DNA Double-Crossover Molecules[†]

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ABSTRACT: DNA molecules containing two crossover sites between helical domains have been suggested as intermediates in recombination processes involving double-strand breaks. We have modeled these double-crossover structures in an oligonucleotide system. Whereas the relative orientations of the helical domains must be specified in designing these molecules, there are two broad classes of the molecules, the parallel, DP, and antiparallel, DA, molecules. The distance between crossover points must be specified as multiples of half-turns, in order to avoid torsional stress in this system; hence, there are two further subdivisions, those double-crossover molecules separated by odd, O, and even, E, numbers of half-turns. In addition, the parallel molecules with odd numbers of half-turns between crossovers must be divided into those with an excess major or wide-groove separation, W, or those with an excess minor- or narrow-groove separation, N. We have constructed models of all five of these classes, DAE, DAO, DPE, DPOW, and DPON. DPE molecules containing 1 and 2 helical turns between crossovers have been constructed; the DAE molecule contains 1 turn between crossovers, and the DAO, DPOW, and DPON molecules contain 1.5 helical turns between crossovers. None of the parallel molecules is well-behaved; the molecules either dissociate or form multimers when visualized on native polyacrylamide gels. In contrast, antiparallel molecules form single bands when assayed in this fashion. Hydroxyl radical autofootprinting analysis of these molecules reveals protection at expected sites of crossover and of occlusion, suggesting that all the complexes contain linear helix axes that are roughly coplanar between crossovers. However, the DPOW molecule and the DPE molecule with 2 turns between crossovers show decreased protection in the portion between crossovers, suggesting that their helices may bow in response to charge repulsion. We conclude that the helices between parallel double crossovers must be shielded from each other or distorted from linearity if they are to participate in recombination. We have analyzed the possibilities of branch migration and crossover isomerization in double-crossover molecules. Parallel molecules need no sequence symmetry beyond homology to branch migrate, but the sequence symmetry requirements for antiparallel molecules restrict migration to directly repetitive segments that iterate the sequence between crossovers. Crossover isomerization appears to be a very complex process in parallel double-crossover molecules, suggesting that it may be catalyzed by topoisomerases if it occurs within the cell.

The Holliday (1964) crossover junction is one of the key paradigms in the molecular biology of recombination. The structure contains four strands of DNA forming four double-helical arms that flank a central branch point. The Holliday junction is unstable in small molecules, because it can resolve to two duplex molecules by an isomerization process known as branch migration (Thompson et al., 1976). The junction has been modeled in recent years by an analog system, four-arm immobile DNA junctions, whose sequences prevent them from branch migrating (Seeman, 1982; Kallenbach et al., 1983). Immobile junctions have been characterized by numerous physical and enzymatic studies (Kallenbach et al., 1983; Seeman et al., 1985; Wemmer et al., 1985; Marky et al., 1987; Cooper & Hagerman, 1987, 1989; Churchill et al., 1988; J.-H. Chen et al., 1988, Mueller et al., 1988, 1990; Duckett et al., 1988; Murchie et al., 1989; S.-M. Chen et al., 1991; Lu et al., 1991, 1992). This work has established that the four double-helical arms of the molecule appear to assemble into two helical stacking domains (Cooper & Hagerman, 1987, 1989; Churchill et al., 1988; Duckett et al., 1988; Murchie et al., 1989). Two strands form a crossover link between helical domains, and the other two strands follow an approximately continuous helical trajectory, each confined within a single domain. The sequence flanking the branch point appears to

determine the particular pairs of duplex segments that stack to form the helical domains (Chen et al., 1988; Duckett et al., 1988). The continuous strands can be oriented roughly parallel or antiparallel to each other. The helical domains assume a structure in which the antiparallel orientation appears to be favored over the parallel orientation (Cooper & Hagerman, 1987, 1989; Duckett et al., 1988; Murchie et al., 1989; Lu et al., 1991).

The DNA double-crossover molecule contains two crossover links between helical domains. This structure has been proposed as an intermediate in relation to the double-strand break model for recombination (e.g., Thaler & Stahl, 1988; Sun et al., 1991). The properties of these molecules are therefore of great interest, and we have sought to construct small synthetic analogs of them and to characterize their structures. The models that are presented in the literature always involve parallel helical domains. However, in light of the finding that antiparallel molecules are more stable than parallel molecules (Cooper & Hagerman, 1987, 1989; Duckett et al., 1988; Murchie et al., 1989; Lu et al., 1991), we have modeled both parallel and antiparallel molecules. It is possible to control the position of crossovers by selecting sequences that will maximize their Watson–Crick base pairing by forming the crossovers, in much the same way that has been done for molecules containing a single branch point (Seeman, 1982; Kallenbach et al., 1983).

Molecules containing multiple crossovers are not as simple to model as those containing a single crossover, because the two crossover points must be phased relative to each other, if they are close enough to be torsionally correlated (e.g., Allison & Schurr, 1979). In molecules with short separations between crossover points, torsional stress or helical disruption is likely to be introduced unless there is an integral number of half-helical turns of DNA between the two crossover points. The molecules containing even and odd numbers of half-helical turns are quite dissimilar: There are two different structural motifs for the antiparallel molecules but there are *three* motifs for the parallel molecules, because there are two different types of parallel associations with an odd number of helical half-turns between crossover points. The five different types of molecules that have been built are illustrated schematically in Figure 1. For clarity, we refer to the portion of a molecule between the crossover points as the central part of the molecule and to parts beyond the crossover points as external parts of the molecule. Note that our drawing convention places arrowheads on the 3′ ends of strands.

The two different antiparallel double-crossover molecules in Figure 1 contain an even number of half-helical turns between branch points, DAE, or an odd number, DAO. The three different parallel double-crossover molecules are also differentiated by the number of half-helical turns they contain between branch points, an even number, DPE, or odd, DPOW or DPON. Parallel double-crossover molecules with an odd number of half-turns between branch points will contain an excess major or minor groove isolated between the two crossovers. This region must therefore be designed to differ from the number of residues per helical turn, N, by approximately ϕN or $(1 - \phi)N$ residues, where ϕ is the golden ratio (0.618), as this is roughly the angular difference between the major and minor grooves of B-DNA (Harel et al., 1986). We use 16 residues between branch points to produce a molecule with an excess major-groove (wide) (DPOW) spacing and 14 residues between branch points to produce a molecule with an excess minor-groove (narrow) (DPON) spacing.

As shown in Figure 1, one must use five strands to build an antiparallel double-crossover molecule of class DAE; the central cyclic strand is almost necessarily nicked, because it is extremely difficult to seal it shut. There is approximate 2-fold backbone symmetry (neglecting the nick) at the center of the molecule perpendicular to the page, as indicated by the lens-shaped symbol at the center of the drawing. The symmetry relates the bold strands to each other, the light strands on the ends to each other, and the central strand to itself. The antiparallel structure of class DAO is shown in Figure 1 containing 1.5 turns between crossover points. Surprisingly, this complex requires only four strands, not five. The 2-fold backbone symmetry in this molecule passes horizontally through the middle of the molecule in the plane of the page. This symmetry is indicated by the horizontal pair of arrows. The symmetry relates the bold strands to the light strands. One central helix contains the 3′ portions of the long strands and the other central helix contains the 5′ portions. The direction of the symmetry axis implies that the two helices that span between the branch points need not be exactly the same length: Model building (Seeman, 1988) suggests that the molecule might be less strained if the helix containing 5′ ends is one nucleotide pair shorter than the helix containing 3′ ends, because this condition appears to result in the shortest angular gap in forming the second crossover.

The parallel molecules all contain a 2-fold symmetry element that passes vertically within the plane of the page, as indicated by the arrows. This potential structural axis can be coincident
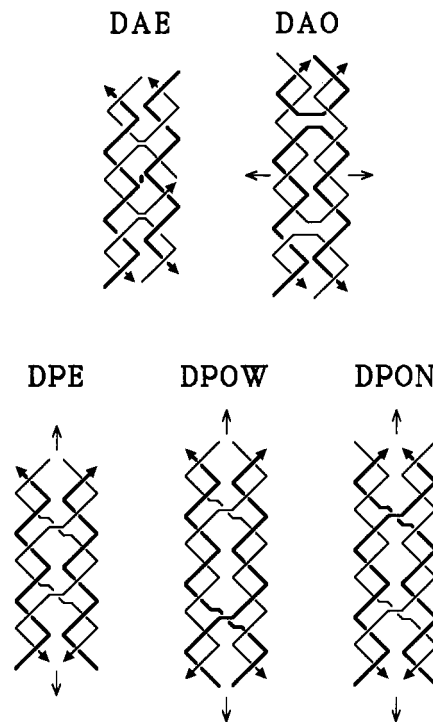

FIGURE 1: Schematic drawings of the five different structural arrangements of double-crossover structures. The structures shown are named by the acronyms describing their basic characteristics. All names begin with D for double crossover. The second character refers to the relative orientation of their two double-helical domains, A for antiparallel and P for parallel. The third character refers to the number (modulus 2) of helical half-turns between crossovers, E for an even number and O for an odd number. A fourth character is needed to describe parallel double-crossover molecules with an odd number of helical half-turns between crossovers. The extra half-turn can correspond to a major-groove (wide) separation, designated by W, or an extra minor-groove (narrow) separation, designated by N. The strands are drawn as zigzag helical structures, where two consecutive, perpendicular lines correspond to a full helical turn for a strand. The arrowheads at the ends of the strands designate their 3′ ends. The structures contain implicit symmetry, which is indicated by the conventional markings, a lens-shaped figure (DAE) indicating a potential dyad perpendicular to the plane of the page, and arrows indicating a 2-fold axis lying in the plane of the page. Note that the dyad in DAE is only approximate, because the central strand contains a nick, which destroys the symmetry. The strands have been drawn with two different thicknesses, as an aid to visualizing the symmetry. In the case of the parallel strands, the bold strands are related to the other bold strands by the 2-fold axes vertical on the page; similarly, the light strands are symmetrically related to the light strands. The 2-fold axis perpendicular to the page (DAE) relates the two bold helical strands to each other and the two light outer crossover strands to each other. The 5′ end of the central double-crossover strand is related to the 3′ end by the same dyad element. A different convention is used with DAO. Here, the dark strands are related to the light strands by the dyad axis lying horizontal on the page. An attempt has been made to portray the differences between the major and minor grooves. Note the differences between the central portions of DPOW and DPON. Also note that the symmetry brings symmetrically related portions of backbones into apposition along the center lines in parallel molecules, in these projections. The same contacts are seen to be skewed in projection for the antiparallel molecules.

with an axis of sequence symmetry if homology is present. In all three cases, the symmetry element relates the bold strands to each other and the light strands to each other. The crossover strands in DPE are the light strands for both branch points, but the DPOW and DPON molecules each contain one bold-strand crossover and one light-strand crossover. Note that the polarities of the chains have been reversed between DPOW and DPON, but the molecules have not simply been turned around. The central cavity visible between branch points in DPOW contains a minor-groove-to-minor-groove contact flanked by two major-groove-to-major-groove contacts; the

central cavity in DPON is a major-groove-to-major-groove contact flanked by two minor-groove-to-minor-groove contacts. A concomitant feature that differentiates DPOW molecules from DPON molecules is the polarity of the strands relative to the central portion of the molecule: The $5' \rightarrow 3'$ direction of each crossover strand at the crossover site points toward the central region in DPOW but points away from it in DPON. Figure 1 cartoons the fact that the backbones are directly opposite each other in the parallel molecules, whereas they are displaced from each other in the antiparallel molecules. This feature renders the parallel molecules qualitatively less stable than the antiparallel molecules.

Here, we report the construction of representative members of each class of molecule, DPE, DPON, DPOW, DAE, and DAO. In addition, we have constructed several variants of the DPE class by varying the lengths of the external arms in a molecule with 1 turn between crossovers and by constructing a molecule, DPE-1, containing 2 turns between crossovers. We have characterized the molecules by native gel electrophoresis, by Ferguson analysis, and by hydroxyl radical autofootprinting. Although it is possible to build molecules of each class, molecules whose helical domains are antiparallel are much better behaved than molecules whose helical domains are parallel.

MATERIALS AND METHODS

Sequence Design. The sequences have been designed by applying the principles of sequence symmetry minimization (Seeman, 1982, 1990), insofar as it is possible to do so within the constraints of this system. The crossover strands of the antiparallel molecules (DAE and DAO) are determined by the selection of the backbone molecule that contains the complement for each nucleotide. The same is true for the parallel molecules separated by an odd number of half-turns (DPOW and DPON), because reversing the crossover strands reverses the polarity of each strand at the crossover site; such a reversal switches the preferred separation of crossovers between 14 and 16 residues per strand, which is not favored by the sequence symmetry associated with immobile junctions. There is no such means of directing the crossover strands for the parallel molecules containing crossovers separated by full turns (DPE). Nevertheless, knowledge of the preferred crossover strands (Churchill et al., 1988) in the immobile junction J1 (Seeman & Kallenbach, 1983) enables one to direct the crossover by choosing the same sequences to flank both junction points. This procedure does not entirely conform to sequence symmetry minimization but appears adequate for the purpose at hand.

It is necessary to make decisions about the number of nucleotide pairs to include between crossover points. Whereas the accepted number of nucleotide pairs in a turn of B-DNA is about 10.5 (Wang, 1979; Rhodes & Klug, 1980), we have selected 10 nucleotide pairs for a single turn, 16 for 1.5 turns, and 21 for 2 turns. Nevertheless, 2 turns may be modeled more effectively in branched systems by 20 nucleotide pairs (Chen & Seeman, 1991). As noted above, we use 14 residues for two minor-groove spacings plus one major-groove spacing, and we use 16 residues for two major-groove spacings plus one minor-groove spacing. We use the same number of nucleotide pairs in both central helices of DAO. The external arms are 8 nucleotide pairs long in DPE, DPE-1, and DAE, 9 in DPOW and DPON, and 11 in DAO. Four variants of DPE have been built, the regular molecule, DPE-8 (called DPE when unambiguous), with external arms containing 8 nucleotide pairs, and three versions with longer external arms, DPE-9, DPE-10, and DPE-11.

Synthesis and Purification of DNA. All DNA molecules in this study have been synthesized on an Applied Biosystems 380B automatic DNA synthesizer, removed from the support, and deprotected using routine phosphoramidite procedures (Caruthers, 1982). DNA strands have been purified by HPLC, utilizing a Du Pont Zorbax Bio Series oligonucleotide column by means of a gradient of NaCl in a solvent system containing 20% acetonitrile and 80% 0.02 M sodium phosphate, as described previously (Wang et al., 1991). Molecules longer than 37 nucleotides are purified from denaturing gels. All molecules are repurified from gels if impurities are detected on denaturing gels.

Formation of Hydrogen-Bonded Complexes. Complexes are formed by mixing a stoichiometric quantity of each strand, as estimated by OD_{260}. This mixture is then heated to 90 °C for 5 min and cooled to the desired temperature by the following protocol: 20 min at 65 °C, 20 min at 50 °C, 30 min at 37 °C, 30 min at room temperature, and (if desired) 30 min at 4 °C. Stoichiometry is determined by titrating pairs of strands designed to hydrogen-bond together and visualizing them by native gel electrophoresis; absence of monomer indicates the end point.

Hydroxyl Radical Analysis. Individual strands of the DPE, DAE, and DAO complexes are radioactively labeled and are additionally gel purified from a denaturing 10–20% polyacrylamide gel. Each of the labeled strands [approximately 1 pmol in 50 mM Tris·HCl (pH 7.5) containing 10 mM $MgCl_2$] is annealed to a 10-fold excess of the unlabeled complementary strands, or it is annealed to a 10-fold excess of a mixture of the other strands forming the complex, or it is left untreated as a control, or it is treated with sequencing reagents (Maxam & Gilbert, 1977) for a sizing ladder. In order to ensure that the monomer of the four-strand species of DPON and DPOW is the predominant species in solution, these molecules are characterized at 1:1:1:1 strand ratios and at concentrations of 450 nM. The DPE molecule with a central region containing two helical turns per domain, DPE-1, is characterized at 80 nM concentration and at 1:1:1:1 strand ratios for the same reason. Under the conditions used, the monomer of DPON, DPOW, and DAE-1 is the dominant species in solution, constituting about 90–95% of the material present. The samples are annealed by heating to 90 °C for 3 min and then cooled slowly to 4 °C. Hydroxyl radical cleavage of the double-strand and double-crossover complex samples for all strands takes place at 4 °C for 2 min (Tullius & Dombroski, 1985), with modifications noted by Churchill et al. (1988). The reaction is stopped by addition of thiourea. The sample is dried, dissolved in a formamide/dye mixture, and loaded directly onto a 10–20% polyacrylamide/8.3 M urea sequencing gel. Autoradiograms are scanned with a Hoefer GS300 densitometer in transmission mode. All features cited in protection patterns are reproduced twice for DPON and DPOW and at least three times for the other complexes.

Polyacrylamide Gel Electrophoresis: Denaturing Gels. These gels contain 8.3 M urea and are run at 55 °C. Gels contain 10% acrylamide (19:1 acrylamide:bisacrylamide). The running buffer consists of 89 mM Tris·HCl, pH 8.0, 89 mM boric acid, and 2 mM EDTA (TBE buffer). The sample buffer consists of 10 mM NaOH and 1 mM EDTA, containing 0.1% xylene cyanol FF tracking dye. Gels are run on an IBI Model STS 45 electrophoresis unit at 70 W (50 V/cm), constant power, dried onto Whatman 3MM paper, and exposed to X-ray film for up to 15 h.

Native Gels. Gels contain 8–20% acrylamide (19:1 acrylamide:bisacrylamide). DNA is suspended in 10–25 μL of a solution containing 40 mM Tris·HCl, pH 8.0, 20 mM acetic
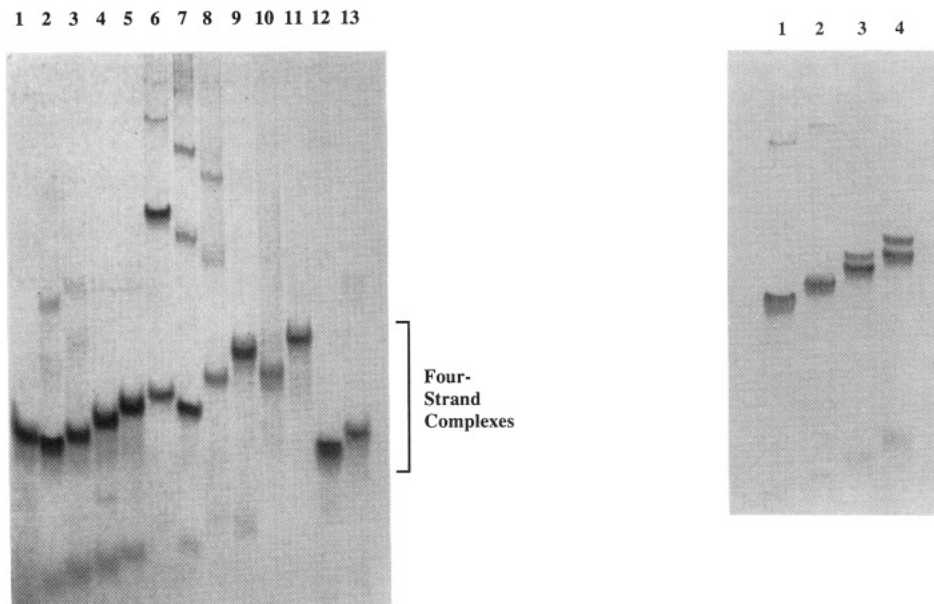
FIGURE 2: Native gels of double-crossover molecules and related junctions. (A, left) Complete set of molecules constructed in this study. This is a native 8% polyacrylamide gel containing stoichiometric mixtures of the complexes. For complexes containing equal-length strands, 1 μg of each strand is present in each complex. For complexes with strands of unequal length, 1 μg of the longest strand is present and proportionately less of the other strands. Lane 2 contains a DPE molecule with one turn in the central region and 8 nucleotide pairs in its external arms. Lane 1 contains a control junction, which contains the domains with the same sequence but lacking the second crossover. Lanes 3, 4, and 5 contain the same DPE molecule, with external arms containing 9, 10, and 11 nucleotide pairs, respectively. Note that the band corresponding to the junction becomes progressively thicker as the external arms lengthen but that an extra band of lower mobility decreases in intensity in response to lengthening arms. Bands corresponding to less than an entire complex can be noted in the more rapidly moving bands near the bottom of the gel. Lane 6 contains a DPOW molecule, lane 7 contains a DPON molecule, and lane 8 contains a DPE-type molecule, DPE-1, containing two helical turns in its central region. Each of these parallel molecules is characterized by the presence of multimers in equilibrium with the four-strand monomer. Lane 9 contains a control junction, whose domains are designed to contain the sequences as DPE-1. Lane 10 contains a DAO molecule with 1.5 turns in its central portion, and lane 11 contains a control junction for the DAO molecule. Lane 12 contains a DAE molecule, composed of 5 strands of DNA, and lane 13 contains a control junction for it. The notation four-strand complexes on the right of the drawing is meant to include the DAE five-strand complex. Note the absence of multimers or breakdown in the lanes containing the antiparallel double-crossover molecules (10 and 12), in contrast to the lanes containing the parallel double-crossover molecules. (B, right) High-resolution gel of DPE molecules with external arms of different lengths. This native 8% gel shows the four DAE molecules of lanes 2–5 in the left panel in more detail. The dimerization of the shorter molecules is evident on this gel. The splitting of the larger molecules into two bands is also clearly seen here.

acid, 2 mM EDTA, and 12.5 mM magnesium acetate (TAEMg buffer); the quantities loaded vary as noted below. The solution is boiled and allowed to cool slowly to 4 °C. Samples are then brought to a final volume of 20 μL with a solution containing TAEMg, 50% glycerol, and 0.02% each bromophenol blue and xylene cyanol FF tracking dyes. Gels are run on a Hoefer SE-600 gel electrophoresis unit at 11 V/cm at 4 °C, and exposed to X-ray film for up to 15 h or stained with Stainsall dye. Absolute mobilities (centimeters per hour) of native gels run at 4 °C are measured for Ferguson analysis; logarithms are calculated to base 10.

RESULTS

Formation of the Complexes. Figure 2A shows a native polyacrylamide gel containing stoichiometric mixtures of representatives each of the five classes of molecules illustrated in Figure 1, as well as a number of control molecules. Lane 2 contains a DPE molecule, all of whose strands are 26 nucleotides long; there are 10 nucleotide pairs on each helix between crossovers and 8 nucleotide pairs in each of the four external arms. Lane 1 contains a control junction designed to possess the same sequence in its helical domains, but it only has a single crossover; two opposite arms contain 8 nucleotide paris, and the other two contain 18 nucleotide pairs. The two continuous helical strands are the same in both the junction and the DPE molecule. The mobility of the DPE molecule in lane 2 is reproducibly different from that of the control junction in lane 1. The difference in mobility suggests that the molecules are different and that the DPE molecule really does contain the two crossovers it is designed to have. If one

of the crossovers does not form, and the 8-mer external regions beyond the second crossover form random mispairs, one would expect a mobility similar to that of the control single junction.

Lanes 3, 4, and 5 contain the same DPE molecule but with external arms of 9, 10, and 11 nucleotide pairs, respectively. The primary molecular band thickens in a characteristic fashion as the external arms lengthen. This may be seen more clearly from the gel in Figure 2B, which is better resolved. The molecular band actually splits into two different species. There is a third, low-mobility band visible in the first two lanes, corresponding to a species migrating as though it contains two copies of each strand; relative to the four-strand species with a single copy of each strand, the mobility of this species is within 6% of the expected mobility of an eight-strand species (data not shown). This material is progressively more pronounced for DPE molecules built with shorter external arms. Charge repulsion between parallel arms is likely to be progressively stronger for longer molecules. The formation of an eight-strand dimeric species can relieve the molecule of this stress by flexing to form a quadrilateral-like structure in which the 5' and 3' ends of strands 2 and 4 pair to different molecules of strands 1 and 3, rather than the same one. Dimers and larger multimers are expected to have the connectivity of oligolaterals, similar to those formed deliberately (Petrillo et al., 1988).

Lane 6 contains a DPOW molecule, and lane 7 contains a DPON molecule. The monomers of both these species are seen to be highly unstable: Bands that correspond to dimers through tetramers or pentamers of the fundamental four-strand unit can be seen above the monomer in these lanes; the

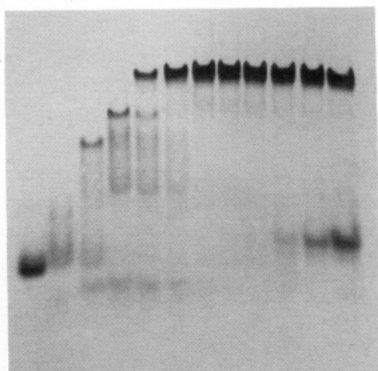|	1	2	3	4	5	6	7	8	9	10	11	12
Strand 1	0	0	0	0	.2	.5	.9	1.0	1.1	1.5	2.0	3.0
Strand 2	0	0	0	1	1	1	1	1	1	1	1	1
Strand 3	2	1	1	1	1	1	1	1	1	1	1	1
Strand 4	0	1	1	1	1	1	1	1	1	1	1	1
Strand 5	0	0	1	1	1	1	1	1	1	1	1	1

FIGURE 3: Stoichiometry determination for DAE. This native 12% gel contains the molecules that compose the DAE complex. At the top of the gel the amount of each strand in micrograms is shown. The gel shows a titration by strand 1 of the complex consisting of strands 2, 3, 4, and 5. Incomplete complexes of branched structures are usually unstable (e.g., Kallenbach et al., 1983), showing a number of dissociation products. These are evident in lanes 4–6 but are clearly absent in lane 8, which contains 1:1:1:1:1 ratios of the component strands. As more of strand 1 is added to the complex, excess strand 1 can be seen as the band of high mobility that appears in lanes 10–12.

plot of log (mobility) of these species versus multimer number is linear (not shown). Lane 8 contains a DPE molecule, DPE-1, with 2 turns (21 nucleotide pairs) in its central region. A well-defined dimer band is also evident in this lane, in addition to a poorly-defined band of intermediate mobility. Lane 9 contains a single junction, analogous to the one in lane 1, whose strands are the same length as the strands of DPE-1 (37 nucleotides). Its mobility is markedly lower than that of DPE-1. The DPON and DPOW molecules force two phosphate–phosphate chain contacts in the central region (Figure 1), and DPE-1 forces three such contacts in that region. None of these molecules is as well behaved as ordinary branched junctions; those complexes form stable monomers without a proclivity to oligomerize (Kallenbach et al., 1983; Wang et al., 1991).

Lane 10 contains an antiparallel double-crossover molecule with three half-turns in its central region (DAO), and lane 12 contains an antiparallel double-crossover molecule with one full turn in its central region (DAE). Lane 11 contains a control single junction for DAO, and lane 13 contains a control junction for DAE; the short arms are adjacent rather than opposite for these junctions. Both double-crossover molecules can be seen to form clean single bands, with no tendency to oligomerize. Their mobilities are distinct from the mobilities of junctions lacking the second crossover.

It is possible to titrate three-strand mixtures (for four-strand complexes) or four-strand mixtures (for DAE) with the missing strand in order to estimate the stoichiometry of the complexes formed, as done previously (Kallenbach et al., 1983; Chen et al., 1988). The results are inherently ambiguous for the species that form multimers (DPOW, DPON, DPE, and DPE-1) but are clear for DAE (Figure 3) and DAO (not shown).

Hydroxyl Radical Autofootprinting Analysis. We have gained considerable insight into the structures of unusual DNA complexes in solution by means of autofootprinting analysis of the chemical attack patterns of hydroxyl radicals generated by Fe(II)EDTA^{2-}. We have used the technique to characterize immobile branched junctions (Churchill et al., 1988), mono-mobile branched junctions (Chen et al., 1988), tethered

junctions (Kimball et al., 1990), five-arm and six-arm branched junctions (Wang et al., 1991), and antijunctions and meso-junctions (Du et al., 1992). The experiment is performed twice for every strand in each complex: First, the strand is labeled, combined with the other strands of the complex, exposed to hydroxyl radicals, and then analyzed by densitometry on a denaturing gel. Second, the labeled strand is complexed with its Watson–Crick complement to form duplex DNA and is put through the same protocol. The two densitometer traces are then compared. Protection of a site, relative to duplex, has been interpreted as occlusion of that site (Churchill et al., 1988); crossover points usually exhibit strong protection. Whereas the double-crossover molecules are closely related to branched junctions, their footprinting patterns contain many of the features of those molecules. In contrast to the antijunctions and mesojunctions (Du et al., 1992), we find strong and unambiguous protection patterns that confirm the expected structures for these molecules. Tethered junctions (Kimball et al., 1990) were originated in an attempt to establish standard protection patterns for parallel and antiparallel Holliday junctions. Double-crossover molecules are even more constrained than tethered junctions.

The same nucleotides flank the DPE crossovers that flank the J1 junction; the penultimate residues are also identical. Consequently, we see an autofootprint (Figure 4A) that is reminiscent of the J1 pattern (Churchill et al., 1988): The dApdC sequences that flank crossovers on strands 2 and 4 show marked protection relative to linear duplex. Likewise, the dTpdG crossover sequences on strands 2 and 4 that flank the junction are strongly protected, although the signals are inherently more striking on the 5′ parts of the strands. Characteristic weaker protection 1 residue 5′ to the junction (Churchill et al., 1988) is also seen. In contrast, virtually no protection is seen for the dCpdT and dGpdA sequences on strands 1 and 3 that pair with the crossovers. Strong protection can be seen on these strands 4–5 residues 3′ to the crossover. This is the point at which one can expect a minor-groove separation to bring the noncrossover strands together, thereby creating a close contact that can diminish access to the backbone (Churchill et al., 1988).

A second molecule of the DPE class, DPE-1, contains crossovers separated by 21 nucleotide pairs. The same sequences are used to direct the crossovers to take place on strands 2 and 4, and the protection pattern (Figure 4B) bears out the success of this strategy. Again, we note protection 4 nucleotides 3′ to the crossover position on the noncrossover strands. The protection is somewhat weaker here than that seen for the DPE molecule with only a single turn of helix between crossovers. Weak protection can also be noted on both these strands approximately 1 helical turn away from this point, around residue 23. Very weak protection can also be seen at residue 19 on strand 2 and at residue 18 on strand 4, approximately 1 turn from the crossover sites.

The other parallel molecules are DPON and DPOW, whose protection patterns are shown in panels C and D of Figure 4, respectively. In both these complexes, each strand is designed to contain a crossover position. This position is in the 3′ part of each DPON strand, at residues 23 and 24; in contrast, it is in the 5′ part of each DPOW strand, at residues 9 and 10. The protection patterns illustrate clearly that these positions are strongly protected, relative to the duplex standards. One would expect a secondary protection pattern 1 turn 5′ to the crossover point in each strand of DPON; this position also corresponds to the contact position 4 nucleotides 3′ to the other crossover on the noncrossover strands. This protection at position 13–14 (weaker protection sometimes at position

DPE

*1   *2   *3   *4

DS

DC

```
1  CGCAATCC TGCCGTAGCC TGAGCACG
   ........ .......... ........
   GCGTTAGG ACGGCATCGG ACTCGTGC  2

4  CGTAAGCC TGACTTGTCC TGATACCG
   ........ .......... ........
3  GCATTCGG ACTGAACAGG ACTATGGC
```

DPE-1

*1   *2   *3   *4

DS

DC

```
1  CGCAATCC TGCCGTACAGCATGTGTAGCC TGAGCACG
   ........ ...................... ........
   GCGTTAGG ACGGCATGTCGTACACATCGG ACTCGTGC  2

4  CGTAAGCC TGACTTGGTCTAGCAGTGTCC TGATACCG
   ........ ...................... ........
3  GCATTCGG ACTGAACCAGATCGTCACAGG ACTATGGC
```

DPON

*1   *2   *3   *4

DS

DC

```
3  GCATTTCGG ACTAACGCTCGTCA GGTACTGGC
   ......... .............. .........
   CGTAAAGCC TGATTGCGAGCAGT CCATGACCG  2

4  GCGTGTAGG ACTAATGATACTCA GGTCTGTGC
   ......... .............. .........
1  CGCACATCC TGATTACTATGAGT CCAGACACG
```

DPOW

*1   *2   *3   *4

DS

DC

```
   GCACAGAGT CCTTGCGAATCCAGCC TGCTTTACG  2
   ......... ................ .........
3  CGTGTCTCA GGAACGCTTAGGTCGG ACGAAATGC

1  GCCAGTAGT CCGTAACTGTTGCTCC TGATGTGCG
   ......... ................ .........
   CGGTCATCA GGCATTGACAACGAGG ACTACACGC  4
```

DAE

*1   *2   *3   *4   *5

DS

DC

```
1  CGCAGACATCC TGCCGTAGCC TGAGGCACACG
   ........... .......... ...........
   GCGTCTGTAGG ACGGCATCGG ACTCCGTGTGC  2

4  GCCAGCGTAGT CCTGTTCAGT CCGACCAATGC
   ........... .......... ...........
   CGGTCGCATCA GGACAAGTCA GGCTGGTTACG  3
```

DAO

*2(5')   *2(3')   *3   *4(5')

DS

DC

*1   *4(3')

DS

DS

```
   GCGTCTGTAGG ACGGCATGCACATCGG ACTCCGTGTGC
   ........... ................ ...........
   CGCAGACATCC TGCCGTACGTGTAGCC TGAGGCACACG

   CGTAACCAGCC TGACTTGGCAGTGTCC TGATGCGACCG
   ........... ................ ...........
   GCATTGGTCGG ACTGAACCGTCACAGG ACTACGCTGGC
```
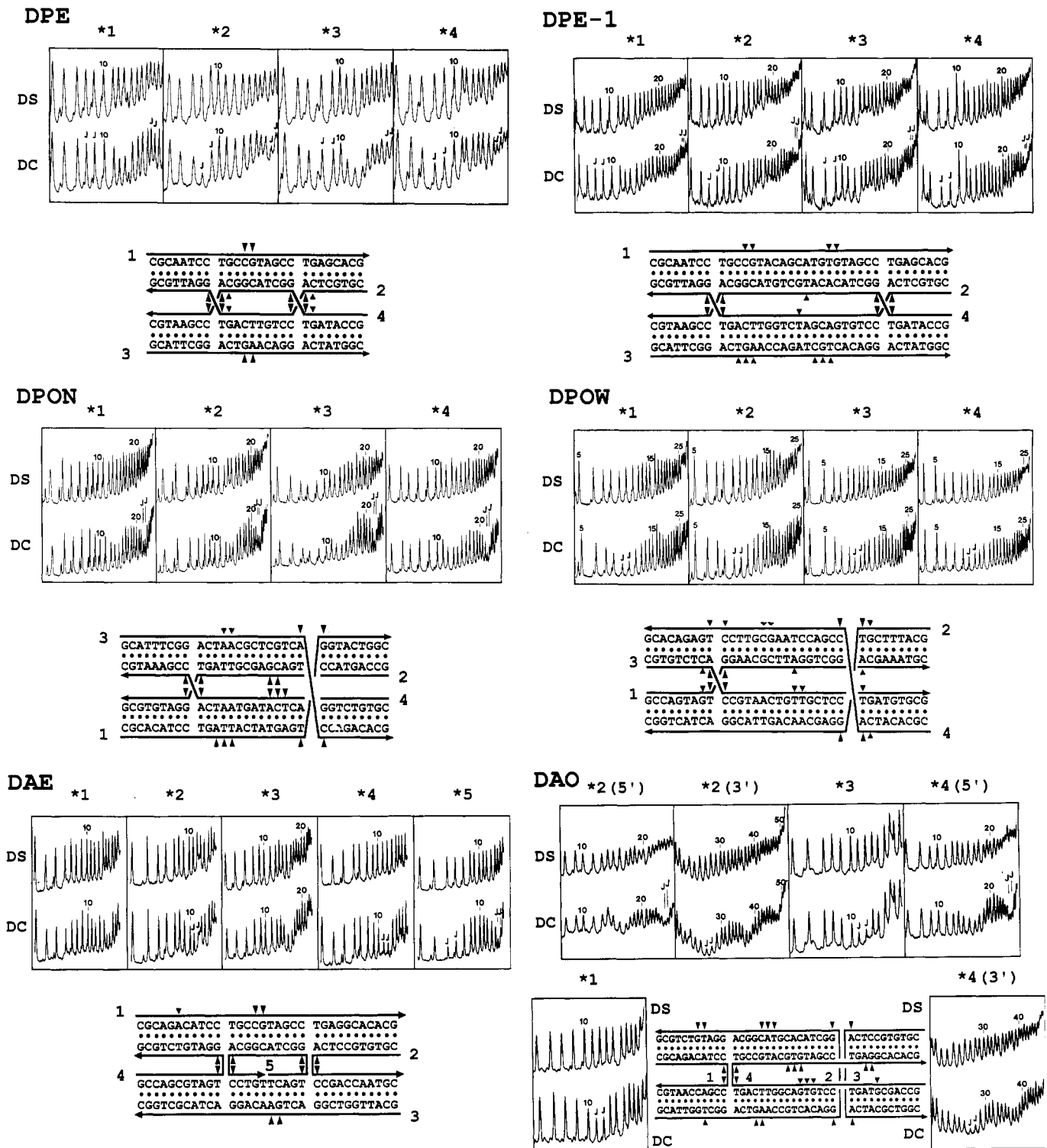
DC   DC

FIGURE 4: Hydroxyl radical cleavage patterns for double-crossover molecules. Each panel of this figure contains two parts, a direct comparison of the hydroxyl radical sensitivity of an individual strands in its complex, DC, compared with the same strand paired to its Watson–Crick complement, DS. The two nucleotides of each strand that flank or potentially flank a crossover junction point are indicated by J symbols. Residue numbers from the 5' end are indicated in the data by Arabic numerals; the hydroxyl radical sensitivity data are displayed 5' → 3' from left to right on the page. Protection at individual sites is indicated in the schematic diagram below the data by triangular indicators, with the extent of protection indicated qualitatively by the size of the triangle. Note that the polarity of the strands in each schematic is indicated by the arrowheads, which denote the *3' ends* of the strands. Strand numbers are indicated by Arabic numerals in the schematic. (A, upper left) Cleavage pattern of DPE. Note the strong protection at the crossover sites and on the crossover strands and weaker protection one residue 5' to that position. Note also the strong protection 4–5 residues 3' to the crossover position on the noncrossover strands. (B, upper right) Cleavage pattern of DPE-1. Strong protection can be seen at the crossover sites, but much weaker protection is seen in the central portion of the molecule than is seen with DPE (compare with panel A). (C, middle left) Cleavage pattern of DPON. The strongest protection can be seen at positions 23 and 24, the crossover position in every strand. Additional protection is evident in the pattern of every strand one turn 5' to the junction, which is 4 nucleotides 3' to the crossover formed by the other pair of strands. (D, middle right) Cleavage pattern of DPOW. The protection at the crossover points is strong, but the protection in the interstrand region is surprisingly weak, in comparison with DPON. (E, lower left) Cleavage pattern of DAE. Protection is seen at all of the crossover positions and at the positions 4 nucleotides 3' to the crossover on the noncrossover strands. (F, lower right) Cleavage pattern of DAO. Six cleavage panels are shown, corresponding to two scans through the long strands. Protection is seen at all of the crossovers and at all of the positions 4 nucleotides 3' to the crossovers on the noncrossover strands. Weaker protection is also seen one turn 5' to the crossover.

12) is evident in all four strands of DPON. By similar logic, one would expect a secondary protection pattern about 1 turn 3′ to the crossover position in each of the DPOW strands, around position 20. Weak protection can be noted on strands 1 and 3, and perhaps 4, but it is absent on strand 2.

We have seen that the antiparallel molecules are better behaved than the parallel molecules when visualized on native polyacrylamide gels. The DAE molecule used for hydroxyl radical studies contains 11 nucleotide pairs in its external arms; the sequences and protection pattern are shown in Figure 4E. One would expect protection at sites that are designed to form crossover positions. In DAE, strands 2 and 4 contain a single crossover site, at residues 11 and 12; strand 5 contains two crossover sites, at residues 5 and 6 and again at residues 15 and 16. Protection is expected and seen at each of these positions (Figure 4E). There is a strong protection pattern 4 nucleotides 3′ to the crossover (residues 15 and 16) on strands 1 and 3, the noncrossover strands. This effect has been noted previously (Kimball et al., 1990), but its structural origin is different from the origin of the protection noted for the parallel molecules. It still results from the occlusion of strands 1 and 3 by identically numbered residues, just as for parallel molecules, but the 2-fold axis between them is perpendicular to the line between crossovers, rather than coincident with it (see Figure 1).

The DAO molecule, in contrast to the DAE molecule, contains only four strands. The molecule used for footprinting contains 11 nucleotide pairs in its external arms. Strands 1 and 3 contain 22 nucleotides, and strands 2 and 4 contain 54 nucleotides; a molecule with 16 nucleotide pairs in its central helices inherently contains pairs of strands whose lengths differ by 32 nucleotides. Every strand contains a designated crossover site at its central residues, 11 and 12 for the first and third strands and 27 and 28 for the second and fourth strands. Figure 4F shows that each of these sites displays the expected strong protection. The central portion of this molecule is composed exclusively of strands 2 and 4. These strands come close to themselves 1 turn past the crossover, and this can be noted by the protection seen in the vicinity of residues 37 and 38 on the panels showing the portion of the strands 3′ to the junction. In addition, one would expect protection 5′ to the junction as well, although this has not been seen previously. The panels showing the portion of the strand 5′ to the junction indeed do show protection in this region, but not exactly a single turn from the junction. Rather, protection centers on positions 15 and 16 on strand 2 and on positions 15–17 on strand 4. The centers of these loci correspond more closely to being 4 residues 3′ to the crossover junctions on their 5′ ends (at positions 11 and 12) than 10.5 residues 5′ to the junctions 3′ to them (at positions 27 and 28). Strong protection is also evident 4 nucleotides 3′ to the junction at the 3′ ends of strands 2 and 4 in the vicinity of position 47. The strength of this protection suggests that the external arms associated with strand 1 are coplanar with each other and that those associated with strand 3 are also coplanar with each other. In the absence of any apparent factor to bend the pairs of helices at the crossovers, the simplest conclusion is that the axes of the two domains are linear and coplanar in this complex.

Ferguson Analysis. The slope of the Ferguson (1964) plot of log (mobility) as a function of polyacrylamide concentration yields information about the friction constant of the molecule (Rodbard & Chrambach, 1971). Tables I lists the intercepts and slopes of the Ferguson plots of the new double-crossover species described here and of control single junctions prepared for comparison with them. Few trends can be spotted in this

Table I: Slopes and Intercepts from Ferguson Plots of Species Described Here[a]

molecule	nucleotides	intercept × 1000	slope × (−1 00)
JAE	26	922	108
DAE	26	907	105
JAO	38	772	110
DAO	38	827	110
JPE	26	877	102
DPE-8	26	914	105
DPE-9	28	879	106
DPE-10	30	889	107
DPE-11	32	881	108
JPE-1	37	716	100
DPE-1	37	833	110
DPON	32	879	109
DPOW	34	886	111

[a] Double-crossover structural types are designated by the names assigned in Figure 1. DPE-1 is a DPE molecule containing 2 turns in its central region, rather than 1. The number of nucleotide pairs in the external arms of various DPE molecules is separated from the name by a dash. "Nucleotides" refers to the number of nucleotide pairs per domain. Structures with names beginning in J are single-crossover molecules, each of whose domains contains the same sequence as the corresponding double-crossover molecule, except that they lack a second crossover. Therefore, the orientations of their domains are not constrained in the same fashion as those of the analogous double-crossover molecules.

table. The junctions analogous to the DPE class of molecules have a lower slope than the double-crossover molecules, perhaps because their domains are not constrained to be parallel. In contrast, the slopes of the DAO molecule and its corresponding junction are similar. The slope of the DPE molecules increases as the external arm length increases, which is not surprising.

DISCUSSION

Complex Formation. It is instructive to compare the assembly of double-crossover molecules with the assembly of stable Holliday junction analogs, immobile junctions that contain a single crossover site (Kallenbach et al., 1983). Both these forms are designed to maximize Watson–Crick base pairing (Seeman, 1982), so that unfavorable structural features associated with duplex disruption at the branch points can be compensated. The single-junction molecule is free to assume any conformation available to it. Increasing the lengths of arms is a useful procedure if a single junction is unstable for some reason; for example, this strategy has been useful in the assembly of five-arm and six-arm junctions (Wang et al., 1991).

These features do not pertain to all double-crossover molecules. The structure of the double-crossover molecule immediately confers an overall conformation upon it. There is a single type of unconstrained single-crossover molecule that can assume four canonical structures, including crossover isomers, in which the crossover strands and the helical strands are exchanged (Kimball et al., 1990); by contrast, there are five different types of double-crossover molecules, ignoring crossover isomers. The parallel molecules do not form readily and cleanly at micromolar concentrations. None of the DPE molecules yields a discrete single band on a gel, without either splitting or forming a dimeric molecule. Lengthening the external arms increases the splitting of the molecular band into two bands. Doubling the central portion of the molecule also appears to destabilize the complex, as seen with DPE-1. The DPON and DPOW molecules likewise form multimers at micromolar concentrations. The apparent cause of this behavior lies in the direct apposition of phosphate backbones across the small gap between the two helical domains in the central region. The external arms might be thought to be

somewhat more able to relieve this stress by partially destacking at the crossovers; nevertheless, the appearance of multiple species in DPE molecules with longer external arms does not support this suggestion. It is possible that the doublets seen when the external arms are lengthened represent the opposite crossover isomer: Weak protection is visible at the branch-point position on the nominal noncrossover strands of DPE-11 (data not shown). Strong protection seen on the two crossover strands argues against the disruption of the crossover in these molecules. (Added in proof: The mobility of the control junction corresponding to DPE-11 differs from those of both DPE-11 bands.)

Antiparallel molecules are much better behaved than parallel molecules. The DAE molecule is difficult to construct in an unnicked fashion, although we have been able to make small quantities by chemical ligation (Ashley & Kushlan, 1991) procedures (data not shown). The DAO molecule is the easiest antiparallel molecule to fabricate, because it contains only four strands. It is stable and well-behaved, presumably because its phosphates are not directly opposite each other in the central region or beyond.

It is also useful to compare the double-crossover molecules to antijunctions and mesojunctions (Du et al., 1992). These DNA arrangements are also often ill-behaved, in that they tend to form multimers of their fundamental repeating arrangement, except at 100 nM concentrations. Their behavior is reminiscent of the parallel double-crossover molecules described here. In addition, one of the mesojunctions, $^{1}4_{2}$, contains two parallel helices, but they are connected by only single-strand linkages, not by two-strand crossovers.

Structural Features. The hydroxyl radical protection patterns for these molecules are clear and unambiguous. In contrast to the dynamic antijunction and mesojunction structures, which are characterized by weak protections at many (often incompatible) sites (Du et al., 1992), the patterns at crossover sites here are strong and readily interpreted. Crossover site protection is noted in all the places that it is expected for molecules containing two coplanar parallel or antiparallel helical domains. Likewise, the protection 4 nucleotides 3' to the crossovers on the noncrossover strands is often seen, as predicted from canonical models (Churchill et al., 1988). The protection on the noncrossover strands is seen to be much stronger here than in single-crossover junctions (Churchill et al., 1988; Chen et al., 1988) and stronger than in some of the tethered junctions (Kimball et al., 1990). We also report the first crossover strand protection a full turn 5' to a junction in an antiparallel molecule (DAO); however, its shifted position makes it unclear whether this protection is actually noncrossover strand protection 3' to another junction.

These molecules constitute baseline structures that provide support for the notion that protection from Fe(II)-EDTA^{2-}-generated hydroxyl radical attack results from occlusion. Even though tethered junctions can be made extremely tight (Kimball et al., 1990), the constraints in that system are subject both to fraying at the tether and to crossover isomerization, which can relieve tension. The protection seen here for noncrossover strands in DPE is stronger than the protection seen in analogous tethered junctions; it is at least as strong for DAE and DAO at these positions as it is in tethered junctions. The relative weakness of the noncrossover protection in the single-crossover molecule suggests either that the conventional junction is nonplanar or that it is a dynamic structure, only some of whose conformations occlude those positions. The finding that antiparallel junctions are only 1.1–1.6 kcal/mol more stable than parallel junctions (Lu et al., 1991) supports the latter explanation.

The protection noted suggests that the axes of the helical domains in all cases are parallel or antiparallel, coplanar, and roughly linear between crossover points in DAE, DAO, DPE, and DPON. The entire DAO molecule may form a coplanar complex, not just the central portion. By contrast, weak protection is seen in the central portions of DPOW and especially DPE-1. One might expect that the central portion of these complexes would show a level of protection similar to that near the double crossovers, but weaker protection is seen. This finding suggests that the two central helices may be bowing, so as to reduce the occlusion of the central site. Whereas the central portions of DPE-1 and DPOW are the longest of the parallel molecules, increased distortion is more likely than in DPE and DPON.

Double Crossovers in Recombination. Our main goal in building double-crossover molecules has been to model a structure suggested to be involved in double-strand break mediated recombination. We have shown that antiparallel double-crossover molecules are more stable than parallel molecules; this preference is also seen for single-crossover molecules (Cooper & Hagerman, 1987, 1989; Duckett et al., 1988; Murchie et al., 1989; Lu et al., 1991). Indeed, the constraints on the double-crossover system appear to exacerbate the instability of parallel molecules. Nevertheless, evidence for the involvement of antiparallel molecules in double-strand break mechanisms is lacking.

There are two ways to accommodate parallel double-crossover molecules in recombination processes. One way is to invoke protein molecules whose binding energies and structural features can mask or neutralize charge repulsion between parallel double helices. The second way is to exploit the bowing suggested by the finding that protection is weak in the central portions of DPOW and DPE-1. Double-crossover molecules with longer central sections are inherently able to flex more easily than those with short central sections (e.g., Hagerman, 1988); molecules whose double crossovers are separated by enough nucleotide pairs could lessen the inherent structural repulsion by this means. It is only plausible to invoke the involvement of parallel double-crossover structures in recombination if the juxtaposition of like charges is relieved by flexibility or by neutralizing factors.

There are two key isomerizations of Holliday structures that are important for recombination, branch migration and crossover isomerization. Branch migration relocates the branch point, and crossover isomerization interchanges the crossover and helical strands that form the branch point. It is useful to discuss these structural transformations in the context of double-crossover molecules. Branch migration in homologous double-crossover structures must be coupled so as to maintain the separation of crossover points that are within torsional correlation (Allison & Schurr, 1979): Independent migration of the branch points will supercoil the DNA between them. In principle, both parallel and antiparallel double-crossover structures can branch migrate. In parallel structures, this could occur by rotating the helical domains (Figure 1) without straining the conventional model of bases rotating about axes parallel to the helix axes (Meselson, 1972). Branch migration in the antiparallel case would entail concerted movements of crossover strands around their bending sites, each moving like a rope around a pulley. Migrating bases on these strands would thereby rotate about axes perpendicular to the axes of double helices. Thus, branch migration in DAE molecules would involve concerted clockwise or counterclockwise movements of the three strands depicted as forming the crossovers, with the two helical strands shifting accordingly. Whereas all four strands of the DAO structure contain bends,
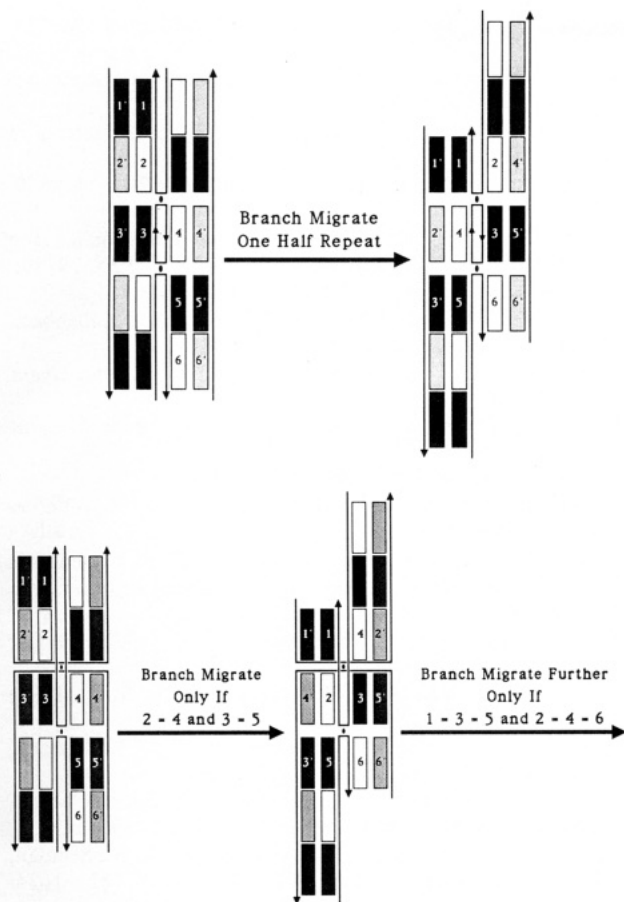
FIGURE 5: Branch migration in antiparallel double-crossover molecules. (A, top) DAE molecules. The five strands of a DAE molecule are shown as linear molecules; no attempt is made to indicate helicity. Arrowheads represent 3′ ends on the helical molecules (straight lines) and on the linear crossover molecules (narrow U-shaped molecules) and the 5′ → 3′ direction in the central circular molecule. Sequences on the crossover molecules and the central molecule are represented by black and white rectangles, and their complements on the helical molecules are represented by dark gray and light gray rectangles, respectively. The sequence symmetry axes necessary for branch migration are indicated by the two lens–shaped objects on the ends of the central cyclic molecule. The numbers indicate sequence blocks. Thus, the black rectangles represent the *same* sequences everywhere, and so do the white sequences. The transformation from the left side of the figure to the right indicates the counterclockwise migration of the crossover strands and the central strand about the bends in the molecules; the extent of the migration is half the central sequence. The nature of the re-pairing is indicated by the numbers. Migration in the opposite direction would occur in a similar fashion. (B, bottom) DAO molecules. The same conventions apply as in the top panel, and the direction of migration is also the same. The overlap of strands to create the central region is indicated by the two thin strands in the middle and the two wide strands on the outside. This is a diagrammatic distortion, and the viewer should refer to Figure 1 for a more realistic representation of the structure. As in the top panel, migration can proceed further only if all similarly colored boxes have the same sequence. Note that after migration 2 pairs with 4′ and 4 pairs with 2′ in both panels, but the postmigratory positions differ in the two isomers. Migration in the opposite direction is similarly constrained.

they would all shift clockwise or counterclockwise around their bending points.

The key to branch migration is sequence symmetry. In the case of parallel double-crossover molecules, there is a single axis of homologous sequence symmetry, coincident with the presumptive structural symmetry axes shown in Figure 1. Thus, branch migration in parallel double-crossover molecules requires *no* sequence symmetry beyond homology. Antiparallel double-crossover molecules are more complex. There are two axes of sequence symmetry that must be satisfied

simultaneously if branch migration is to occur. As shown in Figure 5A, the central strand of DAE is a rolling circle: Branch migration can occur only if the sequences of the helical (unbent) strands are complementary to the sequences of this strand; they must contain direct repeats in order to migrate more nucleotides than the circle contains. The potential structural symmetry axis of DAE (Figure 1) can only be an axis of sequence symmetry if the central rolling circle is itself a tandem repeat. Figure 5B illustrates that the same requirements apply to DAO molecules: All duplexes must contain the repetitive sequence that forms the region between crossovers. The potential structural symmetry axis of DAO (Figure 1) can only be an axis of sequence symmetry at special points in branch migration, where both helices in the central region are self-complementary and their dyads coincide with the structural axis. The repetitive sequences are a consequence of the need to satisfy simultaneously two sequence symmetry axes (Figure 5). This feature can be utilized to create immobile symmetric junctions (S. Zhang, T.-J. Fu, and N. C. Seeman, manuscript in preparation).

Crossover isomerization is a structural transformation that exchanges the helical strands and the crossover strands. It is easy to model this isomerization in antiparallel single junctions by exchanging stacking partners between the helical domains (e.g., Seeman, 1988). The ease of accomplishing this transformation in antiparallel double-crossover molecules is a function of the separation of the crossover points. If they are so far apart that the DNA flanking them is flexurally uncorrelated (e.g., Hagerman, 1988), the same situation pertains as in single junctions. However, if they are close together, so that the helices between them cannot bend readily to permit restacking, the factors favoring crossover isomerization will encounter a structural counterforce. In the work reported here, the crossover points are so close together that we have been able to specify the crossover isomer directly.

It is possible to accomplish crossover isomerization in parallel single junctions without braiding the crossover strands (Sobell, 1974): This is done by first converting them to antiparallel junctions (inverting one helical domain), isomerizing, and then converting back. In double-crossover molecules, this series of operations passes an external arm through the cavity between helices in the central region. Clearly, braid-free isomerization is not feasible in parallel double-crossover molecules if their crossover points are close enough together to prevent these operations. The braid-removal operation introduces a supercoil in the central region: A double-crossover isomerization, necessitating the removal of two braids, entails the introduction of supercoils of opposite sign, leaving the region relaxed. An isomerization at only one crossover will interconvert DPE- and DPO-type molecules, insofar as crossover-strand identity is concerned, but without changing the separation characteristic of these structural motifs; hence the system will be stressed until a half-turn of branch migration or another crossover isomerization relaxes the system. As noted above, crossover isomerization of DPON and DPOW molecules interconverts them, again stressing the molecules. If crossover isomerization occurs in double-crossover molecules, the features described here suggest the possible involvement of topoisomerases; these enzymes could achieve by strand passage the large-scale motions otherwise required to relax parallel double-crossover molecules. The crossover isomerization discussed above with regard to the DPE model systems constructed here may involve alternate assembly or denaturation, rather than isomerization through the routes discussed here.

Recently, strong crossover preferences have been noted in asymmetric single junctions (Cooper & Hagerman, 1987, 1989; Churchill et al., 1988; Chen et al., 1988; Duckett et al., 1988). It has been suggested that crossover isomer equilibration is a possible explanation for the slow rates of branch migration (Mueller et al., 1988). If strong crossover preferences bear up in symmetric crossover structures, the difficulty of crossover isomerization in double crossover molecules could lead to complex situations: For example, if two junctions favor the same crossover isomer at one position and opposite isomers at one adjacent position, but not the other, the direction of migration could be influenced by sequence.

We make no suggestion here that antiparallel double-crossover structures are intermediates in the biological process of recombination. Double-crossover structures are more stable in coupled antiparallel double-crossover systems than in their parallel counterparts. If the central helices are shielded from each other by separation or neutralization, the energetic differences between parallel and antiparallel molecules are likely to be small (Lu et al., 1991). Unlike antiparallel molecules, parallel molecules place no sequence requirements on the migratory process other than homology. Having modeled both parallel and antiparallel systems, we have shown that some sort of energetic investment must be made if parallel structures are involved. Nevertheless, there is no reason to expect this investment to be beyond the means of the cell.

Generalized Structures. The double-crossover structures described here are closely related to Holliday junctions, but nevertheless they constitute a new class of DNA structures. Structures containing only a single branch require only three separate helical fragments (Ma et al., 1986; Duckett & Lilley, 1990; Guo et al., 1990; Leontis et al., 1991), but double-crossover structures require the fusion of two four-arm junctions. Triple and higher crossover structures forming two long helical domains can be imagined readily. These structures would be constrained to being either parallel or antiparallel, but they could apparently mix even and odd varieties between the different crossovers. From the results reported here, parallel structures involving multiple crossovers are unlikely to be formed easily. In principle, multiple-crossover structures are not limited to constructions involving only four arms about each crossover. In addition, the multiple-crossover structure need not be confined to the two-domain motif: It is possible to model parallel or alternately antiparallel helices connected by multiple crossovers that form a two-dimensional lattice.

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