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Dissecting the resolution reaction of λ integrase using suicide Holliday junction substrates

Sok Hong Kho[1] and Arthur Landy[2]

Division of Biology and Medicine, Brown University, Providence, RI 02912, USA
[1]Present address: National University of Singapore, 10 Kent Ridge Crescent, Singapore 0511
[2]Corresponding author

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A reciprocal strand exchange between two DNA helices generates the crossed-strand intermediate, or Holliday junction, which is common to many pathways of homologous and site-specific recombination. The Int family of recombinases are unique in their ability to both make and resolve Holliday junctions. Previous experiments utilizing 'synthetic' *att* site Holliday junctions to study the mechanisms associated with the cleavage, transfer and ligation of DNA strands have been confined to studying reciprocal strand exchanges (a pair of temporally overlapping strand cleavages). To circumvent this limitation, we have designed synthetic suicide Holliday junctions that make it possible to monitor individual DNA strand cleavage events. These substrates contain a pre-existing nick in the vicinity of the Int binding site; when Int introduces a second nick into these substrates, the 5'OH nucleophile required for ligation (in either the forward or reverse reaction) is lost by diffusion, thus trapping the covalent protein–DNA intermediate. The results indicate that resolution (involving two partner Ints) is stimulated by additional 'cross-core' Ints as a result of enhanced cleavage rates, and not as a result of enhanced co-ordination of cleavage. Several models for the role of the 'cross-core' Ints during resolution are discussed, as well as the usefulness of these substrates for studying additional aspects of the Holliday junction resolution reaction.

Key words: Holliday junction/λ integrase/resolution reaction/ suicide substrates

Introduction

The Holliday junction (HJ) is a central intermediate in both homologous and site-specific recombination (Holliday, 1964; Broker and Lehman, 1971; Sobell, 1972; Meselson and Radding, 1975; Orr-Weaver *et al.*, 1981; Hsu and Landy, 1984; Kitts and Nash, 1987; Nunes-Düby *et al.*, 1987). It is generated when one strand in each of two DNA partners undergoes a reciprocal exchange so that the two DNA duplexes are joined by a pair of crossed strands. It is resolved by cleaving the second pair of strands, to generate recombinant duplexes, or by cleaving the original two strands, to regenerate the starting duplexes. Branch migration by the HJ leads to resolution products containing heteroduplex DNA in the region between the first and second pair of strand exchanges [for recent reviews, see Smith (1988) and West (1992)].

The original view of HJs, based on theoretical model building, has been greatly expanded by physical studies with small immobile junctions that cannot branch migrate because of sequence differences between the duplexes (Sigal and Alberts, 1972; Kallenbach *et al.*, 1983; Hsu and Landy, 1984; Cooper and Hagerman, 1987, 1989; Churchill *et al.*, 1988; Duckett *et al.*, 1988, 1990; Murchie *et al.*, 1989, 1991; Lu *et al.*, 1992). The actual configuration of HJs during recombination is likely to be largely determined or strongly influenced by the proteins involved in their formation and resolution. Some of these proteins, such as recA, are primarily involved in the formation of HJs, while others, such as T7 endo I, T4 endo VII and RuvC, only carry out resolution (Mizuuchi *et al.*, 1982; de Massy *et al.*, 1984; Lilley and Kemper, 1984; Dickie *et al.*, 1987; Dunderdale *et al.*, 1991; Iwasaki *et al.*, 1991). The first protein shown to have the capacity to both make and resolve HJs was Integrase (Int), the protein that carries out the site-specific recombination of bacteriophage λ (Hsu and Landy, 1984; Kitts and Nash, 1987; Nunes-Duby *et al.*, 1987). Two other proteins, Cre and FLP, also have this capability, which is likely to be a general feature of the 30-member Int family of recombinases (Argos *et al.*, 1986; Hoess *et al.*, 1987; Meyer-Leon *et al.*, 1988).

The Int-dependent site-specific recombination pathway of bacteriophage λ is responsible for the integration and excision of the phage chromosome during the transitions between lysogeny and vegetative growth. Recombination between specific '*att*' sites on the phage (*att*P) and bacterial chromosomes (*att*B) results in an integrated prophage bounded by the left (*att*L) and right (*att*R) prophage *att* sites, that are themselves the substrates for excisive recombination [for reviews, see Craig (1988), Landy (1989) and Thompson and Landy (1989)]. Both integrative and excisive recombination are initiated by a reciprocal 'top' strand exchange that generates a HJ, which is then resolved to recombinant products by exchange of the 'bottom' strands (Figure 1) (Nunes-Düby *et al.*, 1987; Kitts and Nash, 1988). The sites of top and bottom strand exchange are separated by a 7 base pair (bp) 'overlap' region whose precise sequence is not critical as long as it is the same in both partners (Mizuuchi *et al.*, 1981; Craig and Nash, 1983; Weisberg *et al.*, 1983; Bauer *et al.*, 1985). The overlap region plus the flanking Int binding sites involved in strand exchange (core-type Int binding sites) are referred to here as the 'core region'. Beyond the core region of *att*P there are additional protein binding sites for the accessory proteins IHF, Xis and Fis, and for a different class of Int binding sites (arm-type sites) (Ross *et al.*, 1979; Craig and Nash, 1984; Yin *et al.*, 1985; Thompson *et al.*, 1987; Moitoso de Vargas *et al.*, 1988). Int protein accomplishes cutting, exchange and re-ligation of the DNA in the absence of cofactors by conserving

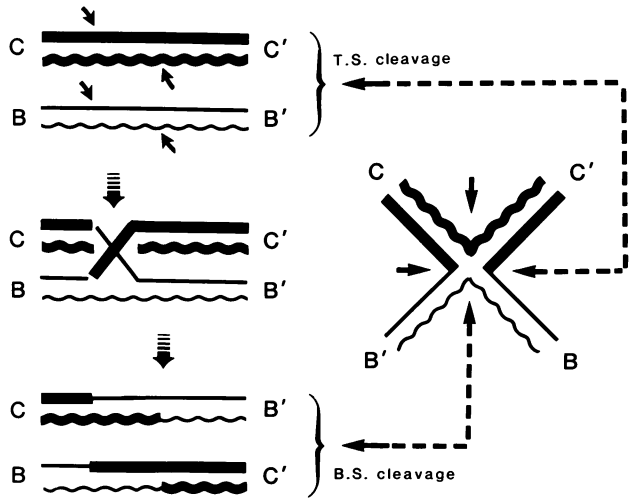

Fig. 1. Formation and resolution of a HJ. Two DNA helices containing the *att* sites CC′ and BB′ undergo reciprocal exchange of top strands (straight lines) at the left boundary (↖) of the 7 bp overlap region to form the HJ recombination intermediate, which is illustrated both as a crossed-strand and open planar structure (middle panel). The HJ can also be made synthetically, as illustrated in Figure 2. The HJ is resolved to products CB′ and BC′ by reciprocal exchange of the bottom strands (wavy lines) at the right boundary (↘) of the overlap region (B.S. cleavage). Alternatively, it can be resolved by exchange of the top strands to (re)generate the CC′ and BB′ helices (T.S. cleavage).

the bond energy in a covalent phosphotyrosine linkage with the 3′ end of the cut strand (Craig and Nash, 1983; Pargellis *et al.*, 1988; Mizuuchi and Adzuma, 1991). A recent model from the laboratory of M.Jayaram, for this reaction in the closely related FLP system, proposes that the active site for cleavage is composed of two protomers bound to adjacent DNA sites (see Discussion) (Chen *et al.*, 1992).

One useful approach to studying the mechanisms associated with strand cleavage and ligation has been the construction of 'synthetic' *att* site HJs. By annealing the appropriate DNA strands containing *att* site sequences from the core region, it is possible to construct four-way junctions analogous to the top strand exchange of either integrative or excisive recombination (Figure 2) (Hsu and Landy, 1984). Int resolution of the synthetic *att* site HJ yields both pairs of resolution products with approximately the same efficiency (Figure 1). However, this symmetry is disturbed by a single base pair of heterology that confines the crossover to one side of the 7 bp overlap region and greatly favors resolution on that side (de Massy *et al.*, 1989). Another observation made with synthetic HJs is that a pair of partner Ints is not sufficient to carry out resolution; the reaction appears to additionally require a third Int binding site on the opposite side of the overlap region (a 'cross-core' Int) (Franz and Landy, 1990a).

One of the obstacles to studying these and other aspects of the HJ resolution pathway stems from the inability to monitor individual cleavage events (as opposed to a co-ordinated pair of cleavages resulting in fully duplex resolution products). Because the phosphotyrosine bond between Int-family proteins and DNA is extremely sensitive to nucleophilic attack, a single Int cleavage that does not participate in strand exchange is rapidly reversed, by resealing the original nick. To circumvent this problem, we have adapted the device of suicide substrates that have proven informative in studying synapsis and HJ formation in several site-specific recombination pathways (Nunes-Duby *et al.*, 1987, 1989). The essential feature of the suicide substrates is that they contain a nick, or an end, positioned such that cleavage by the recombinase protein generates a small oligonucleotide that diffuses away and thereby traps a covalent protein–DNA intermediate. We reasoned that analogous suicide substrates for HJs might make it possible to disengage the closely coupled steps of cleavage and ligation during the resolution phase of recombination. In this report, we describe the construction and application of suicide HJs, and address some of the interactions necessary for the Int-dependent pathway of HJ resolution.

Results

Construction and features of the Holliday junctions

The HJs in this study contain only the four core-type Int binding sites at the locus of strand exchange. As in several previous studies of the resolution reaction, the distal arm-type Int binding sites have been replaced by non-*att* DNA sequences (Figure 2) (Hsu and Landy, 1984; Franz and Landy, 1990a,b). To obtain stable HJs that cannot branch migrate, it is necessary to use a different DNA sequence in each of the four arms. These arm sequences are thus structurally, but not functionally, equivalent to the four arms of a canonical *att* site HJ. We have shown that substituting different non-*att* arm sequences at the same position does not affect the resolution reaction (B.Franz and A.Landy, unpublished results).

The four core-type Int binding sites of *att*P and *att*B each have a slightly different sequence; however, it has been shown that the two *att*B sequences, B and B′, can be replaced by the *att*P sequences, C and C′ (Bushman *et al.*, 1984). This substitution has also been used in constructing the HJs in this work in order to simplify the execution and interpretation of the experiments, and to focus on the relative positions of the Int binding sites within the HJ (rather than small differences in affinity for Int).

To facilitate reference to the various constructions, the four core sites are labeled in a clockwise direction around the HJ as C1, C2′, C3 and C4′ (Figure 2). One pair of partner Int binding sites (C1 and C3) lies to the left of the overlap region and comprises the cleavage sites for 'top' strand exchange. The other pair (C2′ and C4′) lies to the right of the overlap region and comprises the cleavage sites for 'bottom' strand exchange. In the HJs used in this work, the left and right pairs of partner Ints are functionally equivalent. However, they can be distinguished and are labeled differently on the basis of their relationship to: (i) the non-symmetrical 7 bp core sequence; (ii) a single labeled end or nick (see below); and (iii) sequence differences in the adjacent non-*att* DNA. Cross-core binding sites are defined as the inverted repeat elements on opposite sides of the 7 bp overlap region. In contrast to the partner Int binding sites, there are two different kinds of cross-core Int binding sites in a HJ. This can be seen by following the chemical polarity of the DNA strands within the HJ; C1 shares a top strand with the C4′ cross-core Int and it shares a bottom strand with the C2′ cross-core Int (Figure 2).

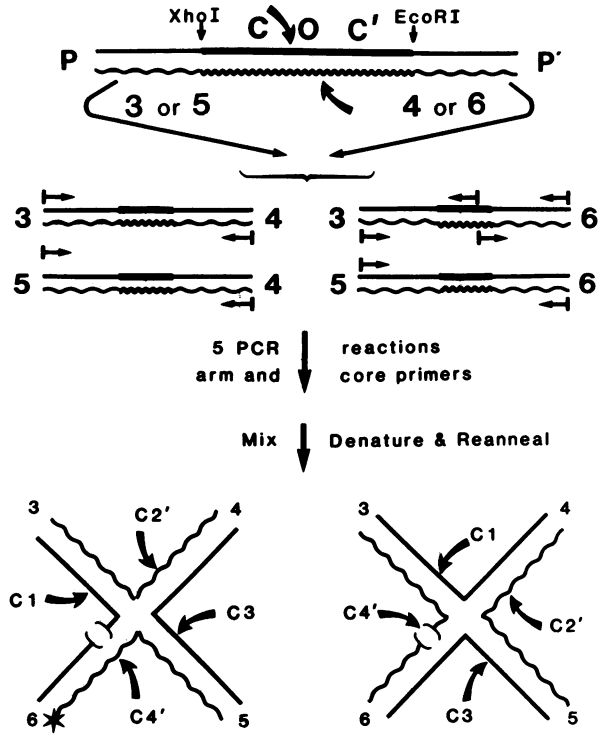

Fig. 2. Construction of nicked HJs. The core region of *att*P contains Int cleavage (◥◤) and binding (C and C′) sites separated by the 7 bp overlap region (O), and flanked by engineered *Xho*I and *Eco*RI sites. The P arm was replaced by DNA sequence 3 or 5 and the P′ arm was replaced by DNA sequence 4 or 6 to generate four different plasmids containing the core cassette (Nunes-Duby *et al.*, 1987; Franz and Landy, 1990a). For some HJs, the plasmids carried modified core cassettes and/or different arm sequences, as indicated (see Table I). Four plasmids are used as templates in separate PCR reactions to generate fragments containing the core cassette and flanking arm sequences. Two primers (⊢and⊣) for the arms of plasmids 3−4, 5−4 and 5−6 were used to generate the unnicked strands (see Table II). The nicked strands were made from plasmid 3−6: one PCR reaction contained the bottom strand (⊢) arm 3 primer and the top strand (⊣) core primer; another PCR reaction contained the top strand arm 6 primer and the bottom strand core primer. The 5′ termini of the two core primers determine the site of the nick in the final HJ; these were always kinased with cold ATP so that the nick contains a 5′ phosphate. The five PCR products are mixed, denatured and annealed. The resulting HJs (χ-forms), which comprise ~25−40% of the total product, are separated from the renatured linear fragments by gel electrophoresis. Two different HJs are formed in the annealing reaction, one is uniquely labeled with ^{32}P (*) by virtue of kinasing the 5′ end of one of the primers (the bottom strand primer for arm 6, in the example illustrated here). See Materials and methods for the details, and Table I for the clones, DNA sequences and primers used to construct the different HJs. The top (C1 and C3) and bottom (C2′ and C4′) strand cleavage sites are uniquely specified with respect to the asymmetric 7 bp overlap sequence, as described in the text.

Many of the HJs used in this study have less than the full complement of four Int binding sites. When non-*att* DNA replaces an Int binding site, it is referred to as 'Δ' or 'Δ′', on the left or right side of the core, respectively. According to our shorthand notation, a HJ (χ-form) with all four Int binding sites is labeled 4X; one in which the C1 Int binding site has been changed to a Δ sequence is labeled 3XC2′C3C4′, etc. (Figure 3A). In some constructions, one or more of the Int binding sites has been shifted 2 bp toward the center of the HJ. The case where two partner sites are shifted is equivalent to decreasing the overlap region from 7 to 5 bp.

HJs containing 1−4 core-type Int binding sites were made by denaturing and annealing four appropriate DNA duplexes, as described in Materials and methods (Hsu and Landy, 1984; Franz and Landy, 1990a). The suicide HJs containing one strand with a nick in the overlap region are made in a similar manner, except that five duplexes are required in the annealing mixture (Figure 2). The DNA plasmids, fragments and primers used to make each of the HJs are listed in Tables I and II. Unless indicated otherwise, all of the suicide HJs used in these experiments contain a single nick in the top strand, opposite the cleavage site of C4′, and a single ^{32}P label at the 5′ end of the bottom strand of C4′. The same relative positions of the nick and label are maintained when one or more Int binding sites are replaced by Δ or Δ′ sequences. To indicate a HJ with a nick, an N is added to our shorthand notation, e.g. 3NXC2′C3C4′ (Figure 3B). In all cases, the nick contains a 5′ phosphate in order to prevent any covalent interactions with Int at this site.

Resolution and cleavage products

When the ^{32}P-labeled *att* site HJ used here is resolved by reciprocal strand exchange at the C1 and C3 partner Int sites, only one (551 bp) of the two products is radioactively labeled (Figure 3A). Similarly, a single labeled 370 bp fragment is diagnostic of reciprocal strand exchange at the C2′ and C4′ partner sites. Note that the resolution products are identified in the autoradiograms by the Int cleavage sites used to generate them and their size in base pairs. In the case of nicked HJs, the labeled cleavage product is predicted to have a covalently bound Int as a consequence of the suicide feature, i.e. the covalent phosphotyrosine cleavage product is trapped, due to the loss of an appropriately positioned nucleophile. To identify products with covalently bound Int, a duplicate of each reaction mixture was treated with SDS and KCl. Protein and covalent protein−DNA complexes are precipitated and recovered from the pellet after centrifugation, while free DNA is recovered in the supernatant (Trask *et al.*, 1984; Pargellis *et al.*, 1988). These two fractions, along with the untreated reaction, were analyzed by PAGE (Figure 3B).

The suicide HJs make it possible to study Int cleavage at a single Int binding site and the resulting products will depend on the location of the Int cleavage relative to the nick. In the case of a single Int binding site at C1 (1NXC1), Int cleavage is on the same strand as the nick and only a three base fragment is lost by diffusion. The resulting 'gapped' HJ, covalently bound to Int, migrates slightly more slowly than the substrate (Figure 3B). Int cleavage at the C2′ position generates a short unlabeled duplex, corresponding to the C1-containing arm, and a labeled three-armed structure covalently bound to Int (Figure 3B, 1NXC2′). Int cleavage at C3 generates two two-armed structures corresponding approximately to the normal resolution products resulting from top strand cleavage (Figure 3B, 1NXC3). The labeled product of Int cleavage at C4′ is a short duplex covalently bound to Int (Figure 3B, 1NXC4′). It appears that Int binding and/or cleavage on the strand opposite the nick (position C4′) is less efficient than the other sites and, therefore, more aberrant cleavages are observed at the Δ

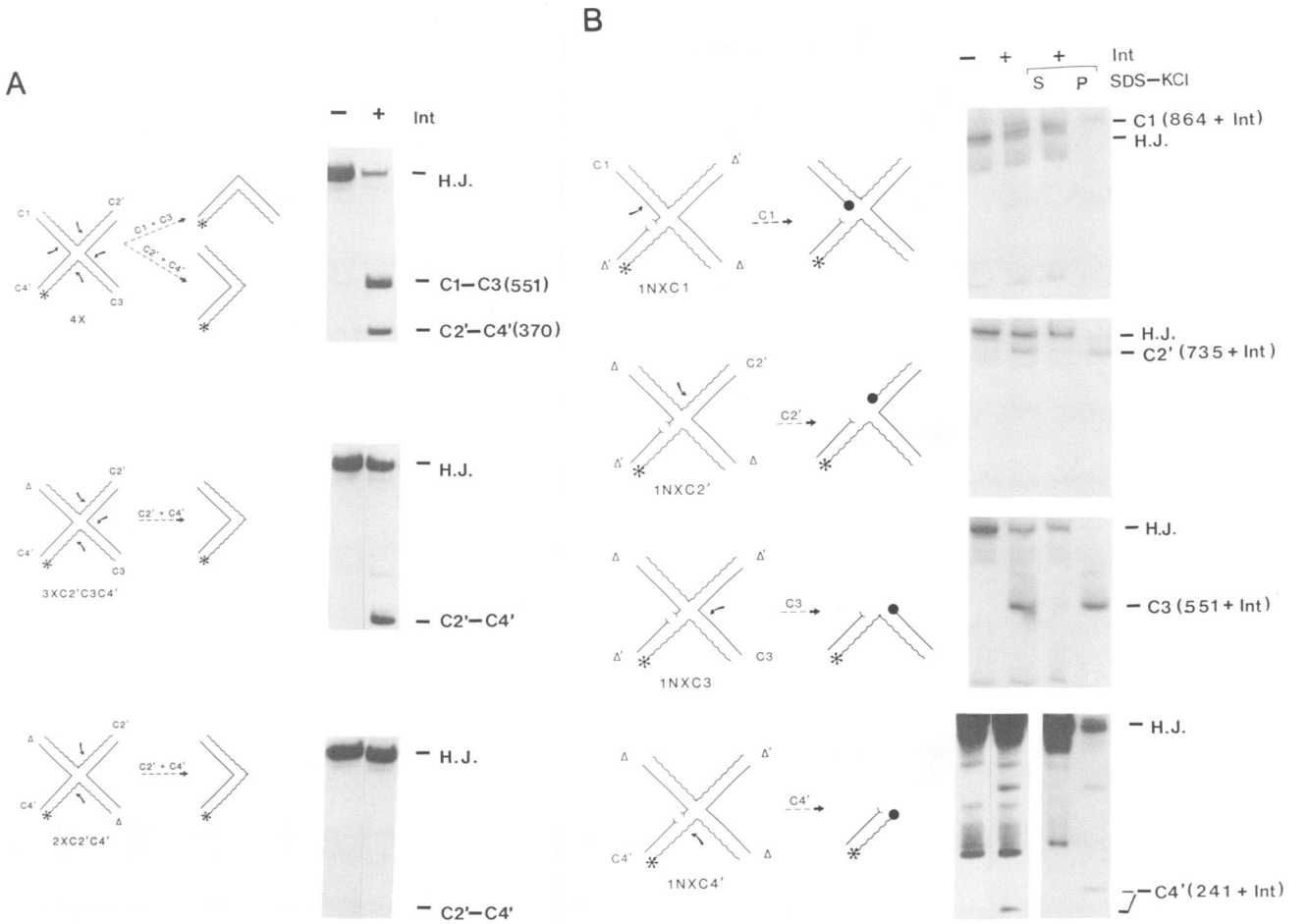

Fig. 3. Int resolution and cleavage products of intact and nicked HJs. (**A**) The structures of three intact HJs with four (4X), three (3XC2′C3C4′) and two (2XC2′C4′) Int binding sites are diagrammed. The shorthand notations are described in the text. They have arm sequences 3, 4, 5 and 6 as described in Figure 2. The C1 Int binding site has been replaced by a non-*att* sequence (Δ) in 3XC2′C3C4′; both C1 and C3 are replaced by non-*att* sequences (Δ) in 2XC2′C4′. The four clones for making each HJ are listed in Table I; e.g. 3XC2′C3C4′ is made from fragments of clones pBF 9-34, 504, 506 and 9-36. The Int cleavage sites (↘ ↗) of top (—) and bottom (〰) strands are shown. The HJs are labeled with ^{32}P (*) at the 5′ end of the bottom strand of arm 6. The diagram shows only the ^{32}P-labeled resolution products of each HJ. The HJs were incubated with (+) or without (−) Int for 35 min and then analyzed by gel electrophoresis and autoradiography (see Materials and methods). The resolution products are identified by the Int cleavages that generated them: C1−C3 in the top strand and C2′−C4′ in the bottom strand. Their sizes, in base pairs, are indicated in parentheses. In this and other gels there are minor bands in addition to the HJ, but they do not react with Int. (**B**) The structures of four suicide HJs with a single Int binding site at different locations relative to the nick are diagramed. Three of the Int binding sites have been replaced by non-Int binding sequences (Δ or Δ′) such that each HJ has only one Int binding site (C1, C2′, C3 or C4′). The four clones for making each HJ are listed in Table I; e.g. 1NXC1 is made from fragments of clones pBF 8-34, 17-54, 17-56 and 8-36. The single Int cleavage site in each HJ is either in a top or bottom strand. The HJs are all labeled with ^{32}P (*) at the 5′ end of the bottom strand of arm 6. Only the ^{32}P-labeled cleavage products are shown; they all have a covalently attached Int (●). The HJs were incubated with (+) or without (−) Int for 50 min. One Int reaction was then analyzed directly by electrophoresis, the other Int reaction was used for SDS−KCl precipitation of covalent protein−DNA complexes (see Materials and methods). The pellet (P) was dissolved in 10 mM Tris−HCl (pH 7.5) and, along with the supernant (S) and untreated samples, was analyzed by gel electrophoresis and autoradiography.

sites with this HJ. It should be noted that in the very long incubations used in Figures 3 and 4, aberrant cleavages at Δ sites and secondary cleavages at Int binding sites have an exaggerated prominence.

The structures and cleavage products of suicide HJs containing four, three or two Int binding sites are shown in Figure 4. In each case, all of the products predicted by cleavage at the individual Int binding sites were obtained and each of the labeled products from these HJs can be found as a product from one of the suicide HJs containing a single Int binding site (Figure 3B). These substrates thus provide an assay for the individual cleavage events in HJs containing different arrangements of Int binding sites.

Relative efficiencies of resolution and cleavage with different numbers of Int binding sites

It had previously been reported that a HJ containing only two Int binding sites was not resolved, but that three Int binding sites did allow resolution (Franz and Landy, 1990a), as seen in Figure 3A. The relative efficiencies of these resolution reactions are compared in Figure 5A, where it is seen that a HJ with three Int sites is resolved >80 times faster than one with only two Int binding sites. Addition of a fourth Int binding site results in another 4-fold increase in the rate of resolution.

Since the resolution reaction requires the simultaneous, or near simultaneous, cleavage by two Int molecules, it was

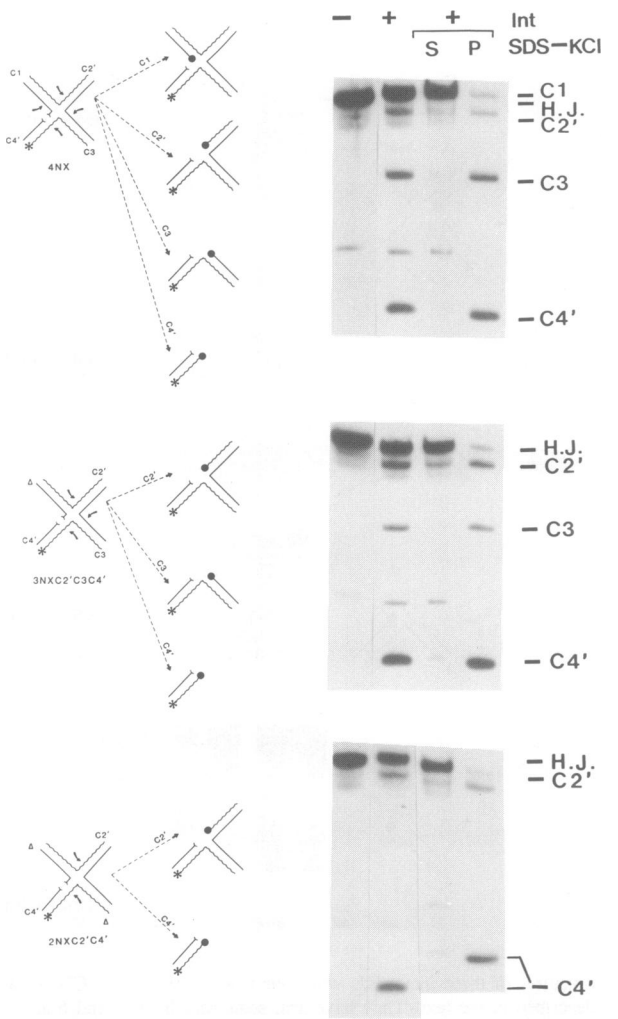

Fig. 4. Cleavage products of suicide HJs with four, three or two Int binding sites. The experimental protocol and labeling of the diagrams and autoradiograms are similar to Figure 3B, with the exception of the incubation times. The 4NX and 3NXC2′C3C4′ HJs were both incubated with Int for 6 min and the 2NXC2′C4′ HJ was incubated with Int for 40 min (the S and P lanes were run on different gels). The products (which all have covalently bound Int) are identified by the site of Int cleavage and their size in base pairs. The relative amounts of the cleavage products in these long incubations reflect the accumulation of secondary and tertiary Int cleavages—all of which ultimately lead to the C3′ and then the C4′ cleavage products (since C4′ is the labeled arm).

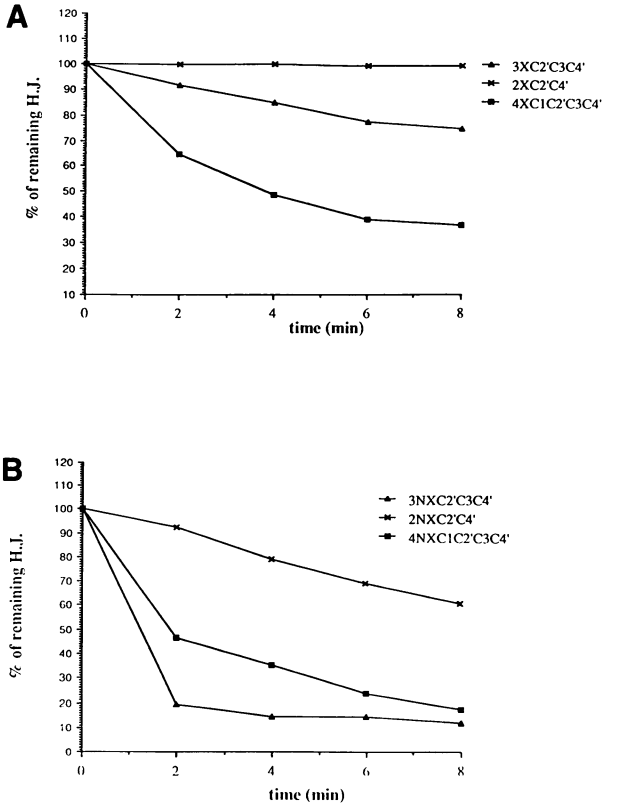

Fig. 5. Time course of the disappearance of nicked and intact HJs with four, three or two Int binding sites when incubated with Int. The amount of uncleaved HJ after incubation with Int for the indicated times was determined by gel electrophoresis and autoradiography as described in Materials and methods. (**A**) Int resolution of HJs 4XC1C2′C3C4′ (−■−), 3XC2′C3C4′ (−▲−) and 2XC2′C4′ (−×−), containing four, three and two Int binding sites, respectively. (**B**) Int cleavage of nicked HJs 4NX (−■−), 3NXC2′C3C4′ (−▲−) and 2NXC2′C4′ (−×−), containing four, three and two binding sites, respectively. The lower rate for 4NX compared with 3NXC2′C3C4′ is discussed in the text. In 3NXC2′C3C4′, most of the products come from C2′ and C4′ cleavage, the C3 cleavage product contributes ~5% to the total (see the text).

of interest to examine the cleavage rates in a suicide HJ where it is possible to monitor cleavage by an individual Int, independent of the activity of any other Int molecules on the HJ. The most striking difference between the cleavage and resolution assays is that whereas the intact HJ with two partner Int binding sites seemed refractile to resolution (co-ordinated cleavage by two Ints), the suicide HJ clearly shows that single Int cleavages are taking place (Figures 3B and 4). Presumably, in an intact HJ these individual cleavage events are rapidly reversed by re-ligation. Comparison of the rate of resolution of 2XC2′C4′ with the rate of total C2′ and C4′ cleavages of 2NXC2′C4′ shows that the sum of individual cleavage reactions (in the suicide substrate) occurs ~80 times more frequently than two temporally overlapping cleavages (as required for resolution in the intact HJ) (Figure 5). The addition of one or two Int binding sites to the two-Int suicide HJ results in an ~10-fold enhancement in the rate of HJ cleavage. We presume that the nicked four-Int HJ has a lower (apparent) 'cleavage' efficiency than the three-Int HJ because the cleaved arm is held more tightly in the four-Int complex, thus allowing more time for the re-ligation (back reaction). (In all of these reactions, it is the difference between cleavage and re-ligation that is actually being assayed.)

The striking differences between the cleavage and resolution reactions raise the question of whether the suicide HJ is a valid device for learning something about the resolution of the intact HJ. To address this question, we constructed a suicide HJ in which we could measure resolution, i.e. two concerted cleavages. In previous experiments, the ^{32}P label was on the same arm as the nick, i.e. the C4′ arm, in order to best observe single cleavage events. However, with this configuration it is not possible to observe the double cleavage product (at C2′ and C4′) as a radioactive product. Therefore, in the following set of

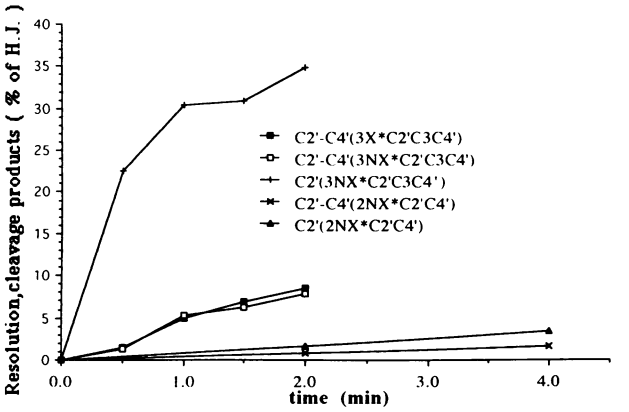

Fig. 6. Comparison of the rates of single and double Int cleavages. The HJs used here have the ^{32}P label at the 5′ end of the bottom strand of arm 4 (all others in this paper are 5′-labeled in the bottom strand of arm 6) (see Figure 2). The arm 4 labeling is denoted by an asterisk in the name. The appearance of the indicated products of resolution or cleavage after incubation with Int was determined by gel electrophoresis and densitometry of autoradiograms, as described in Materials and methods. The amount of ^{32}P-labeled product at each time point is plotted as the percent of the total radioactivity in the reaction: the products of Int cleavage at C2′−C4′ in 3X*C2′C3C4′ (−■−), 3NX*C2′C3C4′ (−□−) and 2NX*C2′C4′ (−×−); the products of Int cleavage at C2′ in 3NX*C2′C3C4′ (−|−) and 2NX*C2′C4′ (−▲−). To facilitate comparisons, the absolute rates shown here have been multiplied by 1.3, which is the factor for normalizing the C2′−C4′ resolution rates in this experiment and that in Figure 5B.

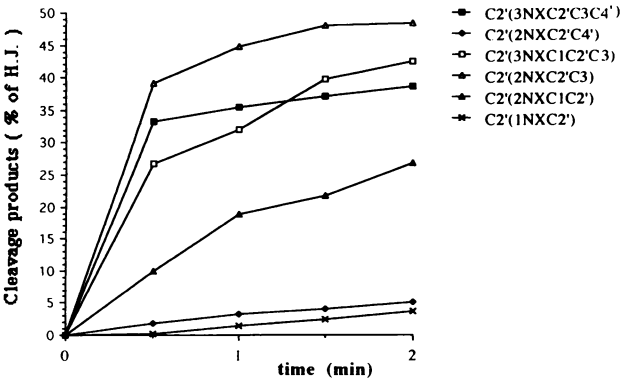

Fig. 7. Cross-core stimulation of Int cleavage. The amount of C2′ Int cleavage product was quantitated as described in Figure 6B for the following HJs: 3NXC2′C3C4 (−■−), 2NXC2′C4′ (−♦−), 3NXC1C2′C3 (−□−), 2NXC2′C3 (−△−), 2NXC1C2′ (−▲−) and 1NXC2′ (−×−).

experiments, the ^{32}P label was placed on the C2′ arm. HJs labeled in this position are denoted by a '*' (3NX*C2′C3C4′) (Figure 6). The rate of formation of the double cleavage product (equivalent to the resolution product) from this suicide HJ with three Int binding sites was compared with the rate of formation of the C2′−C4′ resolution product of the intact HJ, also containing three Int binding sites (3X*C2′C3C4′). As shown in Figure 6, the rate of resolution at C2′−C4′ is the same for both the nicked and intact HJs. It should also be noted (and will be discussed below) that C3 stimulation of the single Int cleavage at C2′ is much greater than its stimulation of concurrent C2′−C4′ cleavages. Although the single cleavage at C4′ is not readily measured with this HJ, in substrates with the label in a different position, it can be seen that its stimulation by C3 is much less than the C2′ stimulation (data not shown).

Mapping the site responsible for cleavage stimulation

It was observed above that HJ cleavage is considerably more efficient if more than one Int binding site is present. By monitoring Int cleavage at C2′, we now ask whether all Int binding sites are equally effective at stimulating this reaction. The C2′ site has been chosen for monitoring because in a suicide HJ containing two or more Int binding sites C2′ cleavage by Int is the most efficient (data not shown) (also see Discussion). These experiments are carried out on a shorter time scale than the experiments in Figure 3B that were designed to characterize the products from the inefficient cleavage of single-site HJs. As seen from the data in Figure 7, Int cleavage at a single C2′ site is very inefficient, and addition of a second Int binding site at the position of the partner Int, C4′, provides hardly any stimulation. However, when the second Int binding site is at the position of a cross-core Int, the stimulation of C2′ Int cleavage is very dramatic. The cross-core Int at C1 stimulates C2′ cleavage ~10-fold and stimulation by the C3 cross-core Int is even more pronounced: >30-fold higher than that of the single C2′ site. The two cross-core Ints together, C1 and C3, do not stimulate Int at C2′ any more than the C3 Int alone. The inability of Int at C4′ to stimulate C2′ cleavage does not appear to involve an inhibitory component since the addition of C4′ does not diminish the C3-stimulated cleavage of C2′ Int (Figure 7).

The C3-stimulated Int cleavage at C2′ does depend critically on the spatial relationship between Int at C3 and C2′. To address this, we constructed HJs in which the cross-core distance between the C3 Int binding site and the C2′−C4′ partner Int binding sites was reduced from 7 to 5 bp (see Materials and methods). With this change of spacing and concomitant axial rotation, the C3 cross-core stimulation of C2′ Int cleavage is almost completely abolished (data not shown). The loss of cross-core stimulation is not due to a loss of Int binding at the shifted C3 site, since Int cleaves C3 in this HJ even better than in a normal HJ.

Constraining the crossover

Several models for the stimulation of resolution by a cross-core Int are considered in the Discussion. Among these is the possibility that the cross-core Int functions to drive the crossover toward the position where two partner Ints can carry out the co-ordinated cleavages leading to resolution. To test this model, we constructed HJs in which the position of the crossover was constrained by non-homologies within the 7 bp overlap region, as originally described by de Massy and Weisberg (de Massy *et al.*, 1989). Two different HJs were constructed by annealing the appropriate DNA strands, as described in Materials and methods. One HJ, called f(TSE), has a heterology at position +2, such that the crossover is constrained to the left side of the overlap region (Figure 8, left panel). As originally shown by de Massy and Weisberg, this constraint of the crossed strands to the left side of the overlap region limits resolution to top strand exchange (cleavage at C1 and C3). Another HJ, called f(BSE), also has a heterology at position +2; however, in this case the crossover is constrained to the right side of the

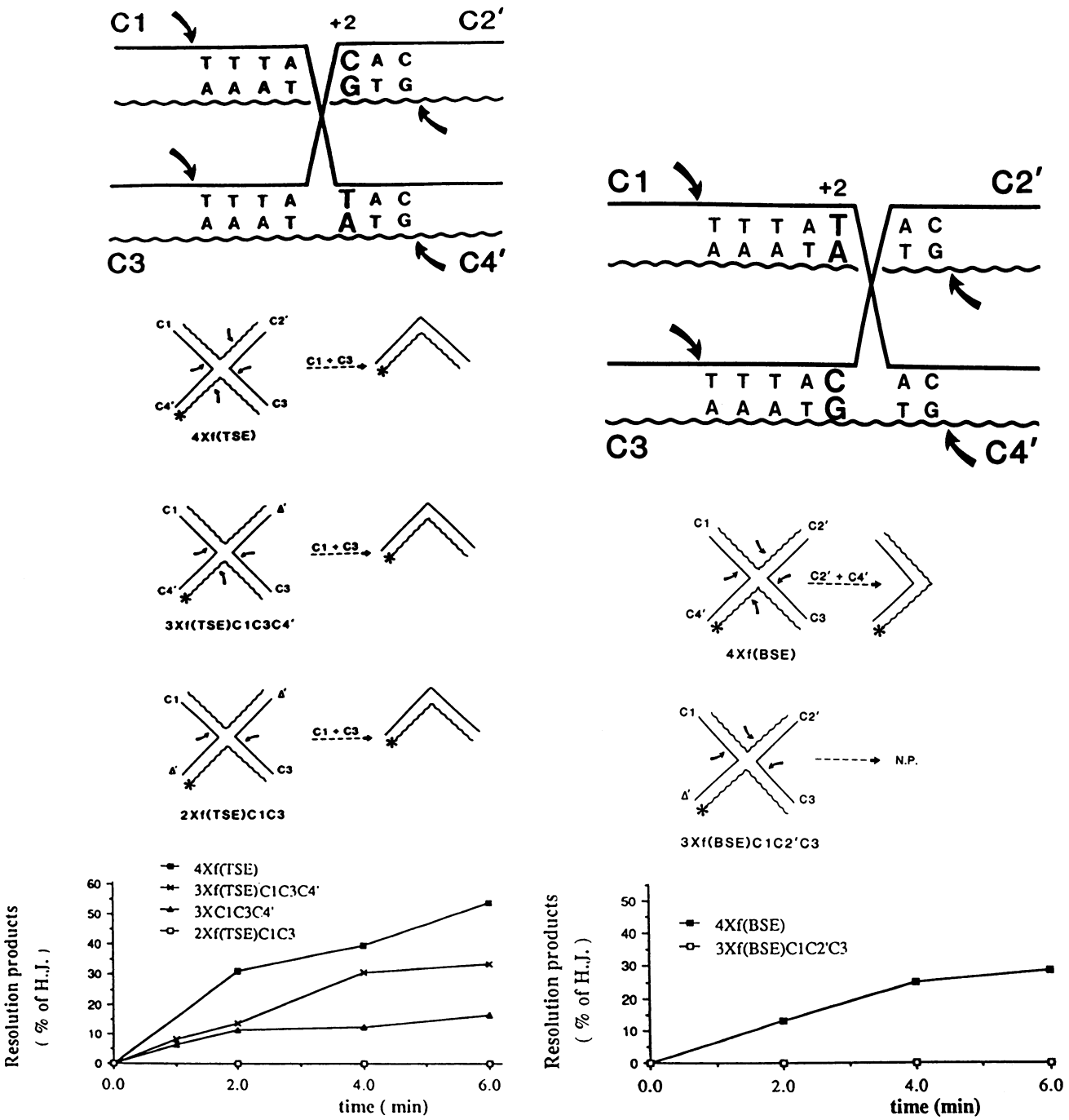

Fig. 8. Structures and Int resolution products of HJs with the crossover restricted to the left or right side of the overlap region. Left panel: the overlap sequences in each of the four HJ strands were chosen so that maximum base pairing (the most stable structure) is only achieved when the crossover is to the left of position +2 (top). Three different HJs were constructed with this overlap sequence [denoted f(TSE) for fixed top strand exchange] and containing four, three or two Int binding sites (diagrammed, along with their respective ^{32}P-labeled Int resolution products). The amount of total ^{32}P-labeled top strand resolution product (TSE) was quantitated at different times as described in Figure 5 for the following HJs: 4Xf(TSE) (−■−), 3Xf(TSE)C1C3C4′ (−×−), 3XC1C3C4′ (−▲−) and 2Xf(TSE)C1C3 (−□−). The HJs 3Xf(TSE)C1C3C4′ and 2Xf(TSE)C1C3 have one or both of their Int binding sites replaced by non-Int sequences (Δ′8 or Δ′11) (see Table I). Right panel: the overlap sequences were chosen so that maximum base pairing is only achieved when the crossover is to the right of position +2 (top). Two different HJs were constructed with this overlap sequence [denoted f(BSE) for fixed bottom strand exchange] and containing four or three Int binding sites (diagrammed along with their respective ^{32}P-labeled Int resolution products). The amount of total ^{32}P-labeled Int resolution product was quantitated at different times, as described in Figure 5 for HJs 4Xf(BSE) (−■−) and 3Xf(BSE)C1C2′C3 (−□−). HJ 3Xf(BSE)C1C2′C3 is not expected to generate any resolution product (see the text).

overlap region, and only bottom strand exchange takes place (Figure 8, right panel).

Superimposed on the changes in the overlap region that restricted branch migration, we constructed a family of HJs containing four, three or two Int binding sites. In the f(TSE) HJ, the Int binding sites were disposed so that there were always two partner sites on the side containing the crossover, e.g. 4Xf(TSE), 3Xf(TSE) C1C3C4′ and 2Xf(TSE) C1C3

(Figure 8, left panel). The Int resolution products of these three HJs, along with the products from the parental 'unconstrained' HJs, were analyzed by gel electrophoresis and plotted as a function of time. The most important conclusion from these experiments is that resolution of the HJ with only two Int binding sites, 2Xf(TSE)C1C3, is not rescued, or even facilitated, by confining the crossover to the left side of the overlap region. The effectiveness of a single base pair difference between the two overlap regions in confining branch migration, and the consequences of this limitation, are highlighted by testing a HJ with two conflicting programs for resolution. In this HJ, resolution is programmed to the left by the configuration of Int binding sites (C1 and C3 partner sites and a C2′ cross-core site) and is simultaneously programmed to the right by constraints on branch migration [f(BSE) 3Xf(BSE)C1C2′C3; Figure 8, right panel]. The result is no resolution. The failure to see any stimulation of resolution in the two-Int site HJ is not likely to be an artifact resulting from the heterology in the overlap region, since the efficiency of resolution with these HJs containing four or three Int binding sites is no lower than with the fully homologous HJs.

Discussion

In a suicide HJ, the 5′OH nucleophile required for ligation (in either the forward or reverse reaction) is lost by diffusion, thus trapping the cleaved intermediate. Although the introduction of a nick into a HJ is very likely to have an effect on its conformation and properties, there are several lines of evidence indicating that useful insights about the Int-dependent resolution reaction can be obtained from these structures. First, the rate of temporally overlapping partner cleavages in a nicked HJ with three Int binding sites is the same as the rate of resolution in the analogous intact HJ (Figure 6). Second, nicked HJs containing a single Int binding site are cleaved by Int uniquely at that site, as has been observed for analogous suicide recombination and half-*att* substrates (Figure 3) (Nunes-Duby *et al.*, 1987, 1989). The only exception to this pattern was observed for the 1NXC4′, where the nick is within the Int binding site and across from the Int cleavage site. (It is not surprising that binding and/or cleavage at this Int site is significantly depressed, thus opening the way for low-level aberrant cleavages.) Finally, changes in the size of the overlap region(s) produced significant perturbations in the patterns of cleavage products, as would be expected for a higher order structure with a specific architecture and a specific set of protein−protein interactions (data not shown). These experimental results suggest that the nicked substrates maintain many of the functionally relevant features of a normal HJ recombination intermediate. Additionally, the Int cleavage patterns of the suicide HJs 'make sense' in the context of what is currently known about this recombination pathway.

To reduce the number of variables in these experiments and to make valid comparisons between HJs with different numbers of Int binding sites, the same nick location (C4′) has been retained and the same Int binding site (C2′) has been used to assay cleavage, wherever possible. The C2′ Int binding site was chosen because its cleavage rates are higher than those at C1 and C3, which are further from the nick (see also below). It should be noted that in these, or any other cleavage assays, it is the difference between the rate of cleavage and the rate of re-ligation that is actually being observed.

The results presented here confirm and quantitate the earlier observation that in a HJ containing only two partner Int binding sites, resolution is ~80-fold less efficient than that same reaction in a HJ with three or four Int binding sites (Figure 5). In contrast, the total cleavage efficiency at two-partner Int sites is depressed ~10-fold relative to those same cleavages in a HJ with three or four Int binding sites. The stimulation by a third Int does not appear to be due to increased Int binding at the two-partner Int sites; however, the experiments on this question are not definitive. In gel mobility shift experiments, the amount of Int protein required to convert all of the HJ DNA to a complex of lower mobility was not altered significantly by the presence or absence of a third Int binding site (data not shown). A more definitive experiment calls for gel electrophoresis conditions that separate HJ complexes with different numbers of bound Int protomers. In Int titration experiments, the third Int stimulation persisted even at apparently saturating concentrations of Int, where Int binding should no longer be rate limiting (data not shown). A reservation in interpreting these results stems from the difficulty in demonstrating an Int saturation plateau because of Int inhibition at high concentrations.

One class of models for the stimulation of resolution and cleavage by the third Int is based on the experiments of de Massy and Weisberg showing that the direction of resolution is strongly biased toward that side of the overlap region where the crossed strands reside (de Massy *et al.*, 1989). In a HJ with only two bound partner Ints, it is possible that the favored position of the crossed strands is on the side away from the bound proteins. In this case, the role of the additional Int(s) might be to drive the crossed strands away from the unfavorable location. The results shown here argue against this class of models. Even when the crossed strands were confined to the same side of the overlap region as the two partner Ints, a third Int was still required for efficient resolution and the extent of stimulation by the third Int was approximately the same as with a fully homologous overlap region (Figure 8).

These experiments also extend the conclusions of de Massy and Weisberg. In the HJ with three Int binding sites, one of the two resolution pathways is eliminated and it is thus possible to ascertain whether resolution actually depends on a favorably positioned branch point, as opposed to favoring one of two competing reactions. When the crossover is confined, as in 3Xf(BSE)C1C2′C3, resolution is reduced by >100-fold (despite the presence of a cross-core Int at C2′) (Figure 8, right panel). The location of the branch point is thus shown to be critical for a single resolution event, even in the absence of a competing reaction.

Models for the cross-core stimulation of resolution can be grouped into two classes. In one class, the role of the additional Int protomer(s) is to co-ordinate the two (otherwise independent) DNA cleavage events that are necessary for resolution. The other class of models includes those mechanisms in which the additional Int protomer stimulates DNA cleavage and/or reduces the rate of re-ligation (the back reaction). The data presented here argue against the first class

of models and support the second. A primarily cleavage-co-ordinating function is not favored by the observation that C3 Int stimulation of the single Int cleavage at C2′ is very different from (greater than) its stimulation of concurrent partner (C2′−C4′) cleavages (Figure 6). Additionally, the C3 stimulation of Int cleavage at C4′ is different from (less than) that at C2′ (data not shown).

The second class of models is favored by the fact that Int cleavage at C2′ is stimulated ~10-fold by a C1 cross-core Int and ~30-fold by a C3 cross-core Int (Figure 7). The specificity of this stimulation is highlighted by the fact that the two cross-core Ints (C1 and C3) differ in their ability to stimulate C2′ Int cleavage and also by the requirement for canonical spacing in the overlap region (data not shown). In a suicide HJ designed to monitor resolution (3NX*C2′C3C4′), it was found that cleavage at C4′ was the rate-limiting step in resolution (concurrent Int cleavages at C2′ and C4′). The simplest interpretation of these data suggests that the cross-core Int functions by enhancing the rate of cleavage (or by decreasing the rate of re-ligation). This is consistent with the observation that the enhancement of resolution by a cross-core Int (~80-fold) is similar to the product of the enhancements of individual cleavages (~100-fold) (from the initial slopes in Figure 5).

There are two classes of models for the mechanism of the cross-core stimulation of Int cleavage. One class consists of activation-type mechanisms in which the intrinsic cleavage activity of an Int protomer is enhanced by the cross-core Int. As discussed above, this class of models encompasses both the stimulation of cleavage and the retardation of re-ligation (back reaction). Activation could be through protein−protein interactions and allosteric effects on the cleaving Int, and/or it could be mediated by alterations in DNA structure. One example of such DNA distortions has been suggested by the Sadowski laboratory for the FLP recombination system where cross-core FLP binding has been shown to induce a bend in the overlap region (Schwartz and Sadowski, 1989, 1990; Kulpa *et al.*, 1993).

The second class of models consistent with these data is prompted by experiments with the FLP recombination system. As a result of complementation studies, the Jayaram laboratory has proposed that the active site for DNA cleavage is composed of two protomers bound to adjacent DNA sites: one activates the phosphodiester bond, while the other provides the tyrosine nucleophile (cleavage in '*trans*') (Chen *et al.*, 1992). Strand exchange is completed when the 5′OH of an invading partner strand attacks the phosphotyrosine linkage to form a newly ligated DNA strand (ligation in '*cis*') (Pan and Sadowski, 1992). Recent experiments from the Gardner laboratory have shown that complementation patterns similar to those seen with FLP are observed in the λ system (Han *et al.*, 1993). According to this 'composite active site' model, HJ resolution (two cleavage events) would require four Int protomers and resolution of a HJ with only three Int binding sites would involve a fourth Int being pulled into the complex by protein−protein interactions. This is consistent with the observation that two cross-core Ints are more efficient at stimulating resolution than one (Figure 8, left panel), while an individual cleavage event does not seem to enjoy additional stimulation by a second cross-core Int (Figure. 7). It is also interesting to note that cross-core stimulation of C2′ Int cleavage was ~5-fold higher by the C3 than the C1 Int (Figure 7) and this is also the pairwise combination proposed for forming the 'composite active site' (Chen *et al.*, 1992).

One aspect of the resolution reaction that has not been explored in the present studies concerns the role of HJ conformation and possible changes in conformation during resolution. A particularly interesting observation by Meyer-Leon *et al.* (1990) suggested that, in the closely related FLP system, there appears to be an obligatory temperature-dependent isomerization between the formation and resolution of the HJ. An interesting observation in the studies reported here is that, despite their similar affinity for Int, all four binding sites are cleaved with different efficiencies in the nicked HJ. The lowest efficiency, observed with Int at C4′, is probably a trivial consequence of reduced Int cleavage (and/or binding) at a site with a nick. The fact that Int cleavages at C1 and C3 are similar, and less efficient than that at C2′, is more interesting and may be related to the conformation of the nicked HJ. In the suicide HJs used here, with the nick at the right side of the overlap region, a cleavage on the left side of the overlap region (C1 or C3) will be 'protected' by the hydrogen bonds of at least 7 bp that hold the structure together and thereby allow more time for re-ligation (the back reaction). This 'protection' is independent of the location of the crossed strands within the overlap region. In contrast, the number of base pairs 'protecting' an Int cleavage at C2′ varies from 0 bp (if the crossover is on the right side of the overlap region) to 14 bp (if the crossover is on the left). Branch migration towards the nick, or preferential residence of the crossover at the nick, should thus make the C2′ Int binding site extremely sensitive in the cleavage assay, as observed here. Cleavage efficiencies might also be influenced by protein−protein interactions if they lead to differential retention of a cleavage product and thereby allow more time for re-ligation. Future experiments in which the location of the nick is altered may provide useful insights regarding HJ conformation(s) during resolution and the possible role of cross-core Ints in stabilizing specific conformations.

Materials and methods

Construction of clones

All of the plasmids were derived from pSN2 which contains the λ *att*P site from −251 to +242 (relative to position 0 of the core) on a *Hin*dIII−*Bam*HI fragment in a pBR322 backbone [see Landy and Ross (1977) for *att* site co-ordinates] (Nunes-Duby *et al.*, 1987). The P and P′ arms of pSN2 were replaced by non-*att* DNA and only the core region from −15 (*Xho*I) to +12 (*Eco*RI) was retained (see Table I). The P arm was replaced either by 'arm 3' or 'arm 5' sequences and the P′ arm was replaced by either 'arm 4' or 'arm 6' sequences as described previously (Franz and Landy, 1990a,b). In some constructions, the *Xho*I−*Eco*RI 'core cassette' was replaced by synthetic oligonucleotides with Δ or Δ′ sequences in place of the core-type Int binding sites C or C′, respectively. The names and characteristics of the resulting plasmids and sequences around the core are shown in Table I. Nucleotide sequences were confirmed by DNA sequencing using the Sequenase kit (United States Biochemicals). Plasmid purification, kinasing, ligations and transformations were performed as described in Sambrook *et al.* (1989).

Preparation of synthetic Holliday junctions

The HJs were made from polymerase chain reaction (PCR) fragments by modification of previously described methods (Hsu and Landy, 1984; Franz and Landy, 1990a,b). PCR with the appropriate templates and primers (Table I) was used to generate the following fragments: arms 3−4 (313

Table I. Clones and core sequences used to make Holliday junctions

cores	pBF clones Arms						DNA sequences at core from −15 to +17
	3–4	3–6	5–4	5–6	3–P′	5–P′	
COC′	304	306	504	506	302		C′ T C G A G C A G C T T T T A T A C T A A G T T G G A A T T C *Xho*I *Bbv*I ↑ *Eco*RI
Δ9OC′	9-34	9-36	9-54	9-56			Δ9 C′ C T C G A G A C G T A G T T T T A T A C T A A G T T G G A A T T C ↑
COΔ′8	8-34	8-36	8-54	8-56	8-32		C Δ′8 C T C G A G C A G C T T T T A T A C T C G G A A C G A A T T C ↑ *Xmn*I
COΔ′11		11-36		11-56			C Δ′11 C T C G A G C A G C T T T T A T A C T G G T C A C G A A T T C ↑
Δ′9OΔ′8	17-34	17-36	17-54	17-56			Δ′9 Δ′8 C T C G A G A C G T A G T T T T A T A C T C G G A A C G A A T T C ↑ *Xmn*I
CDC′			7-54	7-56			C D C′ C T C G A G T C C A G C T T T T A T A C T A A G T T G G A A T T C *Ple*I ↑
CJC′				20-56	20-32	20-52	C J C′ C T C G A G C A G C T T T T A T **c** A C T A A G T T G G A A T T C ↑
CJΔ′8					32-32	32-52	C J Δ′8 C T C G A G C A G C T T T T A T **c** A C T C G G A A C G A A T T C ↑ *Xmn*I

A large number of the pBF clones have been described previously (Franz and Landy, 1990a,b), the remainder were constructed for this study. Each pBF clone has a specific core sequence and the indicated pair of arms. For example, pBF 9-34 has the Δ9 0 C′ core and arms 3 and 4. The nucleotide sequences (from −15 to +17) of the different core regions are shown (Landy and Ross, 1977): the 7 bp overlap sequences are in italics; non-wild-type overlap regions are labeled D or J, Int binding sites are underlined; the two cleavage sites of Int at the boundaries of the overlap region are shown as arrows; mutations within the Int binding sites are indicated as small letters; the mutation in the overlap region is shown as an outlined letter C at +2 of J type overlap; the D core has a 5 bp overlap region. Also shown are the relevant restriction sites.

Table II. PCR primers used to make Holliday junctions

Primer	5′ end co-ordinates[a]	Strand	Sequence
3	−129 of arm 3	top strand	GGACTCTATCGACATATGGC
4	+184 of arm 4	bottom strand	CTGCCACCATACCCACGCCG
5	−310 of arm 5	top strand	CGCAAAAAAGGGAATAAGGGC
6	+241 of arm 6	bottom strand	TCCGAATACCGCAAGC
P′I	+57 of P′ arm	bottom strand	ACCTGTTCGTTGCAACAAATTG
C′RI-6	+5 of wild-type core	top strand	TAAGTTGGAATTACCCGGGTCG
Δ′8RI-6	+5 of COΔ′8 core	top strand	TCGGAACGAATTCCC
XCO	+4 of wild-type core	bottom strand	GTATAAAAAAGCTGCTC
XΔ90	+4 of Δ90C′ core	bottom strand	GTATAAAACTACGTCTCGAG

[a]Relative to position 0 in overlap.
Co-ordinates and the designation of 'top' and 'bottom' strands are from Landy and Ross (1977).

bp); arms 3–6 (370 bp); arms 5–6 (551 bp); arms 5–4 (494 bp). For nicked HJs, two oligonucleotides were used to prime synthesis in opposite directions from the Int cleavage site of C′ (+4), so that two more fragments could be made to form the nick (see Figure 1B). Primers C′RI-6 or Δ′8RI-6 are for the top strand, from +5 toward the *Eco*RI site and into the arm beyond. Primers XCO or XΔ9O are for the bottom strand, from +4 through the overlap region and beyond the *Xho*I site. Both the C′RI-6 and Δ′8RI-6 primers were kinased at the 5′ end so that the nick in the resulting HJ has a 5′ phosphate (see Nunes-Duby *et al.*, 1987). The sequences, co-ordinates and locations of the primers are shown in Table II and Figure 2. In some constructions where the P′ arms are present, as in pBF 20-32, 20-52, 32-32 and 32-52, the P′I primer was used; this primer starts at +57 and does not include the arm binding sites of Int. The f(TSE) HJs were constructed with DNA from clones with wild-type overlap sequences and altered sequences of the J type (Table I).

PCR fragments were generated from 1 ng purified plasmid as template and 20 pmol of each primer using the GeneAmp Kit in a Perkin-Elmer DNA thermal cycler as recommended by Perkin-Elmer Cetus. Four PCR fragments were required to make each intact HJ, whereas five fragments were used to make a nicked HJ (Figure 1A). One of the primers was labeled at the 5′ end with [γ-^{32}P]ATP, to generate HJs radioactively labeled in one strand. The PCR fragments were used without purification. For intact HJs, 1 pmol of the radioactively labeled fragment and 4 pmol of each non-radioactive fragment were combined in 140 μl; for nicked HJs, 3 pmol of the four non-radioactive fragments were used. A 25 μl stock solution of NaOH and EDTA was added to 140 μl of DNA fragments to achieve a final solution of 0.2 M NaOH and 0.02 M EDTA. After denaturation at room temperature for 20 min, the DNA was neutralized by the addition of 8 μl of 5 M acetic acid and 27 μl of 50 mM Tris–HCl (pH 7.8); 10 mM EDTA. The sample was incubated at 65°C for 5 min and cooled to room temperature over ~4 h by turning off the water bath. The HJs were separated from other species by gel electrophoresis on 1.8% agarose and visualized by autoradiography. They were eluted from the gel and purified by Geneclean as recommended by Bio 101 (La Jolla, CA).

***In vitro* resolution and cleavage assays**

Resolution or cleavage reactions were carried out with 0.1 pmol of HJ DNA and 0.5 μg of sonicated salmon sperm DNA in 20 μl of 50 mM Tris–HCl (pH 7.5), 10 mM EDTA, 75 mM NaCl, 10 mM DTE and 1 mg/ml bovine serum albumin (BSA). The reaction was initiated by the addition of purified Int, incubated at room temperature for the indicated times and stopped by the addition of loading dye which was 45 mM Tris–borate (pH 8.3), 2.5 mM EDTA, 1% SDS (w/v) and 10% Ficoll (w/v). The kinetic experiments were carried out at the optimal Int concentration, which was determined by titration. For SDS–KCl precipitation of covalent protein–DNA complexes, 0.1 vol of 10% SDS was added to the reaction which was then incubated for 5 min at 37°C. After the addition of 0.1 vol of concentrated KCl to 200 mM final, the reaction was chilled on ice for 10 min and then centrifuged (Trask *et al.*, 1984; Nunes-Duby *et al.*, 1987). The supernatant was loaded onto the gel with loading dye, as was the pellet after being resupended in 20 μl of 10 mM Tris–HCl (pH 7.5). All reactions were analyzed by electrophoresis on composite gels that were 1.2% agarose, 2% polyacrylamide, 0.028% bis, in a gel running buffer consisting of 45 mM Tris–borate (pH 8.3), 2.5 mM EDTA, 0.1% SDS (w/v). The agarose was melted with the running buffer before polyacrylamide and bis were added. SDS was added next, and only shortly before APS (0.25%) and TEMED (1.6 mM). Following electrophoresis at 250 V for ~2.5 h, the gel was dried. Autoradiography of the gels and quantitation by laser densitometry were carried out in the linear range of the film and densitometer as described previously (Thompson *et al.*, 1987).

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