

# Mapping the $\lambda$ Integrase bridges in the nucleoprotein Holliday junction intermediates of viral integrative and excisive recombination

Wenjun Tong<sup>a,1,2</sup>, David Warren<sup>a,1</sup>, Nicole E. Seah<sup>a</sup>, Gurunathan Laxmikanthan<sup>a</sup>, Gregory D. Van Duyne<sup>b,3</sup>, and Arthur Landy<sup>a,3</sup>

<sup>a</sup>Department of Molecular Biology, Cell Biology, and Biochemistry, Division of Biology and Medicine, Brown University, Providence, RI 02912; and <sup>b</sup>Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104

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The site-specific recombinase encoded by bacteriophage  $\lambda$  [ $\lambda$  Integrase (Int)] is responsible for integrating and excising the viral chromosome into and out of the chromosome of its *Escherichia coli* host. In contrast to the other well-studied and highly exploited tyrosine recombinase family members, such as Cre and Flp, Int carries out a reaction that is highly directional, tightly regulated, and depends on an ensemble of accessory DNA bending proteins acting on 240 bp of DNA encoding 16 protein binding sites. This additional complexity enables two pathways, integrative and excisive recombination, whose opposite, and effectively irreversible, directions are dictated by different physiological and environmental signals. Int recombinase is a heterobivalent DNA binding protein that binds via its small amino-terminal domain to high affinity arm-type DNA sites and via its large, compound carboxyl-terminal domain to core-type DNA sites, where DNA cleavage and ligation are executed. Each of the four Int protomers, within a multiprotein 400-kDa recombinogenic complex, is thought to bind and, with the aid of DNA bending proteins, bridge one arm- and one core-type DNA site. Despite a wealth of genetic, biochemical, and functional information generated by many laboratories over the last 50 y, it has not been possible to decipher the patterns of Int bridges, an essential step in understanding the architectures responsible for regulated directionality of recombination. We used site-directed chemical cross-linking of Int in trapped Holliday junction recombination intermediates and recombination reactions with chimeric recombinases, to identify the unique and monogamous patterns of Int bridges for integrative and excisive recombination.

site-specific recombination | regulation of directionality | recombinogenic architectures | molecular machines

The tyrosine recombinase family, which includes the well-studied and highly exploited Cre, Flp, and  $\lambda$  Integrase (Int) recombinases, is responsible for such diverse functions as chromosome segregation, chromosome copy number control, gene expression, conjugative transposition, gene dissemination, and viral integration and excision [for reviews, see Mobile DNA II (1) and the in preparation Mobile DNA III]. The virally encoded  $\lambda$  Int recombinase is responsible for integrating and excising the  $\lambda$  chromosome into and out of the chromosome of its *Escherichia coli* host in response to a variety of physiological and environmental signals (2). Although all members of this family use the same isoenergetic chemistry and strand exchange mechanisms to execute DNA rearrangements, Int (in contrast to Cre and Flp) depends on an ensemble of accessory DNA bending proteins and carries out a recombination, between *att* site target DNAs, that is highly directional and tightly regulated (3–8).

Int is a heterobivalent DNA binding protein that binds to high-affinity “arm-type” DNA sites via its small amino-terminal domain (NTD), and to “core-type” DNA sites, where DNA cleavage and ligation takes place, via a central core binding domain (CB) and a C-terminal catalytic domain (CAT); the latter two domains are referred to here as the CTD. Each of the four Int protomers,

within a multiprotein 400-kDa recombinogenic complex, is thought to bind and bridge one arm- and one core-type DNA site; the bridging interactions are facilitated by accessory DNA bending proteins IHF, Xis, and Fis. Differential occupancy of the 16 DNA protein binding sites (encoded by 240 bp of *att* site DNA) generates two overlapping ensembles that differentiate integrative from excisive recombination, as diagrammed in Fig. 1.

Despite a wealth of genetic, biochemical, functional, and structural information, generated by many laboratories over the last 50 y, it has not been possible to determine which of the five arm-type DNA sites is paired via an Int bridge with which of the four core-type sites. This deficiency has been the major obstacle in understanding the architectures responsible for regulated directionality of integrative and excisive recombination. In this report, we address the need for a direct determination of the  $\lambda$  Int bridging patterns using site-directed chemical cross-linking of Int to trapped Holliday junction recombination intermediates, and we confirm the observed Int bridges in recombination reactions with chimeric recombinases.

## Results

**Trapping Recombination Intermediates.**  $\lambda$  Int is known to form off-pathway complexes and is also capable of promoting noncanonical recombination reactions under some conditions (9, 10). We

## Significance

The bacteriophage encoded  $\lambda$  Int protein is distinguished from other well-studied and widely exploited tyrosine recombinase family members as a heterobivalent DNA binding protein. With the help of accessory DNA bending proteins, Int bridges two different classes of DNA sites within the unique 400-kDa recombinogenic complexes of integrative and excisive recombination. The absence of any overarching investigations or structural models for these key complexes stems from the inability to determine which core-type and arm-type binding sites are joined to one another by Int-mediated bridges. We solved this long-standing problem using site-directed chemical cross-linking of Int in trapped Holliday junction intermediates and recombination reactions with chimeric recombinases, to identify the unique patterns of Int bridges for integrative and excisive recombination.

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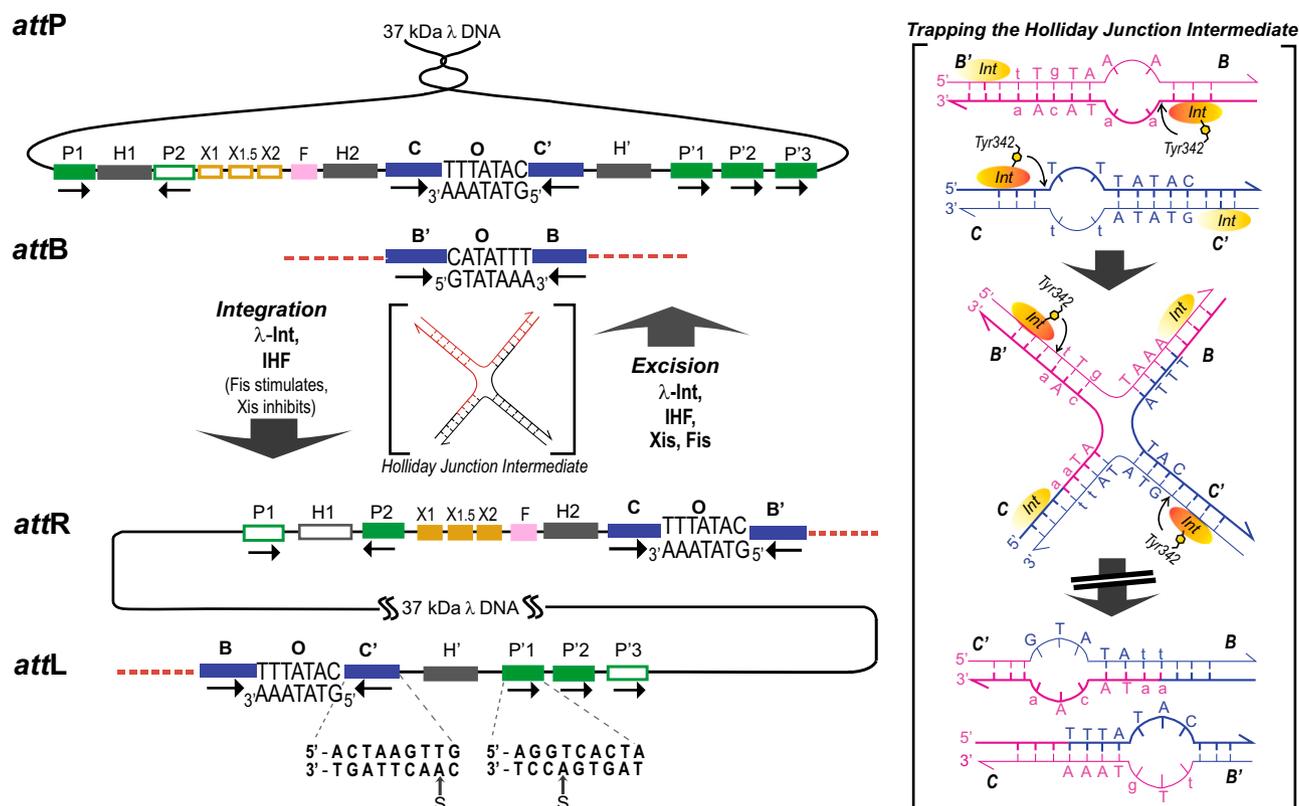
The authors declare no conflict of interest.

<sup>1</sup>W.T. and D.W. contributed equally to this work.

<sup>2</sup>Present address: School of Chemistry and Chemical Engineering, Nanjing University, Nanjing, Jiangsu 210093, China.

<sup>3</sup>To whom correspondence may be addressed. Email: vanduyne@mail.med.upenn.edu or Arthur\_Landy@brown.edu.

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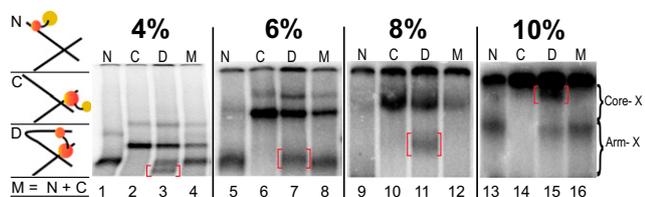
**Fig. 1.** The overlapping ensembles of protein binding sites that comprise *att* site DNA and the DNA modifications used for cross-linking. Integrative recombination between supercoiled *attP* and linear *attB* requires the virally encoded Integrase (Int) (29) and the host-encoded accessory DNA bending protein IHF (30, 31) and gives rise to an integrated phage chromosome bounded by *attL* and *attR*. Excisive recombination between *attL* and *attR* to regenerate *attP* and *attB* additionally requires the phage-encoded Xis protein (which inhibits integrative recombination) (32) and is stimulated by the host-encoded Fis protein (33). Both reactions proceed through a Holliday junction intermediate that is first generated and then resolved by single-strand exchanges on the left and right side of the 7-bp overlap region, respectively. The two reactions proceed with the same order of sequential strand exchanges (not the reverse order) and use different subsets of protein binding sites in the P and P' arms, as indicated by the filled boxes: Int arm-type P1, P2, P'1, P'2, and P'3 (green); IHF, H1, H2, and H' (gray); Xis, X1, X1.5, and X2 (gold); and Fis (pink). The four core-type Int binding sites, C, C', B, and B' (blue boxes) are each bound in a C-clamp fashion by the CB and CAT domains, referred to here as the CTD. These core sites are where Int executes isoenzymatic DNA strand cleavages and ligations via a high energy covalent 3'-phospho-tyrosine intermediate. The CTD of Int and the tetrameric Int complex surrounding the two overlap regions are functionally and structurally similar to the Cre, Flp, and XerC/D proteins. The bottom line shows the locations of the cysteine modifications (S) within the core- and arm-type consensus sequences (see main text and Tables S1 and S2). (Right) DNA sequence changes made in the 7-bp overlap regions to trap Holliday junction intermediates (lowercase letters). Following the first pair of Int cleavages (via the active site Tyr) on one side of the overlap regions (arranged here in antiparallel orientation) the "top" strands are swapped to form the HJ; this simultaneously converts the unpaired (bubble) bases to duplex DNA. On the other side, the sequence differences between the two overlap regions strongly disfavor the second ("bottom") strand swap that would resolve the HJ, because this would generate unpaired bubbles in the product (11, 12). This diagram applies to both integrative and excisive recombination (even though the labels refer to integrative recombination).

therefore decided to study complexes that were trapped in the course of a canonical, bona fide recombination reaction (as opposed to assembling complexes from individual components). The most attractive candidate for this is the Holliday junction intermediate (HJ), the four-way DNA junction generated from two partner DNAs by the first pair of DNA strand exchanges and then resolved to two product DNAs by the second pair of DNA strand exchanges, i.e., the midpoint between reactants and products.

We efficiently trapped stable HJ complexes as described previously and diagrammed in Fig. 1 (11, 12) by recombining two *att* site partners with the following two features. On the left side of their respective overlap regions, each partner has an unpaired 2-bp heteroduplex bubble, such that strand exchange between them creates an HJ with fully base paired DNA. Reversal of this top-strand exchange would result in reformation of the unpaired heteroduplex bubbles and is thus energetically disfavored. On the right side of their respective overlap regions, the two partners differ in 2 bp of DNA duplex sequence. Therefore, the right hand strand exchange, which is required to resolve the HJ, would create a 2-bp heteroduplex in each recombinant product and is

energetically disfavored. Thus, resolution of the fully duplex HJ in either direction is energetically disfavored and rarely occurs. We have characterized the stable nucleoprotein HJ complexes extensively in a wide variety of experiments, as described in *SI Text* and Fig. S1.

**Chemical Cross-Linking of Int Bridges in the HJ Complex.** There are potentially four arm-core Int bridges in the integrative pathway and three in the excisive pathway, which requires only three arm-type sites. To map the Int bridges, we adapted the disulfide trapping technology developed in the Verdine laboratory (13, 14) to introduce disulfide cross-links at the protein-DNA interfaces between an Int NTD and its cognate arm-type site and between an Int CTD and its cognate core-type site. As described below, we used site-directed mutagenesis to introduce appropriately positioned Cys residues at the NTD and/or CTD DNA binding surfaces of an Int in which the two surface accessible Cys residues (C25S and C197S) had been mutated to generate an Int with two buried Cys residues (verified to be nonaccessible in the native protein) (*SI Text*). Additionally, we incorporated sulfhydryl groups



**Fig. 2.** The effect of acrylamide composition on the relative electrophoretic mobility of an HJ with a doubly cross-linked Int bridge. Excisive recombination reactions were carried out between *att* site partners designed to trap HJ intermediates, as described in the main text. Each pair of recombining *att* sites contains a cystamine in the P'2 arm site and a cystamine in the B core site, two sites determined in other excisive recombination experiments (cf. Fig. 3, lanes 1–4) to be bridged by the same Int. A  $^{32}$ P-label was incorporated at the 5' terminus of the P' arm. Each of the four recombination reactions in a set contained a  $\lambda$  Int with a reactive Cys in the DNA binding pocket of either the NTD (N), the CTD (C), or both (D, for dual); the fourth reaction contained a mixture (M) of the N and C Ints. Following recombination, disulfide bond formation was promoted by adding hydrogen peroxide, which was then quenched by the addition of catalase. Reactions were terminated by the addition of SDS, fractionated by SDS/PAGE, and visualized on a phosphorimager. In each set of four reactions, the band unique to the dual labeled (bridging) Int is highlighted with a red bracket, and bands corresponding to complexes singly cross-linked at an arm or core site are labeled in the margin. See *Materials and Methods* and *Results* for details. (Left) Cartoon depicts the cross-linked species expected in lanes N, C, D, and M.

with a two-carbon tether at N6 of appropriately positioned adenosine residues in the DNA of selected arm- and/or core-type sites.

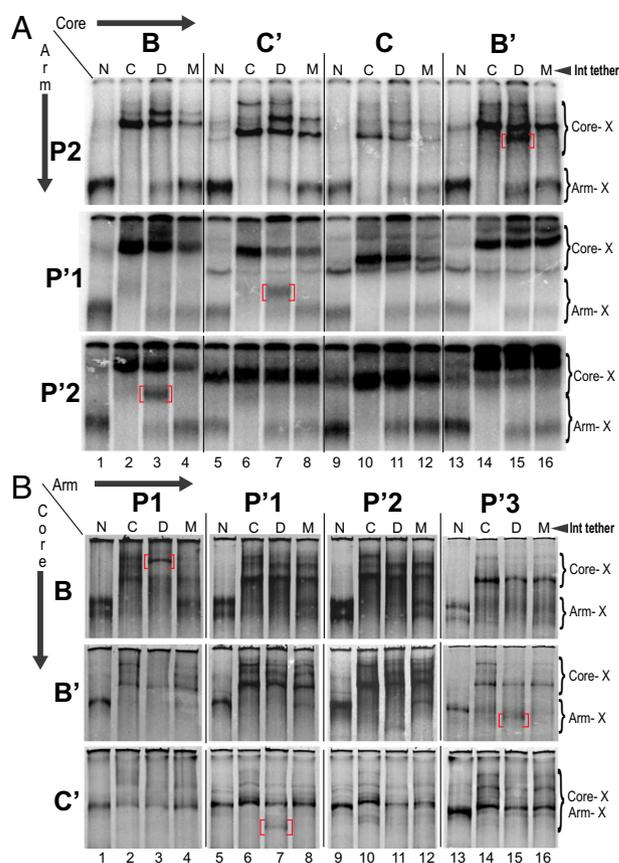
Candidate amino acid and nucleotide residues that were juxtaposed at the protein–DNA interfaces were identified by inspecting the crystal structures of several Int–DNA complexes (15, 16). From these candidates, we constructed and characterized 14 different single Cys substitutions in the NTD or CTD domains and 12 oligonucleotides with appropriately positioned cystamine modifications in the P'1 arm or C' core sites (Tables S1 and S2).

Based on the results of these analyses, we chose substitution N20C (hereafter called N-Cys) and position 4b of the arm-type consensus recognition sequence as the best-matched pair of targets for generating disulfide cross-links between the NTD and arm sites. We chose substitution G283C (hereafter called C-Cys) and position 8b of the core-type consensus recognition sequence as the best-matched pair of targets for generating disulfide cross-links between the CTD and core sites (Fig. 1 and Tables S1 and S2). We also constructed an Int in which the NTD-Cys and CTD-Cys were combined to give the double mutant, (N+C)-Cys. Each of the three Cys-mutant proteins is more than 70% as proficient as WT Int for recombination.

We generated an ensemble of HJ-trapping *att* sites with all possible combinations of a single cystamine-labeled arm-type site coupled with a single cystamine-labeled core-type site for disulfide cross-linking experiments, resulting in 12 *att* sites for excisive recombination (4 core sites and the 3 arm sites required for excisive recombination) and 12 *att* sites for integrative recombination (4 arm sites and 3 core sites, the C core site could not be labeled with cystamine at the required position in the bottom strand of the supercoiled *attP* substrate in our protocol; *Materials and Methods* and *SI Text*). We reasoned that an (N+C)-Cys Int, doubly cross-linked to both an arm- and a core-type site, would have an identifiably distinct electrophoretic mobility in SDS/PAGE compared with an N-Cys or C-Cys Int singly cross-linked to only one type of DNA site. The doubly cross-linked Int can only be generated when the single cystamine arm site and the single cystamine core site are bridged by the same Int. Indeed, the doubly cross-linked complex is identified as a band in an SDS gel that appears only in the lanes corresponding to reactions with (N+C)-Cys Int and not in the lanes from reactions where the reactive Cys resides only in the NTD or only in the CTD. The unique bands in the (N+C)-Cys Int lanes were shown to be HJs because they could be labeled with  $^{32}$ P

from any one of the four core sites. As an additional control, we included a reaction containing a mixture of the single N-Cys and single C-Cys Ints, which cannot give rise to the doubly cross-linked, Int-bridged, product.

In Fig. 2, all four excisive recombination reactions contained identical DNA substrates, in which the single cystamine-modified core site was B and the single cystamine-modified arm site was P'2, chosen on the basis of the data in Fig. 3A. Each reaction contained different Int protomers: N-Cys, C-Cys, (N+C)-Cys, or a mixture of N-Cys and C-Cys. Aliquots of each reaction were analyzed by electrophoresis on SDS polyacrylamide gels of 4%, 6%, 8%, or 10%. All four gels show a single band that is unique to the (N+C)-Cys reaction (labeled lane D), consistent with results expected for an Int mediated bridge between the P'2 arm and the B core. This experiment highlights the well-known effect of shape on relative electrophoretic mobilities and anticipates our observation that the position of the double cross-link within the HJ influences its relative electrophoretic mobility (relative to



**Fig. 3.** Mapping specific Int bridges by disulfide cross-linking. Excisive and integrative recombination reactions were carried out with the indicated Int proteins and processed as described in Fig. 2. Each pair of recombining *att* sites in a set of four reactions contains a single reactive cystamine in the indicated arm site and a single reactive cystamine in the indicated core site. A  $^{32}$ P-label was incorporated at the 5' terminus of the P or P' arm carrying the cystamine. The band unique to the bridging Int (D lanes) is highlighted with a red bracket, and bands corresponding to complexes singly cross-linked at an arm or core site are labeled in the margin. (A) Sets of four excisive recombination reactions between *attL* and *attR*, in which one of the three arm sites required for excision (y axis) and one of the four core sites (x axis) is labeled with cystamine. (B) Sets of four integrative recombination reactions between supercoiled *attP* and *attB*, in which one of the three tested core sites (y axis) and one of the four arm sites (x axis) is labeled with cystamine. The C core site could not be labeled with cystamine at the required position in the bottom strand of the supercoiled *attP* substrate (*Materials and Methods* and *SI Text*).

singly and other doubly cross-linked complexes) in a way that depends on the gel composition. Because of this phenomenon, and the potential for comigration of bands, each of the experiments described below was analyzed on several different percentage gels (only one of which is shown in the experiments below). We also anticipated that the doubly cross-linked product would be generated in relatively low yield because many of the singly cross-linked products will derive from (abundant) non-HJ complexes, such as unrecombined substrate.

For the excisive recombination experiments shown in Fig. 3A, an *att* site, containing a single cystamine modification in one of the three arm-type sites required for excisive recombination (*y* axis), was recombined with *att* sites in which each of the four core sites (*x* axis) was modified with cystamine. This cycle was repeated with the cystamine label in each of the remaining arm sites. Each lane in these gels corresponds to a separate recombination reaction with its own <sup>32</sup>P-labeled substrate; however, for each reaction condition (lane), the patterns of the bands and their relative intensities are very reproducible. The results show a unique band corresponding to an Int bridging B–P'2, C'–P'1, and B'–P2, leaving the C core site as the one that does not form an Int bridge with one of the three arm-type sites required for excisive recombination.

In a similar experiment for integrative recombination (Fig. 3B), there is a unique band corresponding to an Int bridging P1–B, P'1–C', and P'3–B'. In lane 11, there is a weak unique band for the P'2 reactions, but its intensity relative to the reference band in the same lane (singly cross-linked CTD) was reproducibly four times weaker than the unique band in the P'3 reactions. We infer, and confirm in the genetic experiments described below, that P'2 forms an Int bridge with the C core site, which could not be labeled with cystamine at the required position in the bottom strand of the supercoiled *attP* substrate (*Materials and Methods* and *SI Text*).

**Confirmation of the HJ Int Bridges in Complete Recombination Reactions.** Our strategy for genetically testing the Int bridges in a full recombination reaction depends on a previously constructed and characterized chimeric recombinase in which the amino-terminal domain of  $\lambda$ -integrase was fused with the Cre recombinase

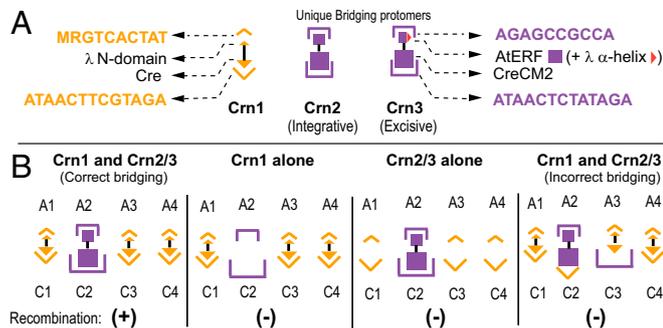


Fig. 4. Confirmation of the Int bridges in complete recombination reactions. (A) Schematic of unique specificity bridging enzymes. Chimeric recombinase Crn1 (17) possesses the NTD of  $\lambda$  Integrase (small gold triangle), which binds to the DNA consensus sequence MRGTCACATAT, fused to the body of Cre (large gold triangle), which binds to the modified *loxP* (*lot*) site, ATAACCTCGTAGA. Crn2 possesses the NTD of AtERF (small purple box), which binds to the consensus GCC box sequence, AGAGCCGCCA, fused to the body of CreCM2 (large purple box), which binds to the *lotM7* sequence, ATAACCTATAGA. Crn3 is the same as Crn2, but the  $\alpha$ -helix of AtERF has been replaced by a structurally analogous  $\alpha$ -helix from  $\lambda$  Integrase (red triangle), which interacts with Xis (*SI Text*). The gold and purple color coding of the two DNA specificities is preserved in B. (B) Cartoon of the logic and possible outcomes of the experiments. In each of the excisive and integrative recombination reactions shown in Fig. 5, one of the arm-core pairs has the DNA sequences of the unique Crn2/3 specificity (purple), as indicated, whereas all of the other arm and core sites have the Crn1 specificity (gold). Recombinant product (+) is observed only when the complete and correct Int bridging pattern is achieved.

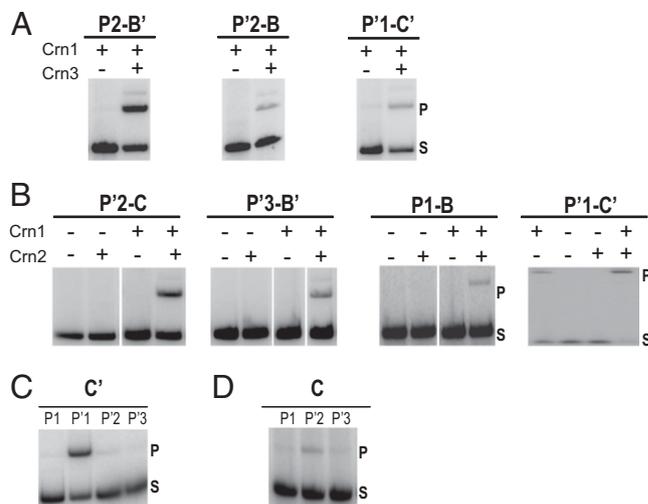


Fig. 5. Excisive and integrative recombination reactions carried out with a mixture of two chimeric recombinases and *att* site substrates containing the indicated unique arm and unique core specificities. In each of the excisive (A) and integrative (B) recombination reactions, one of the arm-core pairs identified by chemical cross-linking has the DNA sequences of the unique Crn2/3 specificity (purple in Fig. 4), as indicated, whereas all of the other arm and core sites have the Crn1 specificity (yellow in Fig. 4). In C and D, all of the integrative reactions contained both Crn1 and Crn2. The unique core specificity was fixed either at C' (C) or at C (D), and the unique arm specificity was moved to a different arm site in each of the recombination reactions, as indicated. All of the *att* sites in D had Crn2 specificity at the previously characterized P'1–C' bridged pair, so there are only three recombination reactions. Integrative recombination reactions contained supercoiled *attP*, linearized <sup>32</sup>P-labeled *attB*, and IHF. Excisive recombination reactions contained linear *attL*, linear <sup>32</sup>P-labeled *attR*, and accessory proteins IHF and Xis. All integrative and excisive recombinations require IHF or Xis, as shown previously (17). Recombinase concentrations were optimized for each set of experiments and typically were in the range of 50–100 nM; the gel lanes shown in A and B have been selected from a large titration gel to present the most informative concentrations. Each DNA substrate was at 6 nM. The reactions were terminated by the addition of SDS; electrophoresed on SDS/PAGE gels; and visualized on a phosphorimager (*Materials and Methods* and *SI Text*).

(17). Here we refer to this recombinase as Crn1. Substrates for Crn1, called *lot* sites, were generated by appending the appropriate P and P' arms of the  $\lambda$  *att* sites to modified *loxP* Cre target sites. The resulting four *lot* sites, *lotP*, *lotB*, *lotL*, and *lotR* (analogous to *attP*, B, L, and R), are recombined by Crn1 in a regulated directional reaction that depends on the  $\lambda$  arm-type sites and accessory proteins (IHF and Xis) in patterns identical to that of  $\lambda$  Int-mediated recombination (17).

From Crn1, we created a recombinase, Crn2, with different arm- and core-type specificities. The arm-type DNA recognition domain of Crn1 (residues 16–55) was replaced with the G box DNA binding domain of the ethylene responsive factor from *Arabidopsis thaliana* (AtERF1) (residues 4–50) (Fig. S2A and C). Like the Int NTD, this domain adopts a monomeric DNA binding fold consisting of a three-stranded, antiparallel  $\beta$ -sheet that is packed against a carboxyl-terminal  $\alpha$ -helix (18, 19). The core-type DNA recognition specificity of Crn1 was changed by introducing the five amino acid changes of the well-characterized CreCM2 mutation, which has a DNA recognition specificity different from, and essentially nonoverlapping with, the WT Cre (20, 21). To enable Crn2 for excisive recombination, the carboxyl-terminal  $\alpha$ -helix of the  $\lambda$  integrase NTD (residues 41–55 in Crn1), which has been shown to interact with Xis (22, 23), was used to replace the corresponding  $\alpha$ -helix (residues 47–62) of Crn2 to generate Crn3 (Fig. 4A and Fig. S2). Crn3, which can be used in place of Crn2, was difficult to purify and was therefore only used for the excisive recombinations.



imperfect protein–DNA interfaces inherent in the design of these experiments. Thus, where the bridging patterns, and the models described in ref. 27, predict a more rigidly held Int bridge, such as P'1–C' in integration, the genetic signal is relatively strong and the cross-linking signal is relatively weak. Conversely, where the bridging pattern predicts a more loosely held Int bridge, such as P1–B in integration, the genetic signal is relatively weak and the cross-linking signal is relatively strong. Indeed, the loosely held P1–B bridge figures prominently in the architectures described in the companion paper (27).

The monogamous relationship of each arm-core bridged pair throughout the course of the recombination reaction makes it possible to extrapolate from the patterns observed in the HJ recombination intermediate to those predicted for the pre-synaptic recombination partners and the post-HJ recombination products. Inspection of Fig. 6 reveals that for excisive recombination, the presynaptic partners have only intramolecular bridges, suggesting that Int bridging is not a driving force in synapsis of *attL* and *attR*. In contrast, integrative recombination has two intermolecular bridges that are responsible for the capture of *attB* by supercoiled *attP*, as predicted by Richet and Nash (28).

Our results do not address the question of whether DNA cleavage of the top strands precedes synapsis but it may be relevant that, in both integrative and excisive recombination, one of two protomers responsible for the first strand exchange is not held tightly by an Int bridge. In excisive recombination, the Int bound at C does not bridge to an arm site and in integrative recombination the Int bound at B is bridged to P1, which resides on a weakly constrained P arm that is postulated to move during synapsis [see above discussion and companion article (27)].

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