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Resolution of Holliday junctions in genetic recombination: RuvC protein nicks DNA at the point of strand exchange

(DNA repair/resolvase/homologous recombination/methyl phosphonate/nuclease)

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ABSTRACT **The RuvC protein of *Escherichia coli* catalyzes the resolution of recombination intermediates during genetic recombination and the recombinational repair of damaged DNA. Resolution involves specific recognition of the Holliday structure to form a complex that exhibits twofold symmetry with the DNA in an open configuration. Cleavage occurs when strands of like polarity are nicked at the sequence 5′-WTT↓S-3′ (where W is A or T and S is G or C). To determine whether the cleavage site needs to be located at, or close to, the point at which DNA strands exchange partners, Holliday structures were constructed with the junction points at defined sites within this sequence. We found that the efficiency of resolution was optimal when the cleavage site was coincident with the position of DNA strand exchange. In these studies, junction targeting was achieved by incorporating uncharged methyl phosphonates into the DNA backbone, providing further evidence for the importance of charge–charge repulsions in determining DNA structure.**

During genetic recombination and the recombinational repair of DNA damage in *Escherichia coli*, Holliday junction resolution is catalyzed by RuvC protein, a junction-specific endonuclease (1–3). The interaction of RuvC with Holliday junctions involves both structure- and sequence-specific contacts. Binding results in the formation of a twofold symmetric RuvC–junction complex in which the DNA lies in an open configuration (4). Resolution requires divalent metal ions and occurs by a dual incision mechanism (1, 2, 5) such that symmetrically related nicks are introduced into strands of like polarity at the sequence 5′-WTT↓S-3′ (where W is A or T and S is G or C) (6). Incisions occur in the two strands that form the wide angles in the twofold symmetric protein–DNA complex (4, 7). RuvC has been crystallized and acts as a dimer of two 19-kDa subunits (8).

In this work, we have investigated the location of the cleavage site relative to the site at which DNA strands pass from one duplex to the other. To achieve this, it was necessary to prepare Holliday junctions in which the exchange point was directed to different positions within a region of homology.

Recent studies show that the conformation of a DNA helix is driven by a requirement to minimize electrostatic repulsions between negatively charged phosphates in the DNA backbone (9, 10). Charge–charge interactions also play an important role in the structure of a Holliday junction. In the presence of metal ions, the four DNA helices adopt a two-fold symmetric stacked X structure (Fig. 1*A*) in which the exchanging strands fold back on themselves as they cross between the helical stacks, while the continuous strands exhibit an unbroken helical axis (12). The pair of exchanging strands, therefore, lie in close proximity at the point of strand exchange and experience strong phosphate–phosphate repulsions (Fig. 1*B*). Using immobile Holliday junctions, Duckett *et al.* (13) showed that replacement of two opposing phosphate groups at the crossover with (uncharged) methyl phosphonates (Fig. 1*C*) directed the isomeric form of the junction, as the neutral groups preferentially adopted positions on the exchanging, rather than the continuous, pair of strands. Since the phosphates in the exchanging strands approach closest and are energetically most unfavorable directly at the point of strand exchange (Fig. 1*B*), we reasoned that phosphate neutralization might also localize a Holliday junction within a region of homology. Using this technique, migrational isomers were prepared with the junction point positioned at different sites. Chemical probes indicated that junction position was maintained upon formation of the RuvC–junction complex, allowing us to determine the effect of junction location on resolution efficiency.

MATERIALS AND METHODS

Proteins and DNA. Synthetic Holliday junctions containing 4 bp of homology (X4 and its derivatives) were prepared by annealing four 41-nt oligonucleotides, as described (4). Junction X4 was made from oligonucleotide 1 (5′-GCCGTGATCACCAATGCAGATTGACGAACCTTTGCCCACGT-3′), oligonucleotide 2 (5′-GACGTGGGCAAAGGTTCGTCAATGGACTGACAGCTGCATGG-3′), oligonucleotide 3 (5′-GCCATGCAGCTGTCAGTCCATTGTCATGCTAGGCCTACTGC-3′), and oligonucleotide 4 (5′-GGCAGTAGGCCTAGCATGACAATCTGCATTGGTGATCACGG-3′). Oligonucleotides were synthesized on an Applied Biosystems model 394 machine, deprotected, purified by denaturing PAGE, eluted, and recovered using SEP-PAC C_{18} cartridges (Millipore). Methyl phosphonate substitutions were synthesized using methyl phosphoramidites (Glen Research, Sterling, VA). Strand 1 of each junction was 5′-^{32}P-labeled prior to annealing, using T4 polynucleotide kinase (Pharmacia) and [γ-^{32}P]ATP (Amersham). Annealed DNAs were purified by gel electrophoresis and their concentrations were determined by calculation of specific activity using the DE81-filter-binding method (4).

RuvC and T7 endonuclease I were purified to homogeneity as described (14, 15).

Hydroxyl Radical Analyses. ^{32}P-labeled Holliday junctions (4 ng) were incubated with RuvC in 50 mM Tris·HCl, pH 8.0/0.5 mM $MgCl_2$/1 mM dithiothreitol/bovine serum albumin (100 μg/ml). After 15 min at room temperature, reaction mixtures were treated with hydroxyl radicals and processed as described (5). ^{32}P-labeled products were analyzed on 12% denaturing polyacrylamide gels.

Resolution Assays. Reactions (20 μl) contained ^{32}P-labeled DNA (3 ng) and RuvC or T7 endonuclease I in cleavage buffer [50 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/1 mM dithiothreitol/bovine serum albumin (100 μg/ml)]. Incubation was at 37°C for 5–30 min. For kinetic analyses, ^{32}P-labeled DNA was

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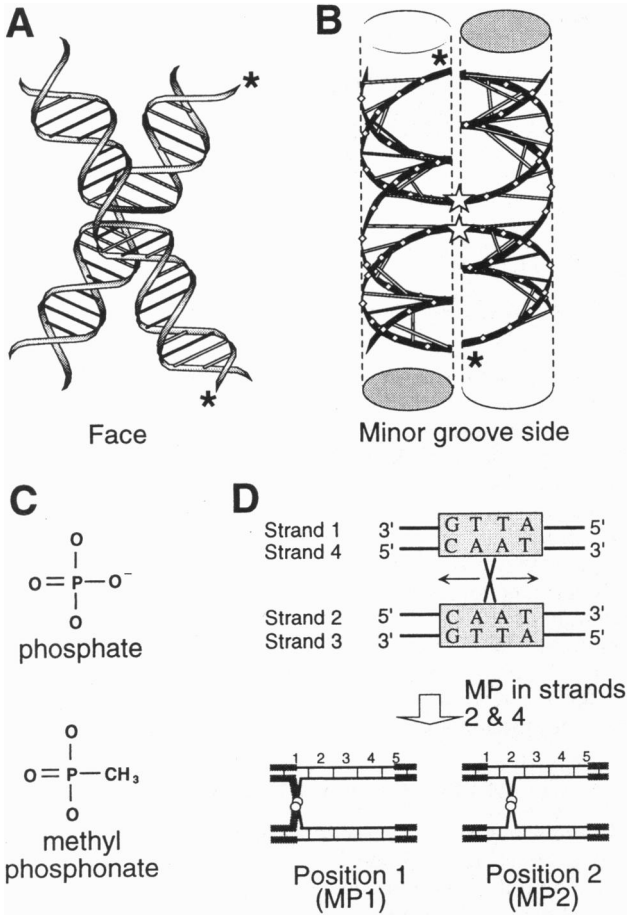

FIG. 1. Rationale for crossover targeting. Ribbon model of the right-handed antiparallel-stacked X structure of the Holliday junction showing face view (*A*) and the view from the minor groove side (*B*). The two continuous DNA strands approximate B-form DNA. The exchanging strands form an acute angle (≈60°) at the point of exchange between interacting helices (5′ ends marked by asterisks). The phosphates in the DNA strands are shown in *B*. Close approach of the exchanging strands suggests that these will experience unfavorable electrostatic repulsions between the phosphates at the point of strand exchange (indicated by stars). [Drawings adapted from Bhattacharyya *et al.* (11).] (*C*) Comparison of negatively charged phosphates and methyl phosphonate analogs. (*D*) Synthetic Holliday junctions were constructed with a 4-bp homologous core flanked by 18-bp heterologous arms (junction X4). The crossover can migrate (as indicated by arrows) within the region of homology. Methyl phosphonate-substituted junctions were constructed with substitutions at positions 1, 2, 3, 4, or 5 (junctions MP1, MP2, MP3, MP4, and MP5, respectively) in strands 2 and 4 in an attempt to make the five possible migrational isomers.

preincubated with excess RuvC (300 ng) at 37°C for 5 min in cleavage buffer (60 μl) from which $MgCl_2$ was omitted. The cleavage reaction was then initiated by addition of $MgCl_2$ to 10 mM, and at the indicated times samples were taken and stopped by addition of excess EDTA. Products were analyzed by electrophoresis through 8% neutral or 12% denaturing gels essentially as described (5). ^{32}P-labeled DNA was detected by autoradiography or quantitated using a Molecular Dynamics model 425E PhosphorImager with IMAGEQUANT software.

RESULTS

Construction of Migrational Isomers. Synthetic Holliday junctions were prepared by annealing four oligonucleotides and contained a 4-bp region of homology containing the RuvC cleavage consensus sequence (junction X4; see Fig. 1*D*). The 4-bp homologous core translates into five possible junction positions or migrational isomers (Fig. 1*D*). Strands 2 and 4 of the junction were synthesized with methyl phosphonate linkages (MP) at each potential crossover position in turn (junctions MP1, MP2, MP3, MP4, and MP5), in an attempt to position the junction at the five substituted sites (see Fig. 1*D*). To determine whether inclusion of the methyl phosphonate purturbs the interaction of RuvC with the DNA, we compared the affinity of RuvC for each junction by using band shift assays (5). We found that junctions MP1–MP5 were bound with efficiencies similar to that observed with the unmodified junction (data not shown).

To determine whether the methyl phosphonates were successful in directing the position of the junction point and whether junction position was maintained upon formation of the protein–DNA complex, RuvC–junction complexes were probed with hydroxyl radicals. Previously, we showed that the binding of RuvC to substrates with fixed junction points resulted in an enhanced reactivity at sites located 1 or 2 nt to the 3′ side of the junction (7, 16). Hypersensitive sites therefore provide accurate markers for the position of the junction within the protein–DNA complex.

When the five methyl phosphonate-substituted junctions (MP1–MP5) and the unmodified junction (all 5′-^{32}P-end-labeled in strand 1) were treated with hydroxyl radicals in the presence of RuvC, specific DNA sites were found to be hypersensitive to radical attack (Fig. 2*A*, lanes c, f, i, l, o, and r). In the absence of RuvC, hypersensitivity was not observed (lanes a, d, g, j, m, and p). The distribution of hypersensitive sites differed between the substituted junctions, indicating that the position of the junction point was affected by the location of the methyl phosphonate groups (summarized in Fig. 2*B*). For three of the five substituted DNAs, MP1, MP2, and MP5, the junction point was situated directly at the position of methyl phosphonate substitution. For example, comparison of the pattern observed with junction MP2 indicated a shift by 1 bp to the 5′ side relative to that observed with MP1 (Fig. 2*A*, compare lanes f and i, and *B*, analyses *i* and *ii*). Similarly, the pattern on junction MP5 indicated that the junction was again positioned at the site of methyl phosphonate substitution (Fig. 2*A*, lane r, and *B*, analysis *v*). In contrast, substitutions made in junctions MP3 and MP4 failed to localize the crossover uniquely (Fig. 2*A*, lanes l and o, and *B*, analyses *iii* and *iv*). Indeed, MP4 appeared to consist of a mixed population of molecules with the junctions at positions 2 and 5.

When the above experiments were performed with junctions that were ^{32}P-labeled in strand 2, only a low level of hypersensitivity was observed. These results indicate that the methyl phosphonate groups were successful in directing isomeric form as well as junction position (data not shown).

These results show that neutralization of charge–charge repulsions can direct the location of a Holliday junction within a region of homology and that the crossover position is maintained upon formation of the RuvC–junction complex—even though the DNA unfolds upon RuvC binding. However, not all attempts to localize the junction point to specific sites were successful (e.g., MP3 and MP4), most likely indicating a dominant effect of DNA sequence. That certain DNA sequences present preferential sites for placement of the crossover was supported by the hydroxyl radical data on the unmodified junction (Fig. 2*A*, lanes a–c). In this case, hypersensitivities were observed at the same sites as in junction MP2 (Fig. 2*A*, compare lanes c and i). It is remarkable that the junction point in the unmodified DNA exhibits a bias to one preferred site, although it had the potential to adopt five possible positions. Presumably this site reflects the most energetically favorable location.

Cleavage by T7 Endonuclease I. To eliminate any possibility that the migrational bias of the junction was altered by formation of the RuvC–junction complex, the location of the

crossover was also analyzed using T7 endonuclease I. This nuclease cleaves Holliday junctions by introducing nicks at the 5′ side of the crossover with little sequence specificity (15, 17, 18). When MP1–MP5 were treated with endonuclease I, the major sites of cleavage correlated well with the predicted location of the Holliday junction (Fig. 3*A*). The results, summarized in Fig. 3*B*, indicate that the two major sites of cleavage in MP1 map to positions located 1 and 2 nt to the 5′ side of the junction point (Fig. 3 *A*, lane b, and *B*, part *i*). In MP2, the pattern was shifted to the 5′ side by 1 base relative to MP1 (Fig. 3*A*, lanes b and d), in keeping with movement of the junction by 1 nt (Fig. 3*B*, compare parts *i* and *ii*). Similarly, the major site of cleavage in MP5 was 4 bases away from that observed with MP1 (Fig. 3 *A*, lanes b and j and *B*, parts *i* and *v*). These results provide independent evidence that methyl phosphonate substitutions direct junction position.

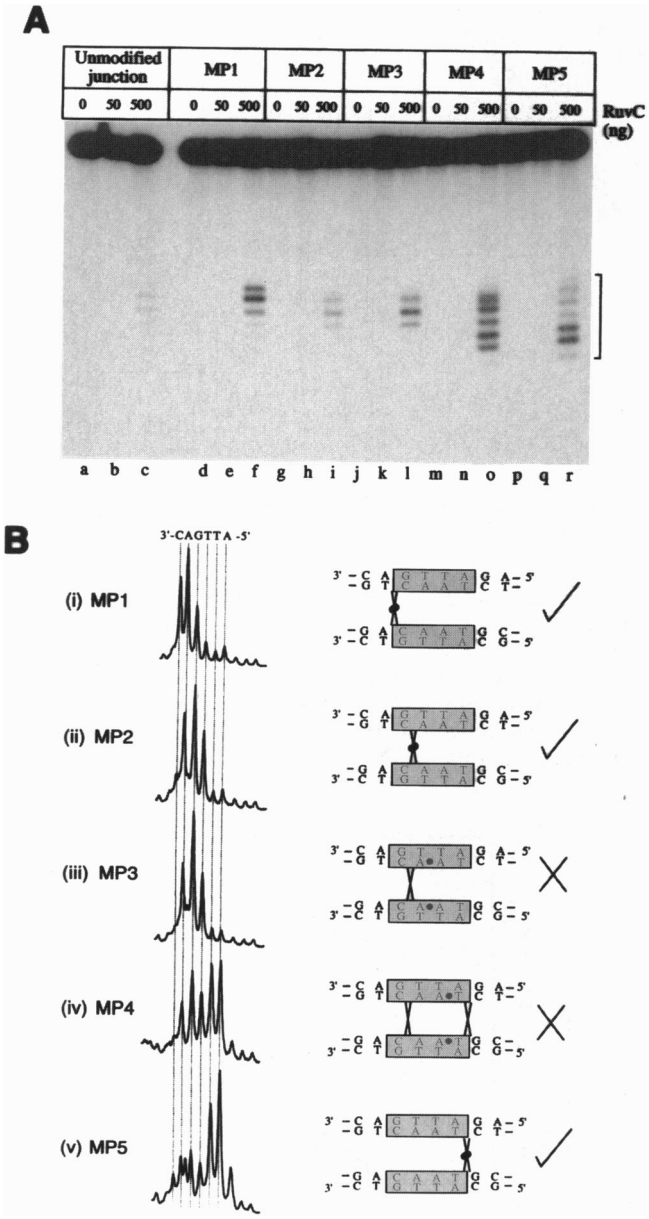

FIG. 2. Determination of crossover location by hydroxyl radical probing. (*A*) PAGE indicating the sites of hypersensitivity. ^{32}P-labeled junction X4 (unmodified) and methyl phosphonate-modified junctions MP1–MP5 were incubated with the indicated amounts of RuvC. Mixtures were treated with hydroxyl radicals and the products were analyzed by denaturing PAGE and autoradiography. The region of hypersensitivity is indicated. (*B*) PhosphorImager analyses of the hydroxyl radical hypersensitivities seen in *A*. The DNA sequence is indicated. On the right is a summary of the crossover position in each junction, as determined from the hydroxyl radical data. The 4-bp region of homology is boxed and the sites of methyl phosphonate incorporation are indicated (solid circles). Checks and crosses indicate whether methyl phosphonate substitution was successful in targeting the crossover. For simplicity in this diagram, the DNA strands are drawn in parallel alignment, but in reality the DNA within the RuvC–junction complex will lie unfolded with twofold symmetry (4).

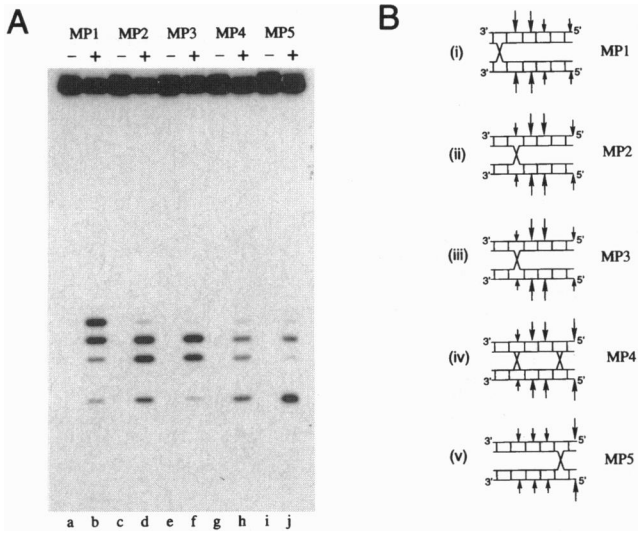

FIG. 3. Cleavage of methyl phosphonate-substituted junctions by T7 endonuclease I. (*A*) ^{32}P-labeled junctions MP1–MP5 (2 ng) were incubated with 3 ng of T7 endonuclease I for 5 min at 37°C. The cleavage products were analyzed by 12% denaturing PAGE followed by autoradiography and sites of cleavage were determined in comparison with marker fragments. (*B*) Summary of the sites of cleavage by endonuclease I relative to the crossover position(s). Large and small arrows indicate strong and weak sites of cleavage, respectively.

Resolution of Migrational Isomers by RuvC. The neutralizing substitutions in junctions MP1–MP5 were made in strands 2 and 4 to ensure that these strands formed the exchanging strands in the protein-free structure (Fig. 1 *A* and *B*). As a consequence, strands 1 and 3, which contain RuvC's cleavage sequence (5′-ATT↓G-3′), form the continuous strands (Fig. 1*A*). Junction unfolding, which occurs upon RuvC binding, repositions the continuous strands containing this sequence to now form the wide angles in the twofold symmetric protein–DNA complex (4) in readiness for cleavage (Fig. 4).

When junctions MP1, MP2, and MP5 were incubated with RuvC, we found that resolution efficiency was directly related to the position of the junction (Fig. 4*A*). Resolution of MP2, in which the exchange point was located directly at the site of cleavage (Fig. 4*ii*), was most efficiently cleaved by RuvC (Fig. 4*A*). With the other junctions, the efficiency of resolution decreased as the distance between the exchange point and the cleavage site increased. In contrast, T7 endonuclease I, which lacks sequence specificity, cleaved the three methyl phosphonate-substituted junctions with similar efficiencies (Fig. 4*B*). Using denaturing PAGE, we observed that the introduction of methyl phosphonates did not alter the position of cleavage by RuvC, since cleavage occurred at the expected site in all junctions, at the sequence 5′-ATT↓G-3′ (Fig. 5, compare lanes b, c, h, i, k, l, and o).

The efficiency of cleavage of each junction was also compared in single turnover kinetic experiments. Excess RuvC protein was prebound to all six junctions (MP1–MP5 and the unmodified junction) in the absence of divalent cations and following addition of Mg^{2+}, the rate of cleavage of each junction was compared (Fig. 6). MP2 and MP3 were cut most efficiently, followed by MP1 and the unmodified junction, and

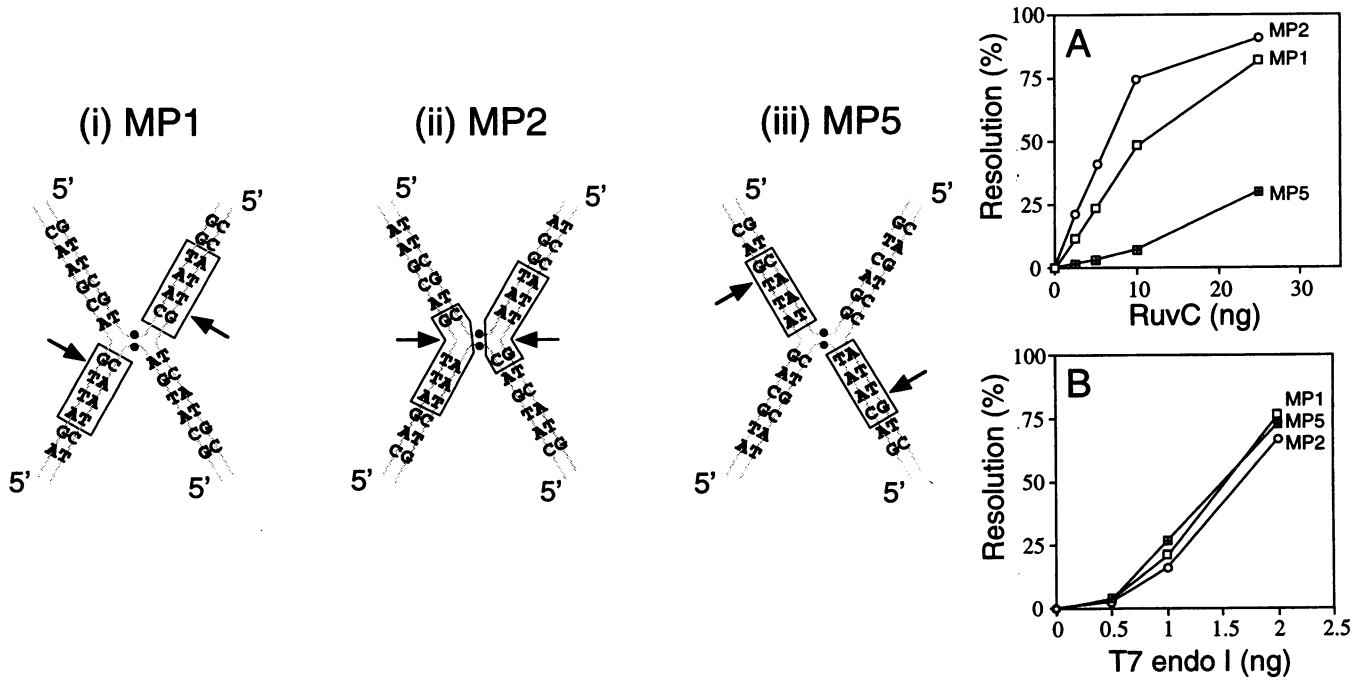

FIG. 4. Resolution of methyl phosphonate-substituted junctions by RuvC and T7 endonuclease I. Schematic diagrams indicating the positions of the Holliday junctions in MP1 (*i*), MP2 (*ii*), and MP5 (*iii*) relative to the site of cleavage (arrows). (*A* and *B*) Resolution of methyl phosphonate-substituted junctions (MP1, MP2, and MP5) by RuvC and T7 endonuclease I, respectively. Reactions were carried out and analyzed by neutral PAGE.

then MP4. Once again, MP5 was cut poorly in comparison with the other junctions. These results demonstrate that the efficiency of cleavage by the *E. coli* Holliday resolvase depends upon the location of the junction relative to the cleavage site and that resolution is most efficient when the junction point is located directly at the site of incision.

DISCUSSION

Charge–Charge Interactions Determine Holliday Junction Structure. By introducing a pair of uncharged phosphate groups (i.e., manipulating the electrostatic forces inherent in the DNA backbone), we were able to direct both the isomeric form and the migrational bias of a synthetic Holliday junction. This observation demonstrates the overriding importance of charge–charge interactions in determining DNA structure and provides a novel tool with which to study Holliday junction structure and protein–DNA complexes formed during general and site-specific recombination. Previously, symmetric junc-

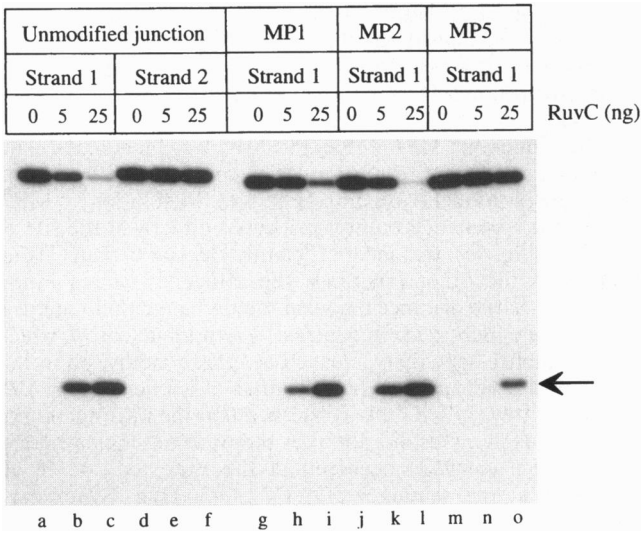

FIG. 5. Cleavage of methyl phosphonate-substituted junctions by RuvC. ^{32}P-labeled junction X4 (unmodified) and methyl phosphonate-substituted junctions MP1, MP2, or MP5 (3 ng) were incubated with RuvC in cleavage buffer at 37°C for 30 min. Reactions were stopped and the cleavage products were analyzed by denaturing PAGE followed by autoradiography. In this experiment, X4 was ^{32}P-labeled in either strand 1 or 2 as indicated.

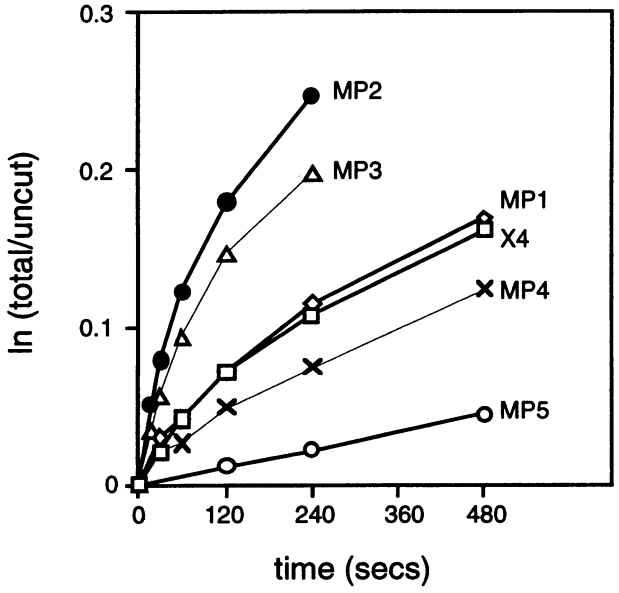

FIG. 6. Relative kinetics of cleavage of methyl phosphonate-modified junctions MP1–MP5 and unmodified X4. ^{32}P-labeled junctions were incubated at 37°C with a predetermined saturating concentration of RuvC to form RuvC–junction complexes. Cleavage was initiated by addition of Mg^{2+}, and at the indicated times thereafter, samples were removed and analyzed by PAGE. Radioactivity was quantitated by phosphorimaging. The natural logarithm of the ratio (total junction/uncleaved junction) is plotted against time.

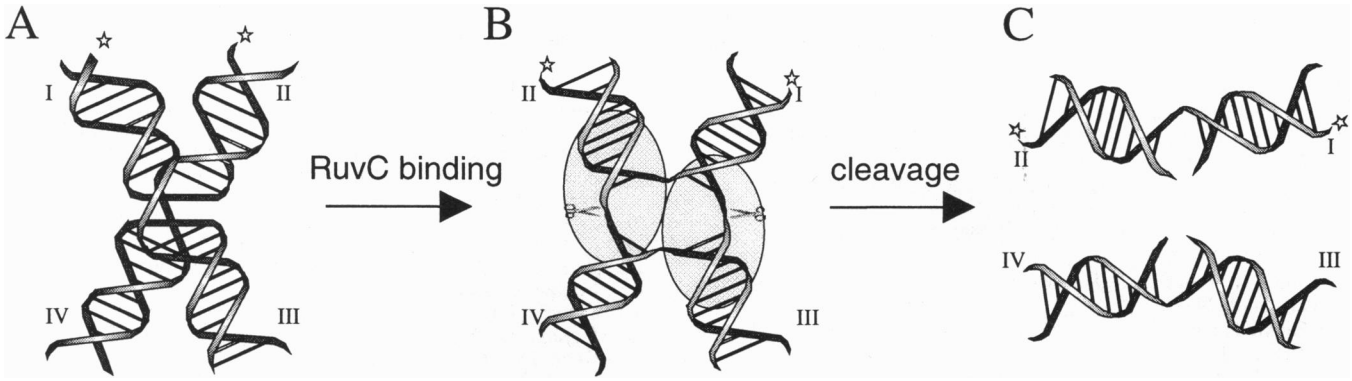

FIG. 7. Schematic model for the binding and resolution of a Holliday junction by RuvC. In this diagram, the continuous DNA strands are indicated with asterisks. The RuvC dimer is shown with monomer subunits facing opposite directions, and the sites of incision are indicated by scissors. For details, see *Discussion*.

tions with fixed crossover points were constructed using DNA molecules that contained two joined DNA crossovers (19, 20). However, in this topologically constrained system, the orientation of the helical domains was fixed such that structural freedom of the junction was forfeited. Similarly, studies of the integrase family of site-specific recombinases have relied upon the introduction of sequence mismatches to limit the position of the crossover (21–23). However, this method suffers the disadvantage that the DNA sequence is altered within the region of homology. Using methyl phosphonate-substituted oligonucleotides, a new class of synthetic Holliday junctions can be constructed that neither constrains the configuration of the junction nor disrupts sequence symmetry. The molecules are straightforward to construct, making this technique one of general applicability.

Resolution of Holliday Junctions by RuvC Protein. Since efficient RuvC-catalyzed resolution requires the sequence 5′-WTT↓S-3′ in two homologous arms of DNA, it was not previously possible to determine the position of the junction point relative to the site of incision. However, using methyl phosphonate-substituted DNAs, we were able to position the junction at different distances from the site at which cleavage occurs. We observed that resolution by RuvC was most efficient when the point of strand exchange was located directly at the site of nicking. This result contrasts with that obtained with bacteriophage T4 endonuclease VII, the prototypic Holliday junction resolvase, which typically cleaves 2 or 3 bp away from the branch point (24, 25).

A Model for Holliday Junction Resolution. The experiments described in this paper allow us to refine a model for the resolution of Holliday junctions by RuvC (Fig. 7). Based on structural studies, it has been shown that protein-free Holliday junctions adopt a twofold symmetric stacked X structure in solution (Fig. 7*A*) (12). In this structure, the arms lie in an antiparallel configuration, with the exchanging strands folding back on themselves as they cross between the helical stacks, while the continuous strands (indicated by asterisks in Fig. 7*A*) exhibit an unbroken helical axis. Binding by RuvC causes the junction to unfold so that DNA strands (formerly defined as continuous) adopt defined positions within the two catalytic domains present in the RuvC dimer (Fig. 7*B*). The crystal structure of RuvC indicates the presence of several positively charged residues within the DNA binding cleft of each monomer (8, 26). These are likely to be involved in interaction with the phosphate backbone, as the DNA fits into the active site. In recent studies of the mechanism of Int-catalyzed site-specific recombination, it was suggested that DNA cleavage immediately at the site of the strand crossover may be sterically hindered due to crowding of the DNA strands in this region (22). With RuvC, steric hindrance is unlikely due to formation of the unfolded or open complex. Indeed, within this complex, base stacking is disrupted as indicated by the increased sensitivity of the DNA to permanganate ions (4). Opening the DNA in this way will also increase the accessibility of the scissile DNA bonds to the active site of the protein.

Cleavage of the junction requires the presence of divalent metal ions and occurs by the introduction of symmetrically related nicks in the DNA strands that constitute the wide angles in the twofold symmetric RuvC–Holliday junction complex (Fig. 7*B*) (4). Cleavage occurs at the site at which the DNA strands pass from one duplex to the other to form nicked duplex products in which 5′-P and 3′-OH termini can be rejoined by DNA ligase (Fig. 7*C*) (5, 14).

A Requirement for Branch Migration. In previous studies with synthetic Holliday junctions (containing either 12 or 26 bp of homology), we observed that the majority of DNA molecules, when bound by RuvC, had their junctions located at defined positions (5, 7, 16). These results indicate that DNA sequence can influence the location of a Holliday junction and that junctions are trapped at energetically favorable locations. The requirement for movement of the crossover to a potential cleavage site reflects the need for specialized proteins that promote branch migration during recombination, thereby overcoming thermodynamic effects (27–30). In *E. coli*, genetic evidence indicates that RuvC-mediated Holliday junction resolution is dependent upon RuvA and RuvB *in vivo* (31, 32). Most likely, RuvAB contribute to resolution by facilitating the movement of Holliday junctions to sites at which RuvC promotes cleavage. The details of this interaction remain to be determined.

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