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Characterization and Comparison of Synthetic Immobile and Mobile Holliday Junctions[1]

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Eight synthetic Holliday junction (HJ) oligonucleotides containing an immobile or a mobile junction were characterized by gel electrophoresis, ultraviolet absorption and circular dichroism (CD) spectroscopy. Four 24-mer deoxyribonucleotides formed stable immobile and mobile HJs in 0.1 M NaCl at 5 μM strand concentration at room temperature. However, the immobile HJ constructed from four 18-mers was less stable, and four 12-mers did not form the HJ structure under the conditions used. A comparison of the melting profiles of the HJs with those of the duplexes corresponding to the arms of four-way junctions indicated that the thermal stability of the HJ was similar to that of the individual arm and the cooperativity of the melting behavior of the HJ was relatively higher than that of the individual arm duplex. The T_ms of the mobile HJs containing 4, 6, 8, and 10 base-pair homologous cores at junctions were essentially identical with that of the immobile HJ of the same size. There is a tendency that the HJ containing a larger homologous core region becomes more resistant to thermal denaturation. The addition of divalent metal cations, Mg^{2+} and Ca^{2+}, to the solutions of the HJs raised their melting temperatures. The difference found for the CD spectra of the HJs which differ only in the arrangement of the HJ depended primarily upon the DNA sequence flanking the junction. The RuvC protein binds to the immobile and mobile HJs, regardless of the presence and the size of the homologous core at the junction.

Key words: CD, Holliday junction, polyacrylamide gel electrophoresis, RuvC protein, UV.

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The Holliday structure, in which two homologous duplex DNA molecules are linked by a single-stranded crossover, is a main intermediate in homologous recombination, which plays an important role in increasing genetic diversity and repairing damaged chromosomal DNA (*1*). In *Escherichia coli*, RuvA, RuvB, and RuvC proteins act on the Holliday structure. Branch migration of the Holliday (four-way) junction (HJ) is promoted by the RuvA-RuvB protein complex (*2, 3*) or by the RecG protein (*4*). After the branch migration, the HJ structure must be resolved to generate recombinant molecules. This resolution of HJ is catalyzed by a specific endonuclease, RuvC, which cleaves HJ at or near the junction point by introducing nicks at identical sites in the two strands with the same polarity and produces unconnected DNA duplexes. Gel filtration has shown that the active form of RuvC in solution is dimeric (*5*). For the cleavage activity of the RuvC protein, divalent cations such as Mg^{2+} and Mn^{2+} are required (*6, 7*). The resulting nicks are linked by DNA ligase (*5, 8*).

To study the molecular mechanisms of the homologous recombination and the structural properties of HJ, various model HJs, such as synthetic HJs constructed from four oligodeoxynucleotides, cruciforms extruded from a plasmid containing an inverted repeat (*5*), and recombination intermediates formed by the RecA protein between a gapped circular DNA and homologous linear DNA (*3*), were analyzed by physical, chemical, and enzymological methods. The RuvC protein requires a homologous sequence, which allows branch migration of the junction, for the HJ cleavage. Therefore, synthetic HJs containing a homologous sequence of more than 12 base pairs have been used for studies of the cleavage reaction by the RuvC protein. On the other hand, immobile HJs lacking the homologous core have been constructed by annealing four oligodeoxynucleotides to prevent spontaneous branch migration and structural polymorphism at the junction point. Although immobile HJs are poor substrates of the RuvC protein, the immobile HJs have been used for the study of the three-dimensional structure of the HJ by various methods. Gel electrophoresis (*9, 10*) and fluorescence resonance energy transfer experiments (*11, 12*) have shown that the four-way junction takes an extended 4-fold symmetric structure in the absence of magnesium ions and takes a stacked antiparallel conformation in the presence of magnesium ions. Furthermore, the structure and the dynamics of synthetic immobile HJs have been analyzed by NMR (*13-17*).

To investigate the precise structure of the HJ which is

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Abbreviation: HJ(s), Holliday junction(s).

resolved by the RuvC protein, resolvable mobile HJ containing a minimal homologous core or resolvable immobile HJ must be constructed. At present, there is little information concerning the influence of sequences at the junction point on the junction conformation. To elucidate the structural properties of the immobile and mobile HJs, we constructed synthetic HJs from four 24-mer strands with variations in the configuration of the base-pairs in the junction core. Here we report the properties of the HJs as evaluated by polyacrylamide gel electrophoresis and ultraviolet (UV) and circular dichroism (CD) spectroscopies.

MATERIALS AND METHODS

DNA and RuvC Protein—Individual oligodeoxynucleotides were synthesized by the routine phosphoramidite procedure on an Applied Biosystems 380B automatic DNA synthesizer. The nucleotide bases were deprotected by heating in 28% ammonium hydroxide overnight at 55°C. The dimethoxytritylated oligomers were purified by reverse-phase C18 HPLC. HPLC was conducted with a Shimadzu LC-6A pump connected to an SPD-6A UV monitor. The reverse-phase HPLC column was a Wakopac WS-DNA (250×4.5 mm i.d.). The eluates were evaporated to dryness in a rotary evaporator. The residues were treated with 80% AcOH at room temperature for 15 min and then the fully deprotected oligomers were purified by reverse-phase C18 HPLC again. Four-way junction DNAs (HJs) were made by annealing the four purified oligomers shown in Fig. 1. For autoradiography, oligomers were labeled at the 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase. Trace amounts of the labeled oligomers were added to the solutions of synthetic HJs and then the mixtures were heated to about 90°C and cooled for annealing.

The RuvC protein was overproduced in the cells of *E. coli* strain BL21 (DE3) carrying plasmid pHS641 (*5*) and purified by the procedure described previously.

Native Polyacrylamide Gel Electrophoresis—The formation of HJ was detected by gel electrophoresis in a 20% polyacrylamide gel (19:1 ratio of acrylamide to bisacrylamide) using 0.4×TBE buffer containing 90 mM Tris-borate, 2 mM EDTA, and 10 mM NaCl, pH 8.3, at 8°C. DNA bands were observed by UV shadowing. Bindings of the HJs by the RuvC protein were assayed using 5% polyacrylamide gels (29:1 ratio of acrylamide to bisacryl-

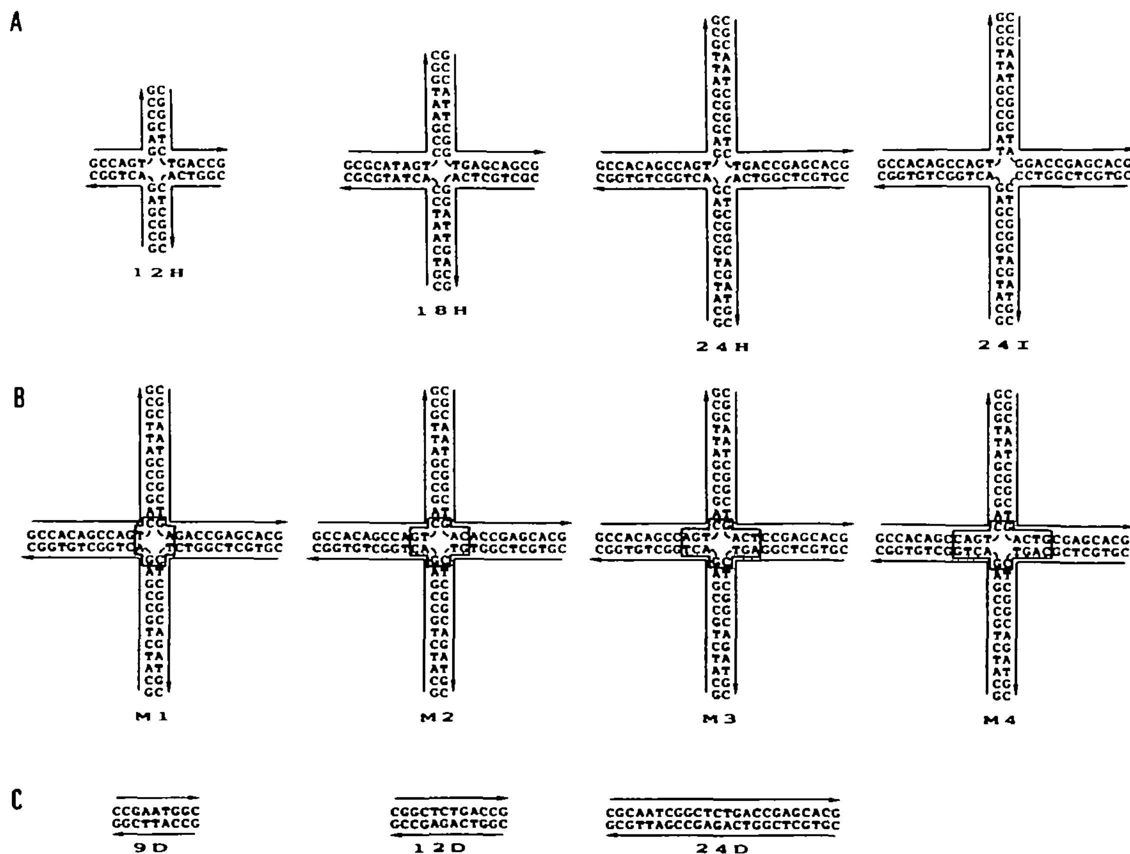

Fig. 1. **Nucleotide sequences of the synthetic Holliday junctions and duplex DNAs which were studied in this work.** (A) Immobile junctions. The HJs (12H, 18H, 24H, and 24I) were constructed from four 12-mer, four 18-mer, and four 24-mer oligonucleotides, respectively. Each arm in the HJ has the same length. The arrows indicate the direction from the 5′ to 3′ ends of oligonucleotides. (B) Mobile junctions. The mobile HJs consist of four 24-mer oligonucleotides. The boxed parts indicate mobile homologous regions. The mobile HJs differ only in the sequences of the right hand arms. The positions of AT and GC base-pairs were the same in all the mobile HJs. However, the mobile HJs were designed, based on 24H, to give inverted repeat sequences flanking the junction with increasing length from M1 to M4. (C) Duplexes. 9D corresponds to the north arm of 18H. 12D and 24D are duplexes composed of one strand of the synthetic four-way junctions (12H and 24H) and their complementary strands.

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amide) for the complex formation. Reaction mixtures (15 μl) containing various amounts of the RuvC protein and ^{32}P-labeled four-way junction in binding buffer [20 mM HEPES-NaOH, (pH 7.6), 50 mM Na glutamate, 2 mM DTT, and 10% glycerol] were incubated at 5°C for 30 min. To each sample, 5 μl of loading buffer [20 mM HEPES-NaOH, (pH 7.6), 40% sucrose, and 0.1% bromophenol blue] was added and the mixtures were immediately applied to the gel. Electrophoresis was carried out in a buffer containing 40 mM Tris-acetate (pH 8.0) and 1 mM EDTA. Gels were dried and DNA bands were visualized by autoradiography.

Spectroscopic Measurements—UV absorption spectra of HJs were recorded on a Shimadzu UV-250 spectrophotometer. The absorbance *vs.* temperature profiles of the HJs and duplexes were measured using a Hitachi 30 spectrophotometer equipped with a JASCO PTC-343 temperature programmer. The temperature was scanned at a heating rate of 1°C/min. CD spectra were recorded on a JASCO J-720 spectropolarimeter. The temperature was controlled using a thermo-jacketed cell and a HAAKE F3 circulating bath.

RESULTS

Four immobile HJs were constructed from 12-mer, 18-mer, and 24-mer oligonucleotides so as to have four arms with the same lengths (Fig. 1A). The four mobile HJs M1, M2, M3, M4 were derived from an immobile HJ (24H), in which the positions of the A and T residues in the AT base-pair and/or the G and C residues in the GC base-pair were inverted at the junction (Fig. 1B). The mobile HJs from M1 to M4 have symmetrical sequences (boxes) as homologous cores containing 4, 6, 8, and 10 base-pairs, respectively.

Formation of the HJ Structures Monitored by Gel Electrophoresis—The electrophoretic mobilities of oligonucleotides in a nondenaturing gel are influenced by molecular size and shape, and the extent of base pairing (*18, 19*). Figure 2A shows that HJs (18H and 24H, lanes g and h) migrated in a native gel with specifically slower mobility than that of a duplex (24D, lane e) formed by a combination of two 24-mer oligonucleotides. Similarly, all four mobile HJs assembled from four 24-mer oligonucleotides migrated with the same mobility as that of 24H (data not shown). However, for the annealed stoichiometric mixture of four 12-mer oligonucleotides, a unique band with a mobility slower than that of the 12D duplex was not observed even at low temperature (8°C) in the presence of NaCl (10 mM). The immobile and mobile HJs constructed from four 24-mer oligonucleotides formed stable complexes at room temperature without NaCl, but 18H migrated as a smear band at room temperature even in the presence of NaCl (10 mM) (data not shown). Thus, the HJs constructed from four 24-mer strands are the smallest stable HJs at room temperature, and may be suitable for further physicochemical studies.

Furthermore, we examined whether or not the synthetic HJs with and without homologous cores could be recognized by the RuvC protein. The binding of the RuvC protein to HJs was detected by gel retardation assay (*5*). The mobilities of the HJs (18H, 24H, M1, M2, M3, and M4) labeled with ^{32}P were reduced by RuvC binding in all cases (Fig. 2, B and C). No difference was detected in the affinities of the RuvC protein for these HJs with different sequences in the core region (Fig. 2C). These results suggested that the immobile and mobile HJs constructed from four 24-mer strands take a proper form that can interact with the RuvC protein.

Stability of HJs—The relative stabilities of different HJs and duplexes were assessed by thermal denaturation monitored by measuring hyperchromism at 270 nm. Figure 3A shows the melting profiles of the immobile HJs and related duplexes. The melting temperatures of 18H and 24H were similar to those of the duplexes 9D and 12D of the same arm length, respectively. This fact indicates that the length of the arms of HJs is the major determinant of the stability. The melting behaviors of the HJs were more cooperative and their degrees of hyperchromism were greater than those of the respective duplexes which showed similar melting temperatures. The melting profile of 12H did not indicate the presence of the HJ structure at the same total nucleotide concentration. Figure 3B shows the melting profiles of four mobile HJs and immobile 24H and Table I gives their melting temperatures. The structural differences among these HJs reside only in the core sequences at the junctions, while the GC contents are the same. These results revealed the tendency that the larger the region of the HJ homology, the higher is the T_m of the HJ and the smaller is the melting cooperativity.

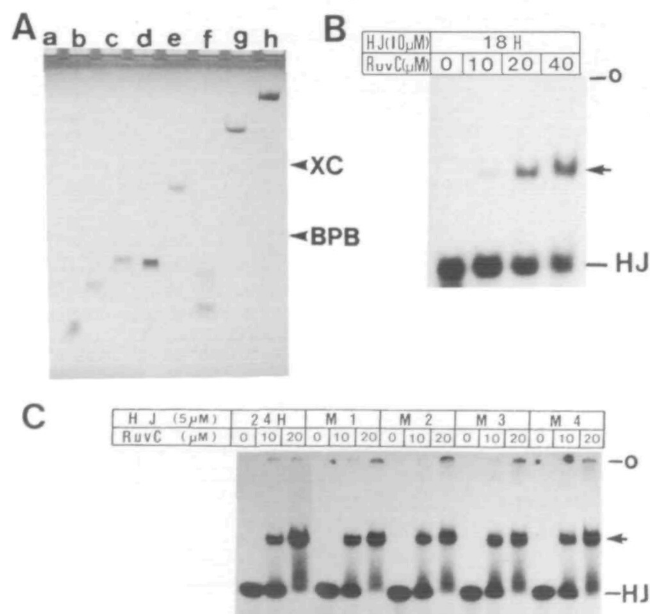

Fig. 2. **Demonstration of formation of HJs and binding of the RuvC protein to HJs.** (A) Electrophoresis of oligonucleotides in a native gel. The first three lanes, a, b, and c, contained single-stranded oligomers, 12-mer, 18-mer, and 24-mer, respectively. The lanes d and c contained the two duplexes, 12D and 24D, respectively. (B) Binding to the synthetic immobile and mobile junctions by RuvC protein. Various concentrations of the RuvC protein were incubated with a ^{32}P-labeled synthetic immobile four-way junction (10 μM). (C) Binding to the synthetic immobile and mobile junctions by RuvC protein. Various concentrations of RuvC proteins were incubated with ^{32}P-labeled HJs. The arrow indicates the HJs retarded due to the formation of complexes with the RuvC protein. The origin of the gel electrophoresis is indicated by O.

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The stability of HJs and duplexes formed by oligonucleotides depends on the strand concentration (*20, 21*). Figure 3C shows plots of the reciprocal melting temperature *vs.* the logarithm of the HJ concentration for 18H and 24H in 0.1 M NaCl and 0.01 M Na cacodylate (pH 7.0), derived from the melting profiles. Neither 18H nor 24H significantly deviated from linear behavior. The simplest assumption is that the HJs composed of four short oligomers undergo a transition in the two-state model of the helix-coil transition (*20*). In fact, we observed the isosbestic points in the UV spectra of the HJs (data not shown).

The stabilization of HJ by adding NaCl, KCl, $MgCl_2$, and $CaCl_2$ to the HJ solution in 0.1 M NaCl and 0.01 M Na cacodylate (pH 7.0) was monitored in terms of the elevation of T_m. Figure 4 shows that the T_m of 18H increased in proportion to the logarithm of the concentrations of the monovalent cations. The divalent cations at 10 mM raised T_m by about 10°C. The degree of T_m elevation was about twice that of the duplex 12D. Similarly, the stability of mobile HJs was improved by the addition of Mg^{2+} (Table I).

Circular Dichroic Spectra—Figure 5A (a-c) shows the temperature-dependent CD spectra of 12H, 18H, and 24H in 0.1 M NaCl and 0.01 M Na cacodylate (pH 7.0), respectively. Under these conditions, 12H did not form a four-way junction. The spectral features of 18H and 24H are the same as those of the B-form DNA. This finding was in agreement with the result obtained for an immobile HJ composed of four 16-mer strands (*20*).

Figure 5B shows the CD spectra of the HJs composed of four 24-mer strands in the absence of Mg^{2+}. Their spectra were compared with each other to ensure that the HJ structures were fully formed at 5°C. Figure 5B-a shows the CD spectra of 24H, 24I, and M1, in which they differ only at the position of two AT and two GC base-pairs at the

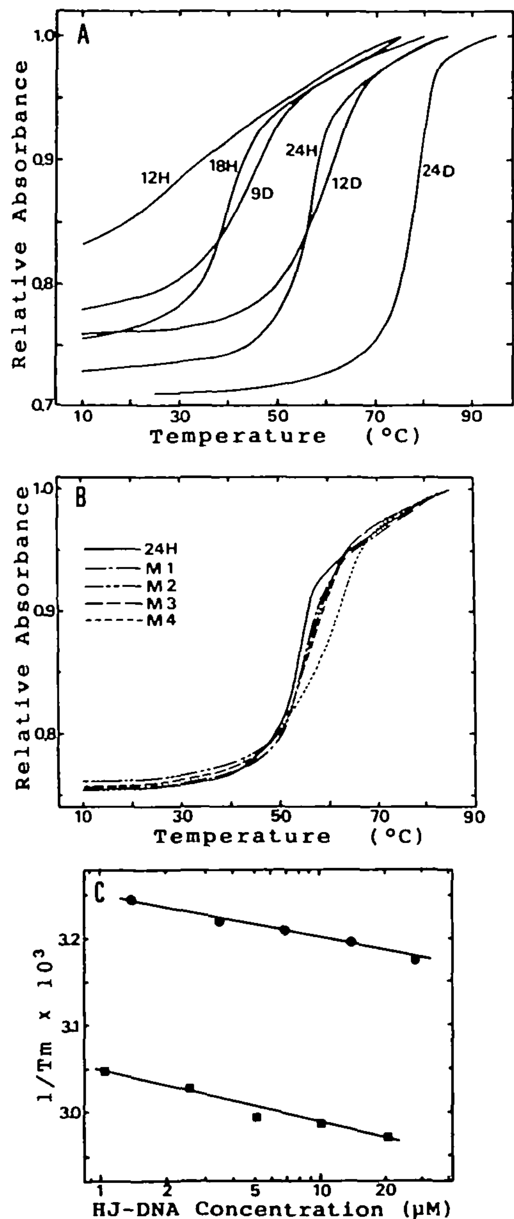

Fig. 3. **Melting behaviors of HJs as measured in terms of UV absorption.** (A) Normalized melting curves for the immobile four-way junctions and duplexes. The melting profiles of the HJs and the duplexes (about 1 A_{260} unit/ml) were measured in 0.1 M NaCl-0.01 M Na cacodylate (pH 7.0) at 270 nm. (B) Normalized melting curves for the mobile four-way junctions. Melting profiles of the HJs (about 1 A_{260} unit/ml) were measured in 0.1 M NaCl-0.01 M Na cacodylate (pH 7.0) at 270 nm. (C) Plots of T_m^{-1} *vs.* log (HJ concentration) for 18H (●) and 24H (■). The T_ms were measured in 0.1 M NaCl-0.01 M Na cacodylate (pH 7.0) at 270 nm.

TABLE I. **T_ms of immobile and mobile HJs in the absence and in the presence of Mg^{2+}.** The T_ms of HJs (1 A_{260} unit/ml) were measured in 0.1 M NaCl or in 0.1 M NaCl-0.01 M $MgCl_2$ (0.01 M Na cacodylate, pH 7.0). ΔT_m indicates the difference in the T_m in the absence and presence of magnesium ions.

HJ	T_m ($-Mg^{2+}$) (°C)	T_m ($+Mg^{2+}$) (°C)	ΔT_m (°C)
24H	54.2	64.6	10.4
M1	54.7	64.8	10.1
M2	55.0	65.5	10.5
M3	55.2	66.0	10.8
M4	62.0	66.1	4.1

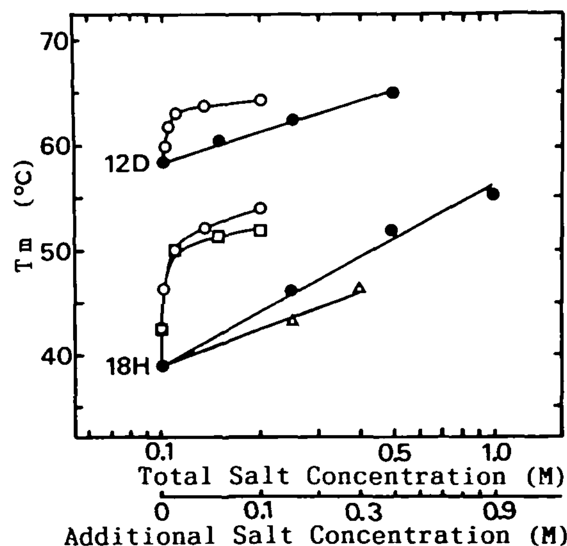

Fig. 4. **Dependence of the T_ms of the four-way junction (18H) and the duplex (12D) on the concentrations and types of metal cations.** The T_ms of 18H and 12D were measured in 0.1 M NaCl and 0.01 M Na cacodylate (pH 7.0) with additional salts, NaCl (●), KCl (△), $MgCl_2$ (○), and $CaCl_2$ (□).

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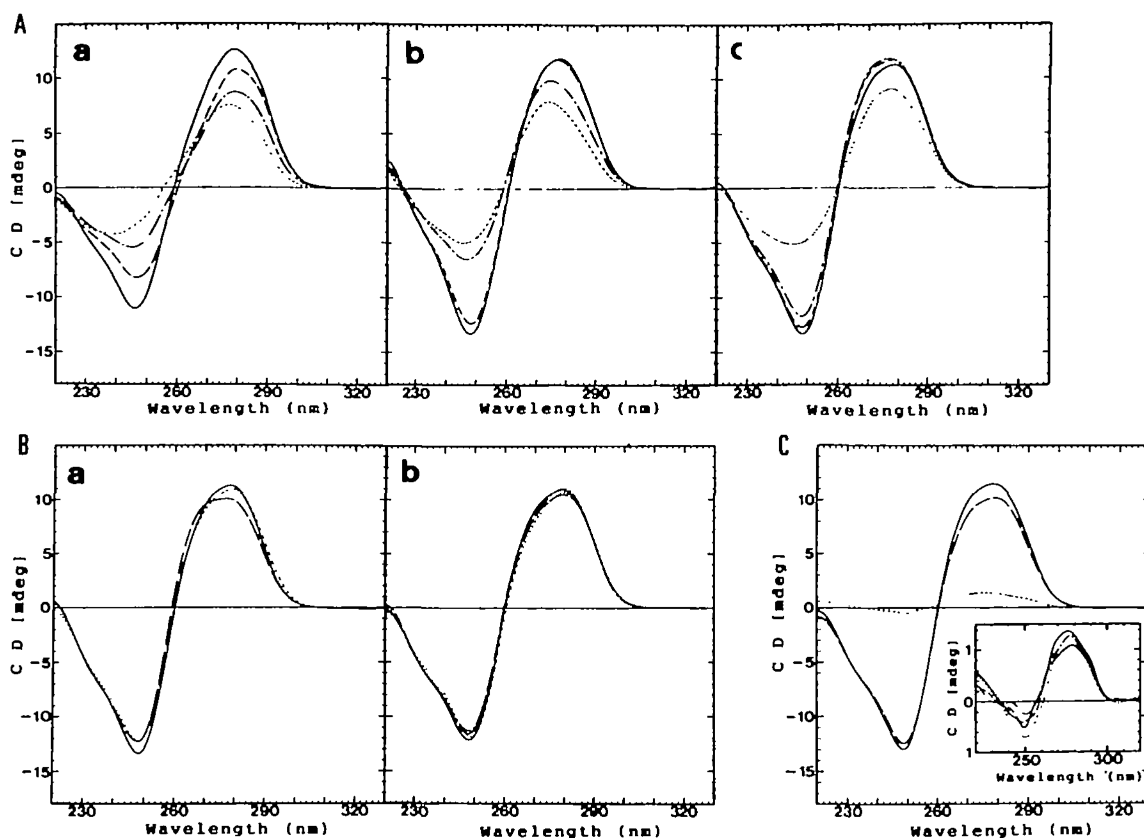

Fig. 5. **CD spectra of HJs.** (A) The CD spectra of immobile four-way junctions, 12H (a), 18H (b), and 24H (c). —, 5°C; – –, 25°C; —-—, 45°C; ----, 65°C in 0.1 M NaCl-0.01 M Na cacodylate (pH 7.0). Oligomer concentration, 1 A_{260} unit/ml. (B) (a), CD spectra of 24H (—), 24I (– –), and M1 (----); (b), CD spectra of four mobile HJs: M1 (—), M2 (– –), M3 (—-—), and M4 (----). Measured in 0.1 M NaCl and 0.01 M Na cacodylate (pH 7.0) at 5°C. Oligomer concentration: 1 A_{260} unit/ml. (C) Difference spectrum (----) of M1 in the absence (—) and presence of Mg^{2+} (– –). The inset displays the difference spectra of M1 (—), M2 (– –), M3 (—-—), and M4 (----) between the values in the absence and presence of Mg^{2+}.

junctions. However, their spectra differed significantly, particularly around 275 nm. This result indicates that the structure of HJ at the junction point is primarily determined by the flanking sequence. There were no remarkable differences among the mobile HJs (Fig. 5B-b).

Figure 5C shows the spectra of 24H in the absence and presence of Mg^{2+} (10 mM), represented by solid and dashed lines, respectively. The difference spectrum is represented by a dotted line. The CD band of 24H around 275 nm was reduced by the addition of Mg^{2+}. This change would be attributable to conformation change at the junction. This phenomenon was also observed for the mobile HJs (inset of Fig. 5C).

DISCUSSION

Four-way junctions (HJs), which are intermediates of homologous recombination, are normally unstable due to branch point migration originated in their internal sequence symmetry. The physical properties of natural HJ are difficult to characterize due to the rapid branch point migration (*22*). Consequently, only synthetic immobile HJs have been physically analyzed. However, since the branch migration is intrinsic to HJ, the HJs containing a homologous core at the junction point should be systematically studied at the oligonucleotide level. A few mobile HJs containing monomobile or bimobile junctions have been analyzed (*23, 24*).

For the formation of stable HJ structure by four strands with 66% GC content, 12 bp arm length was required regardless of immobile or mobile sequences. This result is consistent with the prediction by Seeman and Kallenbach (*25*). The formation of four-strand HJ structures was detected in terms of slow mobility on gel electrophoresis as well as the binding to the RuvC protein. However, the HJs containing homologous sequences gave relatively higher T_m than that of an immobile HJ of the same length and GC content, although the cooperativity of the melting was reduced. This might be explained by an entropical advantage owing to the presence of multiconformers of homologous HJs in contrast to the unique conformation of an immobile HJ.

The increase of the helix-coil transition temperature of DNA is generally due to the reduction of repulsion force between negatively charged phosphate groups. The addition of divalent cations, Mg^{2+} and Ca^{2+}, similarly raised the T_m of the immobile HJ (18H). The stabilization of the HJ by the divalent cations was more effective than that of the duplex. In the comparison of mobile HJs (M1, M2, and M3) and an immobile one (24H), significant differences in the

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elevation of T_m were not observed on the addition of Mg^{2+}. These results suggest that the divalent cations are important for the reduction of repulsion force at the branch point of all HJs, whether mobile or immobile. A fluorescence resonance energy transfer experiment has shown that the addition of Mg^{2+} induced a right-handed non-crossed antiparallel structure (X-structure) at the four-way DNA junction (*10-12*). Therefore, this elevation of stability of HJ might be coupled with a conformation change of HJ from extended forms to a unique antiparallel staked X-structure.

For the mobile HJs, the Mg^{2+} ion has a more significant role. Resolution of HJ by the RuvC protein is stimulated by the presence of Mg^{2+} and Mn^{2+} as divalent metal cofactors (*8*). The present experiment indicates that the mobile and immobile HJs can take conformations which fit the binding pocket of the RuvC protein.

The CD spectra of HJs do not differ from that of canonical B form DNA, but there is a slight difference between the mobile and immobile HJs at around 275 nm. It is not clear whether or not this is simply due to the difference in sequence or conformation. Furthermore, it suggests that the four-way junction structure of the HJ might not be entirely unfolded, and this non-uniformity of any configuration may determine the specificity of the staked X-structure of the HJ in the presence of Mg^{2+}.

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