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The Role of Negative Supercoiling in Hin-mediated Site-specific Recombination*

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A series of biochemical assays were developed and performed to monitor the molecular events that occur during the Hin-mediated DNA inversion reaction. These events can be divided into five different stages: 1) binding of proteins (Hin, Fis, and HU) to DNA; 2) pairing of Hin-binding sites; 3) invertasome formation; 4) DNA strand cleavage; 5) strand rotation and religation. A series of topoisomers of the wild type DNA substrate plasmid (ranging from fully relaxed molecules to those with more than the physiological superhelical density (the physiological superhelical density of pKH336 from *Escherichia coli* DH10B® is −0.072 in this study)) was generated, and the role of negative supercoiling in each step of the inversion reaction was investigated. We found differences in the dependence of the formation of paired Hin-binding sites and of the invertasome formation on the superhelical density of the substrate plasmid. Pairing of Hin-binding sites occurs independently from invertasome formation, and a relatively low degree of negative supercoiling is enough to promote maximal pairing. However, efficient invertasome formation requires higher levels of negative supercoiling.

The Hin recombinase catalyzes site-specific recombination that results in the inversion of a 993-bp[1] region of DNA (H region) in the chromosome of *Salmonella typhimurium* (Zieg *et al.*, 1977; Zieg and Simon, 1980). The H region is flanked by 26-bp DNA sequences (*hixL* and *hixR*) (Zieg and Simon, 1980; Johnson and Simon, 1985). Each of these 26-bp sequences is composed of two imperfect 13-bp inverted repeats. Inversion of the H region causes a switch in expression between alternate forms of flagellin (H1 or H2) resulting in a change in flagellar phenotype known as phase variation (Lederberg and Iino, 1956; Zieg *et al.*, 1977). Hin belongs to a group of site-specific recombinases called DNA invertases which include Gin, Cin, and Pin (Glasgow *et al.*, 1989a). Gin and Cin promote the inversion of the G and C regions in bacteriophage Mu and P1 resulting in changes in host specificity (van de Putte *et al.*, 1980; Iida, 1984). Pin has been shown to mediate the inversion of the P region of the *Escherichia coli* chromosome, but the function of the inversion is not known (Plasterk *et al.*, 1983). The Hin protein has been purified and shown to invert DNA *in vitro* (Johnson and Simon, 1985). Hin has a molecular mass of 22 kDa, exists as a dimer in solution, and binds to *hix* sequences as a dimer (Glasgow *et al.*, 1989b).

Efficient Hin-mediated inversion requires two additional proteins, Fis and HU. Fis is a dimeric protein with a subunit molecular mass of 12 kDa. Its presence stimulates the rate of the inversion reaction 150-fold (Johnson *et al.*, 1986). It binds to two sites within a DNA sequence called the enhancer which can be located almost anywhere on the same plasmid as the *hix* sites (Bruist *et al.*, 1987; Johnson and Simon, 1985). Fis is needed to form the correct synaptic complex in the initial stage of Hin or Gin-mediated inversion. Recently, Kanaar *et al.* (1990) have suggested that Fis is subsequently released from the complex. HU binds DNA nonspecifically, and HU can increase inversion efficiency 10-fold by promoting the bending of DNA between the enhancer and *hixL* and thus facilitating Fis-Hin interaction (Fig. 1, Johnson *et al.*, 1986).

Recent work using electron microscopy to visualize DNA inversion and previous kinetic studies allow us to define a number of discrete intermediate stages in the molecular process of inversion (Fig. 1, Heichman and Johnson, 1990).

1) Protein binding to DNA. Hin binds to *hix* sites and Fis binds to enhancer sequences specifically and independently of each other (Glasgow *et al.*, 1989b). Hin exists in solution as a dimer and most probably bind to DNA as a dimer.

2) Pairing of *hix* sites. Two *hix* sites are brought together by protein interaction resulting in paired *hix* sites. Heichman and Johnson (1990) showed that Hin-Hin interaction probably occur before Hin-Fis interaction in the initial stage of inversion.

3) Formation of invertasome. The paired *hix* sites and Hin complex makes physical contact with Fis bound to the enhancer. This interaction leads to the formation of an intermediate which has been called the invertasome (Heichman and Johnson, 1990).

4) Strand cleavage. Hin makes a two base-staggered cut in the center of each *hix* site. The serine 10 residue of Hin is believed to make a transient phosphate ester linkage with the recessed 5′-phosphate end of the cleaved DNA (Johnson and Bruist, 1989).

5) Strand rotation and religation. It has been suggested that strand rotation can be achieved through exchange of subunits because the DNA strands are covalently attached to the protein (Stark *et al.*, 1989; Kanaar *et al.*, 1990; Heichman *et al.*, 1991). Once in the inverted orientation Hin religates the four DNA strands.

Another requirement for Hin-mediated inversion is negative supercoiling of DNA substrates. Under normal circumstances, inversion does not occur if the DNA is fully relaxed (Johnson *et al.*, 1984). While pairing of *hix* sites occurs at a reduced efficiency on relaxed DNA, invertasome formation

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[1] The abbreviations used are: bp, base pairs; kb, kilobase pairs; SDS, sodium dodecyl sulfate.

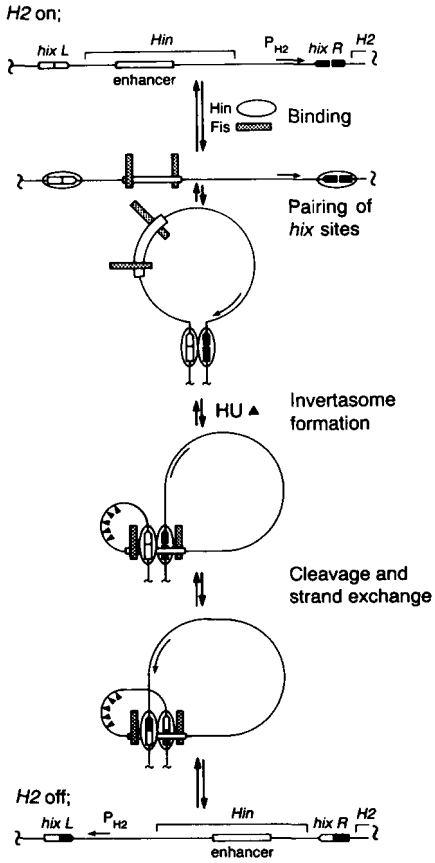

FIG. 1. **Schematic representation of the molecular process of the Hin-mediated inversion reaction.** The H region in the chromosome of *S. typhimurium* is shown. Hin binds to *hix* recombination sites as a dimer while Fis binds independently to each domain of the recombinational enhancer as a dimer (*Binding*). Interaction between Hin dimers bound to two *hix* sites brings two recombination sites close together (*Pairing of hix sites*). A productive synaptic complex is formed at the enhancer between paired *hix* and Hin complex and Fis (*Invertasome formation*). Hin cleavages in the center of each *hix* site followed by strand exchange and religation (*Cleavage and strand exchange*). The resulting recombination product has inverted the genetic information between the *hix* sites. P_{H2}, promoter for the *H2* gene.

has not been reported to occur on relaxed DNA (Heichman and Johnson, 1990).

In order to investigate the role of supercoiling in each step of the inversion reaction, a series of topoisomers of the wild type DNA substrate was generated. The degree of negative supercoiling in these topoisomers varied over a range from fully relaxed to more than physiologically supercoiled. Each step of the inversion reaction, except the binding of Hin to *hix* sites (Hin binds equally well to *hixL* sites on supercoiled and linearized DNA, Glasgow *et al.*, 1989b), was assayed with each set of topoisomers. Strand cleavage and strand rotation-religation were probed by a "cleavage assay" and an "inversion assay," respectively. Protein cross-linking was used to measure pairing of *hix* sites and invertasome formation. We found that pairing of *hix* sites occurred efficiently at relatively lower superhelical density while inversion and invertasome formation required higher levels of superhelical density.

MATERIALS AND METHODS

Proteins and DNA—Construction of the positive control plasmid pKH336 is described in the accompanying paper (Lim *et al.*, 1992). The wild type *hin* gene under the control of the *tac* promoter and *lacI*^{q} was isolated as a 2.5-kb *Eco*RI-*Hin*dIII fragment from pKH66 (Hughes *et al.*, 1988). This fragment was ligated to *Eco*RI-*Hin*dIII-digested pBluescriptII SK+ (Stratagene) to create pHL104. Type I topoisomerase from calf thymus was purchased from Bethesda Research Laboratories Inc (BRL). Proteinase K was from Boehringer Mannheim. *E. coli* strain DH1 harboring pHL104 was used to prepare Hin. Hin was purified with the methods described in Johnson and Simon (1985). Fis and HU were prepared as described by Johnson *et al.* (1986). Quantitation of proteins was performed by the method of Bradford (Bradford, 1976). Hin preparation used in this study was 50% pure estimated by scanning SDS-polyacrylamide gel with a LKB Ultroscan XL laser densitometer and was not contaminated with Fis. HU and Fis were purified to homogeneity. pKH336 was maintained in *E. coli* strain DH10B^{TM} purchased from BRL.

Assays of Reaction Intermediates—The pairing of *hix* sites by Hin was followed after cross-linking with glutaraldehyde. Three-hundred ng of Hin and 1 μg of pKH336 in 25 μl were incubated at 37 °C for 20 min in "cleavage buffer" (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 10 mM Na_2EDTA, 100 μg/ml polycytidylic acid (Pharmacia LKB Biotechnology Inc.), 30% ethylene glycol). One μl of 3% glutaraldehyde was added, and incubation was continued for 1 h at room temperature. In preliminary experiments timed samples were taken and tested for the extent of the reaction. After 5 min, no further increase in paired *hix* sites and Hin complex was found. We therefore concluded that addition of glutaraldehyde both inactivated Hin and cross-linked the products rapidly. We extended the incubation with glutaraldehyde for up to 60 min because it allowed us to conveniently collect a variety of samples and prepare for dialysis. Samples were dialyzed against MS buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl) for 45 min in a Microdialyzer^{TM} (Pierce Chemical Co.). Dialysates were digested with *Cla*I and *Pst*I for 1 h and electrophoresed on 1% agarose gels at 2 V/cm for 16 h in TBE buffer (100 mM Tris-HCl, pH 8.3, 90 mM borate, 2 mM Na_2EDTA). After electrophoresis, the gel was stained with ethidium bromide (0.1 μg/ml). Photographs were made on Polaroid Type 55 film and negatives were scanned with an LKB UltroScan XL laser densitometer. Invertasome formation was also followed after cross-linking with glutaraldehyde. The reaction was initiated by incubation of 300 ng of Hin, 100 ng of HU, and 30 ng of Fis with 1 μg of pKH336 DNA in a total volume of 25 μl of cleavage buffer at 37 °C for 20 min. Cross-linking, dialysis, restriction digestion, and agarose gel electrophoresis were performed exactly as in the assay for *hix* sites pairing. DNA cleavage during the inversion reaction was measured by the method previously described by Johnson and Bruist (1989). Starting conditions were identical to the invertasome formation assay. After incubation at 37 °C for 1 h, 0.5 μl of proteinase K (10 mg/ml) and 0.5 μl of 25% SDS were added and incubation at 37 °C continued for an additional 30 min. Cleaved DNA fragments were analyzed by 1% agarose gel electrophoresis as described for the pairing of *hix* sites assay. The extent of inversion was measured as follows: 300 ng of Hin, 100 ng of HU, and 30 ng of Fis were incubated with 1 μg of pKH336 in 50 μl of "inversion buffer" (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, and 100 μg/ml polycytidylic acid) at 37 °C for 1 h. Proteins were removed by sequential phenol and chloroform extractions, and DNA was precipitated with ethanol. DNA was resuspended in MS buffer, digested with *Pst*I and *Cla*I for 2 h, and electrophoresed in an agarose gel as described for the *hix* sites pairing assay.

Preparation of Topoisomers of pKH336—Variants of pKH336 of defined superhelical density were generated by the method of Keller (1975). pKH336 (6 μg) was incubated with 20 units of calf thymus topoisomerase I (BRL) in the presence of 0–6.4 μg/ml of ethidium bromide in 100 μl of topoisomerase I buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM Na_2EDTA, 30 μg/ml bovine serum albumin) at 37 °C for 1 h. Proteins and ethidium bromide were removed by a phenol extraction and two successive ether extractions, and DNA was precipitated with ethanol. Average linking differences between samples were measured by the standard band counting method (Keller, 1975). Three different concentrations of chloroquine (0.5, 2.5, and 75 μg/ml) in agarose gel were used to count the linking differences generated (Shure *et al.*, 1977). The specific superhelical density (σ) was calculated by a method described in Singleton and Wells (1982). Using 10.6 bp/helical turn (Rhodes and Klug 1980), the linking number of totally relaxed pKH336 (LK_o of pKH336) is 500.

In Gel Digestion of DNA-Protein Complex—An agarose block containing DNA-protein complex was cut out (2 mm W × 10 mm L × 5 mm H) and soaked in 1 ml of a desired buffer at 37 °C for 30 min before enzyme treatment. Final concentration of proteinase K was 2

mg/ml in 50 mM Na_2EDTA, 10 mM Tris-HCl, pH 7.5, 1% SDS, and that of *Eco*RV was 200 units/ml in MS buffer. The incubation continued for additional 2 h at 37 °C after addition of an enzyme. The agarose block was removed from buffer, washed twice with water, and plugged into a well of an agarose gel.

RESULTS

Formation of Paired hix Complex

Plasmid pKH336 (Fig. 2*a*) was used as the wild type DNA substrate for Hin-mediated *in vitro* inversion reactions throughout this study. pKH336 contains two *hixL* recombination sites separated by 900 bp DNA in which the wild type enhancer is located 100 bp away from the left *hixL* site. Efficient inversion of the 900-bp DNA fragment flanked by two *hixL* sites was observed when supercoiled pKH336 was incubated with the necessary proteins, Hin, Fis, and HU in the standard inversion buffer at 37 °C (data not shown).

We have devised a simple method to detect Hin-Hin interactions that would result in bringing the two recombination sites together. The assay is based on use of a protein cross-linker to trap the close contact between Hin dimers bound on two separate *hixL* sites followed by identification of the protein-DNA complex on agarose gel electrophoresis (see Fig. 2*a*). In the assay, interaction between Hin molecules on *hix* sites was initiated by incubation of supercoiled substrate DNA (pKH336) with Hin but not Fis in buffer containing ethylene glycol and no Mg^{2+} (Johnson and Bruist, 1989). The complexed Hin on the substrate DNA was cross-linked by glutaraldehyde which was subsequently removed by dialysis against MS buffer. After dialysis, the material was digested with *Pst*I and *Cla*I and subjected to agarose gel electrophoresis. Two novel bands (labeled T and P′) were observed in a lane where Hin is present; the bands were missing where Hin was omitted (Fig. 2*b*, *lanes 2* and *1*, respectively). The T band migrated slower than linearized pKH336. It was subsequently identified as Hin and paired *hix* complex (see below). The other novel band (P′ band), which migrated with an apparent size of 1.0 kb DNA is a Hin-DNA complex that has a Hin dimer bound to the left *hixL* site of a 0.9-kb *Pst*I-*Cla*I restriction fragment. When the 1.0-kb band (P′) was isolated and treated with proteinase K in the presence of SDS followed by agarose gel electrophoresis (data not shown), it migrated as a 0.9-kb DNA fragment (P band). Thus the retardation was caused by Hin binding.

Two different methods were used to prove that the novel T band represents the expected paired *hix* complex. An agarose block that contained the T band was cut out and divided into 2 aliquots. One portion was digested with *Eco*RV and the other was treated with proteinase K in the presence of SDS (see "Materials and Methods"). *Eco*RV digestion of the protein-DNA complex should produce a 3.4-kb DNA fragment corresponding to the distance between the *Pst*I and *Eco*RV sites in pKH336 (Fig. 2*a*). Proteinase K treatment of the band was expected to generate 4.4- and 0.9-kb fragments, since proteinase K would release *Pst*I-*Cla*I-restricted DNA fragments from the complex. As shown in Fig. 2*c*, both treatments produced the DNA fragments expected. However, the DNA fragments were slightly retarded compared to those generated by control digestion of unreacted pKH336. The combination of glutaraldehyde, SDS (for proteinase K reaction) treatment, and initiation of electrophoresis from a sample in a preformed agarose block may account for this retardation.

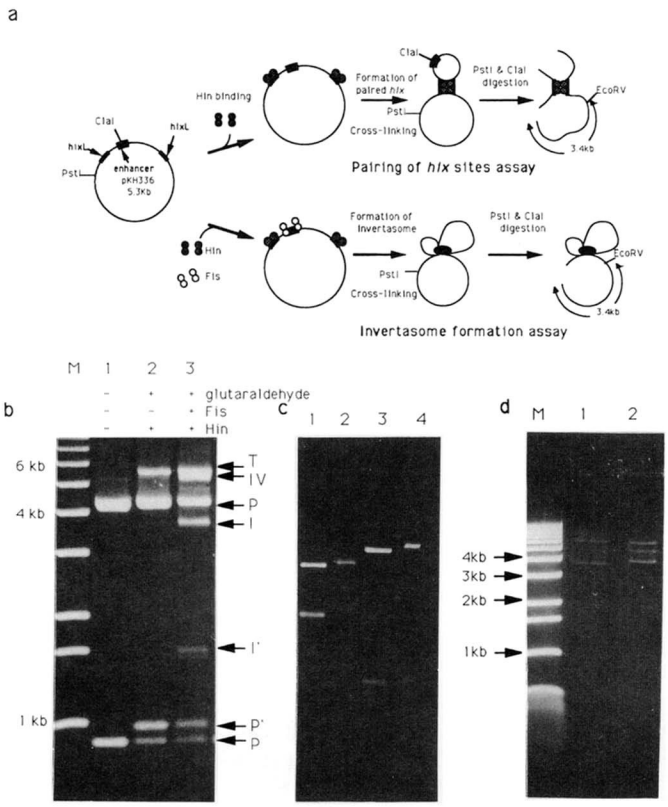

FIG. 2. **Pairing of *hix* sites and invertasome formation assays.** *a*, the wild type substrate plasmid pKH336, schematic representation of the pairing of *hix* sites assay, and invertasome assay are shown. Two *hixL* sites in pKH336 are in inverted orientation. The distance between the two *hixL* sites is 0.9 kb and the distance between *Pst*I site and left *hixL* site is 0.8 kb. The position of the enhancer in pKH336 relative to the two *hixL* sites is the same as they are organized in the chromosome of *S. typhimurium*. *Pst*I and *Cla*I sites are separated by 0.9 kb before inversion. *b*, a typical agarose gel electrophoresis of the pairing of *hix* sites assay (*lane 2*) and the invertasome formation assay (*lane 3*) on pKH336. *Pst*I-*Cla*I digestion of pKH336 generates 4.4- and 0.9-kb DNA fragments (*lane 1*). *Lane M* is the 1-kb ladder (BRL). *T*, paired *hix* sites structure and Hin complex. *IV*, invertasome. *p*, *Pst*I-*Cla*I-digested DNA fragments of pKH336. *P′*, retarded 0.9-kb *Pst*I-*Cla*I-digested pKH336 fragment due to the binding of of Hin to *hixL* site. *I* and *I′* DNA fragments generated by the low frequency inversion during invertasome formation assay. *c*, agarose gel electrophoresis of *Eco*RV-digested T band (*lane 2*) and proteinase K-treated T band (*lane 4*). *Eco*RV-*Pst*I digestion of pKH336 is shown in *lane 1* and *Pst*I-*Cla*I digestion of pKH336 is shown in *lane 3* for comparison. *d*, agarose gel electrophoresis of *Eco*RV-digested IV band (*lane 1*) and proteinase K-digested IV band (*lane 2*). *Lane M* is the 1-kb ladder.

Trapping the Invertasome

The assay for *hix* sites pairing is done in the absence of Mg^{2+} and the presence of ethylene glycol. Those conditions do not allow inversion to go to completion (Johnson and Bruist, 1989); thus, by simply adding Fis to the assay we should be able to trap invertasomes that are formed. When this experiment was performed, and the digested products of the cross-linked plasmid compared to those formed in the pairing *hix* sites assay, three additional bands were identified (*I*, *I′* and *IV* bands, Fig. 2*b*, *lane 3*). The I and I′ bands had the same rate as the *Pst*I-*Cla*I restriction fragments of pKH336 found after one round of inversion had occurred; we assume that the I and I′ bands are generated as a result of low efficiency inversion. Indeed, it has been shown that an intermediate synaptic complex formed in the presence of ethylene glycol in cleavage buffer can produce inversion products if the ethylene glycol is removed (Johnson and Bruist, 1989). Invertasomes that were not cross-linked by glutaraldehyde would have produced inversion products and/or *de*

novo inversion catalyzed by free Hin and Fis molecules may have taken place during the dialysis step. In any event the level of inverted substrates only represents a small fraction of the total DNA. The third novel band (labeled *IV*) that migrated slightly faster than the paired *hix* complex (*T band*) was the protein-DNA complex formed as a result of the presence of Fis in the reaction.

To clearly demonstrate that the IV band was representing the cross-linked invertasome, agarose blocks containing IV bands were cut out from two identical assays. One agarose block was restriction digested with *Eco*RV and the other was treated with proteinase K in the presence of SDS. Enzyme-treated agarose blocks were subjected to gel electrophoresis (see "Materials and Methods"). *Lane 1* in Fig. 2*d* shows the expected 3.4-kb DNA fragment resulting from the *Eco*RV restriction digestion of *Pst*I-digested invertasome structure (also see Fig. 2*a*). The other band which migrated slower than the *Pst*I-*Eco*RV-digested 3.4-kb fragment corresponds to the rest of the protein-DNA complex. Proteinase K digestion of IV band produced six different DNA fragments. Because DNA cleavage occurs at *hix* sites after invertasome formation, we were able to assign these six DNA fragments. The three small fragments resulted from the cleavage of *Pst*I-left *hixL*, left *hixL*-right *hixL*, and *Pst*I-right *hixL* in the order of increasing molecular weight. The other three bands are corresponding vector fragments based on their molecular weight.

The Effect of Superhelical Density (σ) on Four Different Inversion Stages after Protein Binding

Formation of Paired hix Complex—To determine the effect of supercoiling of the DNA substrate on intermediates in the inversion reaction, a series of topoisomers of pKH336 was prepared using topoisomerase I in the presence of different amounts of ethidium bromide. The average linking differences of the generated topoisomers were measured by the band counting method (Keller, 1975, see "Materials and Methods"). The supercoiling of the products ranged from totally relaxed ($\sigma = 0$) to more than the physiological superhelical density. Paired *hix* complex, as shown by the formation of the T band, occurs even if the substrate plasmid is fully relaxed (Fig. 3*a*, *lane 1*). The amount of T band formed on the fully relaxed substrate was 30% of that formed with a template at the physiological superhelical density. By electron microscopy, the number of paired *hix* complex formed on fully relaxed substrate plasmid was 40% of that formed on supercoiled DNA (Heichman and Johnson, 1990). Linearized pKH336 (labeled *L*) might have been formed from a single digestion by either *Pst*I or *Cla*I in Fig. 3*a*.

To determine whether or not the formation of paired *hix* complex has reached equilibrium in 20 min of reaction time in the pairing of *hix* sites assay of Fig. 3*a*, we have investigated the kinetics of the formation of paired *hix* complex on topoisomers with σ of 0 (fully relaxed), −0.016, −0.026, and −0.072 (physiological). In this experiment, all the other variable parameters were fixed and only the time of incubation of Hin with the topoisomers of pKH336 was changed from 0 to 120 min. The amount of T band was measured as a percent of total input DNA and plotted against the time of incubation. Fig. 4 shows the result. Formation of the paired *hix* complex increases with the time of incubation suggesting that the 20-min time point used in the previous pairing *hix* sites assay of Fig. 3*a* reflects the rate of *hix* pairing rather than the equilibrium level of pairing that could form. Fig. 4 also demonstrates that the rate of *hix* pairing at a superhelical density lower than −0.026 is slower than the rate observed with DNA that had a physiological level of supercoiling. The rate is relatively

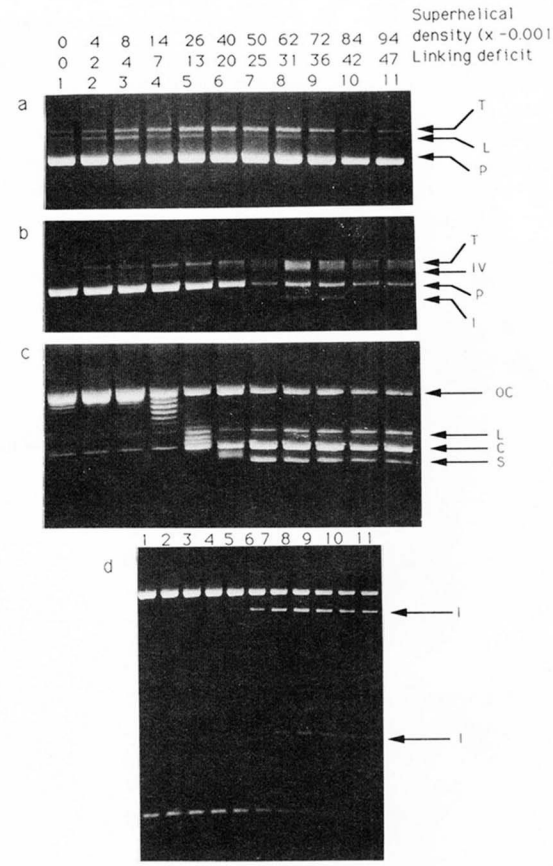

FIG. 3. **Agarose gel electrophoresis of four different assays on topoisomers of pKH336.** *a*, pairing of *hix* sites assay. *b*, invertasome formation assay. *c*, cleavage assay. *d*, inversion assay. *T*, paired *hix* and Hin complex. *L*, linearized pKH336. *IV*, invertasome. *P*, large fragment of *Pst*I-*Cla*I-digested pKH336. *I*, inversion product large fragment. *I'*, inversion product small fragment. *OC*, a combination of nicked open circular and fully relaxed pKH336. *C*, large fragment of Hin-cleaved pKH336. *S*, supercoiled pKH336. The average superhelical density of each topoisomer and corresponding linking deficit are shown above each lane. Because the C band in *lane 5* of *panel c* was mixed with uncleaved topoisomers, the intensity of the band was measured on a film that was taken when the supercoiled topoisomers had moved ahead of the cleaved band. The average superhelical density of the lanes in *panel d* is the same as in *panels a–c*.

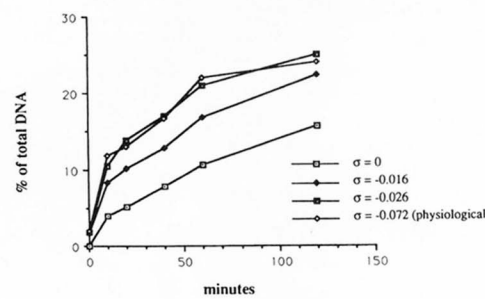

FIG. 4. **Time course of formation of the paired *hix* sites structure on topoisomers of pKH336.**

constant with substrates that have superhelical density greater than −0.026.

Invertasome Formation, Cleavage, Strand Rotation, and Religation

Synaptic complex (invertasome) formation (*IV band* in Fig. 3*b*) started to be detected at $\sigma = -0.026$ (*lane 5*). A very faint

band of inversion (*I band* in Fig. 3*b*) is also first seen at the same density, suggesting that both invertasome formation and complete inversion start at the same range of superhelical density.

Johnson and Bruist (1989) demonstrated that the Hin-mediated DNA cleavage at the center of the *hix* site can be detected in cleavage buffer. Because Hin is connected to cleaved DNA strands via a phosphate ester linkage under these conditions, proteinase K digestion of Hin is necessary to resolve the 4.3-kb cleaved DNA fragments in agarose gel electrophoresis. When this assay was applied to topoisomers of pKH336, the same results were observed for those topoisomers with a superhelical density −0.062 or more (*lanes 8–11* in Fig. 3*c*). At lower superhelical density (*lanes 1–4*), most of the plasmids remain uncleaved. These bands labeled *OC* are a mixture of nicked open circular plasmids which constitute at least 10% of each topoisomer preparation, and low superhelical density topoisomers (less than four in linking deficit) that could not be resolved in the agarose gel electrophoresis condition used in this assay. *Lanes 4* and *5* in Fig. 3*c* show the resolved topoisomers on which strand cleavage did not occur. A band that moved slightly ahead of the cleaved band (*C band*) in *lane 6* of Fig. 3*c* is also the mixture of unresolved, uncleaved topoisomers. Linearized plasmid (*L band*) on which only one *hix* site is cleaved is also observed. A small amount of strand cleavage at both *hixL* sites (8% of cleavage observed at physiological superhelicity) was detected even when the plasmid was fully relaxed (Fig. 3*c*). Because this amount of cleavage was observed regardless of the presence of Fis on relaxed plasmids (data not shown) and this cleavage does not lead to inversion (Fig. 3*d*, *lane 1*), we suggest that this cleavage occurs without invertasome formation. There is a dramatic change in the level of strand cleavage at the superhelical density where invertasome formation starts to occur. To measure the completed reaction, inversion buffer was used under standard inversion assay conditions. Fig. 3*d* shows that strand rotation-religation as seen by the accumulation of inversion products (the *I* and *I′ bands*) also starts to occur at the average σ value of −0.026.

Fig. 5 summarizes the amount of stage-specific bands (T band in paired *hix* complex, IV in invertasome formation, C band in cleavage, and I band in inversion) formed at each superhelical density. Paired *hix* complex increases as superhelical density increases and reaches a plateau at a σ of −0.026, which corresponds to an average linking deficit of 13. The succeeding steps in inversion after pairing of *hix* sites start to occur at a density of −0.026 and reach maximum efficiency at a density of −0.062 (linking deficit of 31). These data demonstrate that lower superhelical density is enough to drive pairing of *hix* sites reaction at maximum efficiency, but maximum efficiency of invertasome formation requires higher superhelical density. Furthermore, these data suggest that the formation of paired *hix* complex is independent of invertasome formation. To clearly demonstrate the relationship between pairing of *hix* sites and invertasome formation, the densitometer-scan profiles of each lane of the invertasome formation assay were overlapped and compared (Fig. 6). This profile shows that pairing of *hix* sites (*T*) reaches maximum at (*lane 5*) $\sigma = -0.026$ even with Fis in the reaction demonstrating that the formation of invertasome does not interfere with the formation of paired *hix* complex at this superhelical density. There are differences in both the height and the left shoulder of T between *lanes 4* and *5* showing that not only the level of pairing *hix* sites increases but the invertasomes start to form in *lane 5*. Between *lanes 5* and *6*, only the difference in height of the left shoulder of T is observed, indicating that the formation of paired *hix* complex has reached its maximum at *lane 5* and the formation of invertasomes increases in *lane 6*. These data are consistent with the notion that pairing of *hix* sites precedes and is necessary for invertasome formation. A superhelical density of more than −0.072 does not seem to interfere with any of the inversion steps.

Effects of Distance between Two hix Sites on the Formation of Paired hix Complex

To determine the effects of the distance between two *hixL* sites on formation of paired *hix* complex, we performed pairing of *hix* sites assays on pKH336 linearized by two different restriction enzymes and also on fully relaxed closed circular pKH336 (Fig. 7). *Pst*I or *Cla*I digestion of pKH336 separates the two *hixL* sites by 0.9 or 4.4 kb, respectively (Fig. 2*a*). The linear distance between the two *hixL* sites in fully relaxed pKH336 is the same as when they are located on pKH336 linearized by *Pst*I. The amount of paired *hix* complex on *Cla*I-linearized pKH336 was 30% of that formed on *Pst*I-linearized pKH336. There was almost no difference in the level of paired *hix* sites structure between fully relaxed and *Pst*I-linearized pKH336 (Fig. 7). These results clearly demonstrate that the distance between *hixL* sites determines the frequency of bringing Hin dimers together.

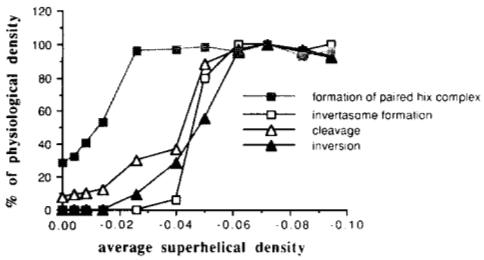

FIG. 5. **Dependence of four different stages in the Hin-mediated inversion reaction on the average superhelical density of pKH336.** Stage-specific bands in Fig. 3 were quantitated using a densitometer. To compensate for uneven exposure of an agarose to UV light during photography, quantitation of each stage-specific band was expressed as percent of the total except for the inversion assay. A previously published method of calculating the number of inversions/DNA molecule was used to quantitate each lane of the inversion assay (Bruist and Simon, 1984). Stage-specific band formation is represented as percent of that formed at the physiological density (Fig. 3, *lane 9*).

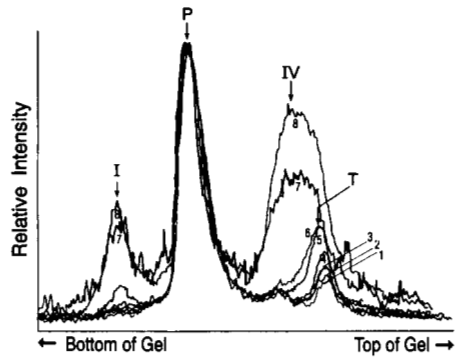

FIG. 6. **Overlapped gel scan profile of the invertasome formation assay (Fig. 3*b*).** Gel scan profiles of each lane of Fig. 3*b* (from *lanes 1* to *8*) were overlapped so that the *P band* bands were lined up perfectly. *I*, inversion product large fragment. *P*, large fragment of *Pst*I-*Cla*I-digested pKH336. *IV*, invertasome formation. *T*, paired *hix* sites and Hin complex. The *numbers* indicate corresponding lanes in Fig. 3*b*.

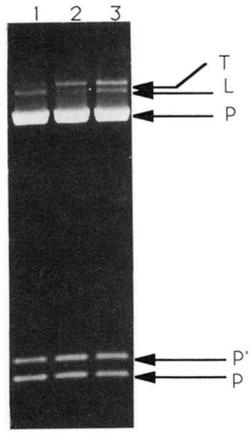

FIG. 7. **Agarose gel electrophoresis of pairing of *hix* sites assay on linearized pKH336.** The pairing of *hix* sites assay was performed on *Cla*I-linearized pKH336 (*lane 1*), *Pst*I-linearized pKH336 (*lane 2*), fully relaxed pKH336 (*lane 3*). *T*, paired *hix* sites and Hin complex. *L*, linearized pKH336. *P*, *Pst*I-*Cla*I fragments of pKH336. *P'*, retarded 0.9-kb *Pst*I-*Cla*I fragment due to the binding of Hin to *hixL* site.

DISCUSSION

A series of pKH336 variants was generated whose superhelical density ranged from totally relaxed to greater than the physiological density. The dependence of each step in the reaction upon superhelical density was examined. The possibility that Hin has a different binding affinity for *hixL* sites on DNA with different superhelical density has been addressed by Glasgow *et al.* (1989b) who showed that Hin binds equally well to supercoiled and linearized DNA. We have developed simple assays to probe the formation of paired *hix* complex and the formation of invertasomes. Strand cleavage and strand rotation-religation were assayed by previously published methods.

Pairing of *hix* sites was assayed by cross-linking physically associated Hin molecules on DNA and resolving the cross-linked protein-DNA complex on agarose gel electrophoresis (Fig. 2*b*). Although glutaraldehyde may not be an ideal cross-linking agent, we have demonstrated that it can be used to measure levels of paired *hix* sites structure by having all the other parameters fixed except the time of incubation (Fig. 4). The level of paired *hix* complex on fully relaxed DNA is 30% of that observed with DNA at the physiological superhelical density and increases with negative supercoiling. This suggests that supercoiling promotes the interaction between Hin molecules bound to *hix* sites. Supercoiling could act in two ways. (*a*) It increases the chance of random collision between Hin molecules. We showed that when the substrate DNA is linearized, the distance between *hix* sites determines the frequency of bringing bound Hin molecules close together. A slithering motion of supercoiled DNA (Benjamin, and Cozzarelli, 1986) can efficiently increase the *hix* site encounter frequency. (*b*) Or supercoiling could act by changing the structure of the bound Hin dimer to form more stable associations between Hin molecules.

The fact that maximum formation of paired *hix* complex occurs at the superhelical density where invertasome formation starts to occur (Fig. 4) suggests that pairing of *hix* sites can occur independently from the formation of invertasome. Maximum invertasome formation was observed also at a σ value of -0.062 (31 linking deficit), suggesting that supercoiling plays a role in formation of the invertasome structure. Our work suggests that random collision between Hin molecules brings two *hixL* sites close enough to efficiently form a presynaptic complex. However, it has been suggested that productive synaptic complex formation (invertasome formation) is directed by the topology of precisely interwound DNA strands and DNA-binding proteins (Johnson *et al.*, 1987). It was proposed for the analogous Gin-mediated inversion system that three cis-acting DNA sequences (two recombination sites and the enhancer) could be brought together at a supercoiling branch point with the correct configuration of DNA strands necessary for an inversion to occur (Kanaar *et al.*, 1989).

The role of supercoiling in invertasome formation can be inferred if one considers the situation at superhelical density of -0.026 where few invertasomes are formed in spite of the fact that pairing of *hix* sites has already reached its maximum and Fis is present bound to the enhancer. To explain why few invertasomes are formed at $\sigma = -0.026$, we propose that it is supercoiling that establishes the invertasome by promoting or stabilizing the physical contacts between Hin and Fis. Supercoiling could act in three possible ways. First, supercoiling may change the phasing of two Fis dimers which are separated by 48 bp (4.5 helical turns of linear DNA). It has been shown that the relative position of Fis dimers is critical in the Hin-mediated inversion reaction (Johnson *et al.*, 1987). Changes in the helical twist of DNA as a result of negative supercoiling could change the relative geometrical position of Fis dimers at the enhancer. Fis dimers repositioned by supercoiling may be able to make effective contacts with Hin in the paired *hix* structure. It is possible that at a superhelical density of -0.026 the relative position of Fis dimers is not optimal to make a contact with Hin in the paired *hix* structure in order to form invertasomes. Second, negative supercoiling can enhance the formation of DNA bending by Fis at the enhancer. Although it is not clear why bending is required for the function of enhancer, the ability of the enhancer to be bent by Fis was shown to be critical in the inversion reaction[2] (Hubner *et al.*, 1989). Third, negative supercoiling could decrease the effective distance between Fis and Hin in the paired *hix* structure so that physical contact of proteins is now possible. Indeed, the only physical parameter that changed significantly over the range of σ value from -0.02 to -0.06 is the radius of superhelix in the electron microscopic study of plasmids with different superhelical density (Boles *et al.*, 1990). Fig. 4 shows that invertasome formation sharply increases at around this range of superhelical density. The radius of superhelix decreases from 150 to 60 Å and remains almost the same at higher densities (Boles *et al.*, 1990).

Negative supercoiling promotes the Hin-mediated inversion reaction at two different levels. First, it increases paired *hix* site formation. Second, it may aid in formation of the productive synapsis by promoting and/or stabilizing physical contact between Hin in the paired *hix* structure and Fis dimers. The contact between Hin and Fis at the enhancer would enable Fis to drive Hin into a conformation where concerted DNA strand cleavage could occur.

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