The Mechanism of Phage λ Site-Specific Recombination: Site-Specific Breakage of DNA by Int Topoisomerase

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Summary

We demonstrate that the topoisomerase activity of bacteriophage λ Int protein introduces single-strand breaks into duplex DNA at specific sites. Strand breakage is accompanied by the covalent linkage of Int to DNA. The linkage connects a residue in Int to the 3' phosphate of DNA at the site of breakage; the other breakage product has a 5' OH terminus. Int is the first procaryotic topoisomerase shown to break DNA in this manner. We find that in att sites, Int breaks DNA within the 15 bp homologous core. These sites of Int topoisomerase action result from the interaction of Int with "junction-type" recognition sequences (CAACTTNNT), and Int topoisomerase acts between the 7th and 8th bases of this sequence. The sites of breakage within the cores of attP and attB coincide exactly with positions where breakage and reunion occur during Int-dependent recombination. These results indicate that Int topoisomerase executes strand exchange during recombination.

Introduction

The integration/excision cycle of phage λ is mediated by recombination between specific regions, called attachment (att) sites (for recent reviews, see Nash, 1981; Weisberg and Landy, 1983). Integration is accomplished by reciprocal recombination between attP, the specific site on the phage chromosome, and attB, the specific site on the bacterial chromosome. The integrated phage DNA, called the prophage, is bounded by the hybrid sites attL and attR. Excision occurs by reciprocal recombination between attL and attR, which regenerates attP and attB. The critical reaction in recombination is strand exchange; in this step the parental DNA strands are broken and then rejoined in novel combinations to produce recombinant molecules. Understanding the mechanism of strand exchange requires identification of the sites of breakage and reunion and the protein(s) that execute these reactions.

Where in *att* sites does breakage and reunion occur? Nucleotide sequence analysis (Landy and Ross, 1977) and subcloning studies (Hsu et al., 1980; Mizuuchi and Mizuuchi, 1980; Mizuuchi et al., 1981) have confirmed and extended earlier genetic studies (Gottesman and Weisberg, 1971) that showed that functional *att* sites are composed of the core, a region of homology shared by all *att* sites, and the arms, flanking sequences that extend beyond the core. Thus each *att* site can be written as a tripartite structure consisting of the core, 0, and two flanking arms: attP = POP', attB = BOB', attL = BOP', and attR = POB'. The initial sequence analysis of parental and prophage att sites showed that strand exchange occurs within the 15 bp core region (Landy and Ross, 1977). Recently, the point of strand exchange within the core has been more precisely mapped. Mizuuchi et al. (1981) determined the positions of breakage and reunion in the phosphodiester backbone during in vitro recombination. This analysis revealed that these positions are fixed in each strand of an *att* site but that the position of breakage and reunion in one strand is displaced from the position of breakage and reunion in the complementary strand.

Integration requires only two proteins: a phage-encoded protein, Int (Nash et al., 1977; Kikuchi and Nash, 1978), and a host-encoded protein, Integration Host Factor (IHF; Kikuchi and Nash, 1978; Miller et al., 1979; Nash and Robertson, 1981). Which of these executes the breakage and rejoining reactions of strand exchange? Three pieces of evidence suggest that Int is intimately involved in these reactions. First, DNA protection experiments have shown that Int can bind specifically to the cores of attP and attB-i.e., the sites of strand exchange (Ross et al., 1979; Ross and Landy, 1983). Second, Int has the capacity to break and rejoin DNA strands; it has topoisomerase activity (Kikuchi and Nash, 1979). Finally, Int has the capacity to resolve artificially constructed recombination intermediates (Hsu and Landy, cited in Nash, 1981 and Weisberg and Landy, 1983). It is appealing to suggest that the topoisomerase activity of Int executes strand exchange (Kikuchi and Nash, 1979) because the capacity of a topoisomerase to conserve the free energy of a phosphodiester bond during strand breakage could account for the observation that the breakage and reunion steps in recombination occur in the absence of a high-energy cofactor (Mizuuchi et al., 1978). However, it has been difficult to assign unambiguously the execution of strand exchange to Int topoisomerase because of the apparent lack of specificity of this activity. Int topoisomerase relaxes plasmids that lack att sites as efficiently as it relaxes plasmids that contain att sites (Kikuchi and Nash, 1979). In this report, we demonstrate that Int topoisomerase breaks DNA at specific sequences. We find that Int topoisomerase acts within the cores of attP and attB and that these sites of Int topoisomerase breakage are at precisely those positions where strand exchange occurs. This strongly implies that Int topoisomerase executes the breakage and rejoining reactions that promote strand exchange during recombination.

Results

Int Breaks DNA at Specific Sites

The ability of Int to break DNA at specific sites was detected by incubating uniquely end-labeled DNA fragments with Int and examining the products by gel electro-phoresis. In the experiment shown in Figure 1, an *att*B-



Figure 1. Specific Breakage of a DNA Fragment Containing *att*B by Int The *att*B-containing restriction fragment Bcl I (-138) to Hinc II (~+385) from pBB'105 was 5'-end labeled at the Bcl I site with ³²P. In agreement with the convention shown in Figure 5, the strand labeled by this procedure is called the top strand of the *att* site. Lane 1 contains the products of an A+G DNA sequencing reaction of this fragment. The nucleotide sequence of this DNA is presented in Landy and Ross (1977) and a portion of the sequence is also shown in Figure 5. The sequence is numbered with 0 as the central base of the 15 bp common core. The fragment was incubated with varying concentrations of Int for 60 min, treated with proteinase K for 45 min, and processed as described in Experimental Procedures. Lane 2 = 0 Int; lane 3 = 2.6 µg/ml Int; lane 4 = 0.9 µg/ml Int; lane 5 = 0.3 µg/ml Int; lane 6 = 0.09 µg/ml Int; and lane 7 = 0.03 µg/ml Int. The arrow indicates the expected mobility for a DNA fragment made by cleavage between nucleotides -3 and -2.

containing fragment labeled at one 5' end was incubated in the presence (lanes 3–7) and absence (lane 2) of Int, treated with proteinase (the requirement for proteinase digestion is considered in detail below), phenol-extracted, and electrophoresed through a denaturing polyacrylamide gel. Incubation with Int results in the appearance of a single new fragment, indicating that Int can break this DNA. Because the substrate fragment is labeled at its 5' end, the detected fragment is 5' to the site of breakage; such a 5' breakage product has a new 3' terminus. The ability of Int to break DNA is an intrinsic activity of Int; it does not require any other components of Int-dependent recombination such as IHF or a partner *att* site.

Int-dependent DNA breakage is limited at low Int concentration (Figure 1, lane 7) and increases to a maximum as the concentration of Int is increased (Figure 1, lanes 5 and 6); further increase in Int concentration does not increase the amount of DNA broken. The presence of IHF at saturating concentrations of Int does not significantly alter the amount of Int-dependent breakage observed (data not shown). The amount of Int-dependent breakage observed is small; we estimate that at any time less than 1% of the substrate DNA is broken by Int. One explanation for the apparent inefficiency of the breakage reaction is that the breakage products we detect reflect only the steadystate population of intermediates in breakage and reunion reactions (see below).

Int-Dependent Breakage Results in the Covalent Linkage of Int to DNA through a 3' Phosphate Linkage

When 5' Int-dependent breakage products are examined as in the experiment of Figure 1, appearance of the breakage products requires treatment of the incubation mixture with proteinase before phenol extraction. Although SDS is also included in the proteinase digestion mixture, its presence is not essential but leads to a several-fold increase in yield (data not shown). Furthermore, when proteinase treatment is omitted, treatment of the incubation mixture with heat (boiling for 2 min) or alkali (NaOH to a final concentration of 0.1 N) prior to phenol extraction does not produce detectable 5' breakage products (data not shown). We have considered two explanations for these results. One possibility is that proteolytic digestion of Int triggers DNA breakage. A second possibility, for which we provide evidence below, is that the 5' breakage products are attached to Int at their 3' termini. The linkage of protein to the broken DNA might cause these fragments to partition into the phenol phase (Langeveld et al., 1978; Prell and Vosberg, 1980) and thereby not be recovered with the aqueous phase during workup of the sample. Even if such a protein-DNA complex were recovered, it might fail to enter the gel or fail to form a band of unique mobility. In either case, proteinase digestion would liberate the DNA fragment and permit its detection.

The experiment shown in Figure 2 provides support for the idea that Int is linked to the 3' terminus of the 5' breakage product. In this experiment, DNA that had been incubated with Int was treated with different amounts of proteinase before phenol extraction. As the proteinase concentration is decreased, a single 5' breakage product is replaced by another, slower one. Thus the mobility of a fragment produced by Int breakage depends on the degree of digestion of the fragment with proteinase. This is not the result expected if proteolytic digestion were required only to trigger Int-dependent breakage. In that case, limiting the degree of digestion should simply reduce the amount of the breakage product detected and should not alter its mobility. Instead, we interpret the change in mobility to mean that protein is attached to the DNA fragment and that higher proteinase concentrations digest away more of this attached protein, permitting the DNA fragment to migrate more rapidly through acrylamide. Because our



Figure 2. Digestion of a 5' Int-Dependent Breakage Product with Varying Concentrations of Proteinase K

The same end-labeled DNA fragment described in Figure 1 was used. Lane 1 contains the products of a T+C DNA sequencing reaction of this fragment and the sequence is numbered as described in Figure 1. The fragment was incubated with 2.3 µg/ml Int for 60 min, the incubation mixture made 0.2% (w/v) in SDS, treated with varying concentrations of proteinase K for 15 min, and then processed as described in Experimental Procedures. Lane 2 = 93 µg/ml proteinase K; lane 3 = 39 µg/ml proteinase K; lane 4 = 19 µg/ml proteinase K; lane 5 = 3.7 µg/ml proteinase K; and lane 6 = 0.7 µg/ml proteinase K. As reported in the text, no cleaved fragment was seen when proteinase K was omitted. The arrow indicates the expected mobility for a DNA fragment made by cleavage between nucleotides -3 and -2.

experiments are performed with highly purified Int protein, we believe the attached protein must be Int. Since we demonstrate below that Int-dependent breakage creates a new 5' OH terminus, it is simplest to suggest that Int is linked to DNA through the 3' phosphate at the site of breakage.

It appears that even our most severe proteinase treatment leaves some residues of Int attached to the DNA fragment. As discussed below, the phosphodiester bond that is broken by Int can be precisely determined by analysis of 3' breakage products. Therefore, it is possible to predict the expected size of the 5' breakage product (for the substrate fragment used in Figures 1 and 2, this position is marked by an arrow). Even at the highest proteinase concentrations, the 5' Int-dependent breakage



Figure 3. Identification of a Phosphodiester Bond Broken by Int

The 3' end of the bottom strand of the *attB*-containing fragment Bam HI (-17) to Eco RI (+26) from pMM290 was labeled with ³²P at the Bam HI site. Lanes 2 and 6 contain the products of a T+C DNA sequencing reaction of the fragment, and lanes 3 and 5 contain the products of an A+G DNA sequencing reaction of the fragment. The nucleotide sequence is numbered as in Figure 1 and the sequence for a portion of this fragment is presented in Figure 5. The fragment was incubated with or without 2.0 μ g/ml Int for 60 min, the incubation mixture treated with proteinase K for 20 min, and processed as described in Experimental Procedures. Lane 1 = -Int.

product observed (lane 2) migrates more slowly than expected.

Int-Dependent Breakage of DNA Produces 5'OH Termini

When a DNA fragment labeled at its 3' end is used as the substrate for Int-dependent breakage, the broken fragment that is detected is 3' to the site of breakage and has a new 5' terminus. In the experiment of Figure 3, a fragment containing *attB* was labeled at the 3' end of its bottom strand; incubation with Int results in the appearance of a new fragment (lanes 4 and 7). This fragment migrates through a denaturing polyacrylamide gel to a position midway between two fragments produced by Maxam-Gilbert sequencing of the substrate DNA fragment (lanes 2, 3, 5, and 6). The products of Maxam-Gilbert sequencing reactions have 5' phosphate termini (Maxam and Gilbert, 1980). A DNA fragment with a 5' OH terminus is expected to migrate more slowly than a phosphate-terminated fragment of the same length and faster than a phosphate-



Figure 4. Int-Dependent Breakage Produces a New 5' OH Terminus

The same end-labeled fragment described in Figure 3 was used. Lanes 7, 11, and 13 contain the products of an A+G DNA sequencing reaction of the fragment and lanes 8, 10, and 14 contain the products of a T+C DNA sequencing reaction. The nucleotide sequence is numbered as in Figure 1 and the sequence for a portion of this fragment is also shown in Figure 5. The fragment was incubated with or without Int at 2.0 µg/ml Int for 60 min, the incubation mixtures treated with proteinase K for 20 min and then processed by phenol extraction and ethanol precipitation as described in Experimental Procedures. The resulting pellets were then treated with polynucleotide kinase and/ or calf intestine alkaline phosphatase as described in Experimental Procedures. Lane 1 = -Int; lane 2 = +Int; lane 3 = +Int and +kinase; lane 4 = +Int, +kinase, and +phosphatase; lane 5 = +Intand +phosphatase; lane 6 = + int and +kinase; lane 9 = -Int; lanes 12 and 15 = +Int and +kinase.

terminated fragment that is a single nucleotide larger (Ross et al., 1979). That Int breakage produces a 5' OH terminus is proven directly in the experiment of Figure 4. Here the mobility of a 3' Int-dependent breakage product is shown to be altered by treatment with polynucleotide kinase and ATP so that it migrates faster than does the untreated breakage product (lanes 2 and 3), comigrating with products of Maxam-Gilbert sequencing reactions (lanes 11-13). The amount and direction of the shift in mobility are exactly as expected if polynucleotide kinase added a phosphate group to a 5' OH terminus introduced by Int. Two other pieces of evidence support the conclusion that the 3' Int breakage product has a 5' OH terminus: treatment of a 3' breakage product with phosphatase does not alter its mobility (lane 5); and the phosphate added to the 5' OH terminus of a 3' breakage product by incubation with polynucleotide kinase and ATP can be removed by subsequent treatment with phosphatase (lane 4).

Is the new 5' terminus produced by Int breakage attached to protein? The 3' cleavage product that contains the 5' terminus is detected at similar yields when either proteinase, alkali, or heat treatment is applied after incubation with Int and prior to phenol extraction (data not shown). The contrast with the 5' breakage product, for which heat and alkali are ineffective agents for breaking the covalent bond between protein and DNA (see above) suggests that the 3' side of the Int-dependent break in DNA is not covalently attached to protein. However, we cannot rule out the possibility that Int is covalently linked not only to the 3' terminus but also the new 5' terminus and that the latter bond is alkali- and heat-labile.

Int Breaks DNA within the Cores of attP and attB

Comparison of the mobility of a 3' Int breakage product with the mobility of the products of Maxam-Gilbert sequencing reactions of the substrate fragment allows identification of the phosphodiester bond broken by Int. In the experiment of Figure 3, the 3' breakage product of the bottom strand of an attB-containing fragment (lanes 4 and 7) migrates between the sequencing reaction products of the substrate DNA designated +5 and +6 (lanes 2, 3, 5, and 6). (These numbers refer to nucleotide positions within the att core-see Figures 1 and 5). If the 5' terminus of the 3' breakage product were phosphorylated (as in the experiment of Figure 4), it would comigrate with the fragment designated +5 in the sequencing ladder. This fragment has the sequence (3')....pTpApTpGp (5') (Landy and Ross, 1977; Maxam and Gilbert, 1980). Therefore, Int must break the bottom strand of the core of attB by cleavage of the phosphodiester bond between nucleotides +4 and +5.

By methods exactly like those shown in Figure 3, we have examined the ability of Int to break the top and bottom strands of *att*P and *att*B: Int can break each of these strands. Int-dependent breakage occurs at a single site in each strand and this site lies within the core. Int-dependent breakage occurs at identical positions in the cores of *att*P and *att*B. The arrows in Figure 5 indicate which phosphodiester bonds in the core are broken by Int. The top strand is broken between nucleotides -2 and -3, whereas the bottom strand is broken between nucleotides +4 and +5 so that the breakage event results in covalent



Figure 5. The *att* Core: Phosphodiester Bonds Broken by Int Topoisomerase, Int Junction-Type Recognition Sequences and the Positions of Strand Exchange

The nucleotide sequence of the *att* core is shown and is numbered as described in Figure 1 (Landy and Ross, 1977). The arrows indicate the phosphodiester bonds broken by Int topoisomerase. The sequences in lower case letters at the upper left and lower right are Int junction-type recognition sequences (Ross and Landy, 1983). Filled circles indicate phosphorus atoms in a recombinant *att* site that are derived from one parent and open circles indicate phosphorus atoms derived from the other parent (Mizuuchi et al., 1981). Lettering of differing intensity is used to distinguish bases derived from each parent when a 7 bp heteroduplex region is formed in a recombinant *att* site (Mizuuchi et al., 1981; Nash, 1981; Weisberg and Landy, 1983).

linkage of Int to the 5' breakage product through a 3' phosphate bond, the 3' phosphate donor in the top strand is a T (at position -3) whereas the donor in the bottom strand is an A (at position +5).

Int Introduces Single-Strand Breaks into Duplex DNA

Because of the use of denaturing polyacrylamide gels, the experiments described so far do not determine whether Int promotes breakage of each strand of the att core independently or whether both strands in a single DNA duplex are broken simultaneously. The experiment of Figure 6 addresses this question. Figure 6A shows an analysis of the products by electrophoresis through a denaturing polyacrylamide gel; Int breaks a small amount of the substrate DNA in the top strand of attP. Figure 6B shows an analysis of the same reaction mixtures by electrophoresis through a nondenaturing polyacrylamide gel. If Int introduces double-strand breaks, the broken pieces would be held together by a 7 bp duplex. Since such a short ATrich duplex is not expected to be stable under our conditions (Wallace et al., 1979), Int-dependent breakage should yield a new, shorter labeled fragment. No Int-dependent breakage product is detected in Figure 6B. We conclude that, unless the remnant of Int left after proteolysis holds the duplex together, Int cleaves only one strand of a DNA duplex at a time.

The experiment of Figure 6 also examines the time course of Int-dependent DNA breakage. Int-dependent breakage is detected in the first time point taken, after 15 min of incubation (6A, lanes 3 and 6), but increasing the time of incubation to 240 min (lane 5) does not lead to an increase in the amount of Int-dependent breakage product observed.

Discussion

We have found that Int breaks DNA at specific sites. We believe that this breakage reflects the action of Int topoisomerase because Int breakage is accompanied by covalent linkage of Int to DNA. Formation of a covalent link between protein and DNA is a characteristic feature of the action of topoisomerases (Wang and Liu, 1979; Gellert, 1981). Such a linkage preserves the bond energy of the DNA backbone so that rejoining of the broken DNA strands can occur in the absence of an external energy source. When Intdependent breakage occurs. Int becomes linked to the strand that is 5' to the breakage site through a 3' phosphate bond; the other product DNA strand has a new 5' OH terminus. In this regard, Int is unlike all previously studied procaryotic topoisomerases, which link protein to a 5' DNA phosphate (Gellert, 1981). However, the linkage observed for Int is identical to that seen with several eucaryotic topoisomerases-e.g., rat liver nicking-closing enzyme (Champoux, 1977a) and HeLa cell topoisomerase I (Edwards et al., 1982). Other biochemical similarities between the topoisomerase activity of Int and these eucaryotic Type I topoisomerases have been discussed elsewhere (Kikuchi and Nash, 1979), and it has been suggested (Kikuchi and Nash, 1979; Been and Champoux, 1981; Halligan et al., 1982) that the eucaryotic enzymes may have recombination functions.

Two other pieces of evidence support the hypothesis that site-specific DNA breakage by Int reflects the topoisomerase activity of this protein. First, the specific breaks introduced by Int appear to affect only one strand of the duplex. This is consistent with the observation that the topoisomerase activity of Int relaxes circles of duplex DNA by transiently breaking one strand of a DNA duplex (Nash et al., 1981). Second, the amount of Int-dependent breakage product detected does not increase as the time of incubation is increased, but quickly reaches a constant value (Figure 6). This is the behavior observed for cleavage of DNA by other topoisomerases (Champoux, 1976) and presumably reflects the capacity of a topoisomerase to continuously break and reseal DNA, generating a steadystate population of broken intermediates. Another indication for equating cleavage with a topoisomerase intermediate, demonstration of the reversible nature of the cleavage, has not yet been observed.

Int Topoisomerase Acts at a Specific Nucleotide Sequence

Int is a specific DNA-binding protein that can recognize two different nucleotide sequences (Ross and Landy, 1982, 1983). Int binds to several sites in the arms of *att*P (Ross et al., 1979; Hsu et al., 1980); this binding is directed by the "arm-type" Int recognition sequence (the minimal consensus sequence for arm-type Int binding is $^{C}_{A}AGTCAC-$ TAT; Ross and Landy, 1982). We have not detected any Int-dependent breakage at these Int arm-binding sites or at any other position in the arms of *att*P. Thus the arm-



Figure 6. Int-Dependent Breakage Introduces Single-Strand Breaks into DNA

The 3' end of the top strand of the *att*P containing fragment Hind III (-160) to Bam HI (+242) from pPH54 was labeled at the Bam HI end with ³²P. Digestion of this fragment with Dde I and MnI I produces the fragments labeled +5 and +123. A fragment produced by an Int-dependent break within the core would have a mobility similar to that of the +5 fragment. The parental DNA was incubated with varying amounts of Int for varying times, the incubation mixtures treated with proteinase K for 20 min and then processed as described in Experimental Procedures. Lane 1 = -Int, no incubation; lane 2 = -Int, 240 min incubation; lane $3 = 2 \mu g/mI$ Int, 15 min; lane $4 = 2 \mu g/mI$ Int, 60 min; lane $5 = 2.0 \mu g/mI$ Int, 240 min; and lane $6 = 0.3 \mu g/mI$ Int, 15 min; lane 7 = products of an A+G DNA sequencing reaction of the fragment; lane 8 = products of a T+C DNA sequencing reaction of the fragment. (A) electrophoresis through a nondenaturing polyacrylamide gel.

type sequence directs Int to bind to specific sites on DNA but does not provoke the action of Int topoisomerase. Int also binds to the core regions in *att*P and *att*B (Ross et al., 1979; Ross and Landy, 1983). This binding is directed by the "junction-type" Int recognition sequence (the minimal consensus sequence for junction-type Int binding is CAACTTNNT; Ross and Landy, 1983). The cores of *att*P and *att*B each contain two copies of this sequence located in inverted orientation at the core–arm junctions (see Figure 5); thus, the core region of each *att* site contains two Int binding sites.

Figure 5 shows that the positions of Int topoisomerase action in the core are located within the junction-type Int recognition sequences. At both the right and left core-arm junctions, Int topoisomerase acts between the two unspecified nucleotides of the recognition sequence CAACTTNNT. This suggests that recognition of the junction-type sequence by Int promotes both the specific binding of Int and the action of Int topoisomerase at these sites. Because Int-dependent strand breakage occurs in the core of *att*B, which contains only junction-type recognition sequences, the presence of both junction-type and arm-type recognition sequences is not necessary for Int topoisomerase action.

The hypothesis that Int topoisomerase action is provoked by recognition of the junction-type consensus sequence offers a satisfactory explanation for a paradox that had been noted in earlier work. Kikuchi and Nash (1979) found that Int relaxed plasmids that contained 0, 1, or 2 att sites with the same efficiency. This apparent lack of specificity in the action of Int as a topoisomerase is in contrast with its specific action as a recombination enzyme (cf., Krasnow and Cozzarelli, 1983). However, this paradox is resolved if Int topoisomerase acts at isolated junctiontype sequences located in non-att DNA in addition to acting at the junction-type sequences in the cores of att sites. By means of DNA footprinting studies, it has been shown that Int will bind to relatively short and somewhat degenerate junction-type sequences that are commonly found in non-att DNA (Ross and Landy, 1983) and we have observed that site-specific Int-dependent breakage can occur at such sites (unpublished observations).

Although it is simplest to assume that Int binding to the left core-arm junction prompts topoisomerase action at the left side of the core (and similarly on the right) it is possible that Int bound to the left core-arm junction cuts the right junction and vice versa. This ambiguity has been resolved by examining the interaction of Int with single junction-type consensus sequences (Ross and Landy, 1983). Breakage occurs within the junction-type consensus sequence at single junction-type sequences in pBR322 (Craig and Nash, unpublished observations) or in a subcloned single core-arm junction of attP (Ross and Landy, personal communication). Thus recognition of the sequence CAACTTNNT by Int results in the specific binding of Int to this site and provokes the action of Int topoisomerase between the two unspecified bases; the 5' breakage product is (5')....CpApApCpTpTpNp-Int and the 3' breakage product is (5') HONpTp....(3').

Int Topoisomerase Executes Strand Exchange during Int-Dependent Recombination

Mizuuchi et al. (1981) have identified in vitro the positions of strand exchange by determining the parental origin of each phosphate in a recombinant att site. This analysis revealed that breakage and reunion occur at fixed positions within the core and that these positions in the top and bottom strands are staggered so that the recombinant core contains a region of heteroduplex (see Figure 5). The heteroduplex region must be either 5 bp in length (if breakage occurs between nucleotides -2 and -1 in the top strand and nucleotides +3 and +4 in the bottom strand) or 7 bp in length (if breakage and reunion occur between nucleotides -3 and -2 in the top strand and nucleotides +4 and +5 in the bottom strand). A choice between these alternatives could not be made from the Mizuuchi experiment because only the distribution of the phosphates and not of the bases was determined. However, a 7 bp overlap can better account for the nucleotide sequences of the products of integrations into sites other than attB (Mizuuchi et al., 1981; Nash, 1981; Weisberg and Landy, 1983). Figure 5 shows that the positions of Int topoisomerase action in the core lie at one of the two possible points of strand exchange identified by the Mizuuchi experiment; the phosphodiester bonds broken by Int topoisomerase are those that define a 7 bp overlap. The coincidence of the sites of Int topoisomerase action and the sites of strand exchange makes it very likely that Int topoisomerase executes the breakage and rejoining reactions that result in strand exchange during Int-dependent site-specific recombination. It should be pointed out that strand breakage by Int simply identifies the catalytic site involved in recombination. One should be cautious in assuming that details of the topoisomerase cleavage (e.g., cleavage of each strand of att independently) will be observed in the complete recombination reaction.

The identification of Int topoisomerase as the activity that executes strand exchange defines at least two roles for Int in recombination (other roles for Int, dependent on the interaction of Int with the arms of att sites, remain to be defined): Int provides the catalytic site for strand breakage and reunion, and it provides the specificity for strand exchange. The sequence-specific action of Int topoisomerase defines where strand exchange will occur and ensures that corresponding positions in the parental DNAs are broken so that rejoining, and hence recombination, occurs conservatively at fixed internucleotide junctions without nucleotide loss. The mechanism by which strand exchange is accomplished by specific breakage and rejoining of DNA is unknown. Several models have been presented (Kikuchi and Nash, 1979; lkeda et al., 1981; Sherratt et al., 1981). For λ site-specific recombination, the determination of the change in linking number that accompanies strand exchange has provided quantitative support for the Kikuchi-Nash model (Nash and Pollock, 1983).

An important role for topoisomerase activity in other recombination reactions has also been suggested. Topoisomerases may facilitate the pairing of double helices in homologous recombination by overcoming the topological difficulties in such a reaction through transient strand breakage (Champoux, 1977b; Cunningham et al., 1981). Furthermore, site-specific topoisomerases may also mediate strand exchange in other recombination reactions (Reed and Grindley, 1981; Krasnow and Cozzarelli, 1983; Abremski et al., 1983).

Experimental Procedures

Preparation of Plasmid DNA and Int Protein

Plasmid-containing derivatives of E. coli strains 204 (Kikuchi and Nash, 1978) or C600 dam-4, hsd-s, (from J. Hays) were grown and supertwisted DNA was prepared from them as previously described (Nash and Robertson, 1981). The plasmids used were: (1) pBB' 105 in which an Eco RI–Bam HI att B-containing fragment (~-800 to ~+800) replaces the Eco RI–Bam HI fragment of pBR322 (Kikuchi and Nash, 1979); (2) pMM290 in which an attB-containing fragment (-17 to +26) derivatized with an Eco RI linker at +26 and a Bam HI inker at -17 replaces the Eco RI–Bam HI fragment of pBR322 (Mizuuchi et al., 1981); (3) pPH54 in which an attP-containing fragment (-160 to +242) derivatized with a Hind III linker at -160 replaces the Hind III–Bam HI fragment of pBR322 (Hsu et al., 1980). Int protein was prepared as previously described (Kikuchi and Nash, 1978; Nash, 1983).

Preparation of End-Labeled DNA Fragments

³²P-end-labeled restriction fragments were prepared from supertwisted plasmid DNA according to the procedures of Maxam and Gilbert (1980) with modifications as indicated. Supertwisted plasmid DNA was first digested with a restriction enzyme, thus generating the end to be labeled. Restriction enzymes were obtained from Bethesda Research Laboratories or New England Biolabs. After removal of endogenous terminal phosphates with calf intestine alkaline phosphatase (Boehringer Mannheim), the digest was 5' end-labeled with ³²P-γ-ATP (New England Nuclear) using polynucleotide kinase (P-L Biochemicals). Alternatively, the digest was 3' end-labeled with ³²P-α-dCTP or ³²P-α-dATP (New England Nuclear) using the Klenow fragment of E. coli DNA polymerase I (Boehringer Mannheim). A second restriction digestion was performed and the fragments separated on 6% or 7.5% polyacrylamide gels. Fragments were eluted from the gel (without the addition of tRNA to Maxam-Gilbert extraction buffer) and concentrated by ethanol precipitation.

Int-Dependent DNA Breakage

Approximately 0.01 to 0.10 pmole of uniquely end-labeled restriction fragment was incubated with purified Int protein in 100 μ I of breakage buffer (52 mM Tris-HCI [pH 7.4], 70 mM KCI, 10% [v/v] glycerol, 1.1 mM EDTA,

1 [or sometimes 10] mM β -mercaptoethanol, and 200 μ g/ml bovine serum albumin [Pentex]). Immediately before use, dilutions of Int protein were prepared in 50 mM Tris-HCl (pH 7.4), 10% (v/v) glycerol, 1 mM EDTA, 600 mM KCl, and 2 mg/ml bovine serum albumin. The breakage reactions were incubated at 25°C; then 2 µl of 10% (w/v) SDS and 5 µl of 2 mg/ml proteinase K (EM Reagents) were added to each sample and the samples were incubated at 37°C. The samples were then put on ice and 100 µl of stop buffer (0.6 M NH₄Ac, 0.1 M EDTA, and 60 µg/ml sonicated calf thymus DNA) was added. The samples were then extracted with phenol/chloroform/isoamyl alcohol (1:1:0.04) that had been saturated just before use with 50 mM Tris-HCI (pH 8.0)-1.0 mM EDTA; subsequent extractions were performed with chloroform and then ether. DNA was then precipitated from the sample by the addition of 2.5 vol ethanol; the resulting pellet was resuspended with 0.3 M NaAc and reprecipitated by the addition of 2.5 vol ethanol. The resulting pellet was washed with ethanol and then dried under vacuum.

Kinase and Phosphatase Treatment of Int-Dependent DNA Breakage Products

The dried pellets from Int-dependent DNA breakage reactions were resuspended with 35 μ l of denaturation buffer (20 mM Tris-HCl [pH 9.5], 1 mM spermidine, and 0.1 mM EDTA), incubated at 90°C for 2 min, and then chilled in ice water. To each sample were added 10 μ l of kinase buffer (250 mM Tris-HCl [pH 9.5], 50 mM MgCl₂, 25 mM DTT and 25% [v/v] glycerol) and 5 μ l of 25 μ M ATP. Polynucleotide kinase (P-L Biochemicals) or calf intestine alkaline phosphatase (Boehringer Mannheim) was then added as indicated in the figure legends, and the samples were incubated at 37°C for 15 min and then at 70°C for 10 min. When phosphatase treatment followed kinase treatment, calf intestine alkaline phosphatase was added to the sample after the incubation at 70°C and the samples were then brought to 200 μ l by the addition of 10 mM Tris-HCl (pH 8.0)—0.5 mM EDTA and NH₄Ac was added to 0.3 M; the samples were then phenol-extracted and concentrated by ethanol precipitation as described above.

Preparation of DNA Sequence Markers and Electrophoresis Conditions

A+G and T+C DNA sequencing reactions were carried out as described by Maxam and Gilbert (1980). Hydrazine was used to generate fragments broken at T or C residues and pyridinium formate was used to generate fragments broken at A or G residues.

Electrophoretic analysis under denaturing conditions was carried out by resuspending the products of DNA sequencing reactions or the dried pellets from Int-dependent-breakage reactions with 80% (ν/ν) formamide, 50 mM Tris, 50 mM borate, 1.4 mM Na₂ EDTA, 0.1% (w/ν) xylene cyanol, and 0.1% (w/ν) bromphenol blue. Following denaturation by incubation at 90°C followed by chilling in ice water, the samples were electrophoresed through 6%, 8%, or 15% (w/ν) acrylamide (1:20 cross linked)—8.3 M urea gels. Electrophoretic analysis under nondenaturing conditions was carried out by resuspending the dried pellets from Int-dependent breakage reactions with 8.3 mM Tris-HCl (pH 8.0), 0.4 mM Na₂ EDTA, 4.2% (w/ν) Ficoll, 0.3% (w/ν) SDS, and 0.02% (w/ν) bromphenol blue, and electrophoresis through 7.5% (w/ν) acrylamide (1:20 cross linked) gels. The electrophoresis buffer was 89 mM Tris, 89 mM borate, and 2.5 mM Na₂ EDTA. After electrophoresis, the gels were exposed to Kodak XAR-2 film at -70° C with intensifying screens.

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