

Stepwise Dissection of the Hin-catalyzed Recombination Reaction from Synapsis to Resolution

Erin R. Sanders¹ and Reid C. Johnson^{1,2*}

¹Department of Biological Chemistry, David Geffen School of Medicine, University of California, Los Angeles Los Angeles, CA 90095-1737 USA

²Molecular Biology Institute University of California Los Angeles, Los Angeles CA 90095, USA

The Hin DNA invertase promotes a site-specific DNA recombination reaction in the *Salmonella* chromosome. The native Hin reaction exhibits overwhelming selectivity for promoting inversions between appropriately oriented recombination sites and requires the Fis regulatory protein, a recombinational enhancer, and a supercoiled DNA substrate. Here, we report a robust recombination reaction employing oligonucleotide substrates and a hyperactive mutant form of Hin. Synaptic complex intermediates purified by gel electrophoresis were found to contain four Hin protomers bound to two recombination sites. Each Hin protomer is associated covalently with a cleaved DNA end. The cleaved complexes can be ligated into both parental and recombinant orientations at equivalent frequencies, provided the core residues can base-pair, and are readily disassembled into separated DNA fragments bound by Hin dimers. Kinetic analyses reveal that synapsis occurs rapidly, followed by comparatively slow Hin-catalyzed DNA cleavage. Subsequent steps of the reaction, including DNA exchange and ligation, are fast. Thus, post-synaptic step(s) required for DNA cleavage limit the overall rate of the recombination reaction.

© 2004 Elsevier Ltd. All rights reserved.

Keywords: Fis; serine recombinase; site-specific DNA recombination; stoichiometry; synaptic complex

*Corresponding author

Introduction

The site-specific DNA inversion reaction catalyzed by Hin provides a model system for unraveling the molecular mechanisms of genetic recombination by the serine family of recombinases. The Hin recombinase was originally isolated as an activity that controlled inversion of a 1 kb segment of DNA in the chromosome of *Salmonella enterica*.^{1–3} The invertible segment contains a promoter that directs the coordinate expression of *fljB*, a gene encoding the H2 flagellin, and *fljA*, a gene specifying a repressor of the unlinked H1 flagellin gene, *fljC*.^{2,4} By switching the orientation of the promoter, Hin regulates the alternate expression of the cell-surface antigens H1 and H2, a phenomenon termed flagellar phase variation, which enables the pathogen to temporarily escape a host immune response.^{2,5}

The invertible segment contains the gene that

encodes the 21 kDa (190 amino acid residues) Hin protein as well as a 65 bp recombinational enhancer, which contains two binding sites for the Fis regulatory protein.^{6–10} The *cis*-acting enhancer is located within the N-terminal region of the *hin* gene but can function thousands of base-pairs away from and on either side of the recombination sites to stimulate inversion rates by three orders of magnitude.⁷ Two 26 bp recombination sites, *hixL* and *hixR*, flank the invertible segment. Hin binds as a dimer to each *hix* site, which is comprised of two 12 bp imperfect inverted repeats (half-sites) separated by a 2 bp core (Figure 1C).^{7,11} To become catalytically active, Hin must assemble into a tripartite synaptic complex, called an invertasome, which contains the two *hix* sites and the Fis-bound enhancer segment (Figure 1A (b)).¹² Formation of the invertasome by wild-type Hin (Hin-wt) is completely dependent upon DNA supercoiling and occurs with a requisite geometry of the three DNA duplex strands, reflecting assembly at the base of a plectonemic DNA branch.^{13,14} The DNA bending activity of the HU protein facilitates looping of the DNA between the

Abbreviations used: Hin-wt, wild-type Hin.
E-mail address of the corresponding author: rcjohnson@mednet.ucla.edu

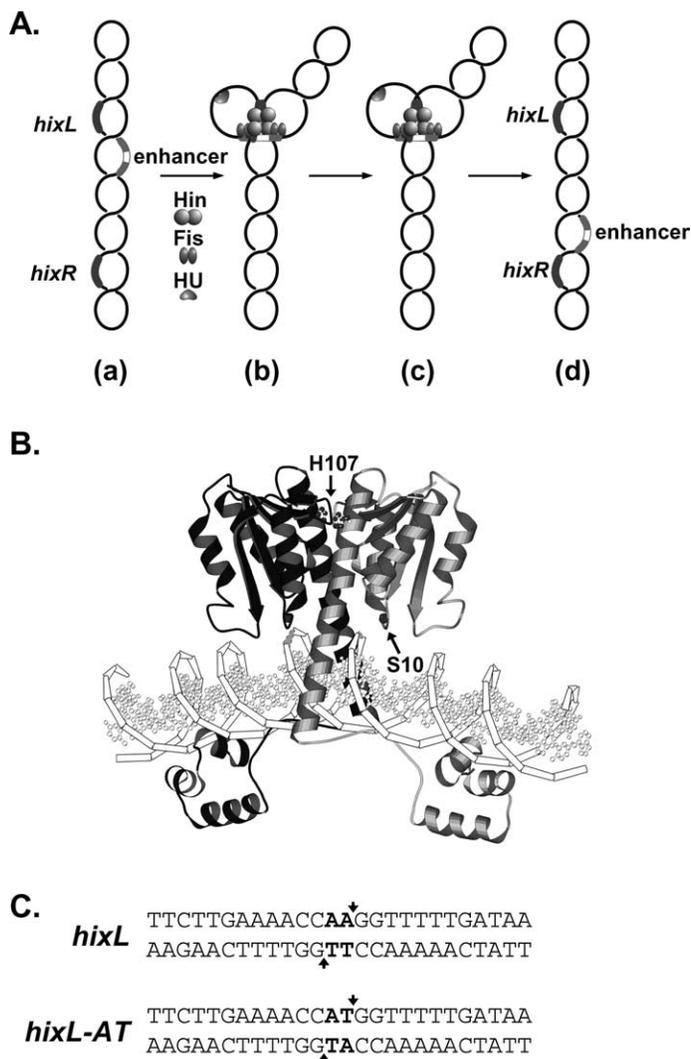


Figure 1. The Hin-catalyzed, site-specific DNA recombination reaction. **A**, Pathway for inversion by wild-type Hin. (a) The supercoiled plasmid substrate contains two *hix* sites and an enhancer. (b) Hin and Fis bind to their respective sites on the DNA and, with the aid of DNA bending by HU, assemble the invertasome complex. (c) DNA cleavage is accompanied by a 180° clockwise rotation of DNA strands catalyzed by Hin. (d) Resolution results in the inversion of the orientation of the DNA segment between the two *hix* sites. **B**, Model of the Hin dimer bound to DNA showing the locations of serine 10 (S10) and histidine 107 (H107).³¹ Grey shading on the DNA ribbon indicates positions of DNA cleavage. **C**, Sequence of *hixL* and *hixL-AT* recombination sites. The core dinucleotides are highlighted in bold, and the positions of the staggered double-strand cleavages by Hin are denoted by the arrows.

enhancer and *hix* sites when the two interacting segments are located close to one another, as arranged in the *Salmonella* chromosome.^{9,15}

Once the invertasome is assembled, four Hin subunits initiate a coordinated nucleophilic attack of the DNA phosphate backbone to create a 2 bp staggered double-strand break at the core dinucleotides within each *hix* site.^{16,17} The cleavage reaction generates a 3'-OH overhang and results in an ester linkage of Hin to the 5' phosphate group through serine 10.^{16,18} The crystal structure of an apo form of $\gamma\delta$ resolvase, a member of the serine recombinase family that shares 40% amino acid residue identity with Hin, revealed that the active-site serine residues within the DNA-bound dimer are not positioned appropriately to initiate the nucleophilic attack.¹⁹ Thus, a conformational change in the dimer that repositions the active sites closer to the phosphate backbone is believed to be a prerequisite for DNA cleavage (Figure 1B). Following cleavage, the DNA strands exchange into the recombinant configuration by undergoing a 180° clockwise rotation, and ligation of the DNA occurs *via* reversal of the serine-phosphodiester

protein-DNA linkage.^{13,14,20} Under certain conditions, multiple rotations of DNA strands can occur prior to ligation.^{13,14,21-23}

As described above, the wild-type Hin reaction is exquisitely regulated, requiring Fis, a recombinational enhancer sequence, DNA supercoiling, and HU. These accessory factors function to ensure that DNA inversion between appropriately oriented *hix* sites on the same DNA molecule is the overwhelmingly favored reaction.³ The requirement for Fis also imparts growth control over the inversion reactions, since Fis is expressed in large amounts only during rapid growth.^{24,25} Other site-specific recombination reactions, such as those mediated by Cre and FLP, do not exhibit these regulatory complexities, which has fostered our advanced understanding of these reactions from both biochemical and structural perspectives.²⁶⁻²⁸

To dissect the enzymology of the Hin recombination system and to facilitate physical analyses of reaction intermediates, we have used a gain-of-function mutant of Hin to develop a simplified *in vitro* recombination reaction. Hyperactive DNA invertases that relieve the normal dependence of

the reaction on the accessory factors have been isolated in the Hin, Gin, and Cin inversion systems.^{17,29,30} The H107Y mutation in Hin has been obtained in screens for Fis-independent recombination, SOS hyperactivation, and suppression of otherwise inactivating mutations (R.C.J. *et al.*, unpublished results).³¹ Hin-H107Y is able to efficiently promote inversions as well as deletions between appropriately oriented *hix* sites without Fis or the enhancer, and substantial Fis-independent recombination can occur without DNA supercoiling (S. K. Merickel, M. J. Haykinson & R.C.J., unpublished results).¹⁷ As is the case for the Gin-M114V mutant,^{21,32} topological analysis of the Hin-H107Y reaction on plasmid substrates indicates that the normally strict control over the configuration of DNA strands for productive recombination is relaxed and that there is a much greater tendency for multiple DNA exchanges to occur from a single reaction. Here, we characterize a minimal recombination reaction employing only the Hin-H107Y mutant recombinase and short *hix*-containing oligonucleotide substrates. We show that stable synaptic complexes are assembled efficiently by a tetramer of Hin in which all four subunits participate in the chemical steps of the reaction. Isolation of the reaction intermediates enables us to individually probe the DNA cleavage, exchange, ligation, and resolution steps of the reaction.

Results

Formation of synaptic complexes by Hin-H107Y

The ability of Hin-H107Y to catalyze recombination in the absence of Fis and in a variety of

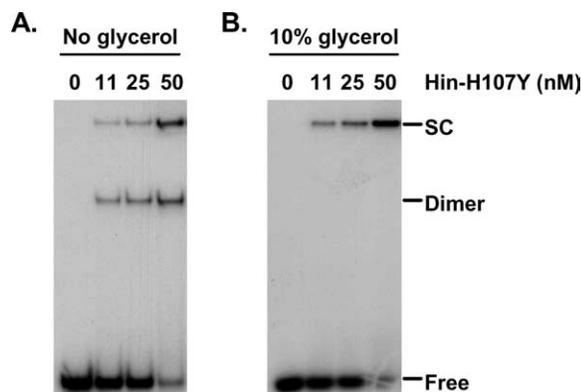


Figure 2. Hin-H107Y binding to *hix*-containing oligonucleotide substrates. The 36 bp *hix* DNA substrates and increasing concentrations of Hin-H107Y were incubated at 37 °C for 30 minutes in Mg²⁺-free buffer containing ethylene glycol. The reactions were then subjected to electrophoresis in native polyacrylamide (acrylamide to bisacrylamide 19:1, w/w) gels with and without 10% (v/v) glycerol as designated. The bands corresponding to a Hin dimer bound to a single *hix* site and the synaptic complex (SC) are labeled.

synapsis topologies encouraged us to develop an intermolecular synapsis reaction using synthetic substrates. Initial evidence of intermolecular synapsis was obtained from standard electrophoretic mobility-shift assays in which a ³²P-labeled 36 bp DNA duplex containing the 26 bp *hixL* site flanked by 5 bp on each side was incubated with Hin-H107Y. A faint band was observed that migrated slower on a native polyacrylamide gel than that of the *hixL*-bound Hin dimer complex (data not shown). Levels of this band increased when the reaction was incubated at 37 °C in Mg²⁺-free buffer containing 25% ethylene glycol (Figure 2A). When aliquots of the same reaction were electrophoresed in a polyacrylamide gel containing 10% glycerol, all of the *hixL*-bound dimer complexes were transformed into the slower-migrating species (Figure 2B). Thus, the presence of glycerol in the gel appears to facilitate conversion into a higher-order form that is stable to electrophoresis.

Higher-order complexes were not obtained with Hin-wt or with the catalytically inactive double mutant Hin-S10G/H107Y, although both Hin proteins efficiently bound as dimers to either a 36 bp or 50 bp *hixL* substrate (Figure 3, lanes 3 and 4 or lanes 7 and 8). However, a similarly migrating higher-order complex has been observed with some other hyperactive Hin mutants (e.g. F88L, M115V, E122Q, and E122Y) to varying extents (data not shown). Taken together, these results suggest that survival of this complex during gel electrophoresis requires a catalytically competent hyperactive Hin mutant (see below).

Reactions employing *hixL* fragments of two different sizes established that the slower-migrating species was a synaptic complex containing two *hixL* sites. As shown in Figure 3, incubation of either 50 bp (lane 2) or 36 bp (lane 6) *hixL* oligonucleotide substrates with Hin-H107Y resulted in electrophoretically separable, higher-order complexes. When reactions were performed for 30 minutes in the presence of equimolar amounts of both substrates, a single intermediate band was also generated (lane 11), demonstrating that the higher-order species was a synaptic complex containing one 50 bp *hixL* substrate and one 36 bp *hixL* substrate. The 50/36 hetero-complex was present at approximately twice the levels of the 50/50 and 36/36 bp homo-complexes, reflecting random pairing of *hixL* sites. This ratio changed to favor the 50/50 and 36/36 homo-complexes over the 50/36 hetero-complex if Hin-H107Y was preincubated with the 50 bp or the 36 bp *hixL* substrates separately for 15 minutes and then mixed together and incubated for an additional 15 minutes prior to electrophoresis (compare lane 9 to lane 11). Only a modest accumulation ($\leq 5\%$) of the hetero-complex occurred when the incubation time was increased from 15 minutes to 60 minutes (data not shown). The small additional amount of 50/36 hetero-complex formed under these conditions is likely

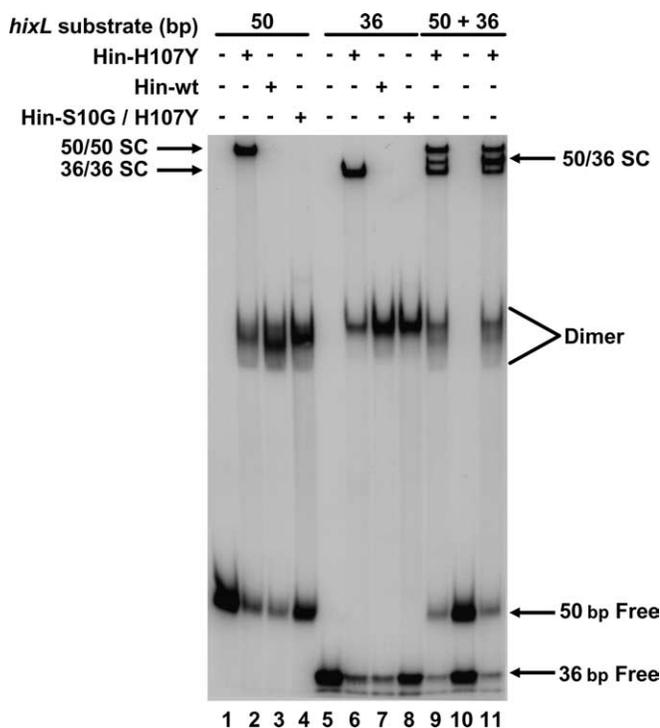


Figure 3. Stable synaptic complexes contain two *hix* sites and require the tyrosine substitution at histidine 107 (H107Y) and the active site residue serine 10 (S10). Reactions employing the 36 bp and 50 bp *hix* substrates and Hin-H107Y, Hin-wt, or the double mutant Hin-S10G/H107Y were electrophoresed in a native polyacrylamide (acrylamide to bisacrylamide 59:1, w/w) gel. The migrations of the dimer and synaptic complex (SC) bands along with the unbound (Free) DNAs are designated. The slower migration of the Hin dimer complexes formed on the 36 bp relative to the 50 bp *hixL* fragments in this gel system presumably reflects differences in overall shapes of the complexes because of the DNA tails. The slightly faster migration of the dimer complexes formed with Hin-wt relative to Hin-H107Y or Hin-S10G/H107Y on the 50 bp fragments (compare lane 3 to lanes 2 and 4) may reflect differences caused by DNA bending.

due to slow exchange of pre-formed synaptic complexes, since the relative distribution of synaptic complexes, *hixL*-bound Hin dimer complexes, and free *hixL* DNA within the population did not change. Taken together, these results suggest that synaptic complexes assembled by Hin-H107Y under the Mg^{2+} -free ethylene glycol conditions are very stable.

The Hin-H107Y synaptic complexes are competent for each chemical step of the reaction

DNA cleavage

The requirement for the active-site serine residue suggested that the complexes may have proceeded through one or more chemical steps in the reaction, which may be coupled to their increased stability during gel electrophoresis. The first chemical step is the coordinated attack of the phosphodiester bonds at the 5' sides of the guanine bases within both strands of *hixL* (Figure 1C) by serine 10 on Hin to form a covalent phosphoserine linkage with the DNA.^{16,17} To determine whether this step had occurred, Hin-H107Y was incubated with ($5'$ - ^{32}P)-labeled 50 bp *hixL*-containing DNA, electrophoresed in a native polyacrylamide gel, and the DNA from the bands representing the dimer and synaptic complex was extracted in SDS buffer. Electrophoresis of the recovered DNA in a denaturing gel revealed that almost all the DNA within the synaptic complex had been cleaved into labeled 26 nt products (Figure 4B, lane 5), consistent with a staggered break at the center dinucleotide of the

hix site (Figure 4A). By contrast, all the DNA extracted from the dimer complex was full length (Figure 4B, lane 4). Thus, the synaptic complexes have progressed through the first chemical step of the reaction.

DNA exchange and ligation

We next asked whether the cleaved synaptic complexes were competent for DNA exchange and ligation. To evaluate ligation, the gel slice containing the synaptic complexes was soaked in Mg^{2+} -containing buffer prior to extraction with SDS. As seen in Figure 4C, the cleaved 19 nt or 26 nt products were ligated into the full-length 36 nt or 50 nt fragments, respectively (compare lanes 2 and 3 or lanes 4 and 5). To distinguish whether the DNA strands could be ligated into a recombinant structure (Figure 4A) or are simply re-ligated into the parental sequence, binding reactions were performed with an equimolar mixture of the 36 bp and 50 bp *hixL* substrates. The reactions were loaded onto a native gel to resolve the 50/36 hetero-complex (e.g. Figure 3, lane 11), and the gel slice containing the hetero-complex was either extracted directly with SDS to monitor DNA cleavage or first soaked in buffer containing Mg^{2+} to promote ligation. As shown in Figure 4C (lanes 6 and 7), these complexes promote ligation into both 43 bp recombinant products and the 50 bp and 36 bp parental sequences.

The *hixL* substrate would be able to ligate into the recombinant configuration only if initial alignment of the sites was parallel because of asymmetry of

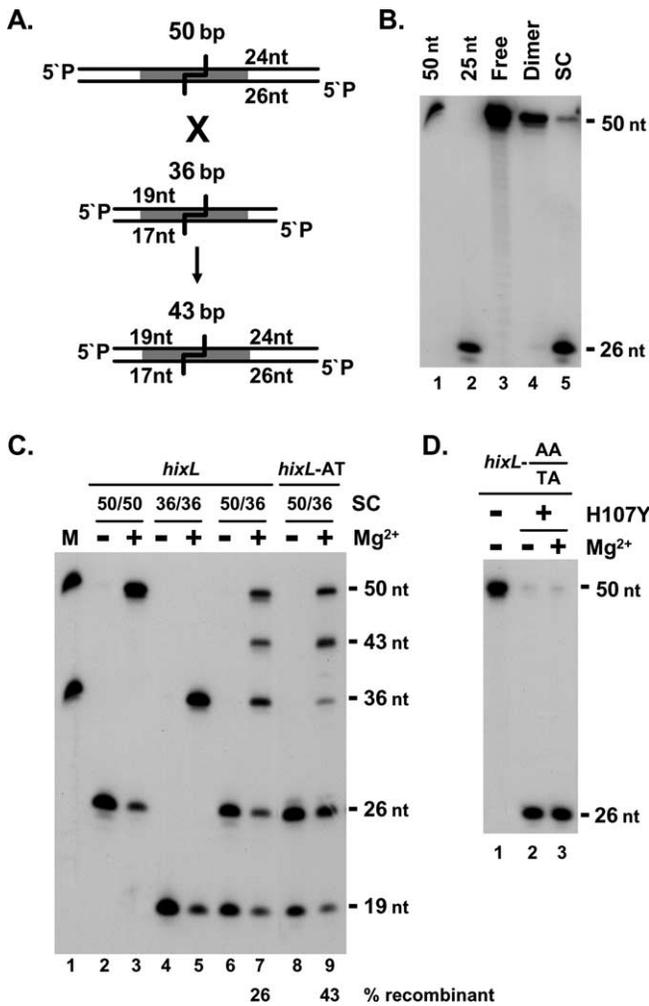


Figure 4. Hin-H107Y synaptic complexes are competent for DNA cleavage, strand exchange, and ligation. A, Schematic representation of the 5'-end labeled 50 bp and 36 bp oligonucleotide substrates showing the 26 bp *hixL* recombination sequence (grey shading) and Hin cleavage sites. Recombination yields a 43 bp product. The lengths of the Hin cleavage products are indicated for each strand. B, Hin-H107Y reactions with 50 bp *hix* substrates were electrophoresed in a native polyacrylamide gel as in Figure 3 (lane 2). The bands corresponding to the free DNA (Free), Hin-bound to a single *hix* site (Dimer) and the synaptic complex (SC) were extracted in the presence of SDS and subjected to further electrophoresis in a denaturing polyacrylamide gel (shown). 50 nt and 25 nt oligonucleotides were included as markers in lanes 1 and 2. C, Bands corresponding to synaptic complexes from reactions employing 36 bp and 50 bp *hixL* and *hixL-AT* sites were isolated from native polyacrylamide gels and either incubated with buffer containing 10 mM Mg²⁺ prior to extraction in the presence of SDS (+), or directly extracted in SDS buffer (-). The DNA recovered from complexes containing two 50 bp *hix* sites (50/50), two 36 bp *hix* sites (36/36), and a 50/36 bp *hix* hetero-complex was then sub-

jected to denaturing gel electrophoresis (shown). Without the Mg²⁺ chase, only Hin cleaved DNA fragments (26 and 19 nt) were present (lanes 2, 4, 6, and 8). After the Mg²⁺ chase, full-length substrates including the 43 nt recombinant product from the hetero-complex were generated (lanes 3, 5, 7, and 9). The number (%) of recombinants among the ligated products is given below lanes 7 and 9. D, The same protocol as in C was followed, except that the substrates were hetero-duplexes of 50 bp *hix* oligonucleotides (*hixL-AA/TA*) containing the *hixL* (top strand) and *hixL-AT* (bottom strand) sequences. Hin-H107Y efficiently cleaved these substrates (lane 2) but failed to ligate them after a Mg²⁺ chase (lane 3).

the core dinucleotide sequence (AA/TT, Figure 1C) where base-pairing and ligation occur. Thus, if synapsis occurs from collisions of *hixL* sites in either parallel or antiparallel orientations, only about 50% of the hetero-synaptic complexes would be expected to be in a parallel orientation that could generate recombinant products. If the DNA strands within these complexes exchanged to an equilibrium mix, a distribution of recombinant and parental duplex forms would be expected at the time of ligation. As shown in Figure 4C (lane 7), 43 bp recombinant duplexes accounted for 26% of the ligated products, indicating that both recombinant and parental forms were represented approximately equally at ligation.

We performed the same mixing experiments described above on 36 bp and 50 bp oligonucleo-

tide substrates containing *hixL-AT* sites. Synapsis of *hixL-AT* sites in either parallel or antiparallel orientations would be expected to be able to support ligation after DNA exchange because of the symmetric nature of the core dinucleotide sequence (AT/TA, Figure 1C),³³ and would result in about twice as many recombinant products as compared to the *hixL* substrates. As shown in Figure 4C (lane 9), 43% of the ligated products with *hixL-AT* sites are in the recombinant configuration. The greater number of recombinants obtained with the *hixL-AT* substrates (43%) compared to the *hixL* substrates (26%) is consistent with synapsis and DNA cleavage occurring after random collision of *hix* sites, followed by DNA ligation into the parental and recombinant configurations at about equivalent frequencies provided base-pairing can occur.

Kinetics of synapsis, DNA cleavage, strand exchange, and ligation

Rates of synapsis, DNA cleavage, exchange, and ligation were individually measured to evaluate the contributions of each step to the overall kinetics of recombination by Hin-H107Y. Aliquots were removed at various times after addition of Hin-H107Y to ($5'$ - 32 P)-labeled 50 bp *hixL* substrates and either: (1) loaded directly onto native polyacrylamide + 10% glycerol gels to obtain levels of synaptic complexes; or (2) denatured by addition of SDS and electrophoresed in a denaturing gel to ascertain levels of DNA cleavage. As described above, the presence of glycerol in the native gel facilitates the conversion of synaptic complexes formed in solution into the cleaved form that is stable to electrophoresis. The number of synaptic complexes that survived electrophoresis reached a maximum within the first minute of incubation (Figure 5A). By contrast, cleavage products accumulated more slowly with only half the number of DNA molecules cleaved that are in synaptic complexes after 25 minutes. These results show that Hin binding to *hix* and synapsis are fast relative to DNA cleavage.

The rate of DNA strand exchange into the recombinant configuration was assessed in the following manner. Approximately equimolar amounts of 36 bp and 50 bp *hixL* substrates were incubated with Hin-H107Y and ethylene glycol under Mg^{2+} -free conditions to accumulate cleaved synaptic complexes. At various times, aliquots of the reaction were removed and a portion was treated with SDS to determine the level of cleavage complexes, and the remainder was chased into ligated products by incubating for two minutes after addition of Mg^{2+} and dilution of ethylene glycol. As quantified in Figure 5B, approximately 80% of the cleaved molecules were ligated within the two minute chase period, regardless of whether cleavage was allowed to proceed for five to 80 minutes. Of these ligated products, 15–17% were recombinants at each time-point. Since the proportion of molecules ligated into the recombinant configuration did not change with time, we conclude that exchange of DNA strands happens either coincidentally or soon after DNA cleavage by Hin.

Mismatched core sequences inhibit ligation but not synapsis or cleavage

Hin-H107Y reactions on equimolar mixtures of 50 bp *hixL* and 36 bp *hixL*-AT substrates failed to generate 43 bp recombinants, although full-length products reflecting re-ligation into the 50 bp and 36 bp parental configurations were obtained efficiently (data not shown). Previous studies with serine recombinases have shown that recombination reactions on plasmids initiated from synaptic complexes between recombination sites with different core sequences generate DNA knots.^{13,14,22–34} It has

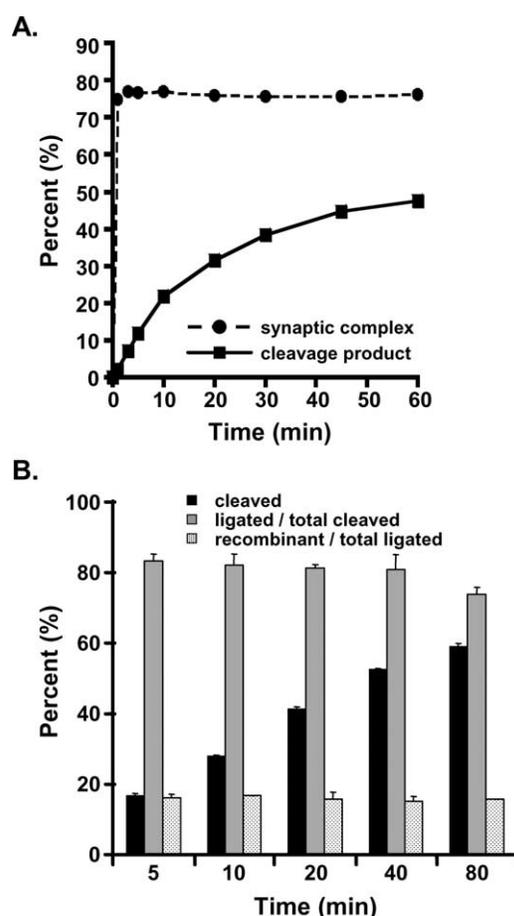


Figure 5. Kinetics of synaptic complex formation, *hix* cleavage, ligation and recombination by Hin-H107Y. A, Hin-H107Y was added to a reaction mix containing 36 bp *hixL* fragments and at various times aliquots were: (1) loaded onto a native polyacrylamide gel containing 10% (v/v) glycerol to measure synaptic complex formation; and (2) quenched with SDS and loaded onto a denaturing polyacrylamide gel to measure cleavage. The percentage of DNA in a synaptic complex (circles) and cleaved by Hin (squares) is plotted against incubation time (in minutes). B, Bar graph depicting the percentage of DNA substrates that are cleaved (dark shading), subsequently ligated after a two minute Mg^{2+} chase (light shading), and in the recombinant configuration (dots) at various times after addition of Hin-H107Y to reactions containing equimolar amounts of 36 bp and 50 bp *hixL* sites. Following electrophoresis in denaturing gels, levels of cleaved products were quantified and plotted as a percentage of the total DNA. Levels of ligated parental products were plotted as a percentage of the total cleaved products, and levels of recombinant products were plotted as a percentage of the total ligated products. The data plotted in A and B represent averages of two independent experiments.

been proposed that the knots are formed because the inability to base-pair at the crossover site after a single exchange of DNA strands prevents ligation. A second exchange that restores the parental sequence of the recombination sites, but ties a knot in the plasmid DNA, is required for ligation. The ability to undergo two or more iterative

rounds of exchange without ligation and *de novo* cleavage at each step has been argued to be strong evidence for recombination mediated by rotation of protein subunits that are linked covalently to the DNA ends. However, an alternative model considers an ability of the recombinase to ligate a mismatched core, creating a hyperactive substrate that supports rapid initiation of another recombination reaction. In the latter model, a complete reaction cycle need proceed through only a single DNA exchange step. Thus, a truly processive mechanism that involves multiple subunit rotations may not occur.

To help distinguish between these models, Hin-H107Y was incubated with 50 bp *hix* substrates that contained a mismatched base-pair within the core nucleotides (*hixL*-AA/TA). These substrates were generated by annealing 50 nt *hixL* top strand and 50 nt *hixL*-AT bottom strand oligonucleotides. Hin-H107Y assembled synaptic complexes with the *hixL*-AA/TA heteroduplex substrates efficiently, and the *hix* sites within the complexes were cleaved (Figure 4D, lane 2). However, unlike reactions with homoduplex recombination sites, no evidence of ligation was obtained after addition of Mg^{2+} (lane 3). These results indicate that Hin is able to cleave *hix* sites containing a mismatched core but that ligation is prevented under conditions where base-pairing at the 2 bp core region cannot be achieved. Thus, iterative cycles of DNA exchange probably occur without an intermediate ligation step, in support of the subunit rotation model.

Stoichiometry of Hin subunits within the synaptic complex

The ability to purify catalytically active synaptic complexes from polyacrylamide gels allows us to directly determine the number of Hin protomers present in the complex. For these experiments, we assembled complexes using fluorescein-labeled 36 bp *hixL* substrates (FAM-36 bp *hixL*) and Hin-H107Y^{HMK} containing a C-terminal kinase tag that was labeled at a predetermined specific activity with ^{32}P . The reactions were electrophoresed together with known concentrations of FAM-36 bp *hixL* oligonucleotides, and the numbers of DNA molecules and Hin molecules were determined by quantitative fluorimaging and Čerenkov counting, respectively. As expected for a dimer of Hin bound to a single *hixL* site, the calculated ratio of Hin to DNA molecules was 2.1 ± 0.2 . The calculated ratio for the synaptic complex was 2.3 ± 0.3 , which, when combined with the information from the mixing experiments above that demonstrated there are two *hixL* sites in the complex (Figure 3), implies that there are four Hin monomers in the complex. Thus, the active synaptic complex is composed of a tetramer of Hin subunits bound to two *hix* sites.

If the cleaved synaptic complex is comprised of a tetramer of Hin subunits, all four Hin subunits

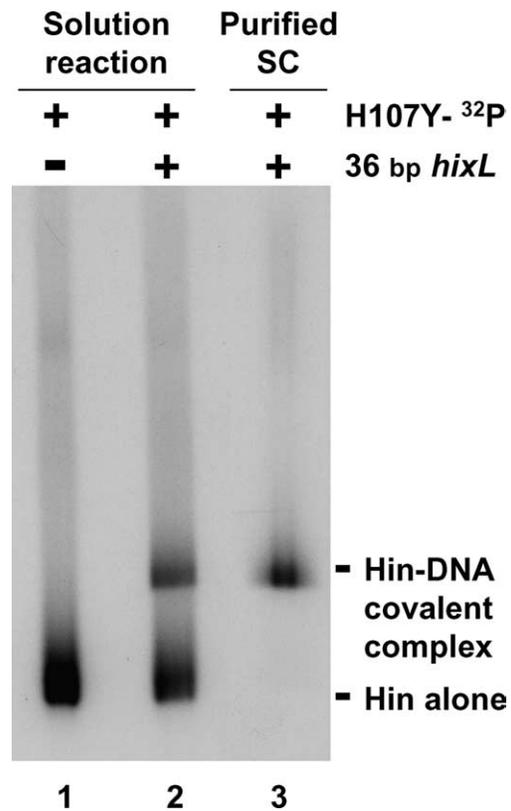


Figure 6. All four Hin subunits in the cleaved synaptic complex are covalently associated with *hix* DNA. A, ^{32}P -labeled Hin-H107Y^{HMK} was incubated with 5'-FAM-labeled 36 bp *hixL* substrates in Mg^{2+} -free buffer containing ethylene glycol. In lane 2, an aliquot was quenched with SDS and applied directly onto a denaturing (SDS) gel. The sample in lane 3 (SC) was first electrophoresed in a native polyacrylamide gel to isolate synaptic complexes, as detected by FAM-fluorescence and ^{32}P phosphorimaging. The Hin-DNA complex then was extracted from the synaptic complex band in the presence of SDS and applied to the denaturing gel. Lane 1 of the autoradiogram shows the migration of the ^{32}P -labeled Hin-H107Y^{HMK} after a mock reaction with no DNA substrates.

would be predicted to be associated covalently with each of the four DNA strands *via* the 5' phosphoserine linkage. To confirm this prediction, complexes assembled with ^{32}P -labeled Hin-H107Y^{HMK} and 36 bp *hixL* substrates were isolated from polyacrylamide gels and extracted with SDS. The products of the gel-isolated synaptic complexes were then subjected to SDS-PAGE (Figure 6, lane 3). An aliquot of the unpurified reaction that was quenched with SDS was loaded directly (lane 2) together with a mock reaction done in the absence of *hix* DNA (lane 1). As shown in Figure 6, all of the ^{32}P -labeled Hin purified from the synaptic complex, but only a portion of the Hin protein in the total reaction mixture was associated covalently with the DNA, as reflected by the retarded electrophoretic migration. These results confirm that all four subunits of the Hin tetramer are participating chemically in the reaction.

Resolution of the tetrameric synaptic complex into *hix*-bound dimers

We were interested in tracking the fate of the Hin tetramer upon ligation and resolution. At the completion of the reaction, the Hin subunits might dissociate, releasing the DNA for subsequent binding by another Hin dimer, or the tetramer might stay intact but release the DNA. Alternatively, the two Hin dimers may remain bound to each *hixL* site either in a synaptic complex or as *hixL*-bound Hin dimer complexes. To investigate the mechanism of resolution, we assembled synaptic complexes under standard ethylene glycol, Mg^{2+} -free conditions. The reactions were then diluted into buffer to give a final concentration of 15% ethylene glycol, 10 mM $MgCl_2$, and aliquots were loaded onto a native polyacrylamide gel at various times. To ensure that any free Hin subunits, including those that may be released from the synaptic complex, did not rebind the ^{32}P -labeled *hixL* substrates, 100-fold molar excess unlabeled competitor *hixL*

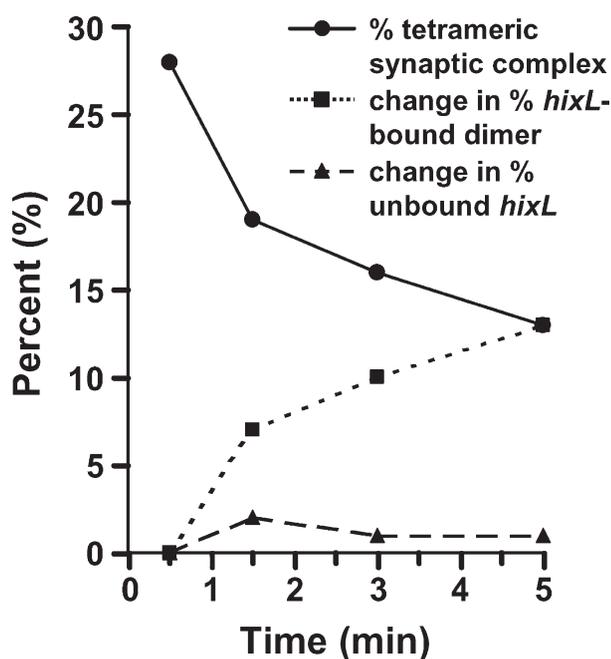


Figure 7. Resolution of the tetrameric synaptic complex into *hix*-bound Hin dimers. To assemble synaptic complexes, Hin-H107Y was incubated with an equimolar mixture of *hixL* substrates in Mg^{2+} -free buffer containing 25% (v/v) ethylene glycol. The reaction mix was then diluted with buffer containing $MgCl_2$ to a final concentration of 15% ethylene glycol and 10 mM available Mg^{2+} plus 100-fold molar excess of unlabeled *hixL* oligonucleotides. Aliquots were applied to a native polyacrylamide gel at various times after the Mg^{2+} chase. The percentage of tetrameric synaptic complexes or the change in the percentage of *hixL*-bound Hin dimers and unbound *hixL* sites are plotted as a function of time beginning 30 seconds after the Mg^{2+} chase. The data are representative of two independent experiments.

DNA was added to the chase buffer. The graph in Figure 7 demonstrates that levels of tetrameric synaptic complexes decreased coordinately with the increase in *hixL*-bound Hin dimer complexes following the Mg^{2+} chase, whereas levels of unbound *hixL* DNA remained largely unchanged. We conclude that the cleaved synaptic complexes disassemble rapidly into predominantly single *hixL*-bound Hin dimer complexes upon shifting to a lower concentration of ethylene glycol and addition of Mg^{2+} . When complexes were diluted into buffer containing Mg^{2+} and only 5% ethylene glycol, resolution was too fast to determine a kinetic correlation and was complicated by the fact that a rapid change in the concentration of ethylene glycol caused the release of a significant fraction of Hin dimers bound to a single site (data not shown).

H107Y accelerates synaptic complex formation in the absence of DNA cleavage

Previous studies have implied that the assembly of *hix* sites into synaptic complexes on supercoiled plasmid DNA molecules is not the step limiting recombination with the wild-type enzyme. This conclusion has been based on the ability to obtain crosslinked synaptic complexes efficiently in the absence of Fis or the enhancer, even though there is only an extremely low rate of cleavage and no detectable recombinants are formed (Figure 8).^{12,35} The H107Y mutation in Hin enables these synaptic complexes to become catalytically active. We wondered if the H107Y mutation enhanced the rate of synapsis in the absence of the increased stabilization obtained by formation of the serine-phosphodiester protein-DNA linkage.

Hin mutants containing the active-site mutation S10G with or without H107Y were incubated with supercoiled plasmid DNA for increasing periods of time and subjected to a five minute crosslinking reaction with glutaraldehyde. After digestion with restriction enzymes, the chi-form *hixL* synaptic complexes were resolved on an agarose gel (Figure 8A and B). Hin containing the H107Y mutation formed crosslinked synaptic complexes at initial rates that were over twofold faster than Hin without H107Y (Figure 8C). Maximal numbers of complexes were obtained after just three minutes at 37 °C with Hin-S10G/H107Y. Complexes formed with Hin-S10G continued to increase over the 20-minute incubation such that the relative numbers of complexes formed by the cleavage-defective Hin without the H107Y mutation exceeded those with H107Y. We conclude from these experiments that the H107Y mutation does enhance the rate of formation of synaptic complexes that are stable to electrophoresis after crosslinking and restriction digestion. The stimulation of synaptic complex formation observed in this assay, however, cannot account for >100-fold stimulation of recombination by H107Y in the absence of Fis.

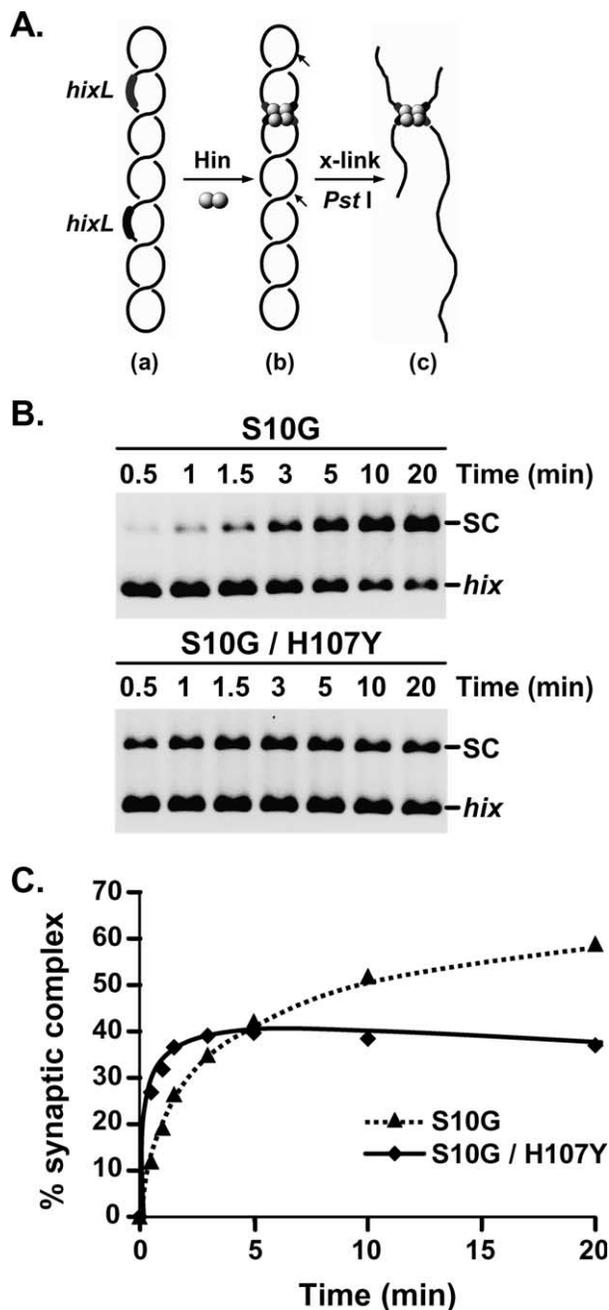


Figure 8. Effect of the H107Y mutation on synaptic complex formation with plasmid DNA in the absence of cleavage. **A**, Schematic representation of the *hixL* plasmid synthesis assay. (a) Hin was incubated for increasing amounts of time with pMS634, which contains two *hixL* sites. (b) Following brief crosslinking with glutaraldehyde, the reactions were subjected to digestion by PstI (cut sites indicated by small arrows). (c) The chi-form *hixL* synaptic complex (SC) was visualized by electrophoresis in agarose gels. **B**, Results of time-course experiments with Hin mutants S10G and S10G/H107Y. The portions of the gels that contain the crosslinked *hixL*-containing fragments (SC) and the largest uncross-linked fragment containing a single *hixL* site (*hix*) are shown. **C**, The percentage synaptic complex formed by each Hin mutant as a function of time is plotted. The data are an average of two independent experiments.

Discussion

We have used a hyperactive mutant of Hin to establish a minimal, but efficient, recombination reaction by a DNA invertase. The reaction supports all the fundamental catalytic and mechanical steps of the Hin recombination pathway. Staging of the reaction using this system permits each of these steps to be examined individually (Figure 9). By contrast, the Fis-dependent wild-type recombination reaction is rapid and highly concerted under standard conditions, which has hindered analysis of the individual steps. Moreover, the use of simple oligonucleotide substrates, as opposed to supercoiled plasmid DNA, enables us to easily examine the importance of specific features of the DNA (e.g. the effects of mismatched core nucleotides) and to perform reactions using chemically modified DNAs. The minimal reaction is amenable to scaling up for structural studies of reaction intermediates.

The Hin recombination pathway: comparison of Hin-H107Y with wild-type

Binding and synapsis

Direct stoichiometry measurements show that Hin-H107Y binds as a dimer to each *hix* site. Earlier binding studies using the wild-type enzyme have implied that Hin binds from solution as a monomer or a dimer but that detergent-sensitive cooperative forces stabilize binding of an apparent dimer to the palindromic *hix* site.^{11,31} Synapsis of *hixL*-bound Hin-H107Y dimers *in trans* to form a Hin tetramer occurs rapidly, as judged from the fact that maximal numbers of synaptic complexes are obtained when reactions are loaded onto an electrophoresing polyacrylamide gel containing glycerol as quickly as possible after mixing (Figure 5A). Collisions where the *hix* sites are configured in either a parallel or antiparallel orientation appear productive for synapsis, cleavage, and exchange (see below).

Previous studies have implied that formation of synaptic complexes on plasmids with the wild-type enzyme is not the rate-limiting step of recombination (Figure 8).^{12,31,35–37} However, the wild-type complexes do not proceed to a stable state where the *hix* DNA is cleaved unless the sites are aligned in the correct configuration in the Fis/enhancer-containing invertasome structure to generate inversion. Thus, Hin-wt synaptic complexes formed without Fis do not survive gel electrophoresis without protein crosslinking. We find that the H107Y mutation results in a modest enhancement to synapsis over the wild-type enzyme rate, as measured by the formation of crosslinked complexes that are not cleaved (Figure 8), but this stimulation is minor compared to the enormous enhancement of Fis-independent DNA cleavage by H107Y.¹⁷ The higher numbers of crosslinked Hin-H107Y synaptic complexes observed at

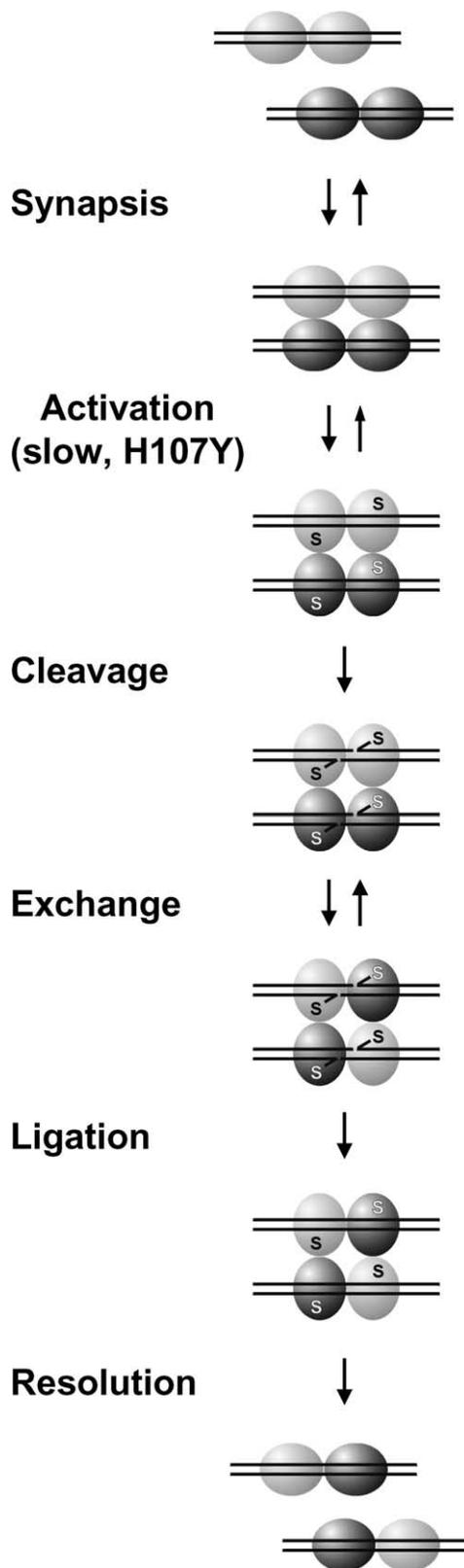


Figure 9. A model of the steps leading to DNA recombination by Hin-H107Y in the absence of the Fis/enhancer system, integrating present and previous work. Hin binds cooperatively as a dimer to a single *hix* site, and two *hix*-bound Hin dimers assemble rapidly into an initial tetrameric synaptic complex. In the context of the H107Y mutation, or when the Fis-bound enhancer has assembled with *hix* sites bound by Hin-wt to form

early times may reflect a propensity for the hyperactive recombinase to isomerize into the catalytically-competent, or activated, state (see below and Figure 9), which may be more amenable for crosslinking.

Catalytic activation and DNA cleavage

Formation of the phosphoserine linkage that results in DNA cleavage occurs only in the context of the synaptic complex and is the slow step of the Hin-H107Y reaction. Indeed, even though initial formation of synaptic complexes is completed within seconds, the number of cleaved complexes continues to increase after a further hour of incubation (Figure 5). Once in the cleaved state, the complexes appear very stable, with re-assorting of recombination sites occurring at a rate of $\leq 5\%$ per hour under ethylene glycol, Mg^{2+} -free conditions (data not shown).

Plasmid-based assays have shown that for both the wild-type enzyme and Hin-H107Y, double-strand DNA cleavage is concerted between sites and is therefore believed to involve a coordinated conformational change between synapsed Hin dimers.¹⁷ We assume that the apo structure of Hin is similar to $\gamma\delta$ resolvase, where the active-site serine residues are not positioned suitably to attack the DNA backbone.¹⁹ Therefore, a considerable conformational change in the subunit structure of the dimer will be a prerequisite for catalysis. In the wild-type reaction, this activation step is where Fis and the enhancer play an essential role. Fis contacts with Hin may induce the conformational change directly, or the Fis-bound enhancer may function as a scaffold to hold the Hin synaptic complex together long enough for Hin to undergo the conformational change. In support of a quaternary change, disulfide linkages across the Hin dimer interface block catalysis.^{31,38,39} Moreover, histidine 107 is located within the dimerization helices (Figure 1B) and is predicted to contact the partner subunit. Thus, the H107Y substitution, as well as many of the other hyperactive DNA invertase mutations that are located in similar positions, is likely altering the chemical properties

an invertasome (Figure 1A (b)), a rate-limiting conformational change by the four Hin protomers occurs that positions the active-site residue serine 10 close to the DNA phosphate backbone. A coordinated nucleophilic attack of each of the four strands of *hix* DNA by the activated Hin protomers covalently links each subunit with the 5' phosphate groups at the cleavage sites. DNA cleavage is followed rapidly by an exchange of a pair of synapsed Hin subunits together with their covalently linked DNA segments. Provided proper base-pairing occurs between the 2 nt core residues after DNA exchange, the serine-phosphodiester linkage is reversed, ligating the DNA strands. The tetrameric synaptic complex is then resolved into two *hix*-bound Hin dimers poised for another round of synapsis and recombination.

of the dimer interface in such a way as to lower the activation energy required to achieve the activated conformation that is poised to initiate DNA cleavage without the activity of Fis. Other substitutions within the dimer interface have been found to provoke uncoordinated cleavage between synapsed *hix* sites on plasmids, and most inhibit catalysis altogether.^{31,37,38,40,41}

DNA exchange, ligation, and synaptic complex resolution

In order for recombinants to form, the cleaved DNA end of one recombination half-site has to move into position to ligate with the appropriate partner half-site. This exchange step has been modeled to involve an accompanying swapping of the Hin subunits that are associated covalently with the DNA ends,^{13,14,20,42,43} and recent data indicate that heterodimers are indeed formed upon DNA exchange (G. Dhar, E.R.S. & R.C.J., unpublished results). Perhaps surprisingly, the DNA exchange step appears rapid relative to cleavage in the Hin-H107Y reaction. This conclusion follows from the observation that the proportion of molecules ligated into the recombinant configuration did not increase with time after DNA cleavage (Figure 5B). Thus, within the timeframes we can measure, the exchange of DNA strands happens coincidentally or soon after DNA cleavage. It should be noted, however, that it is possible that the H107Y substitution may enhance rates of exchange directly because of its propensity to produce complex knotted recombinant products from single reactions (S. K. Merickel & R.C.J., unpublished results).

Our results with mismatched nucleotides over the 2 bp core region where cleavage and ligation occurs demonstrate that local DNA conformational changes that are likely to be induced by the mismatch are well tolerated for synapsis and even DNA scission adjacent to the mispaired site, but that base-pairing is critical for ligation. Experiments with Tn3 resolvase found that a plasmid substrate that created a mismatch in the recombinant configuration after a single 180° rotation was proficient for DNA cleavage but was unable to complete the ligation step.²² These complementary data argue that processive recombinations by serine recombinases are the result of multiple exchanges without intermediate ligation steps. Thus, reactions initiated from complexes containing sites with non-identical core nucleotides fail to ligate after one DNA exchange where the core residues are unable to base-pair; the DNA strands proceed to exchange one or more times to enable re-ligation of the parental sequence. As discussed previously, these properties are consistent with DNA exchange being accompanied by a rotation of subunits.^{13,14,20,42,43}

Ligation, or reversal of the phosphoserine linkage and restoration of the DNA phosphodiester bonds, is rapid for both the Hin-H107Y and the

Fis-dependent wild-type reaction once the concentration of ethylene glycol has been lowered and Mg²⁺ added.¹⁶ Conditions that promote ligation also promote resolution or the physical dissociation of the Hin-H107Y synaptic complexes into the individual *hix*L-bound Hin dimers. The absence of accumulation of free *hix* DNA is consistent with the slow dissociation rates of wild-type Hin from dimer complexes measured previously,⁴⁴ as well as its failure to efficiently catalyze recombination on a second substrate.¹⁷ However, like resolvase,⁴⁵ Hin-wt can catalyze multiple inversions between different *hix* sites on the same DNA molecule efficiently (R.C.J., unpublished results).

Comparison of the Hin-H107Y and resolvase synaptic complexes

Unlike Hin, wild-type Tn3 or $\gamma\delta$ resolvase cannot form even transient synaptic complexes on substrates that contain the resolvase binding sites only at the crossover regions (site I of *res*, which is analogous to a *hix* site). Recently, mutations have been obtained that enable resolvase to form stable synaptic complexes between two site I DNA fragments.^{46–48} These mutations cluster at residues located at and immediately prior to the N terminus of the dimerization helix. The native side-chains of these residues are predominantly solvent-exposed in the dimer and are believed to inhibit interactions between dimers at synapsis. Thus, only when resolvase interactions are stabilized by additional protomers bound to the accessory sites within the full *res* site can site I synapsis and recombination proceed.⁴⁹ The mutated side-chains are proposed to relieve this inhibition and, indeed, the mutations cause resolvase to become an extremely stable tetramer in solution or when bound to DNA.

There are several noteworthy differences between the Hin-H107Y reaction and that of the hyperactive resolvase mutant. The location of histidine 107 on Hin is predicted to be eight residues into the dimerization helix (Figure 1B) and, therefore, not within the region that initiates synapsis (G. Dhar, E.R.S. & R.C.J., unpublished results).^{43,47} As noted above, Hin-H107Y exhibits only a modest stimulation of synapsis in the absence of DNA cleavage, which may not reflect a stimulation of initial interdimer interactions. Moreover, the Hin-H107Y synaptic complexes are assembled *via* collisions of *hix* sites that are each bound by a Hin dimer. We postulate that the H107Y substitution primarily overcomes a rate-limiting step between initial synapsis and DNA cleavage that normally requires the activity of the Fis-bound enhancer (e.g. activation step in Figure 9).¹⁷ The Hin tetrameric complexes become stabilized upon formation of the phosphoserine linkages with DNA, in contrast to the resolvase mutant complexes that are stable without DNA catalysis.

Materials and Methods

Purification of Hin mutants

Wild-type and Hin mutants were cloned into pRJ1518, a derivative of pET11a (Novagen) in which *hin* is expressed from the phage T7 promoter.¹⁷ The heart muscle kinase tag (RRASV) was added to the 3'-end of the Hin-H107Y gene using PCR to produce Hin-H107Y^{HMK}. Hin protein was purified by renaturing inclusion bodies as follows. One liter cultures of RJ3387 (BL21(DE3) *fis* :: *kan-767 endA8* :: *tet*) containing pRJ1518 derivatives were grown in LB to an $A_{600} = 0.5$, and Hin synthesis was induced with 0.5 mM IPTG for two hours at 37 °C. Cells were concentrated 100-fold and lysed by a French press in 50 mM Hepes (pH 7.5), 0.1 M NaCl, 10 mM EDTA, 10 mM DTT, 1 mM PMSF, followed by centrifugation at 3000g for 15 minutes. Urea and Chaps were added to the supernatant at final concentrations of 5 M and 10 mM, respectively, and Hin inclusion bodies were collected by centrifugation at 25,000g for 30 minutes. Inclusion bodies were washed once in pellet buffer (PB) containing 5 M urea, 50 mM Hepes (pH 7.5), 0.1 M NaCl, 1 mM EDTA, 10 mM Chaps, 10 mM DTT, and 10% (v/v) glycerol. Then they were washed a second time in PB (-urea), and finally resuspended in 0.5 ml of PB (-urea), and stored at -80 °C.

Inclusion bodies were solubilized in 6 M guanidine-HCl (GdnHCl), 20 mM Hepes (pH 7.5), 10 mM DTT, 20% glycerol, 50 mM Chaps, and 0.1 mM EDTA at a final protein concentration of 400 µg/ml. Following centrifugation at 25,000g for 30 minutes, the denatured Hin was diluted with an equal volume of 20 mM Hepes (pH 7.5), 1 M NaCl, 10 mM DTT, 20% glycerol, 35 mM Chaps, and 0.1 mM EDTA and subjected to sequential dialyses at 4 °C into the same buffer containing from 2.5 M to 0 M GdnHCl in 0.5 M steps. The refolded Hin was then chromatographed on heparin-Sepharose (Amersham-Pharmacia) as described⁹ and stored at -20 °C in a buffer containing 20 mM Hepes (pH 7.5), 1 M NaCl, 10 mM DTT, 50% glycerol, 4 mM Chaps, and 0.1 mM EDTA.

Hin reactions

Oligonucleotide synapsis reactions were typically performed in 25 µl of 20 mM Hepes (pH 7.5), 80 mM NaCl, 2 mM EDTA, 2–10 mM DTT, 4 mM Chaps, 0.4 µg/µl of polyglutamate, 25% (v/v) ethylene glycol and 0.2 pmol of ³²P-labeled 36 bp or 50 bp substrates containing the *hixL* sequence. Reactions were initiated by addition of 3–5 pmol of Hin and, after 30 to 60 minutes at 37 °C, the samples were applied to a native 6% to 10% polyacrylamide gel with or without 10% glycerol and electrophoresed in 0.5 × TBE. Gels were imaged using a phosphorimager and quantified using ImageQuant software (Amersham).

To assay DNA cleavage, aliquots of the reactions were quenched with 1% (w/v) SDS, followed by incubation with 0.2 mg/ml of proteinase K. For ligation, reactions were diluted to a final concentration of 5–15% ethylene glycol and 10 mM MgCl₂ by mixing pre-warmed chase buffer containing 12.5–25 mM MgCl₂ in binding buffer lacking EDTA and ethylene glycol. Reactions were typically quenched within two minutes by addition of 1% SDS followed by incubation with proteinase K. For "in-gel" reactions, gel slices corresponding to synaptic

complex bands were excised from native gels and crushed and soaked in either 1% SDS to monitor cleavage or 20 mM Hepes (pH 7.5), 20 mM MgCl₂, and 1 mM DTT for 30 minutes at 37 °C before addition of SDS to monitor ligation. The eluted DNA was incubated with proteinase K, extracted with phenol/chloroform, and precipitated with ethanol. Amounts of DNA cleavage and ligation products were quantified by phosphor-imaging after electrophoresis in denaturing 10% polyacrylamide (acrylamide to bisacrylamide 19:1, w/w), 7 M urea, TBE gels.

Plasmid synapsis assays were performed under conditions identical with those used for the oligonucleotides, except that supercoiled pMS634 was added as the substrate.⁷ At various timepoints, aliquots were removed and crosslinked with 0.1% (v/v) glutaraldehyde at 25 °C for five minutes. Unreacted crosslinker was quenched with 35 mM lysine for 15 minutes at 25 °C. Samples were digested with PstI followed by electrophoresis in 1% (w/v) agarose gels. The products were imaged after staining with ethidium bromide, and relative amounts of synaptic complexes were quantified using the ImageQuant software, correcting for differences in fluorescence due to fragment length.

Hin-DNA stoichiometry in the synaptic complex

Hin-H107Y^{HMK} (5 µg) was labeled with ³²P in a reaction containing 20 mM Hepes (pH 7.5), 10 mM MgCl₂, 0.6 M NaCl, 40 mM DTT, 10% glycerol, 1 mCi of [γ -³²P]ATP (7000 Ci/mmol; ICN), and ten units of heart muscle kinase (Sigma). After one hour at 4 °C, the labeled Hin was purified by batch chromatography on heparin-Sepharose (Amersham-Pharmacia). The specific activity of the ³²P-labeled Hin-H107Y^{HMK} was determined as the number of Čerenkov counts (cpm) per quantified amount of protein (moles of Hin monomers) in an excised SDS-PAGE gel slice. Hin-H107Y^{HMK} binding reactions were performed using fluorescein-labeled 36 bp *hixL* oligonucleotides (FAM-36 bp *hixL*) (Sigma-Genosys). Samples were loaded onto native 8% (w/v) polyacrylamide gels with and without 10% (v/v) glycerol alongside a titration of the FAM-36 bp *hixL* oligonucleotides. A fluoroimage (Typhoon, Amersham) of the wet gel was generated to quantify (in moles) the *hixL* sites in the bands corresponding to the Hin dimer or synaptic complex. The moles of ³²P-labeled Hin-H107Y^{HMK} in the bands was obtained by Čerenkov counting of gel slices. The Hin:DNA molar ratios within the Hin-bound *hixL* and synaptic complexes were calculated from four and 16 independent determinations, respectively.

Acknowledgements

We thank Catherine Sohn & Stacy Merickel for purification of Hin mutants. We thank Gautam Dhar and other members of the Johnson laboratory for useful discussions throughout the work, and John Heiss for his help with Figures. This work was supported by NIH grant GM38509.

References

- Kutsukake, K. & Iino, T. (1980). Inversions of specific DNA segments in flagellar phase variation of *Salmonella* and inversion systems of bacteriophages P1 and Mu. *Proc. Natl Acad. Sci. USA*, **77**, 7338–7341.
- Silverman, M. & Simon, M. (1980). Phase variation: genetic analysis of switching mutants. *Cell*, **19**, 845–854.
- Johnson, R. C. (2002). Bacterial site-specific DNA inversion systems. In *Mobile DNA II* (Craig, N. L., Craigie, R., Gellert, M. & Lambowitz, A. M., eds), pp. 230–271, ASM Press, Washington, DC.
- Silverman, M., Zieg, J., Hilmen, M. & Simon, M. (1979). Phase variation in *Salmonella*: genetic analysis of a recombinational switch. *Proc. Natl Acad. Sci. USA*, **76**, 391–395.
- Zieg, J., Silverman, M., Hilmen, M. & Simon, M. (1977). Recombinational switch for gene expression. *Science*, **196**, 170–172.
- Zieg, J. & Simon, M. (1980). Analysis of the nucleotide sequence of an invertible controlling element. *Proc. Natl Acad. Sci. USA*, **77**, 4196–4200.
- Johnson, R. C. & Simon, M. I. (1985). Hin-mediated site-specific recombination requires two 26 bp recombination sites and a 60 bp recombinational enhancer. *Cell*, **41**, 781–791.
- Johnson, R. C., Glasgow, A. C. & Simon, M. I. (1987). Spatial relationship of the Fis binding sites for Hin recombinational enhancer activity. *Nature*, **329**, 462–465.
- Johnson, R. C., Bruist, M. F. & Simon, M. I. (1986). Host protein requirements for *in vitro* site-specific DNA inversion. *Cell*, **46**, 531–539.
- Bruist, M. F., Glasgow, A. C., Johnson, R. C. & Simon, M. I. (1987). Fis binding to the recombinational enhancer of the Hin DNA inversion system. *Genes Dev.* **1**, 762–772.
- Glasgow, A. C., Bruist, M. F. & Simon, M. I. (1989). DNA-binding properties of the Hin recombinase. *J. Biol. Chem.* **264**, 10072–10082.
- Heichman, K. A. & Johnson, R. C. (1990). The Hin invertasome: protein-mediated joining of distant recombination sites at the enhancer. *Science*, **249**, 511–517.
- Heichman, K. A., Moskowitz, I. P. & Johnson, R. C. (1991). Configuration of DNA strands and mechanism of strand exchange in the Hin invertasome as revealed by analysis of recombinant knots. *Genes Dev.* **5**, 1622–1634.
- Kanaar, R., Klippel, A., Shekhtman, E., Dungan, J. M., Kahmann, R. & Cozzarelli, N. R. (1990). Processive recombination by the phage Mu Gin system: implications for the mechanisms of DNA strand exchange, DNA site alignment, and enhancer action. *Cell*, **62**, 353–366.
- Haykinson, M. J. & Johnson, R. C. (1993). DNA looping and the helical repeat *in vitro* and *in vivo*: effect of HU protein and enhancer location on Hin invertasome assembly. *EMBO J.* **12**, 2503–2512.
- Johnson, R. C. & Bruist, M. F. (1989). Intermediates in Hin-mediated DNA inversion: a role for Fis and the recombinational enhancer in the strand exchange reaction. *EMBO J.* **8**, 1581–1590.
- Merickel, S. K., Haykinson, M. J. & Johnson, R. C. (1998). Communication between Hin recombinase and Fis regulatory subunits during coordinate activation of Hin-catalyzed site-specific DNA inversion. *Genes Dev.* **12**, 2803–2816.
- Klippel, A., Mertens, G., Patschinsky, T. & Kahmann, R. (1988). The DNA invertase Gin of phage Mu: formation of a covalent complex with DNA *via* a phosphoserine at amino acid position 9. *EMBO J.* **7**, 1229–1237.
- Yang, W. & Steitz, T. A. (1995). Crystal structure of the site-specific recombinase gamma delta resolvase complexed with a 34 bp cleavage site. *Cell*, **82**, 193–207.
- Stark, W. M., Sherratt, D. J. & Boocock, M. R. (1989). Site-specific recombination by Tn3 resolvase: topological changes in the forward and reverse reactions. *Cell*, **58**, 779–790.
- Crisona, N. J., Kanaar, R., Gonzalez, T. N., Zechiedrich, E. L., Klippel, A. & Cozzarelli, N. R. (1994). Processive recombination by wild-type Gin and an enhancer-independent mutant. Insight into the mechanisms of recombination selectivity and strand exchange. *J. Mol. Biol.* **243**, 437–457.
- McIlwraith, M. J., Boocock, M. R. & Stark, W. M. (1997). Tn3 resolvase catalyses multiple recombination events without intermediate rejoining of DNA ends. *J. Mol. Biol.* **266**, 108–121.
- Merickel, S. K. & Johnson, R. C. (2004). Topological analysis of Hin-catalysed DNA recombination *in vivo* and *in vitro*. *Mol. Microbiol.* **51**, 1143–1154.
- Ball, C. A., Osuna, R., Ferguson, K. C. & Johnson, R. C. (1992). Dramatic changes in Fis levels upon nutrient upshift in *Escherichia coli*. *J. Bacteriol.* **174**, 8043–8056.
- Ninnemann, O., Koch, C. & Kahmann, R. (1992). The *E. coli* fis promoter is subject to stringent control and autoregulation. *EMBO J.* **11**, 1075–1083.
- Van Duyne, G. D. (2002). A structural view of tyrosine recombinase site-specific recombination. In *Mobile DNA II* (Craig, N. L., Craigie, R., Gellert, M. & Lambowitz, A. M., eds), pp. 93–117, ASM Press, Washington, DC.
- Jayaram, M., Grainge, I. & Tribble, G. (2002). Site-specific recombination by the Flp protein of *Saccharomyces cerevisiae*. In *Mobile DNA II* (Craig, N. L., Craigie, R., Gellert, M. & Lambowitz, A. M., eds), pp. 192–218, ASM Press, Washington, DC.
- Rice, P. (2002). Theme and variation in tyrosine recombinases: structure of a Flp–DNA complex. In *Mobile DNA II* (Craig, N. L., Craigie, R., Gellert, M. & Lambowitz, A. M., eds), pp. 219–229, ASM Press, Washington, DC.
- Haffter, P. & Bickle, T. A. (1988). Enhancer-independent mutants of the Cin recombinase have a relaxed topological specificity. *EMBO J.* **7**, 3991–3996.
- Klippel, A., Cloppenburg, K. & Kahmann, R. (1988). Isolation and characterization of unusual Gin mutants. *EMBO J.* **7**, 3983–3989.
- Haykinson, M. J., Johnson, L. M., Soong, J. & Johnson, R. C. (1996). The Hin dimer interface is critical for Fis-mediated activation of the catalytic steps of site-specific DNA inversion. *Curr. Biol.* **6**, 163–177.
- Klippel, A., Kanaar, R., Kahmann, R. & Cozzarelli, N. R. (1993). Analysis of strand exchange and DNA binding of enhancer-independent Gin recombinase mutants. *EMBO J.* **12**, 1047–1057.
- Moskowitz, I. P., Heichman, K. A. & Johnson, R. C. (1991). Alignment of recombination sites in Hin-mediated site-specific DNA recombination. *Genes Dev.* **5**, 1635–1645.
- Stark, W. M., Grindley, N. D., Hatfull, G. F. & Boocock, M. R. (1991). Resolvase-catalysed reactions

- between *res* sites differing in the central dinucleotide of subsite I. *EMBO J.* **10**, 3541–3548.
35. Lim, H. M. & Simon, M. I. (1992). The role of negative supercoiling in Hin-mediated site-specific recombination. *J. Biol. Chem.* **267**, 11176–11182.
 36. Lee, S. Y., Lee, H. J., Lee, H., Kim, S., Cho, E. H. & Lim, H. M. (1998). *In vivo* assay of protein–protein interactions in Hin-mediated DNA inversion. *J. Bacteriol.* **180**, 5954–5960.
 37. Nanassy, O. Z. & Hughes, K. T. (1998). *In vivo* identification of intermediate stages of the DNA inversion reaction catalyzed by the *Salmonella* Hin recombinase. *Genetics*, **149**, 1649–1663.
 38. Lim, H. M. (1994). Analysis of subunit interaction by introducing disulfide bonds at the dimerization domain of Hin recombinase. *J. Biol. Chem.* **269**, 31134–31142.
 39. Hughes, R. E., Rice, P. A., Steitz, T. A. & Grindley, N. D. (1993). Protein–protein interactions directing resolvase site-specific recombination: a structure–function analysis. *EMBO J.* **12**, 1447–1458.
 40. Nanassy, O. Z. & Hughes, K. T. (2001). Hin recombinase mutants functionally disrupted in interactions with Fis. *J. Bacteriol.* **183**, 28–35.
 41. Lee, H. J., Lee, S. Y., Lee, H. M. & Lim, H. M. (2001). Effects of dimer interface mutations in Hin recombinase on DNA binding and recombination. *Mol. Genet. Genomics*, **266**, 598–607.
 42. McIlwraith, M. J., Boocock, M. R. & Stark, W. M. (1996). Site-specific recombination by Tn3 resolvase, photocrosslinked to its supercoiled DNA substrate. *J. Mol. Biol.* **260**, 299–303.
 43. Leschziner, A. E. & Grindley, N. D. F. (2003). The architecture of the gamma delta resolvase crossover site synaptic complex revealed by using constrained DNA substrates. *Mol. Cell*, **12**, 775–781.
 44. Chiu, T. K., Sohn, C. S., Dickerson, R. E. & Johnson, R. C. (2002). Testing water-mediated DNA recognition by the Hin recombinase. *EMBO J.* **21**, 801–814.
 45. He, J., McIlwraith, M. J., Burke, M. E., Boocock, M. R. & Stark, W. M. (2002). Synapsis of Tn3 recombination sites: unpaired sites destabilize synapses by a partner exchange mechanism. *J. Mol. Biol.* **319**, 385–393.
 46. Arnold, P. H., Blake, D. G., Grindley, N. D., Boocock, M. R. & Stark, W. M. (1999). Mutants of Tn3 resolvase which do not require accessory binding sites for recombination activity. *EMBO J.* **18**, 1407–1414.
 47. Sarkis, G. J., Murley, L. L., Leschziner, A. E., Boocock, M. R., Stark, W. M. & Grindley, N. D. F. (2001). A model for the gamma delta resolvase synaptic complex. *Mol. Cell*, **8**, 623–631.
 48. Burke, M. E., Arnold, P. H., He, J., Wenwieser, S. V. C. T., Rowland, S.-J., Boocock, M. R. & Stark, W. M. (2004). Activating mutations of Tn3 resolvase marking interfaces important in recombination catalysis and its regulation. *Mol. Microbiol.* **51**, 937–948.
 49. Grindley, N. D. F. (2002). The movement of Tn3-like elements: transposition and cointegrate resolution. In *Mobile DNA II* (Craig, N. L., Craigie, R., Gellert, M. & Lambowitz, A. M., eds), pp. 272–302, ASM Press, Washington, DC.

Edited by M. Belfort

(Received 17 February 2004; received in revised form 3 May 2004; accepted 5 May 2004)