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Biochemistry

RuvC protein resolves Holliday junctions via cleavage of the continuous (noncrossover) strands

(recombination/DNA repair/resolvase/stacked X-structure/hydroxyl-radical footprinting)

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ABSTRACT **The RuvC protein of *Escherichia coli* resolves Holliday junctions during genetic recombination and the postreplicational repair of DNA damage. Using synthetic Holliday junctions that are constrained to adopt defined isomeric configurations, we show that resolution occurs by symmetric cleavage of the continuous (noncrossing) pair of DNA strands. This result contrasts with that observed with phage T4 endonuclease VII, which cleaves the pair of crossing strands. In the presence of RuvC, the pair of continuous strands (i.e., the target strands for cleavage) exhibit a hypersensitivity to hydroxyl radicals. These results indicate that the continuous strands are distorted within the RuvC/Holliday junction complex and that RuvC-mediated resolution events require protein-directed structural changes to the four-way junction.**

The *Escherichia coli* RuvC protein resolves Holliday junctions *in vitro* (1–4). Resolution occurs by the introduction of symmetrical nicks in two of the four DNA strands to produce nicked duplexes that can be repaired by DNA ligase (2, 5). Although recognition of the Holliday junction is structure-specific, cleavage occurs at a tetranucleotide consensus sequence, 5′-WTT↓S-3′ (where W is A or T, and S is G or C) (6). In recent years there has been increasing interest in the physical structure of Holliday junctions. With model substrates it was shown that the structure of the junction is sensitive to the presence of divalent metal ions which facilitate their folding into a "stacked X-structure," in which the arms of the junction are antiparallel (7–10). The stacked X-structure exhibits two-fold symmetry with two strands approximating B-form DNA (defined as the continuous strands), while the complementary strands are sharply bent where they pass from one helix to the other (7, 8). It is these crossover (or exchanging) strands that are cut by T4 endonuclease VII, a bacteriophage-encoded resolvase (11, 12).

Although genetic recombination requires the parallel alignment of homologous arms during homologous pairing and strand exchange, structural studies of synthetic Holliday junctions indicate that they adopt an antiparallel conformation (13, 14). To provide details of the mechanism of resolution, the interaction of RuvC with a series of conformational isomers of the Holliday junction was analyzed. We found that RuvC cleaved antiparallel junctions by incision of the continuous, rather than the crossover, pair of DNA strands.

MATERIALS AND METHODS

Enzymes and DNA. RuvC was purified as described (2). Oligonucleotides were synthesized by cyanoethyl chemistry and purified by band excision from a denaturing polyacrylamide gel. Synthetic Holliday junctions were prepared by annealing the appropriate oligonucleotides (15). Prior to annealing, one strand was 5′-end-labeled by T4 polynucleotide kinase and [γ-^{32}P]ATP or 3′-end-labeled by terminal deoxynucleotidyl transferase and [α-^{32}P]ddATP (Amersham). Junction X13 was made from oligonucleotide 1 (5′-GCCGTGATCACCAATGGATTGCTAGGACATCTTTGCCCACGT-3′), oligonucleotide 2 (5′-GACGTGGGCAAAGATGTCCTAGCAATCCTGTCAGCTGCATGG-3′), oligonucleotide 3 (5′-GCCATGCAGCTGACAGGATTGCTAGGACGCTAGGCCTACTGC-3′), and oligonucleotide 4 (5′-GGCAGTAGGCCTAGCGTCCTAGCAATCCATTGGTGATCACGG-3′). Constrained junctions were prepared from oligonucleotides in which two strands were linked by a tether of 9 (or 18) thymine nucleotides: oligonucleotide 4-3 (5′-GGCAGTAGGCCTAGCGTCCTAGCAATCCATTGGTGATCACGGTTTTTTTTTCCATGCAGCTGACAGGATTGCTAGGACGCTAGGCCTACTGC-3′), oligonucleotide 2-4 (5′-GACGTGGGCAAAGATGTCCTAGCAATCCTGTCAGCTGCATGGTTTTTTTTTGCAGTAGGCCTAGCGTCCTAGCAATCCATTGGTGATCACGG-3′), and oligonucleotide 1-3 (5′-GCCGTGATCACCAATGGATTGCTAGGACATCTTTGCCCACGTTTTTTTTTTTCCATGCAGCTGACAGGATTGCTAGGdACGCTAGGCCTACTGC-3′). Annealed DNA substrates were purified by gel electrophoresis. Cleavage and hydroxyl radical-hypersensitive sites were mapped by comparison with G+A and T+C sequence ladders.

Band-Shift Assays. Binding reaction mixtures (20 μl) containing 5′-^{32}P-labeled DNA (0.5 ng) and RuvC were incubated on ice for 15 min in binding buffer (50 mM Tris·HCl, pH 8.0/0.5 mM $MgCl_2$/1 mM dithiothreitol with bovine serum albumin at 100 μg/ml). Products were assayed by neutral 5% PAGE (5).

Resolution Assays. Reaction mixtures (20 μl) containing 5′-^{32}P-labeled DNA (0.5–1 ng) were incubated with RuvC at 37°C for 30 min in cleavage buffer (50 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/1 mM dithiothreitol with bovine serum albumin at 100 μg/ml). Products were analyzed by neutral 8% or denaturing 12% PAGE (5). Quantitation of binding and resolution assays was achieved with a PhosphorImager (Molecular Dynamics).

Hydroxyl-Radical Footprinting. Reaction mixtures (140 μl) containing 5′-^{32}P-labeled junction DNA (2 ng) and the indicated amounts of RuvC were incubated in binding buffer for 15 min at room temperature. Treatment with hydroxyl radicals was as described (5).

RESULTS

Construction of Constrained Holliday Junctions. Previously, we showed that Holliday junction resolution by RuvC occurs at the tetranucleotide sequence 5′-WTTS-3′ (6). To investigate whether resolution occurred via cleavage of the continuous or crossing pair of DNA strands, synthetic junctions (42 bp in length; Fig. 1) were prepared in which the

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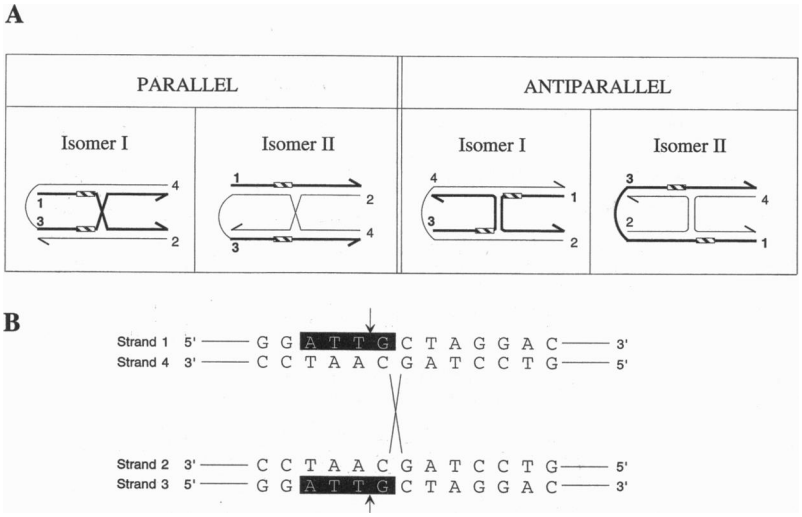

FIG. 1. Design of constrained Holliday junctions. (*A*) Parallel and antiparallel junctions were constructed with 9-nt tethers that linked two of the four oligonucleotides. In isomer I; strands 1 and 3 cross over, and strands 2 and 4 are continuous. In isomer II; strands 2 and 4 cross over, and strands 1 and 3 are continuous. Half arrows indicate 3′ termini and hatched boxes show the location of the consensus sequence. (*B*) Homologous core of the junction X13 indicating the consensus sequence (white on black) and sites of cleavage by RuvC (arrows). The crossover point is arbitrarily positioned within the region of homology.

consensus was located in either pair of strands. With the procedure of Kimball *et al.* (16), a short tether of 9 thymine residues was used to constrain the isomeric form of each junction (Fig. 1*A*). The tether was also used to dictate parallel or antiparallel configuration. Tethering oligonucleotides 2 and 4 ensured that these formed the continuous strands of an antiparallel junction (isomer I, Fig. 1*A Right*), whereas tethering strands 1 and 3 caused these to become the continuous strands of the junction (antiparallel isomer II). In contrast, tethering oligonucleotides 4 and 3 resulted in a parallel structure but did not fix the isomeric form of the junction (Fig. 1*A Left*).

Binding and Resolution of Constrained Holliday Junctions. The interaction of RuvC with constrained and unconstrained junctions was examined by band-shift and resolution assays. Incubation of 5′-^{32}P-labeled junction X13 (unconstrained junction) with RuvC resulted in the formation of a defined RuvC/junction complex (Fig. 2*A*, lanes 1–3). RuvC also bound to the parallel junction (lanes 4–6) and to both antiparallel junctions (isomer I, lanes 7–9; isomer II, lanes 10–12), albeit with lower efficiencies. Whereas only a single protein/DNA complex was evident with the unconstrained junction, two complexes were formed with the tethered junctions (complexes a and b; lanes 4–12).

To limit cleavage of the junction, binding reactions were carried out at low Mg^{2+} (0.5 mM) and temperature (0°C). At 10 mM Mg^{2+} and 37°C, Holliday junctions were resolved by the cleavage of strands of like polarity, giving rise to nicked duplex products as observed by neutral PAGE (Fig. 2*B*, lanes 1–3). RuvC resolved the parallel junction (lanes 4–6) and the antiparallel isomer II junction (lanes 10–12), but the efficiencies were approximately 50- and 10-fold lower, respectively, than that of the unconstrained junction. The resolution product of the parallel junction ran as a 92-mer because the tether prevented separation of the two nicked duplexes. Resolution of antiparallel isomer I was barely detectable (lanes 7–9).

RuvC Cleaves the Continuous Strands. To determine whether the inability to cleave antiparallel II to any significant extent was due to a strand specificity, the sites of cleavage in each junction were mapped. Junctions were uniquely 5′-^{32}P-labeled in each of their three strands and treated with RuvC, and the resulting DNA cleavage products were analyzed by denaturing PAGE (Fig. 3). Comparison with sequence ladders indicated that resolution of the unconstrained junction occurred by nicking strand 1 (lanes 1–3) and strand 3 (data not shown) at the consensus sequence 5′-ATT ↓ G-3′. The same specificity of cleavage was observed for the parallel junction (lanes 4–6 and 10–12).

With the antiparallel (isomer II) junction, strands 1 and 3 are connected by the tether. If concerted cleavage occurs in these strands, only the nick closer to the 5′-^{32}P label (i.e., in strand 1) should be visible, as observed experimentally (Fig. 3, lanes

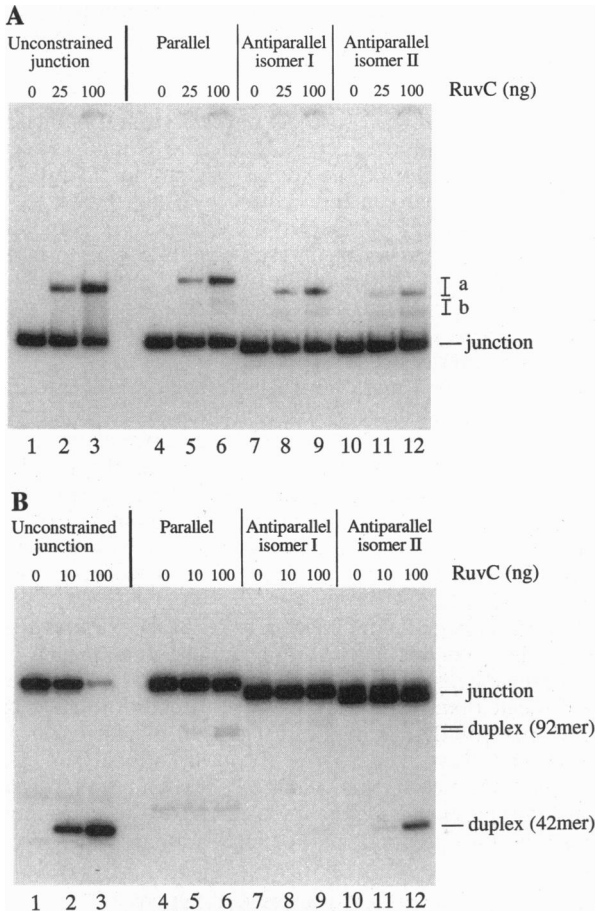

FIG. 2. Binding and resolution of constrained Holliday junctions by RuvC. (*A*) Band-shift assay. Junctions (0.5 ng; Fig. 1) were incubated with RuvC and protein/DNA complexes were analyzed by neutral 5% PAGE. The RuvC/junction complexes (a and b) are indicated. Junctions were 5′-^{32}P-labeled in strand 1, except for antiparallel isomer II (labeled in strand 2). (*B*) Resolution assay. Reactions containing junctions (0.5 ng; 5′-^{32}P-labeled) were incubated with RuvC in cleavage buffer at 37°C for 30 min. DNA products were analyzed by neutral 8% PAGE. The unconstrained junction was labeled in strand 1, and constrained junctions were labeled in the tethered strand.

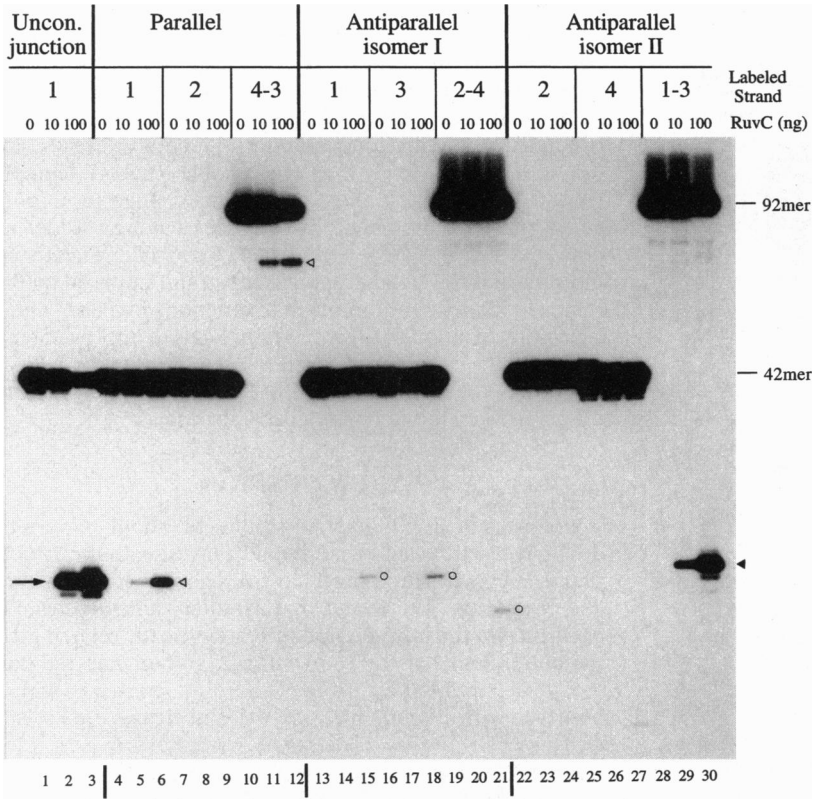

FIG. 3. Cleavage of constrained junctions by RuvC. Parallel and antiparallel junctions were 5′-^{32}P-labeled in each of their three strands (as indicated). The junctions (1 ng) were incubated with RuvC in cleavage buffer at 37°C for 30 min. DNA products were analyzed by denaturing 12% PAGE. The cleavage products from unconstrained (arrow), parallel (◁), and antiparallel (○) isomer I and antiparallel isomer II (▲) junctions are indicated.

28–30). To detect cleavage in strand 3, a junction was prepared in which this strand was 3′-^{32}P-end-labeled. When 5′- and 3′-^{32}P-labeled junctions were treated with RuvC, we observed similar levels of cleavage in strands 1 and 3 at the consensus 5′-ATT ↓ G-3′ (data not shown).

When the cleavage sites in the antiparallel isomer I junction were analyzed, we found that the consensus sequence was nicked at a frequency at least 25-fold lower than that in isomer II (Fig. 3, compare lanes 13–18 with lanes 28–30). Isomer I was also nicked inefficiently in the continuous strands (lanes 19–21), but in this case the nick was found to correspond to a nonconsensus sequence. The results, summarized in Fig. 4, indicate that cleavage was most efficient when the consensus sequence was located in the continuous strands of DNA (e.g., antiparallel isomer II). This result was supported by experiments with antiparallel junctions based upon a different DNA sequence (data not shown).

Induced Hypersensitivity of Continuous Strands to Hydroxyl Radicals. Previously, we showed that binding of a junction by RuvC resulted in a hypersensitivity to hydroxyl-radical attack. Hypersensitivity was strongest in two of the four DNA strands, at sites located 1–2 nt to the 3′ side of the junction point (5). This structure-specific hypersensitivity is sequence-independent and provides a sensitive indicator for the position of the crossover point. To determine whether the hypersensitivity was specific for either the continuous or crossover strands, the two isomeric forms of the antiparallel junction were probed with hydroxyl radicals in the presence of RuvC. In each case, hypersensitivity was limited to two of the four DNA strands. Hypersensitive sites on the antiparallel (isomer I) junction occurred in strands 2 and 4 (Fig. 5, lanes 13–15), whereas the antiparallel (isomer II) junction was hypersensitive in strands 1 and 3 (lanes 22–24). Thus, hypersensitivity is specific for the continuous strands of the junction, the strands in which cleavage occurs.

Hydroxyl-radical treatment of the complex formed with the unconstrained junction also showed a two-fold symmetric pattern, with strong hypersensitivity in strands 1 (Fig. 5, lanes

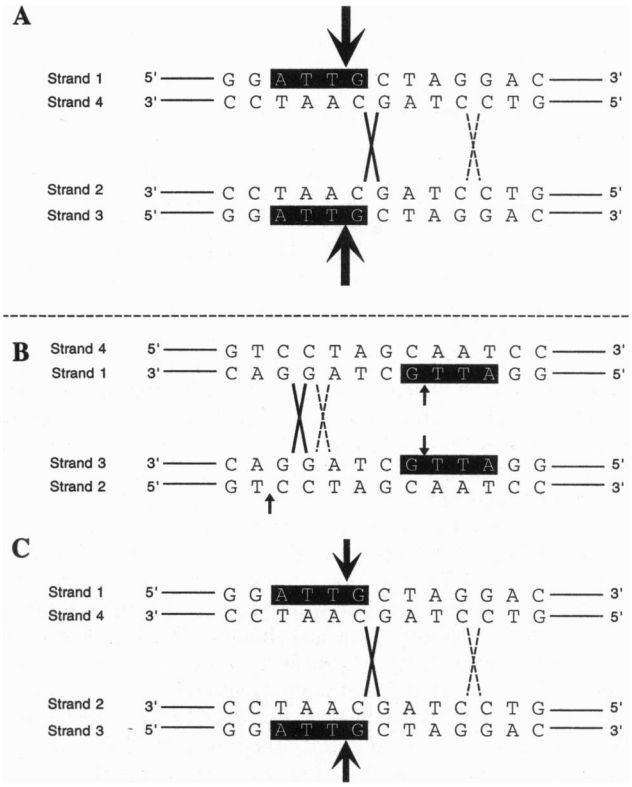

FIG. 4. Summary of the cleavage sites and crossover positions in the unconstrained junction (*A*) and antiparallel isomers I (*B*) and II (*C*). Only the 13-bp homologous core is shown, and the consensus sequence is shown white-on-black. Arrows indicate the sites of cleavage by RuvC, and their size approximates cleavage efficiency. Crossover location, determined with hydroxyl radicals, is indicated. Highly populated crossover positions are indicated by solid lines, and sparsely populated positions by dashed lines. All junctions are drawn in a parallel configuration for comparison purposes. Only the predominant isomeric configuration of the unconstrained junction is shown in *A*.

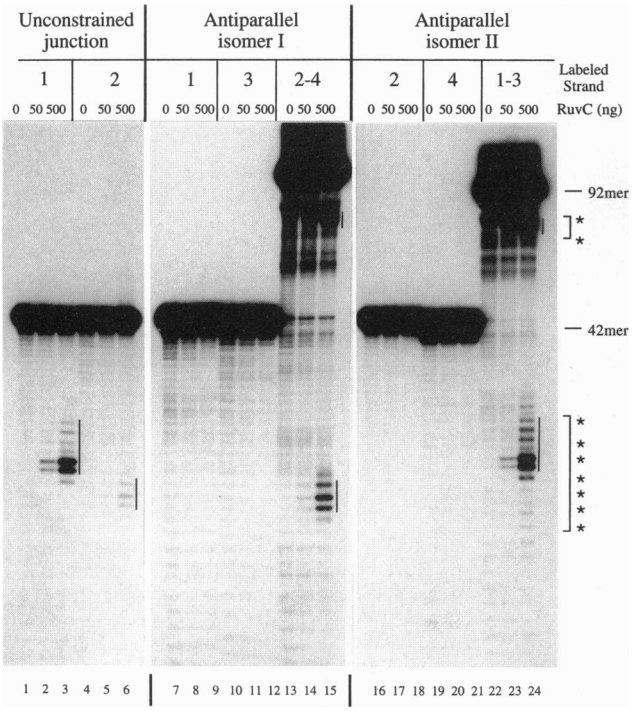

FIG. 5. Hydroxyl-radical footprinting of the RuvC/Holliday junction complex. Junctions (2 ng), 5′-^{32}P-labeled in the indicated strand, were incubated with RuvC, complexes were treated with hydroxyl radicals, and the products were analyzed by denaturing 12% PAGE. Stars indicate hypersensitive regions.

1–3) and 3 (data not shown) and only a weak hypersensitivity in strands 2 (lanes 4–6) and 4 (data not shown). This indicates that the unconstrained junction demonstrates an isomeric bias with strands 2 and 4 crossing over (i.e., isomer II; see Fig. 4*A*).

The sites at which the crossovers were located were mapped from the hypersensitivity data and found to differ in the two antiparallel isomers (compare Fig. 4 *B* and *C*). The position of the crossover in the unconstrained junction was found to be the same as that observed with antiparallel isomer II (compare Fig. 4 *A* and *C*), indicating that the unconstrained junction adopts an antiparallel II-like configuration.

Interaction of RuvC with the Junction Is Affected by Tether Length. Although the results obtained with antiparallel isomer II most closely mimic those seen with the unconstrained junction, none of the constrained junctions served as efficient substrates for RuvC. To determine whether relaxation of the structural constraint improved substrate recognition, related DNA substrates were produced in which the length of the tether was increased from 9 to 18 thymine residues. By use of hydroxyl radicals the position of the crossover site and the isomeric bias of each junction were shown to be unaffected by changing tether length (data not shown). The interaction of RuvC with antiparallel junctions containing a lengthened tether (T_{18}) was significantly improved (Fig. 6 *A* and *B*, compare lanes 6–10 and 11–15). For example, the resolution efficiency of the antiparallel T_{18} junction was ≈3-fold higher than that of antiparallel T_9 (Fig. 6*B*). Similar effects were observed with parallel junctions (data not shown).

Junctions containing the T_{18} tether were found to migrate more slowly through neutral polyacrylamide gels than identical junctions containing the T_9 tether (Fig. 6 *A* and *B*, compare lanes 6 and 11), consistent with a widening of the angle between the tethered arms. Significantly, the unconstrained junction migrated at about the same position as that of the antiparallel T_{18} junction (Fig. 6 *A* and *B*, compare lanes 1 and 11). Since the acute angles in the unconstrained X-structure are thought to average about 60° (7, 10), we estimate that the angles between the tethered arms in antiparallel T_{18} are also about 60°, whereas those in antiparallel T_9 are <60° due to constraints imposed by the shorter tether.

On analysis by neutral PAGE, the complexes formed between RuvC and the constrained T_{18} junctions were also found to migrate more slowly than comparable RuvC/T_9 complexes (Fig. 6*A*, lanes 6–10 and 11–15). This change in mobility is consistent with the RuvC/T_{18} complex having a wider angle between the tethered arms than RuvC/T_9. These results indicate that the tight structural constraint imposed by the T_9 tether limits recognition of these junctions by RuvC and that a partial relaxation of this constraint leads to improved binding and resolution. However, even though greater flexibility was permitted, the interaction of RuvC with T_{18} was less than that observed with the unconstrained junction.

DISCUSSION

We constrained Holliday junctions to adopt a variety of defined structures and used them to investigate the structural requirements of RuvC protein with regard to junction binding and resolution. We found that Holliday junction resolution occurred via the introduction of symmetrically related nicks in the continuous pair of DNA strands, rather than the pair of crossover strands (Fig. 7*A*). This result contrasts with that observed with T4 endonuclease VII, a nuclease often regarded

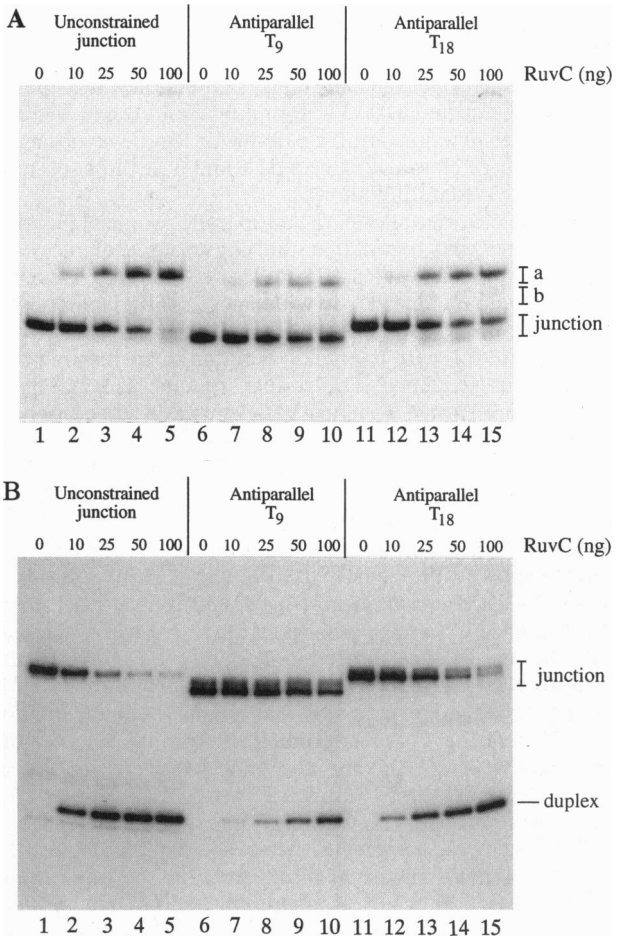

FIG. 6. Effect of tether length on RuvC binding and resolution. Antiparallel (isomer II) junctions (0.5 ng) containing T_9 or T_{18} tethers were incubated with RuvC in binding buffer at 0°C for 15 min (*A*) or in cleavage buffer at 37°C for 30 min (*B*). The RuvC/junction complexes (a and b) are indicated. Reactions were stopped and analyzed as in Fig. 2.

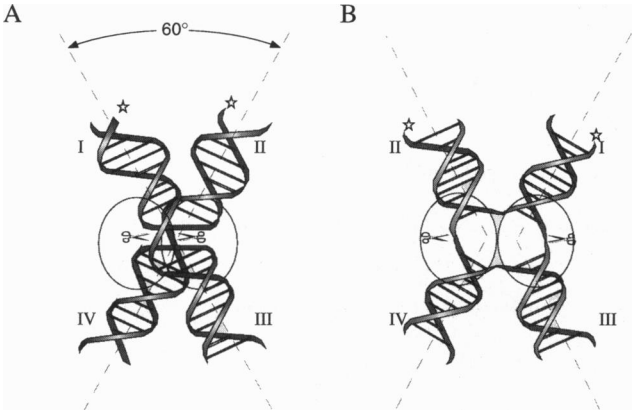

FIG. 7. Model showing cleavage of two of the four strands of an antiparallel Holliday junction by RuvC. Sites of symmetrical nicking are indicated by scissors. (*A*) Resolution of a stacked X-structure. Continuous strands are indicated with stars. (*B*) Resolution of an unfolded junction. In this structure, arms I and II of the structure shown in *A* have been rotated by 180° to indicate the formation of a twofold-symmetric unfolded structure.

as the prototype Holliday junction resolvase (11, 12), and further illustrates the difference between these two nucleases (6, 17, 18).

The binding of a Holliday junction by RuvC induces a hypersensitivity to hydroxyl radicals, characteristic of a distortion to the DNA. Using junctions whose arms are tethered in a particular isomeric form, we have shown that the hypersensitivity occurs in the pair of continuous strands—i.e., the strands in which cleavage takes place. Other studies indicate that RuvC protein shares structural similarities with RNase HI from *E. coli* (19), the RNase H domain of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RTase) (20) and the HIV-1 integrase protein (21). DNA binding by HIV-1 RTase also induces a hypersensitivity to hydroxyl-radical attack, in a reaction dependent upon RNase H activity (22). Induction of structural changes to the DNA may be important for the nuclease activities of these proteins, which could be related by a common ancestor or structural motif.

Although models for general recombination traditionally assume that Holliday junctions adopt a parallel configuration, synthetic Holliday junctions form antiparallel stacked X-structures *in vitro*. Modeling studies indicate that the antiparallel X-structure can be modified to fit the RuvC crystal structure (19) by narrowing the acute angles (60°) of the stacked X-structure. We observed that both parallel and antiparallel constrained junctions served as substrates for RuvC-mediated resolution, although the antiparallel junction was preferred. However, the interaction of RuvC with each constrained junction was poor compared to that with an unconstrained junction. It is likely that a 9-nt tether will impose a stringent structural constraint on the junction, and we found that a relaxation of this constraint, by increasing the tether to 18 nt, led to improved junction recognition by RuvC. However, junctions with lengthened tethers were still bound and resolved with only limited efficiency. We suggest that RuvC requires conformational freedom to induce the formation of a structure different from the stacked X-structure or the modified X that has been modeled into the RuvC dimer. In particular, the improved recognition of junctions containing a longer tether may imply that a widening of the acute angles from 60° is necessary for efficient junction recognition and cleavage (Fig. 7*A*).

Using a gel electrophoretic technique (7), we recently observed that junction binding by RuvC results in the formation of a complex in which the junction is unfolded and exhibits twofold symmetry (unpublished work). Within this unfolded structure, the strands close to the crossover point were found to be hypersensitive to permanganate ions, characteristic of a loss of base pairing. Cleavage of the continuous strands of the antiparallel X-structure as indicated by the present work (Fig. 7*A*) would be analogous to cleavage of the DNA strands that provide the wide angle in such an unfolded junction (Fig. 7*B*).

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1. Connolly, B., Parsons, C. A., Benson, F. E., Dunderdale, H. J., Sharples, G. J., Lloyd, R. G. & West, S. C. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 6063–6067.
2. Dunderdale, H. J., Sharples, G. J., Lloyd, R. G. & West, S. C. (1994) *J. Biol. Chem.* **269,** 5187–5194.
3. Dunderdale, H. J., Benson, F. E., Parsons, C. A., Sharples, G. J., Lloyd, R. G. & West, S. C. (1991) *Nature (London)* **354,** 506–510.
4. Iwasaki, H., Takahagi, M., Shiba, T., Nakata, A. & Shinagawa, H. (1991) *EMBO J.* **10,** 4381–4389.
5. Bennett, R. J., Dunderdale, H. J. & West, S. C. (1993) *Cell* **74,** 1021–1031.
6. Shah, R., Bennett, R. J. & West, S. C. (1994) *Cell* **79,** 853–864.
7. Duckett, D. R., Murchie, A. I. H., Diekmann, S., Von Kitzing, E., Kemper, B. & Lilley, D. M. J. (1988) *Cell* **55,** 79–89.
8. Cooper, J. P. & Hagerman, P. J. (1987) *J. Mol. Biol.* **198,** 711–719.
9. Cooper, J. P. & Hagerman, P. J. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 7336–7340.
10. Murchie, A. I. H., Clegg, R. M., von Kitzing, E., Duckett, D. R., Diekmann, S. & Lilley, D. M. J. (1989) *Nature (London)* **341,** 763–766.
11. Mueller, J. E., Kemper, B., Cunningham, R. P., Kallenbach, N. R. & Seeman, N. C. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9441–9445.
12. Bhattacharyya, A., Murchie, A. I. H., von Kitzing, E., Diekmann, S., Kemper, B. & Lilley, D. M. J. (1991) *J. Mol. Biol.* **221,** 1191–1207.
13. West, S. C. (1992) *Annu. Rev. Biochem.* **61,** 603–640.
14. Radding, C. M. (1991) *J. Biol. Chem.* **266,** 5355–5358.
15. Parsons, C. A., Kemper, B. & West, S. C. (1990) *J. Biol. Chem.* **265,** 9285–9289.
16. Kimball, A., Guo, Q., Lu, M., Cunningham, R. P., Kallenbach, N. R., Seeman, N. C. & Tullius, T. D. (1990) *J. Biol. Chem.* **265,** 6544–6547.
17. Benson, F. E. & West, S. C. (1994) *J. Biol. Chem.* **269,** 5195–5201.
18. Kemper, B., Pottmeyer, S., Solaro, P. & Kosak, H. (1990) in *Structure and Methods. I. Human Genome Initiative and DNA Recombination*, eds. Sarma, R. H. & Sarma, M. H. (Adenine, New York), pp. 215–229.
19. Ariyoshi, M., Vassylyev, D. G., Iwasaki, H., Nakamura, H., Shinagawa, H. & Morikawa, K. (1994) *Cell* **78,** 1063–1072.
20. Davies, J. F., II, Hostomska, Z., Hostomsky, Z., Jordan, S. R. & Mattews, D. A. (1991) *Science* **252,** 1783–1790.
21. Dyda, F., Hickman, A. B., Jenkins, T. M., Engelman, A., Craigie, R. & Davies, D. R. (1994) *Science* **266,** 1981–1986.
22. Metzger, W., Hermann, T., Schatz, O., Legrice, S. F. J. & Heumann, H. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5909–5913.