The analysis of the RuvC Holliday junction resolvase with synthetic Holliday junctions is a critical experimental determination of a single mobile (catalytically competent) substrates. On the other hand, synthetic HJs that are cleavable by RuvC can branch migrate spontaneously within the homologous core sequence (mobile HJs), and experimental determination of a single mobile (catalytically competent) HJ structure among the many isomers would be difficult.

In this study, we investigated the RuvC cleavage requirement for the homologous core sequence and to obtain new insights into molecular mechanisms of the HJ resolution by the protein, we designed a systematic series of synthetic HJs. First, we varied the size of the homologous core sequence from 10 bp with five possible steps of branch point migration (pentamobile HJ) to 2 bp with only a single possible step (monomobile) and analyzed cleavability by RuvC. Second, the sequence specificity for RuvC cleavage was examined in dimobile HJs (4 bp of

The Escherichia coli RuvC protein endonucleolytically resolves Holliday junctions, which are formed as intermediates during genetic recombination and recombination repair. Previous studies using model Holliday junctions suggested that a certain size of central core of homology and a specific sequence in the junction were required for efficient cleavage by RuvC, although not for binding. To determine the minimum length of sequence homology required for RuvC cleavage, we made a series of synthetic Holliday junctions with various lengths of homologous sequence in the core region. It was demonstrated that a monomobile junction possessing only 2 base pairs of the homology core was efficiently cleaved by RuvC. To study the sequence specificity for cleavage, we made 16 dimobile junctions, which differed only in the homologous core sequence. Among them, 6 junctions were efficiently cleaved. Cleavage occurred by introduction of nicks symmetrically at the 3' side of thymine in all cases. However, the nucleotide bases at the 3' side of the thymines were not always identical between the two strands nicked. These results suggest that RuvC recognizes mainly topological symmetry of the Holliday junction but not the sequence symmetry per se, that the thymine residue at the cleavage site plays an important role for RuvC-mediated resolution, and that a long homologous core sequence is not essential for cleavage.

The Holliday junction (HJ) is an important intermediate in the proposed pathway for genetic recombination and recombination repair (Holliday, 1964). In it, two homologous DNA molecules are joined at the single strand crossover by a four-armed DNA structure. Although there have been reports about putative Holliday junction cleavage activities in yeast and mammala (Shinagawa and Iwasaki, 1996), the Escherichia coli RuvC protein is the first cellular enzyme that has been highly purified and shown to resolve HJs in vitro (Dunderdale et al., 1991; Iwasaki et al., 1991), and its three-dimensional structure has been determined by x-ray crystallography (Ariyoshi et al., 1994). Studies using HJ analogs have demonstrated that
homology core; two steps of possible branch point migration freedom) with all possible sequence variations in the homologous core.

MATERIALS AND METHODS

Oligonucleotides—The oligodeoxyribonucleotides were synthesized with an automated synthesizer (ABI 380B). These oligonucleotides were purified by reverse-phase C18 column chromatography.

Enzymes and Chemicals—T4 polynucleotide kinase was purchased from Takara Shuzo (Kyoto, Japan). RuvC protein was purified as described (Iwasaki et al., 1991). The concentration of RuvC was determined by using a molar extinction coefficient of 7.0 × 10^3 M⁻¹ cm⁻¹ at 280 nm.

Construction of Synthetic HJs—Synthetic HJs were prepared by annealing stoichiometric amounts (10 μM each in terms of the junction molecule) of four 24-mer oligonucleotides as described (Shida et al., 1996). Higher concentrations of oligonucleotide were required to form stable HJs from 24-mer oligonucleotides (Shida et al., 1996). One of the four oligonucleotides was uniquely labeled at the 5'-end using [γ-^32P]ATP and T4 polynucleotide kinase before annealing. Sequences of the oligonucleotides are shown in the appropriate figures.

RuvC Cleavage Assay—The standard reaction mixture (10 μl) containing a 5'-^32P-labeled synthetic DNA junction (5 μM) and RuvC (20 μM) in the reaction buffer containing 20 mM HEPESE-NaOH, pH 7.5, 8 mM MgCl₂, 50 mM sodium glutamate, 2 mM dithiothreitol, and 10% glycerol was incubated with RuvC. The DNA products were analyzed by 20% denaturing PAGE and autoradiography. Size markers in lanes S2 and S4 consisted of three possible products of strands 2 and 4, respectively, and were 11–13 bases long. Note that all of the cleaved products of strand 2 of M2–M4 showed the same mobility as that of strand 2 of M1.

10 bp of homologous sequences around the crossover, which allows, respectively, two through five steps of possible branch point migration freedom. An immobile junction, IM, which does not possess a homologous core sequence, was used as a control. RuvC bound to all of the HJs with the same affinity, as judged by a gel retardation assay, and mediated the cleavage of all mobile HJs at almost the same high efficiency (∼95% of the substrates cleaved) except M4, which was less efficiently cleaved (∼70% cleaved), and IM, the cleavage of which was not detected (Fig. 2). To determine the cleavage sites in the junctions, the products were analyzed by denaturing PAGE (Fig. 2). All of the mobile HJs were cleaved by the introduction of single nicks into strands 2 and 4. These cleavage sites were symmetrically related within the homologous core and mapped to the sequence 5'-T↓C-3' in all cases. The cleavage sites are indicated in Fig. 1.

Sequence Specificity for Cleavage of Bimobile HJs—To determine sequence specificity, if any, for RuvC cleavage, we constructed 16 possible bimobile HJ derivatives of M1 (Fig. 3). Only the homologous core sequence differed. Gel shift assay showed that RuvC bound to all of the bimobile HJs with the same affinity (data not shown). However, only 6 HJs (A, 60%; D, 60%; E, 39%; H, 50%; I, 54%; and P, 93%) were cleaved by RuvC with relatively high efficiency, and the other bimobile HJs were not or very poorly cleaved by RuvC (Fig. 4). The cleavage sites of these HJs were determined and are summarized in Fig. 5. All of the nicks were introduced into exactly symmetrical positions at the 3'-side of thymine within the homologous region. However, there was no specificity for the base at the 3'-side of the thymine. In addition, in the case of A, D, H, and P junctions, the rule of symmetrical sequence iden-
homologous core sequences. The sequences of four heterologous arms are the same as M1 of Fig. 1. Arrow, direction from 5' to 3' of the strand; numbers in the box, base pair partners (1 and 2 and 3 and 4) in the homologous core that were varied. B, 16 homologous core sequences (A–P) are possible and are indicated in boxes. Junction E is the same as M1 in Fig. 1.

Fig. 3. Systematic construction of bimobile HJs. A, the junctions consisted of four synthetic 24-mer oligonucleotides with different homologous core sequences. The sequences of four heterologous arms are the same as M1 of Fig. 1. Arrow, direction from 5' to 3' of the strand; numbers in the box, base pair partners (1 and 2 and 3 and 4) in the homologous core that were varied. B, 16 homologous core sequences (A–P) are possible and are indicated in boxes. Junction E is the same as M1 in Fig. 1.

Fig. 4. Resolution of bimobile HJs by RuvC. The DNA products were analyzed by 20% denaturing PAGE. The results of only strands 1 and 2 were shown, because the cleavage sites of the bimobile HJs were symmetrically related (data not shown).

Fig. 5. Diagram of the RuvC cleavage sites in the bimobile HJs. The HJs A, D, E, H, I, and P among 16 possible combinations were cleaved by RuvC. Arrowheads, cleavage sites. Homologous core sequences are enclosed in boxes. The crossover points are arbitrarily positioned.

Cleavage of a Monomobile HJ—Since sequence identity at the 3'-side of the cleavage site between the opposing strands was not observed (e.g. in the A junction, T \( \rightarrow \) T of strand 2 and T \( \rightarrow \) A of strand 4 were the cleavage sites, respectively). This demonstrates that the nucleotide base specificity at the 3'-side of the nicking sites is not a major determinant of RuvC-mediated cleavage. Notably, some of the noncleavable HJs (B, C, L, M, N, and O junctions) also contain thymine in their homologous core sequence; the presence of thymine is obviously not sufficient for cleavage.

Cleavage of a Monomobile HJ—Since sequence identity at the 3'-side of the cleavage site between the opposing strands was not observed (e.g. in the A junction, T \( \rightarrow \) T of strand 2 and T \( \rightarrow \) A of strand 4 were the cleavage sites, respectively). This demonstrates that the nucleotide base specificity at the 3'-side of the nicking sites is not a major determinant of RuvC-mediated cleavage. Notably, some of the noncleavable HJs (B, C, L, M, N, and O junctions) also contain thymine in their homologous core sequence; the presence of thymine is obviously not sufficient for cleavage.

Discussion

The present study has provided several novel clues to the molecular mechanism of HJ resolution by RuvC. We have shown that a monomobile HJ was cleavable by RuvC, and this demonstrates that RuvC introduces nicks at or 1 base away from the point of strand exchange (Figs. 6 and 7). In retrospect, the large homology cores (e.g. 12 bp in synthetic HJs) previously used as substrates (Dunderdale et al., 1991; Bennett et al., 1993; Takahagi et al., 1994) were not essential. However, not all of the bimobile HJs were cleaved; only 6 bimobile HJs among 16 possible combinations were cleaved by RuvC (Figs. 3 and 4). The size of homology core sequence per se is not the key determinant of cleavage. Introduction of nicks occurred symmetrically at the 3'-side of thymine in all cases, with the nucleotide at the 3'-side of the thymine of cleavage sites varying. In addition, nucleotide bases at the 3'-side of the thymine were not always identical between the two cleaved strands (A, D, H, and P in Figs. 3–5; and Q in Figs. 6 and 7). Therefore, these results suggest that the thymine residue at the cleavage site plays an important role in RuvC-mediated resolution and that RuvC recognizes mainly topological symmetry of the Holliday junction but not DNA sequence symmetry at both sides of the thymine at the cleavage site. Since some bimobile HJs with thymine in the homologous core (B, C, L, M, N, and O in Figs. 3 and 4) and immobile HJs (S–X in Fig. 6B) were not cleaved, other molecular requirements in addition to the presence of thymine in the homologous core are critical for resolution by RuvC. Unlike restriction enzymes, which recognize and cleave specific primary sequences per se, RuvC may require a certain sequence context for catalysis to occur. This context may, for example, induce a certain structure that can interact productively with RuvC on forming an RuvC-HJ complex.

The three-dimensional structure of RuvC has been revealed at a 2.5-A resolution by x-ray crystallography (Ariyoshi et al., 1994). The subunits in the RuvC dimer are related by a dyad axis, and thus, the DNA binding cleft and catalytic center in each subunit are located symmetrically in the dimer (Saito et al., 1995). This relative positioning ensures the introduction of symmetrical nicks by RuvC. The computer docking model of RuvC structure with DNAs suggested that the HJ in RuvC consists of two antiparallel, quasicontinuous DNA duplexes linked with at least two unpaired nucleotides. Each DNA du-
plex is inclined by about 80° to the dyad axis of the dimer (Ariyoshi et al., 1994). This model of HJ differs from that of the protein-free stacked X structure of HJ proposed previously (Duckett et al., 1988; Cooper and Hagerman, 1987; von Kitzing et al., 1990). The stacked X structure exhibits 2-fold symmetry with two strands approximating B-form DNA, whereas the complementary strands are sharply bent where they pass from one helix to the other; as a result, all of the base pairings are maintained around the junction. However, RuvC binding can convert the protein-free junction with a folded stacked X structure into an unfolded form with 2-fold symmetry with base pairings around the crossover disrupted (Bennett et al., 1993; Bennett and West, 1995a). These results suggest that configuration of protein-free HJs is converted into a productive form by RuvC binding.

Using synthetic HJs that are constrained to adopt defined isomeric configurations, it has been shown that the nicks are preferentially introduced into the continuous (noncrossing) pair of strands rather than the bent (crossing) pair of strands of antiparallel forms of HJs (Bennett et al., 1995b). The RuvC-HJ computer docking model agrees with this biochemical evidence. In it, the thymines at the cleavage sites are on the continuous strands of antiparallel forms of HJ and are positioned in the hydrophobic pocket just beside the active site of RuvC, and base pairings at the crossover are disrupted so that the phosphodiester bond at the 3'-side of the thymine should become closer to the catalytic site. The thymine-containing sequence context around the crossover; and 3) the HJ can be converted into a productive form in the complex with RuvC, in which the thymine is in the hydrophobic pocket just beside the active site of RuvC, and base pairings at the crossover are disrupted so that the phosphodiester bond at the 3'-side of the thymine should become closer to the catalytic site. The thymine-containing sequence context around the crossover in the synthetic HJs may determine the ability to adopt such a catalytically competent configuration. This model could explain why RuvC does not cleave all of the synthetic HJs that contain a thymine at the junction.

Shah et al. (1994) have shown that RuvC cleaves the HJs at hot spots with a consensus sequence of 5'-AATT↓G-C-3' in vitro. One of our efficiently cleavable bimobile HJs, P, possesses this consensus sequence, and nicking occurred at 5'-TTT↓G-3' in strand 1 and 5'-ATT↓C-3' in strand 3 in this junction (Fig. 5). Therefore, their result does not necessarily conflict with our results. We have found here that HJs that do not possess the consensus sequences were still cleaved by RuvC. They used a HJ made by the RecA protein and then deproteinized. Thus, their substrate can branch migrate over a large DNA sequence, whereas our synthetic HJs have a very limited distance of branch migration, in which two junction points are possible for the monomobile HJ and three junction points are possible for the bimobile HJ. The equilibria among isomers (or choice of junction points) are governed by the sequence contexts around the encountering junction points (Duckett et al., 1988), and they may be quite variable. The consensus sequence might be selected by the two factors: relative stability and cleavable configuration of the junctions with the sequence (with equilibrium biased to such an isomer). However, our cleavable junctions can take only limited numbers of isomers due to the limited junction mobility, which results in relative abundance of the cleavable isomer. It is conceivable that the favorable isomers occur infrequently and rather unstably in a large Holliday structure with freely mobile junctions.

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REFERENCES
Ariyoshi, M., Vassylyev, D. G., Iwasaki, H., Nakamura, H., Shinagawa, H., and Morikawa, K. (1994) Cell 78, 1063–1072
Bennett, R. J., and West, S. C. (1995a) J. Mol. Biol. 252, 213–226

FIG. 7. Resolution of the monomobile HJ by RuvC. The monomobile HJ Q, as shown in Fig. 6, was uniquely 5'-32P labeled and incubated with RuvC. The DNA products were analyzed by 20% denaturing PAGE and autoradiography. Size markers in lanes S2 and S4 consist of three possible products (11–13 bases long) for strands 2 and 4, respectively.

FIG. 6. Monomobile and immobile HJs and their cleavage by RuvC. A, monomobile HJ Q was constructed from four 24-mer oligonucleotides with the same sequences in the flanking regions of four arms as those of IM in Fig. 1. The monomobile homologous core is inclined in the box. Two isomers with different crossover points are possible by spontaneous branch migration. Arrowheads, cleavage sites by RuvC. B, immobile four-way junctions were constructed from four 24-mer oligonucleotides with the same sequences in the flanking regions of four arms as those of IM in Fig. 1. None of the immobile junctions were resolved by RuvC.
Bennett, R. J., and West, S. C. (1995b) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 5635–5639
Bennett, R. J., Dunderdale, H. J., and West, S. C. (1993) *Cell** **74*, 1021–1031
Churchill, M. E. A., Tullius, T. D., Kallenbach, N. R., and Seeman, N. C. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4653–4656
Cooper, J. P., and Hagerman, P. J. (1987) *J. Mol. Biol.* **198**, 711–719
Cooper, J. P., and Hagerman, P. J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7336–7340
Duckett, D. R., Murchie, A. I. H., Diekmann, S., von Kitzing, E., Kemper, B., and Lilley, D. M. J. (1988) *Cell** **55*, 79–89
Dunderdale, H. J., Benson, F. E., Parsons, C. A., Sharples, G. J., Lloyd, R. G., and West, S. C. (1991) *Nature** **354*, 506–510
Holliday, R. (1964) *Genet. Res.* **5**, 282–304
Iwasaki, H., Takahagi, M., Shiba, T., Nakata, A., and Shinagawa, H. (1991) *EMBO J.* **10**, 4381–4389
Murchie, A. I. H., Clegg, R. M., von Kitzing, E., Duckett, D. R., Diekmann, S., and Lilley, D. M. J. (1989) *Nature** **341*, 763–766
Saito, A., Iwasaki, H., Ariyoshi, M., Morikawa, K., and Shinagawa, H. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7470–7474
Shah, R., Bennett, R. J., and West, S. C. (1994) *Cell** **79*, 853–864
Shida, T., Iwasaki, H., Shinagawa, H., and Kyogoku, Y. (1996) *J. Biochem.* **119**, 653–658
Shinagawa, H., and Iwasaki, H. (1996) *Trends Biochem. Sci.* **21**, 107–111
Takahagi, M., Iwasaki, H., and Shinagawa, H. (1994) *J. Biol. Chem.* **269**, 15132–15139
von Kitzing, E., Lilley, D. M. J., and Diekmann, S. (1996) *Nucleic Acids Res.* **18**, 2671–2683
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