The Hin Invertasome: Protein-Mediated Joining of Distant Recombination Sites at the Enhancer

KAREN A. HEICHMAN AND REID C. JOHNSON

The Hin protein binds to two cis-acting recombination sites and catalyzes a site-specific DNA inversion reaction that regulates the expression of flagellin genes in *Salmonella*. In addition to the Hin protein and the two recombination sites that flank the invertible segment, a third cis-acting recombinational enhancer sequence and the Fis protein, which binds to two sites within the enhancer, are required for efficient recombination. Intermediates of this reaction were trapped during DNA strand cleavage and analyzed by gel electrophoresis and electron microscopy in order to determine their structure and composition. The analyses demonstrate that the recombination sites are assembled at the enhancer into a complex nucleoprotein structure (termed the invertasome) with the looping of the three segments of intervening DNA. Antibody studies indicated that Fis physically interacts with Hin and that both proteins are intimately associated with the invertasome. In order to achieve this protein-protein interaction and assemble the invertasome, the substrate DNA must be supercoiled.

THERE ARE NOW MANY EXAMPLES OF DNA BINDING PROteins that manifest their activities at large distances from their own binding sites. Efficient transcription initiation at many eukaryotic and some prokaryotic promoters requires protein binding to enhancer sequences that can be located thousands of base pairs from the start site of RNA synthesis (*1*). It is generally believed that proteins bound to enhancer and promoter sequences distant from one another must interact; however, there is little direct evidence to support this belief.

Convincing evidence for long-range protein-protein interactions has been demonstrated for several bacterial repressors, including those used to regulate the *lac*, *gal*, *ara*, and *deo* operons (*2*). This has been shown by DNA binding assays that demonstrate enhanced binding by a repressor at a low affinity site in the presence of a high affinity site. In some cases, DNA looping has been confirmed by electron microscopy (*3*). The initiation of R6K plasmid DNA replication has been associated with DNA looping to facilitate interactions between proteins bound to the origin and distant sites (*4*). Site-specific recombination and DNA transposition are also likely candidates for long-range protein-protein interactions that would minimally involve the two recombination sites or the ends of the transposon. Indeed, such complexes have been observed in the bacteriophage lambda and resolvase site-specific recombination systems and in the mini-Mu transposon (*5*).

We now show that a tripartite complex, an invertasome, is assembled in the Hin-mediated site-specific DNA inversion reaction. This nucleoprotein complex contains not only the two recombination sites, but also a recombinational enhancer sequence to generate a three-looped structure. The three DNA loops are held together by two types of protein-protein interaction, one involving the same protein species, and the other involving heterologous proteins. Hin is a DNA binding protein from *Salmonella* that catalyzes the site-specific DNA inversion reaction through the association of its dimers bound to the two recombination sites. Another bacterial DNA binding protein, Fis, interacts with Hin in the invertasome to stimulate inversion. These two proteins must initially bind to their distantly located sites before they can interact and assemble into this novel structure.

The Hin inversion system. The Hin inversion reaction regulates the expression of alternate flagellin genes by switching the orientation of a promoter located on an invertible DNA segment (*6*). It is one of a family of site-specific inversion systems that includes the Gin and Cin systems found in bacteriophages Mu and P1, respectively, which function to increase the host range of the phage by altering tail fiber expression (*7*). In vivo and in vitro analyses of these systems have shown that a supercoiled substrate containing three cis-acting sequences, two recombination sites, and a sequence termed the recombinational enhancer is required for efficient inversion (*8, 9*). In the Hin system, the two recombination sites, *hix*L and *hix*R, are 26 base pairs (bp) in length and contain two imperfect inverted repeats separated by two nucleotides (*8, 10*). The Hin protein binds to each of these recombination sites as a dimer and catalyzes a double-stranded cleavage, leaving the central dinucleotide as a two-base overhang (*10, 11*). The Hin protein remains covalently bound to the 5′-phosphates at the cleaved sites and is released during religation in the inverted orientation. The recombinational enhancer is a 60-bp sequence that serves to stimulate the rate of inversion 150-fold (*8*). It can be located within or outside of the invertible segment in either orientation and can function efficiently at distances of several kilobases from either recombination site. However, the enhancer cannot function properly when located within 30-bp of a recombination site. The enhancer contains two Fis binding sites separated by 48 bp that position the Fis dimers on opposite sides of the DNA helix (*12*). The spacing and helical positioning of these binding sites are crucial for the function of the enhancer, although no evidence for interactions between Fis molecules at the two sites has been obtained. The HU protein is also required for efficient recombination, but only under circumstances

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where the cis-acting sites are located very close to one another (*13*). Presumably HU serves to facilitate the looping of intervening DNA and thereby allows interaction between sites. This looping between interacting sites may explain why the recombinational enhancer cannot function when close to a recombination site.

Our model for the formation of the inversion complex is shown in Fig. 1A (*12, 14*). The Hin and Fis proteins each bind to their respective binding sites on the supercoiled substrate molecule. Assembly of the invertasome may proceed by pairing of the *hix* recombination sites by Hin-Hin interactions, with subsequent association of these sites with the recombinational enhancer. Alternatively, an initial interaction could occur between one recombination site and the enhancer, followed by the addition of the second recombination site. In either case, the final structure involves the interaction of all three cis-acting sites mediated by protein-protein interactions. Our experiments were aimed at visualizing these potential intermediates to provide evidence for this model and to distinguish between these two potential pathways.

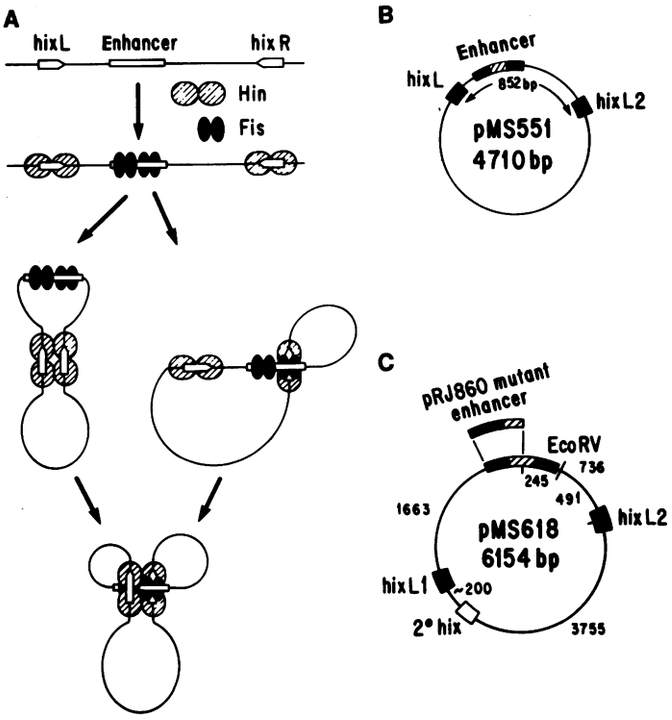

Fig. 1. (**A**) Model of two possible pathways leading to the assembly of the invertasome. Hin and Fis bind to their respective recognition sequences on the DNA substrate molecule. Two routes with potential intermediates leading to the formation of the invertasome are shown. The intermediate on the pathway on the left is the "paired *hix*" structure, and that on the right represents a single recombination site associated with enhancer. The "invertasome" (bottom) contains the two Hin-bound recombination sites aligned at the recombinational enhancer, presumably by Hin-Fis interactions. The enhancer is drawn symmetrically positioned between Hin molecules, although the precise relation of the proteins and DNA sites within the invertasome is not known. The intervening DNA is displaced and is shown as relaxed even though the substrate molecule must be supercoiled. (**B**) The structure of the inversion substrate pMS551 (*8*), which was used for the experiment in Fig. 2. The *hix*L2 site is actually a synthetically derived *hix*L sequence inserted into the Sal I site of this pBR322-based substrate. The region from *hix*L to the enhancer is derived from wild-type *Salmonella* sequences and is inserted into the Cla I site of the plasmid. (**C**) The structure of the inversion substrate pMS618 (*8*), which was used for the experiments in Figs. 3 through 7 and Tables 1 and 2. The numbers between sites refer to length in base pairs. pMS618 is similar to pMS551 except that a 1543-bp fragment from the Tn5 transposon has been inserted between *hix*L1 and the enhancer to exaggerate looping. A cryptic recombination site, 2°*hix*, can serve as an inefficient cleavage site. pRJ860 is a pMS618 derivative with a mutant enhancer that contains only one Fis binding site. It inverts at less than 5 percent of the in vitro rate measured for pMS618 and was created by digesting pMS618 with Cla I and Hind III restriction enzymes (Boehringer Mannheim) and incubated with T4 DNA polymerase (Boehringer Mannheim) to create blunt ends, which were then ligated.

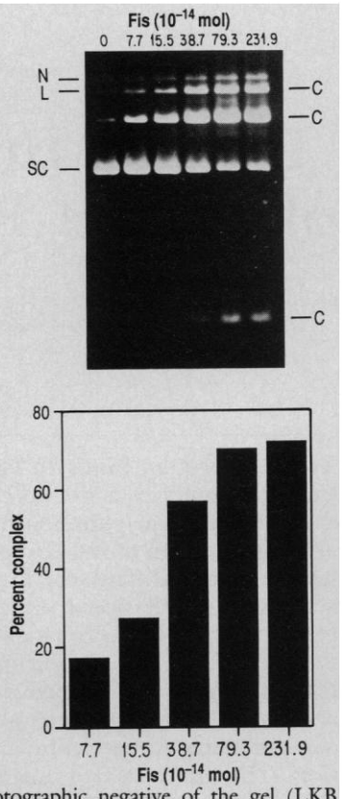

Fig. 2. (**A**) Stoichiometric association of Fis with recombination complexes. In vitro reactions were performed on supercoiled pMS551 substrate DNA under Mg^{2+}-free reaction conditions with increasing amounts of Fis protein essentially as described (*11*). The reactions were done in a 25-μl volume containing 20 mM tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, polycytidylic acid at 200 μg/ml (Pharmacia), 30 percent ethylene glycol, and 0.1 pmol of pMS551. Purified HU (100 ng), and Fis (0 to 28.2 ng, 0 to 231 × 10^{-14} mol) were incubated in the reaction mixture for 5 minutes at 37°C and 80 ng of Hin was added (*28, 29*). The reaction was incubated for 60 minutes at 37°C and terminated by the addition of SDS and proteinase K (Boehringer Mannheim) to final concentrations of 0.5 percent and 150 μg/ml, respectively, and incubation was continued for 10 minutes at 37°C. The samples were subjected to electrophoresis through a 1 percent agarose gel in tris-acetate–EDTA buffer (40 mM tris-HCl, pH 7.5, 60 mM acetate, 35 mM sodium, 1 mM EDTA) at 2.5 V/cm. The amount of complex was quantitated by scanning a photographic negative of the gel (LKB Ultroscan model 2202 densitometer interfaced with an Apple II computer for integration). The locations of the supercoiled (SC), linear (L), and nicked (N) plasmid forms, and the complex (C), or "cleavage fragments," are shown. The linear form also contributes to "complex" values since it results from cleavage at one recombination site (*11*). (**B**) Bar graph relating the amount of Fis added to the reaction as a function of the amount of complex accumulated after a 60-minute incubation.

The inversion reaction can be performed with a purified in vitro system (*13*). Each of the DNA substrates pMS551, pMS618, and pRJ860 (Fig. 1) contains the two recombination sites and the recombinational enhancer with pRJ860 lacking one Fis binding site in the enhancer. Optimal in vitro inversion conditions consist of a buffered salt solution containing Mg^{2+} and a supercoiled DNA substrate. Under these conditions inversion is very efficient, and appreciable amounts of intermediate complexes do not accumulate. However, if the reaction is performed under modified conditions, in the absence of Mg^{2+} and in the presence of EDTA and ethylene glycol, stable complexes can be generated (*11*). When these complexes were treated with various denaturing agents, the invertible segment and vector fragments were released. These fragments (cleavage products) contain the Hin protein covalently associated with the four DNA ends. These complexes accumulate with incubation time and are true reaction intermediates since they can directly become inversion products on addition of Mg^{2+} and dilution of the ethylene glycol (*11*). Thus, these Mg^{2+}-free, modified reaction conditions can be used as a measure of recombination complex formation and as a means of accumulating intermediates for further study.

Stoichiometric association of Fis with the recombination complex. The use of the Mg^{2+}-free conditions has allowed us to determine whether Fis acts catalytically to assemble multiple recombination intermediates or if Fis is required stoichiometrically to

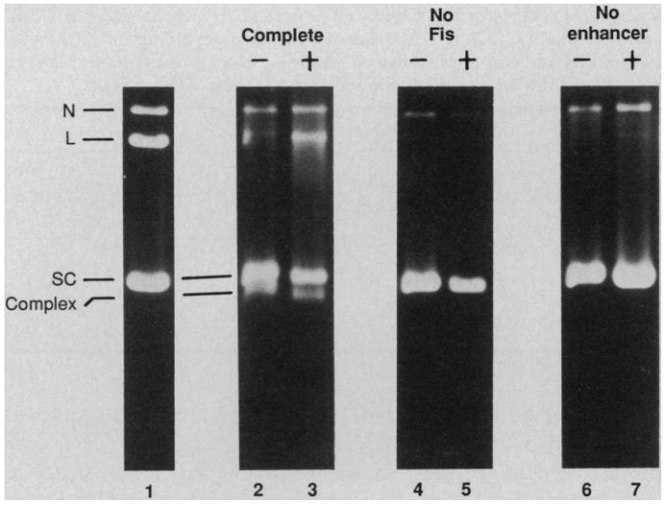

Fig. 3. Identification of a nucleoprotein complex by gel electrophoresis. In vitro reactions were performed on supercoiled pMS618 or pRJ860 substrate DNA under Mg^{2+}-free conditions. The reactions were performed in a 50-μl volume containing 20 mM Hepes-HCl (pH 7.5), 80 mM NaCl, 1 mM EDTA, polycytidylic acid at 550 μg/ml, 25 percent ethylene glycol, and 0.8 pmol of pMS618 or pRJ860. Purified Fis (150 ng) was added to the wild-type and mutant enhancer samples but omitted from the "no Fis" sample, and incubated for 5 minutes at 37°C. HU was not included in the reaction since pMS618 shows only a small stimulation in inversion rates in its presence (*13*). After 5 minutes, 480 ng of Hin was added and the reaction was incubated for 15 minutes at 37°C. A 5-μl sample was stored on ice (lanes 2, 4, and 6). The remaining reaction was further incubated for 15 minutes at 23°C with DSP (Pierce) at a final concentration of 0.02 percent. After the addition of lysine to 5 mM to quench the cross-linking agent, the reaction was passed over a Sepharose 4B mini-column (2 by 0.4 cm) equilibrated with medium salt buffer (10 mM tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$), and peak fractions were collected (40 μl total). A sample was removed to ice (lanes 3, 5, and 7). Each of the samples was subjected to electrophoresis through 0.9 percent agarose, tris-acetate EDTA gels at 2.5 V/cm. The locations of supercoiled (SC), linear (L), and nicked (N) plasmid forms are shown in lane 1. The position of the nucleoprotein complex is indicated. The headings indicate the complete (with pMS618), no Fis (with pMS618), and no enhancer (with pRJ860) reaction conditions. The minus and plus symbols above lanes 2 to 7 refer to the addition of DSP.

form each complex. The reaction was performed with limiting Fis protein, and the number of cleavage products was measured as an indicator of recombination complex formation. If Fis protein functioned catalytically, then suboptimal amounts would eventually generate large numbers of recombination complexes. Instead, we observe that the yield of complexes is proportional to the amount of Fis added (Fig. 2). Time course experiments with varying amounts of added Fis showed that maximal accumulation of cleavage products was reached by 30 minutes after the start of incubation at 37°C. From the 60-minute time points shown in Fig. 2, the number of Fis molecules per recombination intermediate was determined. For the lowest amounts of Fis added, 7.7×10^{-14} and 15.5×10^{-14} mol, we calculate that 4.3 and 5.5 monomers of Fis were bound per recombination complex, respectively. These values are consistent with Fis binding as a dimer to each of its two binding sites. These results indicate that Fis is stoichiometrically required for complex formation and suggest that it may be a stable component of the inversion complex.

A small amount of a complex can be generated in the absence of Fis, yielding cleavage products on protein denaturation (Fig. 2, lane 1) (*11*). However, previous studies have shown that these complexes have not advanced past a rate-limiting step in the reaction since an increased rate of inversion was not observed on the addition of Mg^{2+} and dilution of ethylene glycol. The amount of cleavage products generated without Fis, 13 percent of the total DNA, has been subtracted from the values used to determine Fis stoichiometry.

Detection of nucleoprotein complexes by electrophoresis. The initial evidence for potential inversion intermediates came from gel electrophoresis experiments. We took advantage of our Mg^{2+}-free reaction conditions to arrest the inversion complex in the midst of recombination so that the nucleoprotein complexes could be analyzed with regard to structure and to their requirements for assembly. Electrophoresis of the products of the modified reaction revealed the presence of a proteinase-sensitive species with an unusual mobility when compared to that of the substrate. Experiments with all of the reaction components were compared with those performed without Fis or with a mutant recombinational enhancer (Fig. 3). After these complexes were assembled, a sample of the reaction mixture was incubated with the cross-linking agent dithio*bis*(succinimidyl-propionate) (DSP) to covalently couple interacting proteins. With all of the necessary components, a species that migrates slightly faster than the supercoiled substrate can be seen in both untreated and DSP-treated lanes, but appears to be enhanced with the addition of the cross-linking agent (Fig. 3, lanes 2 and 3). SDS and proteinase treatment of the excised band generated DNA fragments that were the size of the predicted cleavage products. When the same experiment was performed

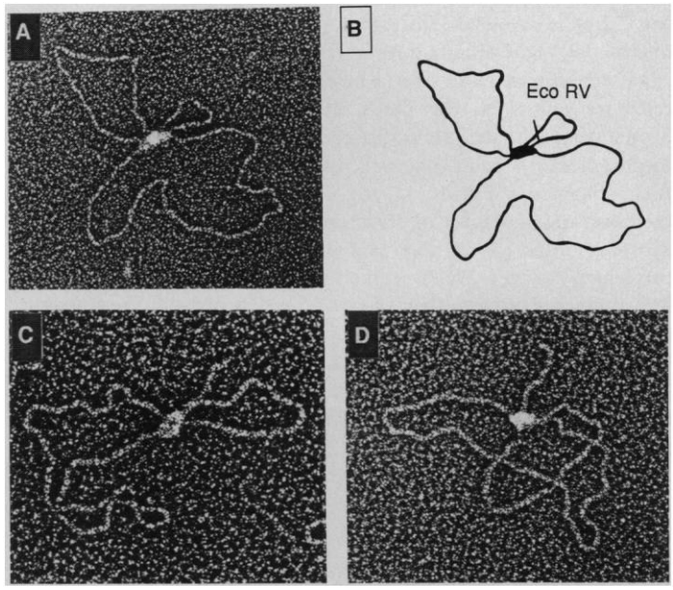

Fig. 4. Electron micrographs of invertasomes. Examples of the invertasome structure are shown above. (**A**) An undigested molecule; (**B**) a tracing of the molecule in panel A with small, medium, and large loops; the location of the unique Eco RV site within the small loop is identified; (**C** and **D**) molecules which have been digested with Eco RV to cleave the small loop into small tail 1 and small tail 2. Expected lengths of the tails and loops are as follows (Fig. 1C): small tail 1 (245 bp; not always visible), small tail 2 (491 bp), small loop (736 bp; small tail 1 + small tail 2), medium loop (1663 bp), large loop (3755 bp). Lengths were calculated from the center of the recombination sites or enhancer. In vitro reactions were performed as described in Fig. 3, except for the following modifications. Ethylene glycol was included to 30 percent; polycytidylic acid was omitted since it created debris on the EM grid; incubations were for 30 minutes at 37°C; and cross-linking included incubation with DSP at a final concentration of 0.015 percent for 15 minutes at 23°C followed by the addition of glutaraldehyde (Polysciences) at a final concentration of 0.03 percent and an additional 5 minutes of incubation at 23°C. Subsequent restriction digestion with Eco RV also included 5 units of topoisomerase I (Bethesda Research Laboratories), and incubation was for 15 minutes at 37°C. Samples were prepared for electron microscopy with the Alcian blue technique as described (*16*). The nucleoprotein complexes were examined with a JEOL JEM 1200 EX electron microscope. Molecular lengths were measured with a Hewlett-Packard HP9825A calculator coupled to an HP9864A digitizer.

Table 1. Quantification of nucleoprotein complexes formed under different reaction conditions. The electron microscope cross-linking experiments were performed under complete conditions, and in the absence of Fis, a functional enhancer, or DNA supercoiling. The substrate pMS618 was used for all experiments except for the mutant enhancer experiment in which pRJ860 was used (Fig. 1C). In the experiment without supercoiling, pMS618 that had been relaxed with topoisomerase was used. Most of the complexes in the "others" column are the described structures lacking a tail or loop.

Reaction conditions	DNA molecules scored			Structures observed (%)							
	Total scored	Molecules complexed (%)	Interpretable complexes measured	Inverta-somes	Paired *hix*	2° *hix* inverta-somes	2° *hix* paired *hix*	*hix* L1-enhancer	*hix* L2-enhancer	2° *hix*-enhancer	Others
Complete	5382	40.1	211	19.0	64.9	5.2	6.6	<1	<1	<1	2.4
Minus Fis	1535	36.3	96	0	84.4	0	5.2	0	0	0	10.4
Mutant enhancer	1211	42.2	96	4.2	77.1	1.0	4.2	2.1	2.1	1.0	8.3
Minus supercoiling	400	13.3	30	0	90.0	0	0	0	0	0	10.0

without Fis or a functional enhancer, the same faster migrating species could not be detected even after cross-linking (Fig. 3, lanes 4 to 7). This nucleoprotein complex can be interpreted as a highly ordered assembly that must have some additional suprastructure, since it migrates faster than the supercoiled substrate even though it is bound by protein. The requirement for Fis and a functional enhancer suggests that an interaction between Hin and Fis is required to generate this species.

Electron microscopy of nucleoprotein complexes. The structure of the cross-linked recombination complexes generated under Mg^{2+}-free reaction conditions was directly determined by electron microscopy. To facilitate interpretation of the structures, the molecules were reacted with one or more restriction enzymes to provide reference points on the DNA substrate molecule to orient the protein binding sites. In addition, the products were treated with topoisomerase to relax supercoiled loops before they were spread on Alcian blue–coated grids (*15, 16*). Figures 4 and 5 show examples of the predominant types of structures that were observed with the substrate pMS618 (see Fig. 1C). The structures were counted and the lengths of the DNA segments were measured (Fig. 6). The measurements indicate that a few of the structures utilize a secondary *hix* site located about 200 bp from *hix*L1 (2°*hix*, see Fig. 1C). Gel electrophoresis of the Mg^{2+}-free reaction confirmed the presence of an infrequently used cleavage site at this location, although the DNA sequence within this region does not show any striking homologies to the *hix* consensus sequence (*8*). Hin binding sites that deviate considerably from the consensus *hix* sequence have been reported (*10*).

Under conditions where the requisite proteins and sequences were included, two major structures were observed. From the measured lengths of the tails and loops, the first major structure (Figs. 4 and 6, A and B) represents the joining of the two recombination sites with the recombinational enhancer to give a structure analogous to that at the bottom of Fig. 1A. Including those complexes containing the 2°*hix* site, approximately 25 percent of the interpretable structures were in this configuration (Fig. 6). We term this complex an "invertasome." Cross-linking reactions in the absence of Fis do not give rise to any observable structures of this nature. Likewise, reactions with pRJ860, the substrate containing a mutant enhancer lacking one Fis binding site, formed far fewer of these structures (Table 1). Therefore, the efficient assembly of the invertasome requires the presence of all of the components needed for in vitro inversion. This structure may be the electrophoretically distinct species described above (also Fig. 3).

The second major structure represents more than 70 percent of the interpretable structures (Fig. 5). The measurements of the tails and loops of these complexes (Fig. 6, A and C) indicate that this structure involves only the pairing of recombination sites. Unlike the invertasome, this complex can be formed in comparable amounts under complete conditions or in reactions lacking Fis or a functional enhancer (Table 1). It is unclear whether this structure is a true recombination intermediate; however, it is conceivable that initial *hix* site pairing by Hin-Hin interactions could precede positioning at the enhancer (Fig. 1A). A nucleoprotein complex band that may correspond to paired *hix* structures can be detected upon gel electrophoresis of reaction products linearized by restriction diges-

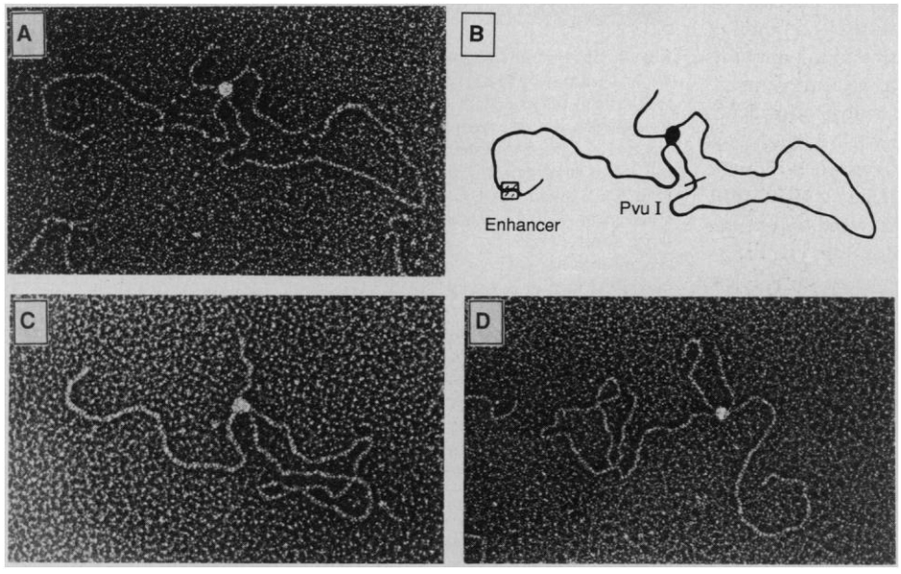

Fig. 5. Electron micrographs of paired *hix* complexes. (**A** and **C**) Molecules digested with Eco RV; (**B**) a tracing of the molecule in (A) identifying the locations of the Pvu I site, the enhancer and the tails and loop; (**D**) a molecule digested with both Eco RV and Pvu I. Digestion with Eco RV yielded the small tail 2 (491 bp), large tail 1 (1908 bp), and the large loop (3755 bp). Digestion with both Eco RV and Pvu I resulted in the small tail (491 bp), small tail 3 (644 bp), large tail 1 (1908 bp), and large tail 2 (3111 bp). Lengths were calculated from the center of the recombination sites. These structures were obtained as described in Fig. 4.

tion. This species is also present in reactions lacking Fis or a functional enhancer.

Electron microscopy of cross-linked structures digested with different restriction enzymes and structures assembled on molecules with the enhancer at different locations showed the presence of the same two types of structure. The same structures could also be found in reactions under optimal inversion conditions (including Mg^{2+}), but they were many fewer in number.

Antibody-tagging of nucleoprotein complexes. Purified antibodies to Hin and Fis were used to determine the protein composition of the cross-linked complexes. The nucleoprotein structures were incubated with antibody (immunoglobulin G, IgG) to Hin or Fis, and then incubated with protein A coupled to colloidal gold. The samples were prepared for electron microscopy after incubation with restriction enzyme and topoisomerase. The invertasome structure was bound by gold particles identifying Hin and Fis, respectively (Fig. 7, A and B). Thirty-eight percent of the invertasomes

A	sm tail 2	large tail	med loop	large loop	Number scored	Percent of interp	Percent of total
Invertasome	424 ±91	–	1857 ±146	3819 ±131	40	19.0	7.6
Paired *hix*	443 ±66	1884 ±114	–	3827 .±120	137	64.9	26.0
2°*hix* invertasome	403 ±103	–	2140 ±137	3575 ±66	10	5.2	2.1
2° *hix* paired *hix*	499 ±55	2139 ±90	–	3516 ±90	14	6.6	2.6

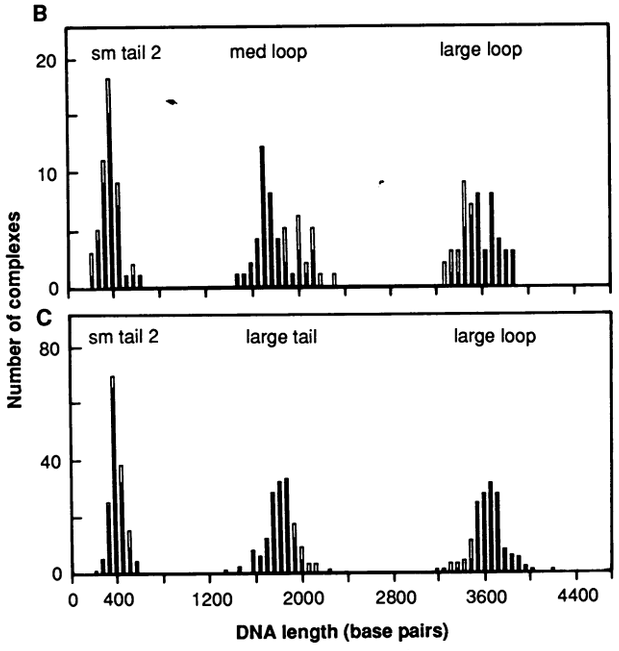

Fig. 6. (**A**) Summary of DNA lengths comprising the nucleoprotein complexes. Sizes of loops and tails measured for the nucleoprotein complexes generated in the presence of all of the required components are shown. For each measurement, the mean value and standard deviation are given. Only structures that could be interpreted and measured unambiguously are included. Calculations for the smallest tail (small tail 1) were not included since it was not visible in most cases. The number of structures measured is shown in the "interpretable complexes measured" column. For each structure, the relative percentage of interpretable complexes is shown. Complexes involving the 2°*hix* site contained a long tail 1 and medium loop that was 200 bp larger and a large loop that was 200 bp smaller (Figs. 1C, 4, and 5). (**B**) Histogram of the measured lengths of the loops and tails of the invertasomes. Shaded boxes refer to structures with *hix*L1 and *hix*L2. Open boxes refer to structures with 2°*hix* and *hix*L2. (**C**) Histogram of the measured lengths of the loop and tails of the molecules containing paired *hix* structures. Shaded boxes refer to structures utilizing *hix*L1 and *hix*L2. Open boxes refer to structures utilizing 2°*hix* and *hix*L2.

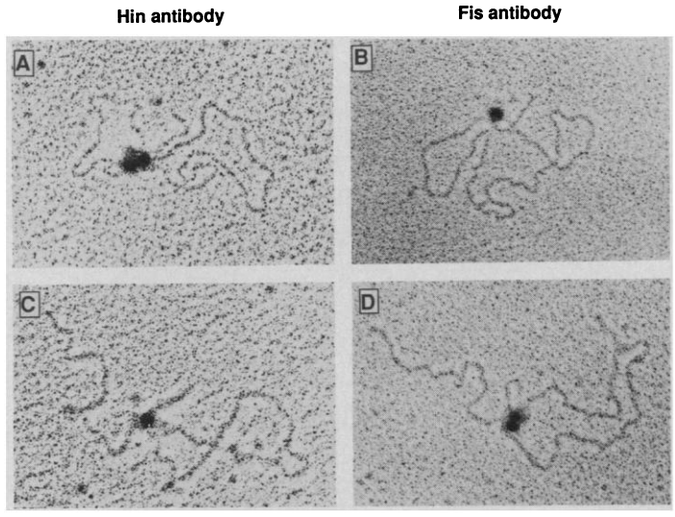

Fig. 7. Immuno-electron microscopy of nucleoprotein complexes. Darkly staining particles of gold identify the presence of Hin and Fis in both invertasomes and paired *hix* structures. (**A**) An invertasome with two gold particles indicating the presence of Hin in the complex; (**B**) an invertasome with one gold particle indicating the presence of Fis; (**C**) a paired *hix* structure with one gold particle indicating the presence of Hin; (**D**) a paired *hix* structure containing two gold particles indicating the presence of Fis. All of the structures have been digested with Eco RV restriction enzyme. In vitro reactions and subsequent cross-linking were performed in exactly the same manner as in Figs. 4 and 5. Reaction of these complexes with antibody to Hin (serum; courtesy of M. Bruist and M. Simon, Caltech) or Fis (purified antibody; courtesy of C. Ball and S. Finkel, UCLA), and protein A–gold was performed with a modification of the procedure described by Dodson *et al.* (*30, 31*). The samples were spread on Alcian blue–treated grids as described (*15, 16*).

incubated with either antibody to Hin or antibody to Fis were bound by one or more gold particles (Table 2). These experiments demonstrate that both Hin and Fis are associated with this structure and imply that a direct interaction occurs between the two proteins.

Unexpectedly, Fis was present in 31 percent of the paired *hix* structures generated in reactions containing Fis (Fig. 7D and Table 2). Antibody to Hin also confirmed the presence of Hin in these complexes (Fig. 7C and Table 2). We believe that the paired *hix* structures containing Fis were cross-linked while the *hix* sites were localized at the enhancer. These structures subsequently lost the enhancer by the release of Fis from its DNA binding site, leaving the covalent attachment of Fis to Hin at the paired recombination sites. As is described below, antibody localization of Fis in the paired *hix* structure required the presence of the enhancer. This provides the first direct evidence for the close physical association of the Hin and Fis proteins in an enhancer-dependent reaction.

In control reactions, fewer than 5 percent of the complexes were found by protein A–gold when antibody was omitted from the reactions (Table 2). When Hin was absent, no complexes were generated. In the absence of Fis, fewer than 5 percent of the complexes incubated with antibody to Fis were bound by gold. Finally, fewer than 5 percent of the free DNA molecules observed under any conditions were bound by gold.

Assembly of the invertasome. In defining the steps involved in the assembly of the invertasome, we have considered three possible routes leading to Hin-Fis interaction: (i) The association of the two proteins in solution with subsequent binding to their respective DNA binding sites; (ii) the initial binding of one protein to its recognition sequence followed by the addition of the second protein in solution to the first protein-DNA complex, or (iii) the binding of each protein to its respective recognition site followed by interaction with each other from widely spaced sites on the substrate molecule.

Table 2. Quantification of immunoelectron miscroscopy of nucleoprotein complexes. The nucleoprotein complexes generated with the substrate pMS618 were reacted with antibody to Hin or Fis and then with protein A–coupled gold. Included are the values obtained with all of the required components as well as values obtained in the absence of Fis or either antibody. NA, not applicable; ND, not done.

Reaction conditions	Invertasomes bound by gold (%)		Paired *hix* bound by gold (%)		Interpretable complexes scored (No.)	Free molecules bound by gold (%)	Free molecules scored (No.)
	Hin Ab	Fis Ab	Hin Ab	Fis Ab			
Complete	37.7	37.8	42.8	30.9	300	3	100
No Fis	NA	NA	ND	4.0	100	1	100
No antibody	3.7	3.7	4.1	4.1	100	3	100

In order to distinguish between these three possibilities, we performed similar antibody-tagging experiments on cross-linked complexes with either a DNA substrate lacking an enhancer sequence or a substrate without recombination sites. Substrates without an enhancer did not show localization of antibody to Fis, and plasmids containing only the enhancer sequence did not show localization of antibody to Hin as assayed by the presence of protein A–coupled gold particles. These results favor the third possibility that the proteins interact to form a stable complex that can be cross-linked only after they are bound to their respective recognition sites. These assays should have detected Hin-Fis associations if the two proteins could interact efficiently in solution before binding DNA or if only one of the proteins must be bound to its DNA substrate to interact with the other.

The question then arises, whether the Hin-bound recombination sites independently interact with Fis bound to the enhancer, or whether the Hin proteins interact before the association with Fis at the enhancer (Fig. 1A). While the first possibility cannot be eliminated at present, our observations are more consistent with the latter route. Structures representing paired recombination sites accounted for more than 70 percent of the molecules assembled in the presence of all of the required components (Table 1). The presence of Fis or an enhancer was not required for the efficient assembly of these paired *hix* complexes. Furthermore, the association of only one recombination site at the enhancer was seen very rarely, even with pRJ860, which contains only one of the Fis binding sites in the enhancer. Some of the structures with one recombination site associated with the enhancer may have been derived from inefficient cross-linking that allowed for the release of a recombination site from the invertasome. We cannot rule out that Hin-Hin cross-linking might be inherently easier to obtain than Hin-Fis cross-linking because of constraints imposed by the cross-linking agents or the available reactive sites on the proteins. Thus, our results could be experimentally biased in favor of paired *hix* sites. The initially paired Hin proteins may require some reorganization to assemble with the enhancer. This restructuring of the Hin complex may be crucial for enhancer activation of recombination.

Role of supercoiling in invertasome assembly. DNA supercoiling is required to obtain Hin-mediated inversion and Fis-dependent DNA strand cleavage (*11, 13*). When the Mg^{2+}-free reaction was performed on the pMS618 substrate previously relaxed with topoisomerase, the total number of complexes was reduced and no invertasomes were observed out of 400 molecules scored (Table 1). The number of paired *hix* structures generated with the relaxed substrate was reduced, from 29 percent (40.1 percent are complexed; of these, 71.5 percent are paired *hix* or 2° paired *hix*) to only 12 percent (13.3 percent are complexed; of these, 90 percent are paired *hix*) of the total number of molecules. Of the paired *hix* structures that could be formed with the relaxed substrate, only 5 percent (subtracting background of nonspecific protein A–gold binding) were associated with Fis protein as assayed by immuno electron microscopy. Taken together, these findings suggest that supercoiling promotes the association of Hin and Fis into a configuration at the enhancer that can be cross-linked. Supercoiling seems to play a lesser role in pairing Hin-bound recombination sites when compared to its importance for invertasome formation. While the stability of cross-linked complexes subsequently incubated with topoisomerase might be reduced, our electron microscopy studies indicate that both invertasomes and paired *hix* structures can be recovered after removal of supercoiling.

Relation to other recombination systems. Electron microscopy, together with gel electrophoresis, has been used to analyze the structure and composition of nucleoprotein complexes formed between DNA sequences during Hin-mediated site-specific inversion. From these studies, the Hin and Fis proteins have been shown to physically interact in order to assemble the two recombination sites at the recombinational enhancer to form a tripartite, three-looped structure called an invertasome. The assembly of the invertasome requires that the substrate molecule be supercoiled.

It is particularly intriguing that the association of Fis and Hin proteins into a cross-linked species requires that both polypeptides be bound to their respective binding sites on DNA. This requirement could simply reflect a weak affinity of the two proteins for each other; this weakness can be compensated by increasing the local concentration of the protein by binding to the same DNA molecule. Alternatively, a conformational change may be induced by binding to DNA, thus promoting the interaction. The finding that Fis-Hin interactions do not efficiently occur with relaxed molecules does not favor these models, but points to the topology of supercoiled DNA as being most important. A particular alignment or interwrapping of protein-bound DNA sites would be strongly favored on supercoiled DNA, and this may be essential for stable interaction between Fis and Hin. Similar ideas have been proposed for other recombination systems that require DNA supercoiling (*17–19*).

The structure of an intermediate complex involving the two recombination sites with the enhancer was postulated from earlier studies of the Hin- and related Gin-mediated inversion systems (*12, 19*). Electron microscopic evidence for a three-looped structure has been obtained in the Gin system (*20*). In our study, the use of modified reaction conditions allowed for the accumulation of large numbers of intermediates that are more difficult to capture under normal reaction conditions. Although nucleoprotein structures could be detected by electrophoresis in the absence of chemical cross-linking, these reagents helped to fortify otherwise unstable structures so that they could be observed with the electron microscope.

Many of the other site-specific recombination reactions that have been analyzed in detail require the function of a single recombinase protein which interacts with the DNA at, and in some cases immediately adjacent to, the positions of strand exchange (*21*). Rather than relying solely on the recombinase, the Hin system utilizes an additional protein, Fis, to promote efficient inversion. Similarly, the chromosomal integration and excision reactions of bacteriophage lambda require not only the integrase (Int) protein, but also accessory proteins such as IHF, Xis, and Fis for recombination (*22–24*). Although there appears to be some flexibility in the exact location of these proteins, their binding sites are all located within 150 bp of the sites of strand exchange (*24, 25*). A major role of these ancillary proteins seems to be in promoting the assembly of reactive complexes, intasomes, at the sites of recombination (*18, 25,*

26). In the case of IHF binding to one of its sites (*26*), the sole function of the protein is to bend DNA, thus facilitating the association of distant Int binding sites without directly interacting with other proteins. Indeed, where the Fis protein is concerned, there appears to be no evidence supporting direct contact with the Int recombinase, unlike our finding that Fis interacts with Hin in the inversion reaction. It therefore seems likely that Fis performs very different functions in promoting these two site-specific recombination reactions.

The phage Mu transposon also contains an enhancer-like sequence that is required to achieve efficient recombination (*27*). The transposase protein appears to interact with two domains within this site in addition to the sites located at the transposon ends. The formation of an intermediate that includes all three of these sequences has been proposed. Thus, DNA transposition reactions may also rely on the formation of nucleoprotein structures analogous to the invertasome.

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28. HU, Fis, and Hin were purified as described (*13*). Fis was purified from an overproducer strain (S. E. Finkel and R. C. Johnson, unpublished data). Hin was purified from a *fis*$^-$ *Escherichia coli* strain.
29. The concentration of Fis was determined (Biorad protein assay) with a known concentration of Fis. The Fis standard was prepared from a solution that had been dialyzed into 0.2 *M* NH_4HCO_3, and a portion was analyzed by quantitative amino acid hydrolysis after lyophilization.
30. After being cross-linked with DSP and glutaraldehyde, the reactions were passed over a mini-Sepharose column equilibrated with medium salt buffer. Peak fractions were collected and incubated with 2.5 μg of purified (DEAE-Sepharose) rabbit antibody to Hin or Fis protein (30 minutes at 37°C). This reaction mixture was then passed over another mini-column equilibrated with protein A buffer (40 mM Hepes-KOH, pH 7.6, 100 mM KCl, 11 mM magnesium acetate). Peak fractions were incubated with 3 μl of *Staphlococcus aureus* protein A (8 μg/ml) coupled to either 10- or 15-nm colloidal gold particles (10-mm size for antibody to Fis, 15 nm for antibody to Hin) (E-Y Laboratories) for 15 minutes at 23°C. This reaction was passed over another mini-column equilibrated with medium salt buffer. Peak fractions were incubated with 12 units of Eco RV and 5 units of topoisomerase I (Bethesda Research Laboratories) for 15 minutes at 37°C.
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