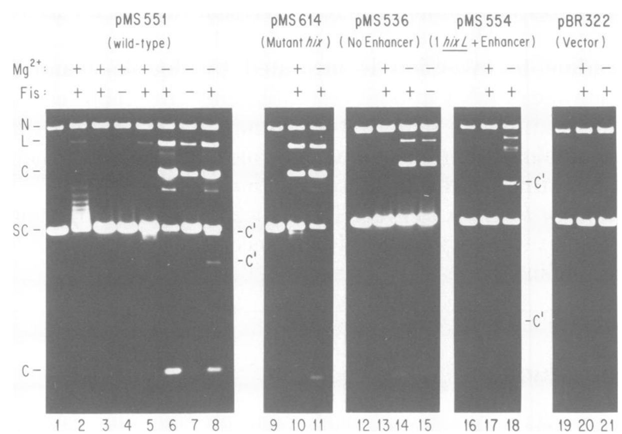

Fig. 2. Hin reactions on different substrates. The plasmids were incubated with Hin, HU (with the exceptions noted below) and Fis as denoted above the lanes. $+Mg^{2+}$ indicates normal recombination solution conditions while $-Mg^{2+}$ indicates reactions performed without Mg^{2+} and in the presence of 10 mM EDTA and 30% ethylene glycol. **Lanes 1, 9, 12, 16** and **19** contain unreacted supercoiled DNA. **Lane 3**, reaction performed in the absence of Hin; **lanes 5** and **8**, reactions performed in the absence of HU. The products of the reactions were electrophoresed in 1% agarose gels and visualized under UV transillumination after staining with ethidium bromide. The locations of the supercoiled (SC), linear (L) and nicked (N) plasmid forms, and the major (C) and some minor (C') Hin-cleaved fragments are denoted.

two other major products (labeled C) migrate at ~850 bp and ~3850 bp. These correspond to the lengths of DNA separating the recombination sites (Figure 1) and suggest that double-stranded cleavages have occurred at these sites. This was confirmed by digesting the products of the Hin-cleavage reaction with *Hin*dIII which cleaves within the invertible segment 212 bp from the center of one recombination site and 640 bp from the center of the other recombination site (Figure 1). The resulting fragments were end-labeled, digested with proteinase K and electrophoresed in a native polyacrylamide gel. As shown in Figure 3, lane 4, fragments corresponding to 212 and 640 bp were obtained, demonstrating that double-stranded cleavages are occurring under these conditions within the recombination sites. In these cleavage reactions, little increase in the amount of molecules migrating as nicked circles is observed (Figure 2) indicating that the cuts are almost exclusively double-stranded. Similar Hin-generated cleavages have been observed in the other wild-type recombination site, *hixR* (data not shown).

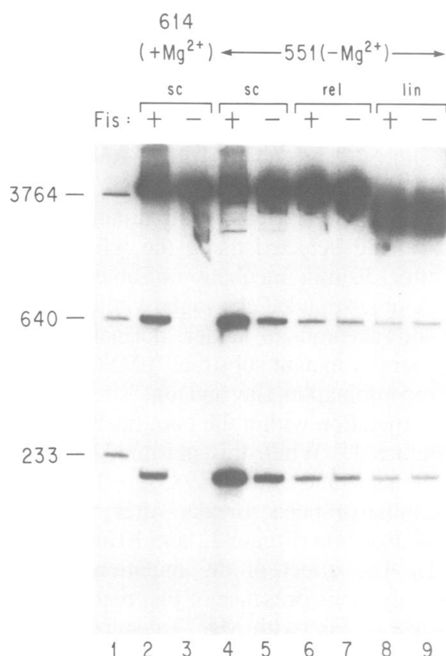

Fig. 3. Polyacrylamide gel electrophoresis of cleavage products. Supercoiled (sc), relaxed (rel) or linearized (lin) pMS551 or pMS614 were incubated under normal recombination conditions ($+Mg^{2+}$) or with 10 mM CDTA +30% ethylene glycol ($-Mg^{2+}$) in the presence or absence of Fis as denoted. Plasmid DNA was relaxed using topoisomerase I and linearized by digestion with *Pvu*II. The DNA was isolated, digested with *Hin*dIII and end-labeled with [α-^{32}P]ATP using reverse transcriptase. The products were electrophoresed in a 5% native polyacrylamide gel after digestion with proteinase K and autoradiographed. **Lane 1** shows end-labeled size markers.

While the most efficient conditions for generating the cleavage products involve the combination of the absence of Mg^{2+} and the presence of high concentrations of ethylene glycol or glycerol, either of these two conditions alone greatly increase the number of cleaved DNA molecules (data not shown). Under normal *in vitro* recombination conditions, only 0.2% of the DNA molecules can be isolated in a cleaved configuration using the wild-type substrate (Table I). Presumably, the absence of Mg^{2+} and the presence of ethylene glycol either induce or stabilize a nucleoprotein complex that is normally only transiently present during the reaction. The cleaved DNA is detected after terminating the reaction using a variety of protein denaturing agents or conditions, followed by digestion with proteinase K to remove covalently bound protein. Quenching the reaction with 0.5% SDS, 0.1 M HCl, 0.1 M NaOH, 0.05% diethylpyrocarbonate (DEP), 75% ethanol, or by incubation at 65°C, or with proteinase K all yield cleaved molecules. While there is some difference in yields depending on the denaturing agent, in general, the faster the reaction is quenched, the greater the yield. Thus, quenching with SDS or HCl results in molecules primarily cleaved at both recombination sites while incubation at 65°C or with proteinase results in molecules primarily cleaved at one

Table I. Double-stranded cleavage and recombination proficiency of substrate molecules

DNA	Topology[a]	Mg^{2+} [b]	Fis[c]	% cleaved[d]	Recombination[e]
pMS551 (wild-type)	sc	+	+	0.2	1.0
	sc	+	−	<0.01	<0.02
pMS614 (AA × AT)	sc	+	+	14.3	<0.02
	sc	+	−	<0.01	<0.02
pMS551	sc	−	+	51.4	0.07
	sc	−	−	11.3	<0.02
pMS551	rel	−	+	4.2	<0.02
	rel	−	−	4.9	<0.02
pMS551	lin	−	+	3.0	<0.02
	lin	−	−	3.3	<0.02
hixL fragment[f]	lin	+	+	<0.01	
	lin	−	+	0.3	
	lin	−	−	0.2	

[a]sc, supercoiled; rel, relaxed using topoisomerase I; lin, linear.
[b]+, normal recombination conditions containing 10 mM Mg^{2+}; −, reaction in the presence of 10 mM CDTA + 30% ethylene glycol.
[c]Fis present (+) or absent (−) from the reaction.
[d]The DNA was 3′ end-labeled at the *Hin*dIII site after reaction for 30 min with Hin, HU and Fis (as denoted), electrophoresed in an acrylamide gel and the radioactivity in each band quantitated. The number of molecules containing double-stranded cleavages at both recombination sites/total number of substrate molecules × 100 is given.
[e]Numbers given are recombination rates relative to supercoiled pMS551 measured under optimal recombination conditions ($+Mg^{2+}$, +Fis) in which the number of inversions per substrate molecule after 2 min was 0.4.
[f]192 bp 3′ end-labeled fragment containing one *hixL* site.

recombination site. These results are most consistent with the DNA being cleaved prior to the time of quenching (Ray and Long, 1976; Vinkler *et al.*, 1978) with the strands being held together via protein linkage. The alternative in which the cleavage of DNA is induced by protein denaturation has been considered in reactions with topoisomerase (e.g. Liu and Wang, 1979). However, cleavage of single-stranded DNA by the eukaryotic topoisomerase I enzyme as well as site-specific cleavage by the lambda Int protein has been detected without the use of protein denaturants (Been and Champoux, 1981; Halligan *et al.*, 1982; S.Nunes-Düby and A.Landy, personal communication). In both these cases, covalent protein–DNA adducts are formed which can undergo further reaction.

The formation of complexes probably requires two recombination sites

The majority of cleaved molecules have double-stranded breaks at both recombination sites although a significant number of linear molecules are observed. This implies that the predominant reaction involves the association of two recombination sites into a complex in which cleavages at the two sites occur. To address this further, we assayed Hin-mediated cleavage of a substrate that contains just one *hixL* site and an enhancer (pMS554). In normal recombination conditions, this plasmid supports no detectable inversion and only a very small amount of topoisomerase activity (Figure 2, lane 17). In reaction conditions containing EDTA and ethylene glycol, several discrete products are produced. These appear to be due to the recruitment of secondary Hin binding sites which are capable of associating with a wild-type *hixL* at a low frequency. The major cleavage products denoted in Figure 2, lane 18 (labeled C′) migrate as 3530- and 1310-bp fragments. Low amounts of similar size fragments can also be seen with the wild-type substrate (e.g. Figure 2, lane 6). These sizes, combined with additional mapping experiments (not shown) position the second site at ~3200 on the pBR322 sequence. This site would thus correspond to one of the major secondary sites (*cixQ3* or *cixQ4*, pBR322 coordinants 3216 and 3248 respectively) that is recruited by the homologous DNA invertase Cin (Iida and Hiestand-Nauer, 1986).

To avoid the problem of secondary Hin binding sites, we incubated Hin with a 192-bp fragment containing only *hixL*. In the absence of Mg^{2+}, extremely low amounts of cutting were observed (Table I). This cleavage is much less than that measured in the absence of Fis on a linear plasmid containing two recombination sites (see below) and may represent the inefficient association of two fragments by Hin. The inefficiency of the reaction with the *hixL* fragment combined with the analysis of pMS554 argue that the generation of cleaved molecules involves the association of two recombination sites.

Effect of HU on complex formation

Cleavage reactions performed in the absence of HU with pMS551 result in a 4- to 5-fold slower rate of cleavage but accumulate large numbers of cleaved molecules over long incubation times (Figure 2, lane 8). The amounts of two minor cleavage products (labeled C′) are significantly greater under these conditions. The sizes of these products are consistent with cleavages at the *hixL* located at the *Sal*I site and one of the secondary recombination sites located at ~3200 on the pBR322 map. Thus, in the absence of HU, formation of complexes containing cleaved DNA involving the recombination site adjacent to the enhancer is decreased and the relative amount of complexes formed involving sites located >650 bp from the enhancer is increased. This is consistent with the effect of HU on inversion with respect to the location of the enhancer (Johnson *et al.*, 1986).

Complexes formed with non-homologous recombination sites

The complexes described above were isolated in the presence of EDTA and ethylene glycol, conditions which are not optimal for inversion. In optimal reaction conditions where Mg^{2+} is present, only extremely small numbers of cleaved molecules can be detected using the wild-type substrate (~0.2% after 30 min incubation, Table I). Complexes generating double-stranded cleavages within the recombination sites do accumulate under normal recombination conditions using a mutant substrate. pMS614 contains one wild-type recombination site and one site with a T to A transversion mutation within the core nucleotides (position +1, see Figure 1). While this plasmid does not support inversion (Johnson and Simon, 1985), ~15% of the DNA contains double-stranded breaks after denaturing and proteinase K digestion (Figure 2, lane 10 and Figure 3, lane 2, Table I). The effect of the mutation in pMS614 is manifested only in the presence of Fis; reactions performed in the absence of Fis (with Mg^{2+}) result in no detectable DNA cleavage (Figure 3, lane 3). As shown below, the pMS614 complexes formed with Fis can be chased into uncleaved substrate by relaxing the supercoiling. A substrate (pMS631) with both recombination sites containing the mutation in pMS614 supports high rates of recombination and does not accumulate cleaved molecules. The failure to detect cleavage products previously with pMS614 (Johnson and Simon, 1985) presumably reflects the use of partially purified components and limiting Fis concentrations resulting in a less efficient reaction.

Complexes formed in the absence of Fis

Complexes generating site-specific cleavages of DNA within the recombination sites can be formed in the absence of Fis under minus Mg^{2+} plus ethylene glycol reaction conditions. After a 30-min reaction period, 11% of the supercoiled plasmids contain double-stranded breaks at both recombination sites (Figure 2, lane 7 and Figure 3, lane 5; Table I). This amount of cleavage is much greater than the amount of inversion seen without Fis (<1% after 30 min, Johnson *et al.*, 1986) and indicates that a Hin-mediated complex capable of site-specific cleavage of DNA can occur in the absence of an active enhancer. The ability of Hin to cleave DNA in the absence of Fis and the enhancer is further demonstrated using pMS536 which contains the two recombination sites separated by 684 bp without an enhancer. As shown in Figure 2, lane 15, a 680-bp fragment representing the invertible segment is generated from pMS536 in the absence of Fis. The addition of Fis has little effect on the amount of product generated from pMS536 (Figure 2, lane 14). The formation of complexes without Fis requires the presence of two recombination sites on the substrate; no cleavages are detected in reactions lacking Fis using pMS554 (data not shown).

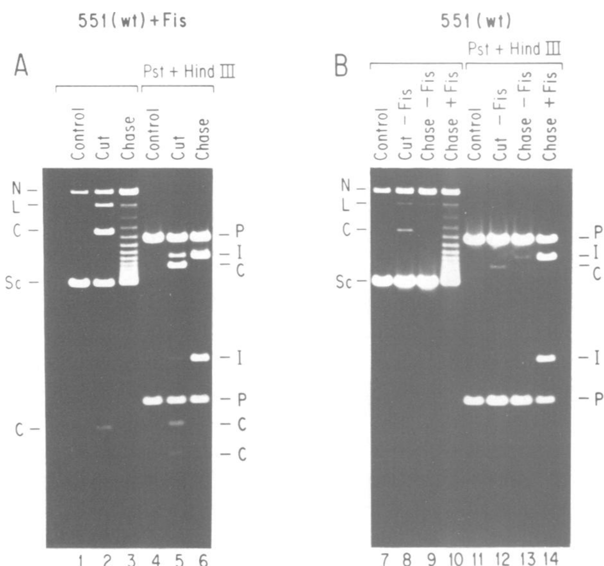

Fig. 4. Cleavage-chase experiments in the presence or absence of Fis. In **lanes 1–3** and **7–10** the samples were electrophoresed in a 1% agarose gel without prior digestion with restriction enzymes. In **lanes 4–6** and **11–14** the samples were digested with *Pst*I and *Hin*dIII prior to electrophoresis to assay inversion. The bands are labeled as follows: N, nicked plasmid form; L, linear plasmid form; Sc, supercoiled plasmid form; C, fragments resulting from Hin cleavage; P, parental orientation of invertible segment; I, inverted orientation. **(Panel A) Lanes 1** and **4**, unreacted pMS551; **lanes 2** and **5**, pMS551 was incubated for 10 min in the presence of Fis under cleavage conditions (CDTA + ethylene glycol); **lanes 3** and **6**, same as previous samples (lanes 2 and 5) except the reaction products were chased for 30 min by adding Mg^{2+} and diluting out the ethylene glycol to a final concentration of 5%. **(Panel B) Lanes 7** and **11**, unreacted pMS551; **lanes 8** and **12**, pMS551 was incubated for 10 min under cleavage conditions in the absence of Fis; **lanes 9** and **13**, same as previous samples (lanes 8 and 12) except the reaction products were chased in the absence of Fis for an additional 30 min by adding Mg^{2+} and diluting out the ethylene glycol to a final concentration of 5%; **lanes 10** and **14**, same as previous samples (lanes 9 and 13) except Fis was present in the chase.

Effect of DNA topology on DNA cleavage by Hin

DNA supercoiling is required in order to generate inversions *in vitro* under normal recombination conditions (Johnson *et al.*, 1986). We asked whether Hin-mediated cleavage could occur on relaxed or linear DNA molecules and whether this cleavage was stimulated by Fis. Hin and HU were incubated with the wild-type substrate pMS551 which had been relaxed using topoisomerase I or linearized at its unique *Pvu*II site (see Figure 1). After a 30-min incubation, 3–5% of the molecules contained double-stranded cleavages with the recombination sites (Figure 3 and Table I). This low amount of cleavage was not increased by the presence of Fis in the reaction. In contrast, Fis increases the number of cleaved molecules on a supercoiled substrate by 4- to 5-fold (Table I). The lack of any stimulation by Fis on the rate of cleavage of linear or relaxed DNA in contrast to supercoiled DNA, suggests that supercoiling is required for Fis activity.

Chasing complexes assembled in the presence of Fis

In order to determine if the complexes are intermediates in the pathway to DNA inversion, we performed a series of 'cleavage-chase' experiments. In the description of these experiments we are assuming that the DNA strands are cleaved within the complex. pMS551 was incubated with

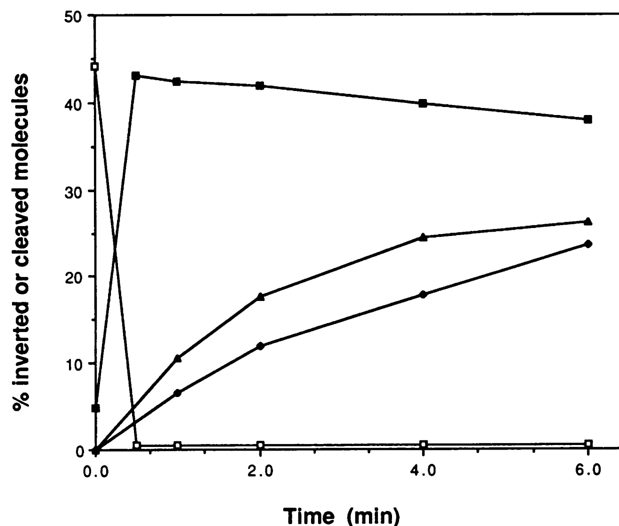

Fig. 5. Rates of ligation and inversion of Hin-cleaved molecules. pMS551 was incubated at 37°C for 5 min in the presence of Fis under cleavage conditions (CDTA + ethylene glycol) and then equilibrated at 25°C for 2 min. At time 0, Mg^{2+} was added and the ethylene glycol was diluted to a final concentration of 5%. Aliquots were taken at the indicated times and the reaction terminated by the addition of DEP to 0.1% final concentration. The DNA was precipitated with ethanol, digested with *Pst*I and *Hin*dIII and electrophoresed in an agarose gel. The percentage of cleaved (open squares) and inverted (filled squares) molecules were quantitated by densitometry. The same protocol was followed except Fis was absent during the cleavage reaction which was performed for 10 min at 37°C. At the start of the chase (time 0), 8% of the molecules contained double-stranded cleavages at the recombination sites; after 1 min of chase, no Hin-cleaved molecules were detected. The number of molecules containing inversions are shown (diamonds). Control inversion reaction at 25°C (triangles). The reaction was initiated by the addition of Hin. The plasmid DNA preparation used in these experiments was 89% supercoiled.

Hin, Fis and HU under cleavage conditions (CDTA + ethylene glycol) and then the reaction products were 'chased' by the addition of Mg^{2+} and dilution of the ethylene glycol. As shown in Figure 4, a small number of molecules (12%) have undergone inversion but ~40% of the molecules are cleaved at the two recombination sites. After a 30-min chase, no cleavage products are detectable and 46% of the molecules contain inversions. This result implies that the eight ends of DNA are held together in a highly specific and stable complex such that they can be efficiently religated when conditions are favorable. This notion is further supported by the fact that the DNA remains topologically constrained after repair of the cleavages (Figure 4, lane 3).

Since the chase time in the experiment in Figure 4 is long (30 min), one cannot distinguish between ligation of cleaved molecules in the recombinant configuration which would directly generate inversion or ligation in the parental configuration followed by a subsequent *de novo* reaction yielding inversion. In Figure 5, the number of cleaved and inverted molecules are measured at various times after the addition of Mg^{2+} and dilution of the ethylene glycol. In this experiment, the cleavage reaction was performed at 37°C for 5 min and the products were chased at 25°C to decrease the rate of the reaction. The control for this experiment is to initiate the reaction with Hin at 25°C without prior incubation in cleavage conditions. Immediately prior to chasing, 44% of the molecules were cleaved at the recombination sites while 4% contained inversions. After

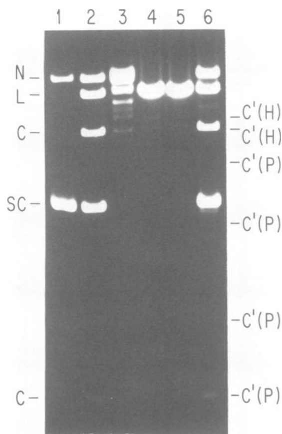

Fig. 6. Effect of relaxing or linearizing pMS614 on the accumulation of Hin-cleavage products. pMS614 was incubated with Fis under normal recombination conditions for 15 min (**lane 2**) to accumulate cleaved molecules. Aliquots were incubated for an additional 15 min in the presence of topoisomerase I (**lane 3**), *Hind*III (**lane 4**), *Pvu*II (**lane 5**) or no additional enzyme (**lane 6**). The reactions were terminated by the addition of SDS to 0.5% final concentration, incubated with proteinase K, and electrophoresed in a 1% agarose gel. **Lane 1** contains unreacted pMS614. The locations of the supercoiled (Sc), linear (L) and nicked (N) plasmid forms and fragments resulting from Hin cleavage (C) are denoted on the left side of the panel. The locations of some of the expected products containing Hin-generated cleavages at one or both recombination sites after digestion with *Hind*III [C'(H)] and *Pvu*II [C'(P)] are denoted on the right side of the panel. An aliquot of the DNA digested with *Hind*III was further digested with *Pst*I to assay the orientation of the invertible segment. No molecules containing inversions were detected (not shown).

just 30 s of incubation in the presence of Mg^{2+}, no cleaved molecules were detected and 43% of the molecules contained inversions. Therefore, repair of the cleaved molecules occurs very rapidly. Furthermore, the number of inverted molecules compared with the control (~5%, see Figure 5) suggests that the majority of cleaved molecules are ligated in the recombinant configuration.

Chasing complexes assembled in the absence of Fis

The experiments in the previous section were performed in the presence of Fis and thus an active enhancer. The same experiments have been performed with complexes generated in the absence of Fis. In Figure 4b, pMS551 was incubated under cleavage conditions for 10 min without Fis which generated a low but detectable number of cleaved molecules (11%). Chasing these products in the absence of Fis again resulted in complete repair of the cleaved molecules but no topoisomerase activity and <2% of the molecules contained inversions. Chasing in the presence of Fis resulted in numerous strand exchanges with 46% of the molecules containing inversions after 30 min. However, unlike that observed in the presence of Fis, prior complex formation without Fis does not result in an increase in the rate on inversion (Figure 5). Therefore, the complexes isolated in the absence of Fis are either not productive for inversion or are in the pathway leading to inversion but have not advanced past a rate-limiting step in the reaction. These experiments do suggest that Hin is capable of cleaving DNA and maintaining the ends in a topologically constrained configuration in which efficient ligation can occur under the appropriate conditions. The ligation of strands in the absence of Fis results in only the parental configuration being regenerated.

Requirement of DNA supercoiling for the maintenance of pMS614 complexes

The enhancer, Fis and DNA supercoiling are all required in order to accumulate cleaved molecules with pMS614 under normal recombination conditions. It would seem likely that inversion is attempted with pMS614 but that complexes containing broken DNA molecules accumulate due to the inability to ligate in the recombinant configuration (see Discussion). We investigated the effects of removing DNA supercoiling on the maintenance of this structure.

Supercoiled pMS614 was incubated under normal recombination conditions with Fis for 15 min to accumulate 45% of the molecules cleaved at one or both recombination sites (Figure 6, lane 2). The plasmid was then incubated an additional 15 min with topoisomerase I (lane 3), *Hind*III which cuts within the invertible segment (lane 4), *Pvu*II which cuts within the vector (lane 5), or no additional enzyme (lane 6). After relaxation or linearization of the plasmid, <5% of the molecules remained cleaved at the recombination sites. These results demonstrate that DNA supercoiling is required to maintain the pMS614 complex. Assuming that the DNA molecules are broken and positioned in the recombinant configuration in these complexes, removal of supercoiling leads to the molecules being religated into the parental configuration. In this respect, supercoiling may be performing several roles: it may be required for the assembly and maintenance of a recombinogenic complex that requires the function of the enhancer, and it may provide the energy to rotate the DNA strands into the recombinant configuration.

Precise identification and nature of cleavage site

Hin catalyzes a 2-bp staggered cut within the center of the recombination site. The protein remains covalently associated with the 5' ends and leaves a free hydroxyl at the 3' ends. This was determined using both the wild-type substrate pMS551 in reactions containing EDTA and ethylene glycol and with the mutant substrate pMS614 under normal recombination conditions. The experiments leading to this conclusion are described below.

Supercoiled pMS551 or pMS614 was incubated with Hin in the presence of Fis and HU and then digested with *Eco*RI or *Cla*I which cleave ~120 bp on either side of *hixL*. The products were labeled at their 5' ends using polynucleotide kinase and [γ-^{32}P]ATP and electrophoresed in a denaturing gel in parallel with the Maxam–Gilbert sequence ladder of fragments labeled at the same position. Since the Maxam–Gilbert chemistry results in the elimination of the reacted base, the chain length represents a fragment of one less than the indicated base on the gel (Maxam and Gilbert, 1980). The migration of the Hin cleavage fragment thus places the cleavage after nucleotide +1 on the top strand of *hixL* and after nucleotide −1 on the bottom strand (reading in the 5' to 3' direction for each strand, see Figure 7, lanes 1–6 and 8–13, and Figure 8).

To determine the nature of the 3' end of the Hin cleavage site, Hin cleaved DNA was incubated with terminal transferase and [α-^{32}P]dideoxyATP. Terminal transferase will only recognize a free 3' hydroxyl as a substrate for nucleotide addition and the use of the chain terminating nucleotide allows for the addition of only one nucleotide. Digestion with *Eco*RI and electrophoresis (Figure 7, lane 7) generated a labeled fragment whose migration was

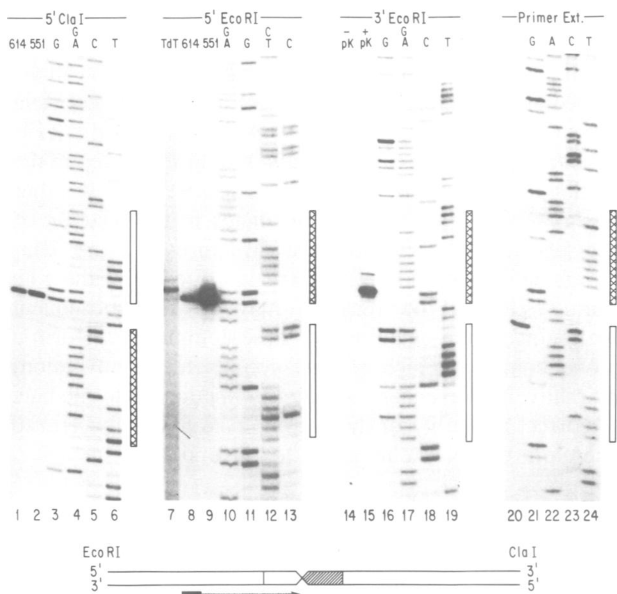

Fig. 7. Determination of precise location and nature of cleavage site. **Lanes 1, 2, 8** and **9**, Hin cleavage reactions were performed on pMS614 under normal recombination conditions or on pMS551 in the presence of CDTA and ethylene glycol. The products were digested with *Eco*RI or *Cla*I as denoted, end-labelled at the 5′ end and electrophoresed in a native polyacrylamide gel. The labeled fragments were extracted from the gel and electrophoresed in a denaturing 8% polyacrylamide–urea gel in parallel with the Maxam–Gilbert chemical sequencing reactions on restriction fragments end-labeled at the same position (**lanes 3–6** and **10–13**). **Lane 7**, Hin cleaved pMS551 was labeled with terminal transferase and [α-^{32}P]dideoxyATP, digested with *Eco*RI and electrophoresed in parallel with the sequencing reactions (lanes 10–13). **Lanes 14** and **15**, a Hin cleavage reaction was performed on pMS551 in the presence of CDTA and ethylene glycol. The products were digested with *Eco*RI, end-labeled at the 3′ position of the restriction site and electrophoresed with or without prior digestion with proteinase K (– or + pK) in parallel with the Maxam–Gilbert chemical sequencing reactions on a restriction fragment end-labeled at the same position (**lanes 16–19**). **Lane 20**, a synthetic oligonucleotide complementary to sequences 64–45 bases to the left of *hixL* as denoted on the bottom of the figure was hybridized to denatured pMS551 which had been subjected to a Hin cleavage reaction in the presence of CDTA and ethylene glycol. The oligonucleotide was extended with reverse transcriptase in the presence of [α-^{32}P]ATP and electrophoresed in parallel with the products of dideoxy sequencing reactions performed using the same primer (**lanes 21–24**). The rectangles denote the nucleotides within the *hixL* recombination site with the crosshatched areas indicating the region inside the invertible segment.

increased by one nucleotide as compared with the 5′ labeled fragment. Therefore, the 3′ end of the Hin cleavage site contains a hydroxyl.

Analysis of the 5′ end of the cleavage site indicates that Hin is covalently associated with the DNA. Fragments labeled at the 3′ end of the *Eco*RI site do not enter the sequencing gel unless first digested with proteinase K. The major species appearing after proteinase K digestion migrates ~4 bases larger than the chain length predicted from the previous experiments employing 5′ end-labeled DNA. This discrepancy is probably a function of a small number of amino acids still remaining associated with the DNA. In order to confirm the 5′ end assignment, an oligonucleotide complementary to the bottom strand of the *Eco*RI–*hixL* DNA segment (see bottom of Figure 7) was used as a primer for synthesis by reverse transcriptase. The primer extension product terminates after the C at nucleotide –2 (Figure 7,

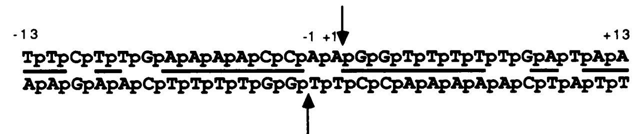

Fig. 8. Location of the cleavage site in *hixL*. Arrows mark the position of Hin-mediated cleavage within the *hixL* sequence.

lanes 20–24). These experiments confirm that the 5′ nucleotide on the bottom strand is the G at –2 (Figure 8) and suggests that Hin is bound in a phosphoester linkage at that site.

Discussion

Under optimal *in vitro* conditions, Hin-mediated inversion is very rapid and efficient. Approximately 50% of the DNA substrate molecules have undergone inversion within 3 min at 37°C. The cleavage-ligation reactions required for inversion are normally highly concerted making the isolation of potential intermediate complexes difficult. Indeed, only 0.2% of the molecules can be isolated that contain site-specific cleavages within the recombination sites in reactions using the wild-type substrate. We have established conditions which promote the accumulation of substantial amounts of complexes that are recognized by the presence of double-stranded cleavages upon quenching the reaction. Efficient formation of these complexes requires the presence of two recombination sites on the substrate molecule and in some cases depends on the nucleotide sequence of the two sites.

Fis-dependent complexes

At least two clearly distinguishable complexes have been identified: those formed in the presence of Fis and those formed in the absence of Fis and thus a functional enhancer. Complexes involving Fis activity are generated by either of two methods. Reactions performed in the absence of Mg^{2+} combined with the presence of ethylene glycol result in a lower rate of inversion and the accumulation of up to 50% of the substrate molecules containing double-stranded cleavages within both recombination sites. The second method involves the use of a mutant substrate, pMS614, which contains a base substitution mutation within the central nucleotides of one of the palindromic recombination sites. We have previously shown that this mutation prevents inversion when present together with the wild-type recombination site (Johnson and Simon, 1985). In this paper, we show that reactions using pMS614 under conditions optimal for inversion also result in the accumulation of complexes containing double-stranded cleavages at one or both recombination sites.

We can consider several stages along the recombination pathway in which the reaction could be blocked to generate the Fis-dependent complexes. (i) The recombination sites may have associated into a 'synaptic complex' in which the nucleoprotein assembly is primed to initiate strand exchange but no cleavages have occurred. Rather, the DNA is associated with Hin in such a way that denaturation of the protein triggers cleavage. (ii) The recombination sites are associated and cleaved but the DNA strands have not been positioned into the recombinant configuration. (iii) The recombination sites are cleaved and positioned into the recombinant configuration with the final ligation step blocked.

As discussed above (see Results), the first possibility is not favored because quenching the reaction using acid would be expected to be too rapid for catalysis involving a phosphoserine linkage with DNA to occur. The last possibility seems the most attractive, especially for the complexes found with pMS614. Non-complementarity at the position of ligation in the recombinant configuration would be expected to inhibit phosphodiester bond formation. The non-complementarity would be sensed by positioning the DNA strands into the recombinant configuration and attempting ligation. It should be noted that a base substitution mutation at the nucleotide immediately outside of the 2-bp overlap region (position +2) in one of the recombination sites has only a small effect on inversion rates (unpublished data).

The DNA strands within the Fis-dependent complexes must be held together in a precise and stable complex such that the topological state of the DNA is maintained. Incubation of preassembled wild-type complexes in optimal inversion conditions directly yields inversion, demonstrating that these complexes are intermediates that have advanced past the major rate-limiting step(s) in the reaction. Since pMS614 does not yield inversion, it is not possible to chase these complexes into recombinant products. However, removal of DNA supercoiling on preassembled pMS614 complexes results in molecules religated back into the parental configuration. These findings suggest that supercoiling is required for the formation and maintenance of the Fis-dependent complexes. Supercoiling may also facilitate the rotation of DNA to position the strands into the recombinant configuration. Negative supercoiling will direct strand rotation in the clockwise direction which is the direction that is consistent with the observed topological changes that have been measured on the products of Gin-mediated inversion (Kahmann *et al.*, 1987; Kanaar *et al.*, 1988). The apparent enhancement of the cleavage reaction by Fis on supercoiled DNA may be a function of the cleaved strands being positioned in a configuration preventing rapid religation. Thus, the rate of cleavage may not be enhanced but rather the length of time the molecules remain unligated.

Fis-independent complexes

Complexes containing double-stranded cleavages at both recombination sites can also be generated in the absence of Fis in reactions lacking Mg^{2+} and containing ethylene glycol, although the rates and yields of these complexes are lower than those generated with Fis (Table I). The DNA strands, which are presumed to be in a cleaved configuration within the complex because of the reasons discussed above, can be resealed by adding Mg^{2+} and diluting the ethylene glycol. Thus, these experiments demonstrate that Hin is able to associate the two recombination sites into a highly precise and stable complex in the absence of an active enhancer. Electron microscopy of crosslinked complexes has also shown that Hin, without a functional enhancer, can mediate the association of recombination sites (R.C.Johnson, M.Dodson and H.Echols, unpublished results). DNA supercoiling is not required for Hin-mediated association of recombination sites, since cleavage at both recombination sites can occur on relaxed or linearized DNA. However, supercoiling may facilitate the association of sites since greater numbers of cleaved molecules are generated with supercoiled DNA than with relaxed or linear DNA in the absence of Fis (Table I).

While Hin can mediate DNA cleavage and ligation, in the absence of a functional enhancer, these reactions do not yield inversion. In addition, the complexes generated without Fis have not advanced past a detectable rate limiting step in the reaction. We therefore cannot directly determine whether these complexes are true intermediates in the reaction or represent inversion-incompetent structures. If the Hin-recombination site complexes are intermediates, the rate limiting step in the reaction may be the subsequent functioning of the enhancer on these complexes to form a second complex which is now competent for inversion. Alternatively, the enhancer may be functioning to mediate the correct assembly of the recombination sites by Hin to generate a complex competent for inversion.

Mechanism of strand exchange

The cleaved DNA is broken in both strands within the central dinucleotide of the recombination sites. The cleavage site is characterized by a 2-bp 3′ protruding end that terminates with a hydroxyl. The 5′ recessed end is covalently associated with the Hin protein. A transient phosphoester linkage with the recombinase during strand exchange will conserve the energy of the broken phosphodiester bond in the DNA, alleviating the need for high-energy co-factors in the reaction. Resolvase and the Gin recombinase also generate cleavages at the analogous location within their recombination site and contain the protein associated with the DNA via a 5′ phosphoester linkage (Reed and Grindley, 1981; Klippel *et al.*, 1988). In both of these systems, biochemical and genetic data have provided evidence that a serine within a conserved block of amino acids in the N terminus is the site of linkage (Reed and Moser, 1984; Hatfull and Grindley, 1986; Klippel *et al.*, 1988). The analogous residue in Hin (serine 10) is implicated as the site of linkage by the inability of a mutant Hin containing a threonine at this position to cleave DNA (K.Heichman and R.C.Johnson, unpublished).

Klippel *et al.* (1988) have reported that Gin-mediated cleavages on restriction fragments are primarily single-stranded. In our experiments the Hin-induced cleavage appears to be primarily or exclusively double-stranded. We have not detected nicking of one strand without the other at appreciable rates under any condition thus far tested. These include reactions using the wild-type or mutant substrates that contain alterations in the recombination sites or enhancer sequence, reactions in the presence or absence of Mg^{2+}, ethylene glycol or Fis, and reactions on supercoiled or linear DNA. The reason for this difference is not known although it could reflect the methods of enzyme preparation or the precise reaction conditions. It has been noted that λ integrase can induce both single- and double-stranded cleavages depending on the reducing conditions (Kitts *et al.*, 1984). Like Hin, site-specific cleavage of DNA by resolvase has been found to be primarily double-stranded (Reed and Grindley, 1981).

Strand exchange mediated through double-stranded cleavages relates the enzymology of Hin to the resolvase family which share significant homology especially in the N-terminal segments where the covalent linkage with DNA occurs. Topological analysis of both the deletion reaction promoted by resolvase and the inversion reaction promoted

by Gin are also most consistent with strand exchange occurring through a double-stranded cleaved intermediate (Boocock *et al.*, 1987; Kanaar and van de Putte, 1987). This mechanism is different from that used by the recombinases belonging to the Int family. Current evidence is most consistent with the Int-related recombinases, including Cre, Flp and the phage integrases, mediating a pair of single-stranded DNA transfers with the recombinase being transiently covalently associated with the 3′ end. A Holliday structure, generated after the first single-stranded transfer, migrates 6−8 bp before it is resolved by a second strand transfer reaction (for review see Craig, 1988).

Conclusion and possible role of the recombinational enhancer

We have shown that Hin is able to associate the two recombination sites into a highly ordered structure. We believe Hin has cleaved the DNA at both recombination sites and is holding the ends together in the complex. Without a functioning enhancer, however, these reactions do not lead to inversion. Complexes assembled on the wild-type substrate in the presence of an active enhancer and DNA supercoiling have advanced past the major rate limiting step(s) of the reaction. Similarly, the accumulation of complexes that depend on the nature of the dinucleotide sequence within the recombination site require the activity of the enhancer and supercoiling. It is tempting to speculate that the broken DNA strands in the latter two complexes are positioned in the recombinant configuration. This leads us to suggest that the enhancer, combined with DNA supercoiling, may be required in part to mediate the rotation of DNA strands subsequent to double-stranded cleavage by Hin within both recombination sites. Ligation in the recombinant configuration will then result in inversion of the intervening DNA segment.

Materials and methods

Enzymes and general methods

Restriction enzymes and proteinase K were purchased from Boehringer Mannheim, topoisomerase I was from Bethesda Research Laboratories, polynucleotide kinase was from Pharmacia, Klenow and AMV reverse transcriptase was from Promega and terminal transferase was from International Biotechnologies, Inc. Purified preparations of Hin, Fis and HU were obtained as outlined in Johnson *et al.* (1986). Agarose gel electrophoresis was performed in Tris phosphate EDTA (TPE) buffer and polyacrylamide gel electrophoresis was performed in Tris borate EDTA buffer as described (Maniatis *et al.*, 1982).

Plasmids

All the various substrate plasmids have the same basic structure as outlined for the wild-type substrate pMS551 in Figure 1. One recombination site (*hixL* sequence) and the enhancer at the wild-type spacing is inserted between the *Eco*RI and *Hin*dIII sites and a second synthetically derived recombination site (*hixL*) is located at the *Sal*I site of pBR322. pMS614 contains a mutant recombination site (Figure 1B) at the *Sal*I site. pMS554 is lacking the second *hixL* sequence at the *Sal*I site and pMS536 contains two synthetically derived *hixL* sites without an enhancer. Details on the construction of these plasmids are given in Johnson and Simon (1985).

Inversion and cleavage reactions

Normal recombination reactions were performed in 20 mM Tris−HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol and 200 μg/ml polycytidylic acid (Pharmacia) containing 50 ng Hin, 30 ng Fis and 100 ng HU/0.1 pmol of supercoiled substrate DNA in a 25 μl volume. Incubations were for 30 min at 37°C unless otherwise noted. Reactions were terminated by the addition of SDS and proteinase K to final concentrations of 0.5% and 150 μg/ml respectively, and incubated an additional 15 min at 37°C prior to electrophoresis. Alternatively, DEP (Sigma) was added to a final concentration of 0.05% and the DNA was ethanol precipitated and digested with restriction enzymes, if required. The samples were digested with proteinase K in the presence of SDS as above prior to electrophoresis. Conditions favoring the cleavage reaction were identical except that the $MgCl_2$ was omitted and 10 mM EDTA or CDTA plus 30% (v/v) ethylene glycol were included. Products accumulated after cleavage reactions were chased by the addition of 5 vols of 20 mM Tris−HCl (pH 7.5), 100 mM NaCl, 15 mM $MgCl_2$ and 1 mM dithiothreitol. The amount of cleavage was quantitated by scanning negatives of gels using a LKB ultroscan model 2202 laser densitometer interfaced with an Apple II computer for integration. DNA fragments labeled with ^{32}P were excised from the gel and the Cerenkov radiation measured using a scintillation counter.

Analysis of the Hin cleavage site

Hin-cleaved fragments radiolabeled at the 5′ end of adjacent restriction sites were prepared as follows: pMS614 was incubated in normal recombination conditions and pMS551 was incubated in cleavage conditions as described above. The reactions were quenched and the plasmids were then digested with *Eco*RI or *Cla*I, dephosphorylated using calf intestinal phosphatase, and labeled with [γ-^{32}P]ATP and polynucleotide kinase as described (Maxam and Gilbert, 1980). The products were electrophoresed in a 5% native polyacrylamide gel containing 10% glycerol. The labeled fragments were eluted from gel slices by diffusion, precipitated with ethanol and resuspended in 80% formamide (v/v), 10 mM NaOH, 1 mM EDTA and electrophoresed in a sequencing gel containing 8% polyacrylamide and 7 M urea after incubating for 2 min at 100°C. 3′ end-labeled fragments were prepared by filling in the 5′ overhang of the restriction site with [α-^{32}P]ATP or CTP using reverse transcriptase or the Klenow fragment of DNA polymerase I. The products were incubated with 200 μg/ml proteinase K, as noted, prior to electrophoresis in a sequencing gel. Fragments labeled at the 3′ end of the Hin cleavage site were generated as follows: Hin-cleaved DNA (0.1 pmol) was precipitated with ethanol, resuspended in 140 mM K cacodylate, 30 mM Tris base, 1 mM $CoCl_2$, 1 mM dithiothreitol (pH 7.2) and incubated with 7.5 units of terminal transferase and [α-^{32}P]dideoxyATP for 30 min at 37°C. The reaction was terminated by extraction with phenol, the DNA collected by precipitation with ethanol and digested with *Eco*RI or *Cla*I. The products were directly electrophoresed in a sequencing gel after denaturing in formamide buffer as above. The Maxam−Gilbert chemical sequencing reactions were performed essentially as described (Maxam and Gilbert, 1980) with some modifications from G.Church (Harvard University). The protocol for the T reaction was from Rubin and Schmid (1980). The primer extension assays were performed on Hin cleaved DNA which had been denatured by incubation with NaOH (0.2 M) for 5 min. A 20-base oligonucleotide (5 pmol) complementary to sequences 45−64 bases to the left of *hixL* as denoted in Figure 7 was hybridized with 0.05 pmol of plasmid DNA in a 10 μl reaction volume and the products were extended with reverse transcriptase in the presence of [α-^{32}P]ATP. Dideoxy sequencing reactions (Sanger *et al.*, 1977) were performed on alkaline denatured DNA using the same primer and hybridization conditions.

The location of the cleavage site was also determined using a modification of the supercoiled DNA footprinting technique involving indirect end-labeling of DNA fragments electroblotted from sequencing gels onto GeneScreen (New England Nuclear) (Church and Gilbert, 1984; Richet *et al.*, 1986).

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