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Structures of helical junctions in nucleic acids

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1. Introduction

Helical junctions in nucleic acids are important in biology. In DNA, their main significance is as intermediates in both homologous and site-specific recombination events. In RNA they are important architectural elements.

Helical junctions may be defined as branchpoints where double-helical segments intersect with axial discontinuities, such that strands are exchanged between the different helical sections. Thus the integrity of junctions is maintained by the covalent continuity of the component strands. Junctions can be perfectly basepaired, such that every base is paired with its Watson–Crick complement, or they can contain mismatches or unpaired bases; the latter can have significant effects on the folding of the structures. A systematic nomenclature exists for the unambiguous description of different junctions (Lilley *et al*. 1995) and some examples are illustrated in Fig. 1.

The purpose of the present article is to review what is known about the structures of helical junctions, and their recognition by proteins. The recent presentation of crystal structures of four-way junctions (Nowakowski *et al*. 1999; Ortiz-Lombardía *et al*. 1999; Eichman *et al*. 2000) provides a good opportunity to examine the current state of knowledge. We can also

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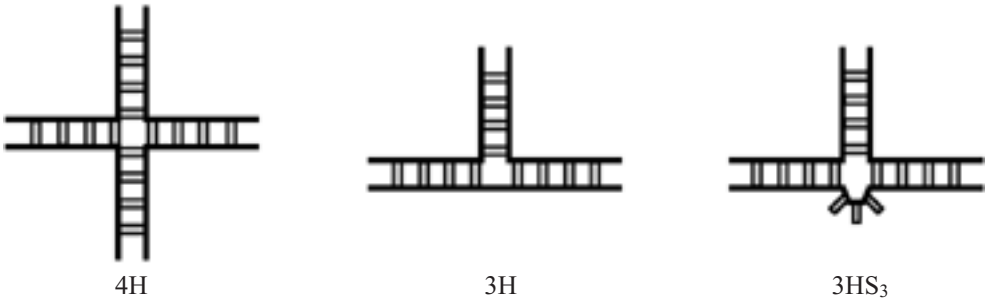


Fig. 1. Some examples of some helical junctions. These are named according to the IUPAC nomenclature for helical junctions in nucleic acids (Lilley *et al*. 1995). The examples shown are a perfect four-way junction (4H), a perfect three-way junction (3H), and a three-way junction with a three base bulge ($3HS_3$).

ask whether there are general principles behind the folding of branched nucleic acid species. Two possible principles emerge.

- *Pairwise coaxial helical stacking*. The formation of branchpoints potentially involves unstacking and exposure of basepairs to solvent. Coaxial stacking of helical arms maximises base stacking interactions, and thus folding based on coaxial stacking might be expected. This is exemplified in the tertiary structure of tRNA (Jack *et al*. 1976; Kim *et al*. 1973). Coaxial stacking can create alternative conformers, the relative stability of which is generally dependent on local sequence.
- *Ion-induced folding*. Nucleic acids are highly charged polyelectrolytes. Electrostatic repulsion between phosphate groups will tend to keep the structure extended in the absence of charge neutralisation, and thus metal ions play a critical role in folding.

We shall see how just how useful are these general principles in the course of this review.

2. The occurrence of helical junctions in nucleic acids

2.1 The four-way DNA junction and genetic recombination

The four-way DNA junction is generally accepted to be the central intermediate in homologous genetic recombination (Holliday, 1964; Broker & Lehman, 1971; Sigal & Alberts, 1972; Potter & Dressler, 1976, 1978; Orr-Weaver *et al*. 1981; Schwacha & Kleckner, 1995). This process is important in the repair of double-strand breaks in DNA, and in providing genetic diversity that is necessary for Darwinian evolution. DNA junctions are the substrates for a number of proteins acting in the later stages of recombination, most of which recognise the structure of the branched DNA. The manner of these interactions determines the genetic outcome of the process. It is an interesting challenge to explain the mechanism of this structure-selective interaction at a molecular level, and it is becoming increasingly clear that the DNA structure plays a role in the detailed function of these enzymes. The four-way junction is also the key intermediate in the integrase class of site-specific recombination events (Hoess *et al*. 1987; Nunes-Düby *et al*. 1987; Kitts & Nash, 1987; Jayaram *et al*. 1988; McCulloch *et al*. 1994). DNA junctions can arise in other ways, including the replication of DNA as exemplified by bacteriophage T4 (Kemper & Janz, 1976).

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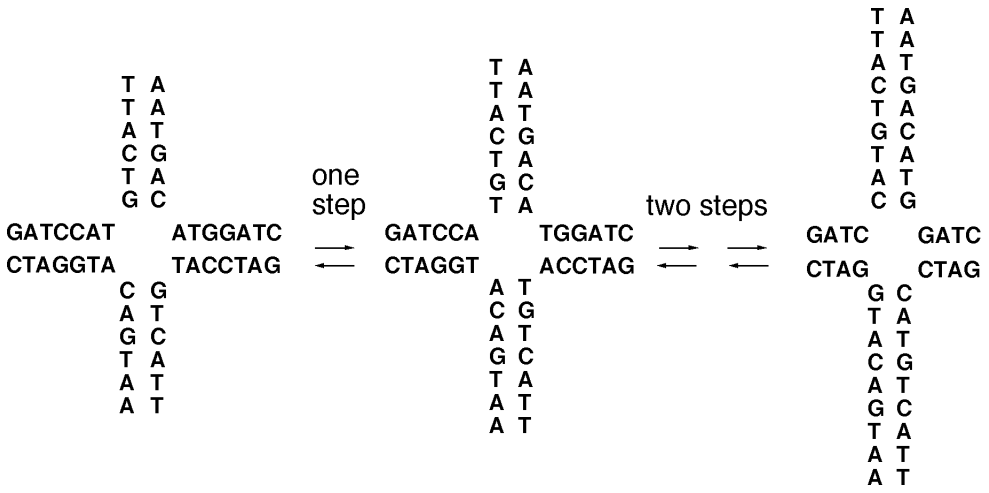


Fig. 2. An illustration of branch migration in a four-way DNA junction. The sequence shown is a fully homologous junction, that can undergo multiple steps of branch migration.

2.2 Helical junctions in RNA

Helical junctions are common structural elements in RNA, and three- and four-way junctions can be found in the secondary structure (confirmed or predicted) of many functional RNA species. These junctions frequently contain one or more single-stranded bases at the point of strand exchange, which would be expected to influence their conformation. Prominent examples of helical junctions can be found in snRNA species (such as the four-way junction of the U1 snRNA (Branlant *et al*. 1981; Guthrie & Patterson, 1988)) and in the hepatitis C virus internal ribosome entry site (Honda *et al*. 1996). A number of functional catalytic RNA molecules are based upon helical junctions, exemplified by the hairpin ribozyme (Hampel & Tritz, 1989), which is a four-way junction (4H) in the tobacco ringspot viral RNA, and the hammerhead ribozyme which can be regarded as an imperfect three-way junction ($HS_1HS_7HS_3$) (Forster & Symons, 1987; Hazeloff & Gerlach, 1988). These junctions are likely to play a key architectural role in the organisation of the tertiary structure of these molecules; a direct correlation between junction structure and ribozyme function has been demonstrated in the case of the hairpin ribozyme (Murchie *et al*. 1998; Thomson & Lilley, 1999).

2.3 Homology and branch migration of four-way junctions

A significant distinction among different four-way junctions lies in their potential ability to undergo branch migration. This process is illustrated in Fig. 2. When a junction is formed by strand exchange between two homologous (i.e. largely identical) DNA duplexes, as in the original Holliday model for recombination (Holliday, 1964), it can undergo a sequential exchange of basepairing in which the branchpoint becomes effectively displaced along the DNA sequence. Branch migration was first demonstrated in bacteriophage (Lee *et al*. 1970; Broker & Lehman, 1971; Benbow *et al*. 1975; Thompson *et al*. 1976a). The length of the region of homology will limit the number of steps of branch migration that are possible, from zero (sometimes called an immobile junction) to thousands. The process of branch migration

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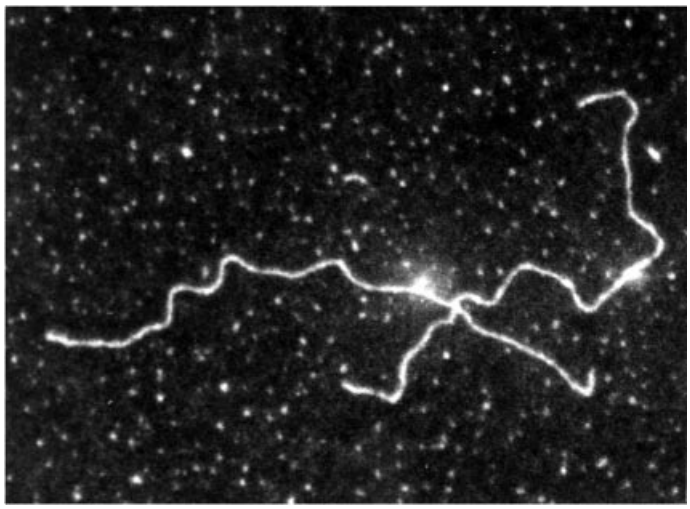


Fig. 3. An electron micrograph of a four-way DNA junction. This was generated by restriction enzyme cleavage of a figure-8 form of a plasmid. Original micrograph provided by Dr H. Potter (University of South Florida).

will be considered in greater depth below. In general, junctions formed in RNA are not capable of branch migration, although this has been suggested in the case of the kissing complex formed by CopA–CopT RNA of plasmid R1 (Kolb *et al*. 2000).

2.4 Forms of helical junctions available for study

The first experimental confirmation of the existence of four-way junctions in DNA came from electron microscopy, where the extension of four helices from a central location could clearly be observed in figure-8 molecules or chi structures derived from plasmid (Potter & Dressler, 1976, 1978) or bacteriophage (Thompson *et al*. 1976b) DNA. An example is shown in Fig. 3. The cruciform is another form of four-way junctions found in DNA derived from natural sources. Cruciforms are formed by intra-strand basepairing at an inverted repeat sequence, and their junctions are recognised by junction-resolving enzymes (Lilley & Kemper, 1984; Mizuuchi *et al*. 1982a). Cruciform structures are characterised by a significant positive free energy of formation ($\geqslant$ 14 kcal mol^{-1} (Mizuuchi *et al*. 1982b; Courey & Wang, 1983; Lilley & Hallam, 1984; Greaves *et al*. 1985)), but they can enjoy a stable existence in negatively supercoiled DNA circles (Gellert *et al*. 1979; Lilley, 1980; Panayotatos & Wells, 1981). This can be a problem for many studies, since the structures will be destabilised if a cleavage is introduced anywhere within the circular DNA; however, this aspect can also be exploited, and cruciforms have ben particularly informative substrates for determining the order of strand cleavage by junction-resolving enzymes (Giraud-Panis & Lilley, 1997).

Isolated four-way junctions have provided the most informative species for structural studies. These can be made from four (Kallenbach *et al*. 1983; Hsu & Landy, 1984) or two (i.e. as a pseudocruciform) (Gough & Lilley, 1985) strands. Chemical synthesis of the component strands allows great flexibility in terms of sequence and functional group modification, and the attachment of exogenous groups (such as fluorophores) to facilitate physical study. Construction of RNA junctions from chemically synthesised strands is also

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now straightforward (Duckett *et al*. 1995b; Walter *et al*. 1998a); component strands of length up to 55 nt can be routinely synthesised using standard *t*BDMS chemistry (Lilley, 2000).

3. The structure of the four-way DNA junction

3.1 The global structure of the junction

In principle it might be expected that a four-way junction could exist in one of two broad classes of structure. The component helices might be held together only by the continuity of the strands, or they might undergo pairwise coaxial stacking. These structures are illustrated in Fig. 4. If the helices are coaxially stacked, there are two possible conformers depending on the choice of stacking partners. If the helices are sequentially numbered as in Fig. 4, then the two possible forms are obtained by stacking helix 1 on helix 4 (together with helices 2 on 3), or alternatively by stacking helix 2 on helix 1 (and thus helices 3 on 4). In earlier literature the different stacking forms were referred to as isomers. However, Altona (1996) has pointed out that no bonds are broken in their interconversion, and that they are therefore better termed conformers. Starting from a structure that is potentially four-fold symmetrical, with four equivalent strands, the symmetry is lowered to two-fold in the stacked forms. Consequently the stacked conformers have two different kinds of strands. Two strands are continuous throughout each pair of stacked helices, with single axes, while the other pair are exchanged between helices. The point where the strands exchange is variously called the crossover, or the point of strand exchange. The nature of the strands becomes exchanged if the stacking partners are swapped – exchanging strands become continuous strands and vice versa in a transition between the two conformers.

Fig. 4 also illustrates another important conformational feature of a four-way junction. Having stacked the helices into pairs, in one or other of the two conformers there is then a question of the angle subtended between the two helical axes. Two extremes are illustrated. If the axes are parallel and the continuous strands run in the same direction, the structure is said to be parallel. The exchanging strands appear to cross at the centre. However, one can imagine rotating one of the two pairs of stacked helices by 180°, such that the continuous strands now run in opposite directions to generate the antiparallel structures. Clearly any rotation between these two extremes is possible in theory. Most of the early genetic literature, and the majority of textbooks, have illustrations of models for recombination that incorporate parallel junctions, but there is no *a priori* reason to assume that this would be the natural conformation for a DNA junction.

Finally, the relative rotation of one helical pair could be clockwise or anticlockwise. Thus right- and left-handed structures are possible for all but the extreme (0 and 180°) structures.

3.2 The stacked X-structure

The structure of the four-way DNA junction was analysed over a decade before crystals were obtained, and before high-resolution NMR methods could be usefully applied to an assembly of this size. The global structure therefore had to be studied by whatever methods could be devised, and this problem has served as a significant stimulus for the development of alternative methods, such as comparative gel electrophoresis (Gough & Lilley, 1985; Cooper & Hagerman, 1987; Duckett *et al*. 1988), fluorescence resonance energy transfer (FRET)

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(Murchie *et al*. 1989), hydrodynamic methods (Cooper & Hagerman, 1989) and enzyme and chemical probing. All these approaches have subsequently proved valuable in the study of other branched species, especially in RNA.

The structure of the four-way DNA junction is dependent on the presence of divalent metal ions. In the absence of added ions the four helices remain unstacked, and the junction is extended (Duckett *et al*. 1988). This structure will be discussed further below. In the presence of divalent metal ions (e.g. $\geqslant 100$ μM magnesium ions) the helices undergo pairwise coaxial stacking into a right-handed antiparallel structure, termed the stacked X-structure. This structure was first proposed in 1988 (Duckett *et al*. 1988), on the basis of comparative gel electrophoresis experiments (Fig. 5), and confirmed soon after by FRET analysis (Murchie *et al*. 1989; Clegg *et al*. 1992). The pairwise coaxial stacking was also consistent with patterns of protection against hydroxyl radicals (Chen *et al*. 1988; Churchill *et al*. 1988), and the shape was in agreement with transient electric birefringence experiments (Cooper & Hagerman, 1989). The coaxial stacking was further supported by the demonstration of *Mbo*II cleavage where the recognition and cleavage sites are located in different helical arms (Murchie *et al*. 1991).

The stacked X-structure is illustrated schematically in Fig. 6. The structure is based on pairwise coaxial stacking of arms, and evidence for the two alternative conformers was obtained (Chen *et al*. 1988; Duckett *et al*. 1988; Murchie *et al*. 1989). The structure is X-shaped, and is closer to antiparallel than parallel. Modelling (von Kitzing *et al*. 1990) suggested that a right-handed form would allow a favourable juxtaposition between DNA strands and grooves; the alignment would be best for a small angle of about 60°. Similar strand–groove alignment has been observed between DNA duplexes packed into crystal lattices (Timsit *et al*. 1989; Lipanov *et al*. 1993). The location of the strands in the grooves is consistent with localised protection against nuclease digestion (Lu *et al*. 1989; Murchie *et al*. 1990). The DNA junction was shown to be right-handed by FRET experiments (Murchie *et al*. 1989). The structure presents two sides of different character. This arises because the four basepairs at the point of strand exchange are oriented in the same direction. On one side of the junction (the major groove side) the point of strand exchange has major groove characteristics, while on the other side (the minor groove side) it has minor groove characteristics.

3.3 The junction has antiparallel character

All early models for recombination assumed a parallel orientation of strands, arising from an expectation that the homologous duplexes would be aligned side by side. For this reason the most controversial feature of the original stacked X-structure model was its antiparallel nature, and some geneticists found the new conformation hard to accept. However, this has now been firmly established beyond all reasonable doubt, most recently in three crystal structures discussed below. The chemical structure of DNA has been in existence almost as long as life itself, and the ability of DNA to fold into a structure that suggests a satisfying mechanism of recombination was probably not a selective pressure when DNA was first adopted as the genetic material. The unconstrained four-way DNA junction has a strong propensity to adopt an antiparallel structure, and Nature is presented with a conformational *fait accompli*. If other conformations are required for the junction to function, then proteins will be required to manipulate the DNA. As we shall see below, this is exactly what happens.

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3.4 The stereochemistry of the four-way DNA junction

3.4.1 Molecular modelling

While the low-resolution approaches were successful in revealing the overall global structure of the four-way DNA junction, this left unanswered the question of the stereochemistry of the crossover. In the absence of higher resolution methods, the only way of approaching this was molecular modelling, the results of which might be used to guide further experiments. There have been a number of such attempts, building up the structure *ab initio* (von Kitzing *et al*. 1990; Srinivasan & Olson, 1994), or derived from crystal structures of juxtaposed duplexes (Timsit & Moras, 1991; Goodsell *et al*. 1995). These exercises demonstrated that stereochemically reasonable structures could be built that preserved full basepairing and stacking. In fact, von Kitzing *et al*. (1990) showed that an acceptable trajectory for the exchanging strands required a change in only one torsion angle on each exchanging strand, viz. the torsion angle ϵ about the $C_{3'}$–$O_{3'}$ bond on the 5′ side of the junction was altered from *t* to g^-. Making this change swivels the phosphate group around and redirects the trajectory of the backbone in just the manner required to make the exchange point (refer ahead to Fig. 8 for more details).

3.4.2 NMR studies

The size of the junction has limited the applicability of NMR studies, but there have been a number of investigations of small four-way DNA junctions (Wemmer *et al*. 1985; Chen *et al*. 1991, 1993; Chen & Chazin, 1994; Pikkemaat *et al*. 1994, 1996; Carlström & Chazin, 1996; Overmars & Altona, 1997; van Buuren *et al*. 2000). These studies have shown that the overall DNA geometry is essentially B-form, with unbroken basepairing at the point of strand exchange. Importantly, evidence for base–base stacking across the exchange point was obtained for a number of junctions (Chen & Chazin, 1994; Pikkemaat *et al*. 1994). Chemical shifts of base and sugar protons in the exchanging strands indicated that the geometry was altered at this site (Overmars & Altona, 1997), consistent with a special stereochemistry at the point of strand exchange. Sequence-dependent stacking conformer bias has been observed in NMR studies (Carlström & Chazin, 1996; Overmars & Altona, 1997; van Buuren *et al*. 2000), discussed further below.

3.4.3 Crystallography

Undoubtedly the biggest advance in understanding the stereochemical structure of the four-way DNA junction in recent times has come from crystallography. Since the stimulation of interest in this structure in the late 1980s there have been a number of serious attempts to obtain crystals of four-way junctions using a variety of strategies, but none was successful. In the past year there have been three junctions crystallised and solved at atomic resolution, yet none of the groups involved had intentionally set out to crystallise junctions; in each case something quite different was the target.

Joyce, Stout and colleagues were trying to obtain a structure of a DNAzyme (Nowakowski *et al*. 1999) – a DNA molecule that catalyses the cleavage of an RNA substrate by a transesterification reaction typical of the small nucleolytic ribozymes. Somehow in the course of the crystallisation process this DNA–RNA assembly mis-hybridised (made possible by the

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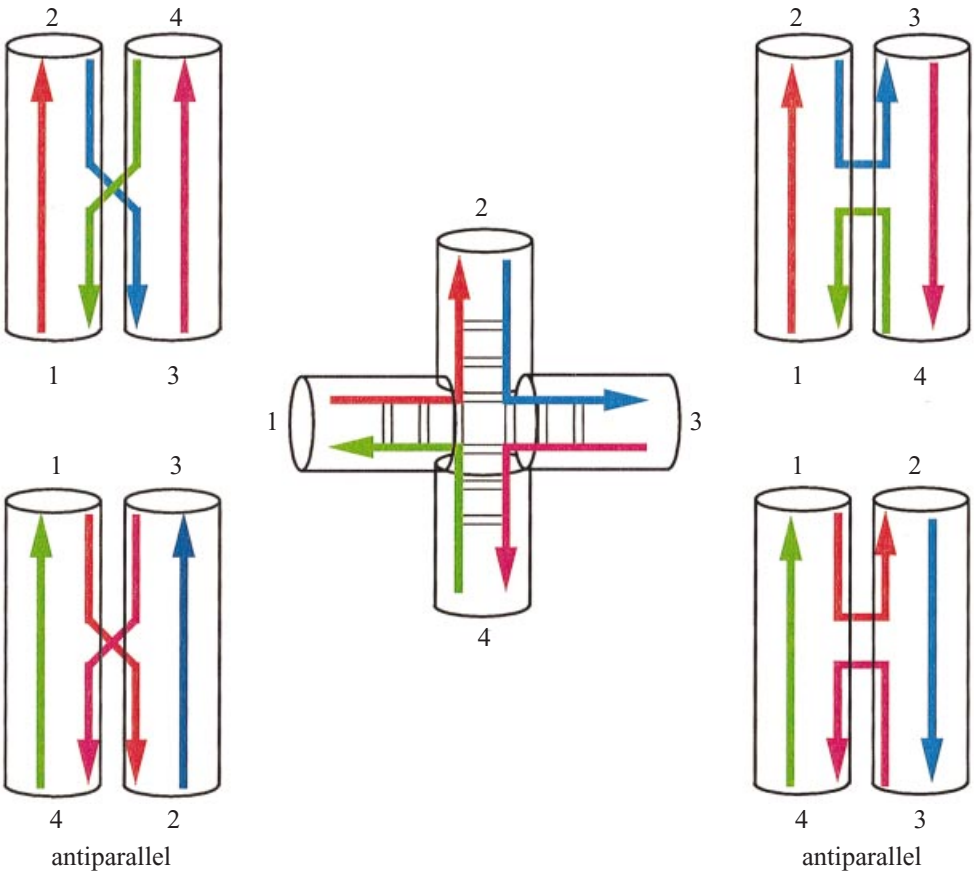


Fig. 4. Possible stacked forms of a four-way helical junction. The centre shows the junction in an unstacked, extended conformation, with the four arms directed towards the corners of a square. The arms are labelled 1 to 4 in a circular manner. The junction may undergo pairwise coaxial stacking of arms in two ways, characterised by 2 on 1 and 3 on 4 stacking (top) or 1 on 4 and 2 on 3 stacking (bottom). Each of these might be rotated in either a parallel (left) or antiparallel (right) manner, although these are clearly extremes. Intermediate rotation can generate either right- or left-handed structures.

presence of a hexanucleotide with two-fold symmetry in the DNAzyme loop) to generate a novel four-strand species containing two four-way junctions related by two-fold symmetry. This rather strange form crystallised in a hexagonal space group, giving diffraction out to a resolution of 3 Å. These junctions were some way from being perfect four-way DNA junctions in a number of respects. First, one component strand in each junction was RNA. Second, one arm terminated with an A·G mismatch at the point of strand exchange. Lastly, one 'arm' of each junction was effectively only a single basepair closed by a four-base loop. Despite these imperfections, the structure is clearly a four-way helical junction (Fig. 7(*a*)), with pairwise coaxial stacking of arms and a right-handed rotation of 55°. Because of the RNA strand, one of the two helices is A-form, while the other is B-form. The structure is based upon an antiparallel orientation of the continuous strands, and thus the exchanging strands do not cross (see Fig. 4).

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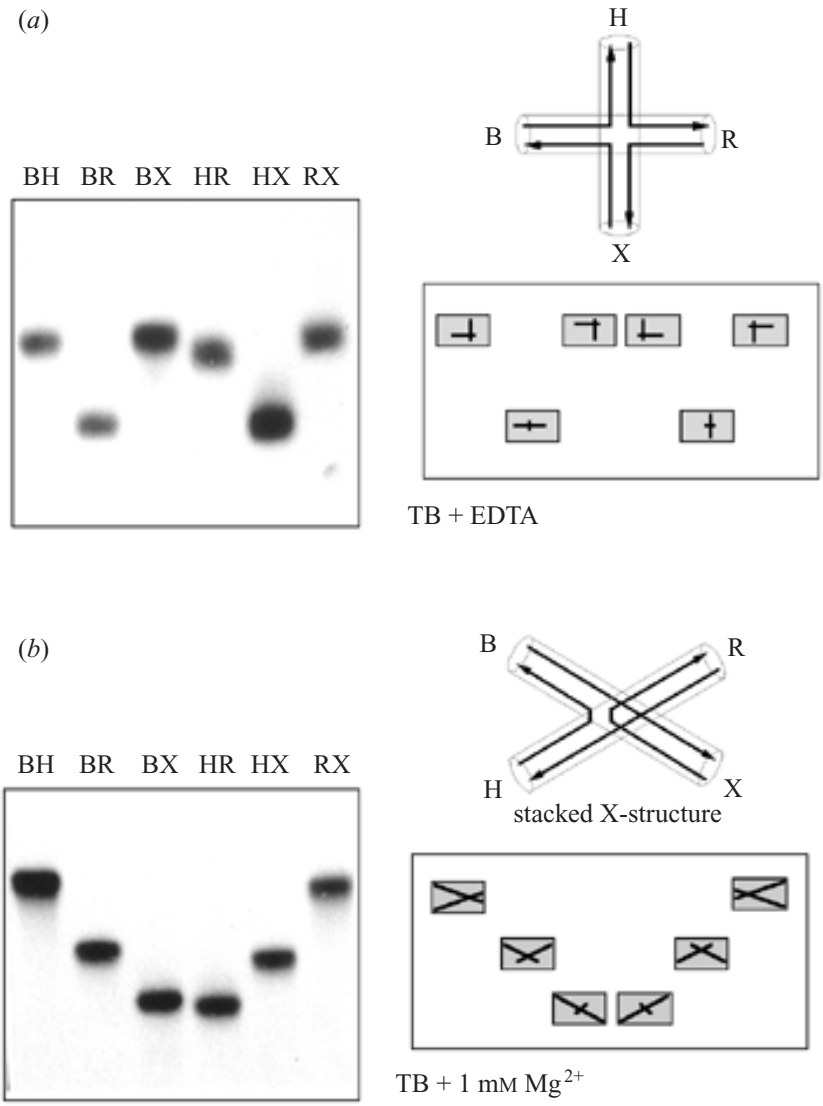


Fig. 5. Analysis of the global structure of a four-way DNA junction by comparative gel electrophoresis. Comparative gel electrophoresis (Gough & Lilley, 1985; Cooper & Hagerman, 1987; Duckett *et al.* 1988) provided the first indication of the global structure of the four-way junction. In this approach, variants of a given junction are prepared with six combinations of two long and two short arms, and their electrophoretic migration in polyacrylamide compared. The four helical arms are named B, H, R and X sequentially around the junction. In the absence of added metal ions the pattern of migration (slow, fast, slow, slow, fast, slow) is consistent with the extended square structure shown. On addition of 1 mM magnesium ions the pattern changes to slow, intermediate, fast, fast, intermediate, slow indicative of the stacked X-structure with B on X stacking as illustrated. The species are named by their long arms, e.g. the species BX has long B and X arms and short H and R arms.

The second four-way junction structure was solved by Coll and colleagues (Ortiz-Lambardía *et al.* 1999), who were trying to crystallise a DNA duplex containing central consecutive A·G mismatches based on the oligonucleotide dCCGGGACCGG. This also associated as a four-way junction from twice the anticipated number of strands, and

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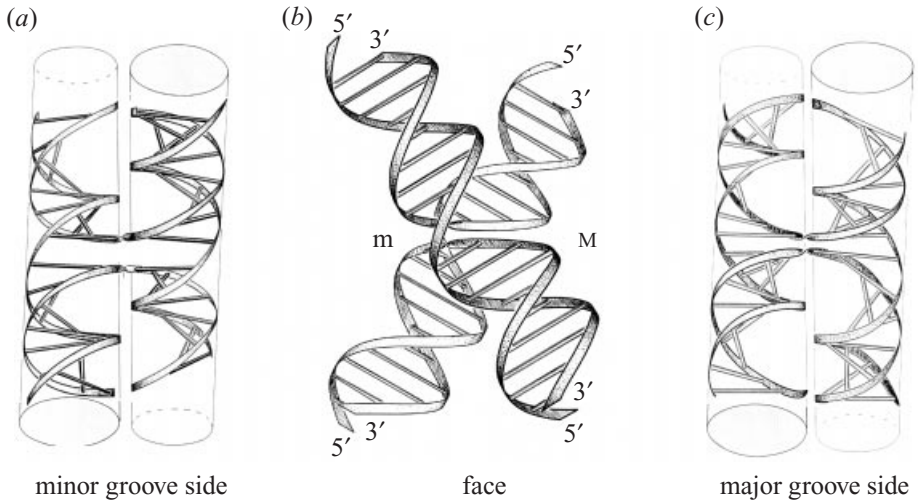


Fig. 6. A ribbon representation of the stacked X-structure. Three views of the structure are illustrated. (*a*) View of the minor groove side. There is a continuous minor groove running through the point of strand exchange; an imaginary ball rolling down the minor groove could pass through the point of strand exchange and continue down the other helical pair. (*b*) The face view. The minor (m) and major (M) groove sides are indicated. Note the juxtaposition of the continuous strands in the major groove of the opposed helix. (*c*) View of the major groove side. Note the continuous major groove running through the point of strand exchange.

crystallised in this form in a monoclinic space group. This junction was formed completely from DNA, and comprised two arms of 4 bp and two of 6 bp, but retained the A·G mismatches at the point of strand exchange in the two longer arms. The resulting structure, which was solved at 2·16 Å resolution by MAD phasing, was again a right-handed antiparallel cross, based on pairwise, coaxially stacked helices (Fig. 7(*b*)).

The most recent structure has been obtained by Ho and coworkers, who were attempting to crystallise a self-complementary DNA duplex containing a psoralen binding site based on the oligonucleotide dCCGGTACCGG. Once again a four-way junction resulted from a hybridisation between four strands. Because of the two-fold symmetry of the sequence, this junction could branch migrate along its length in principle, but in fact it crystallised in a single conformation with a strand crossover localised at the ApC step (Fig. 7(*c*)). The structure has the highest resolution yet, at 2·1 Å, and was solved by molecular replacement using DNA duplexes. In contrast to the previous two structures, perfect Watson–Crick basepairing was present throughout the structure, allowing the smooth trajectory of the backbone to be seen more clearly. As with the other structures, the junction is a right-handed antiparallel cross of pairwise, coaxially stacked helices.

There is good agreement between the three crystal structures on the structure of the junction. Despite differences in helical arm lengths and strand composition, crystallisation conditions, and space group or crystal forms, all three structures are based on pairwise, coaxial stacking of helices, forming a right-handed antiparallel cross. This is exactly the stacked X-structure (Duckett *et al.* 1988; Murchie *et al.* 1989) deduced from the lower resolution methods. This structure allows the strand exchange to take place between minimally perturbed B-DNA duplexes.

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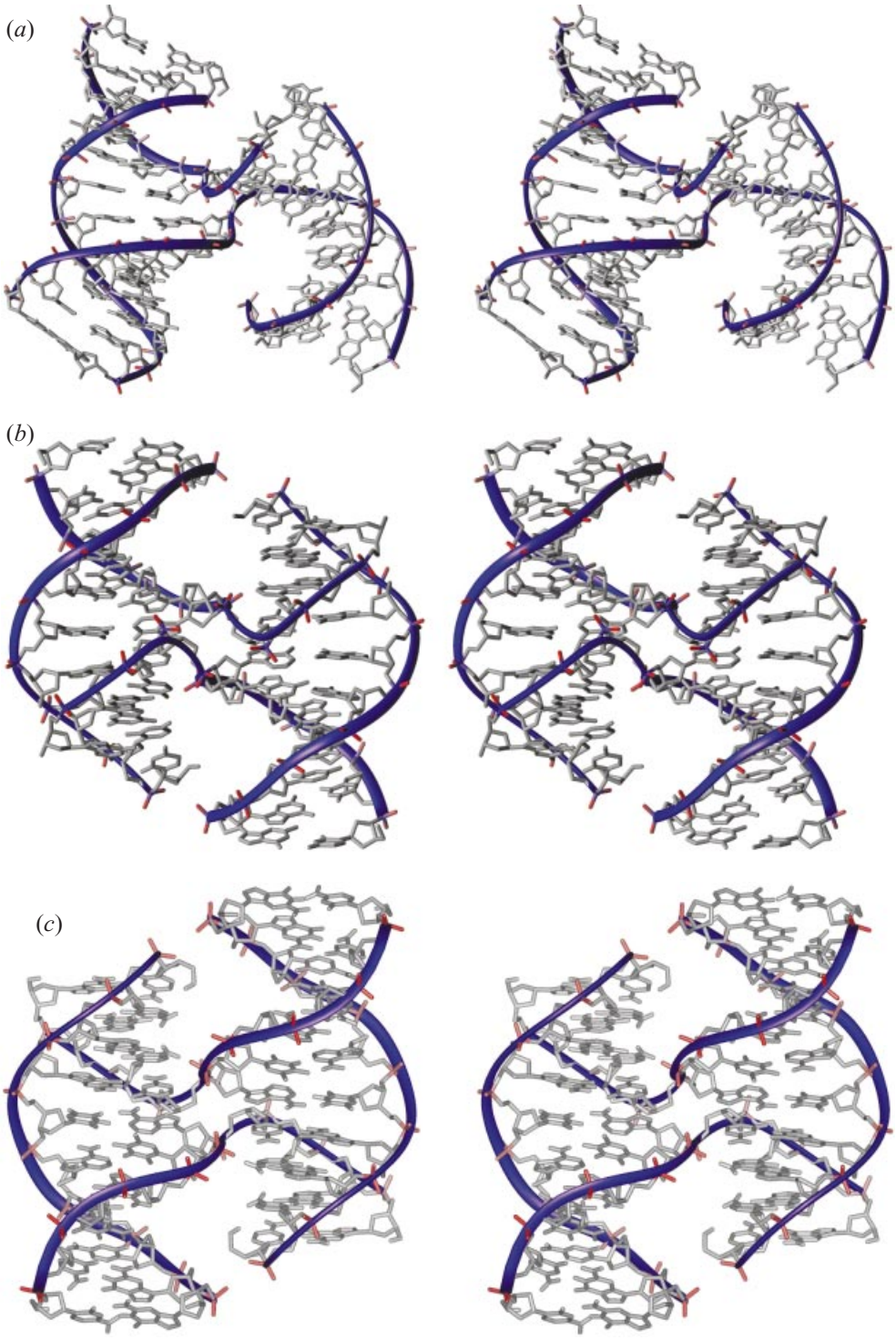


Fig. 7. Three molecular structures of the four-way DNA junction. Stereoviews of the structures have been generated from the coordinates of three different junctions solved by X-ray crystallography. (*a*) The DNA–RNA junctions of the DNAzyme (Nowakowski *et al*. 1999). The view presented is that of the major groove side, from a rotated perspective that shows the angle between the helical axes. The helix

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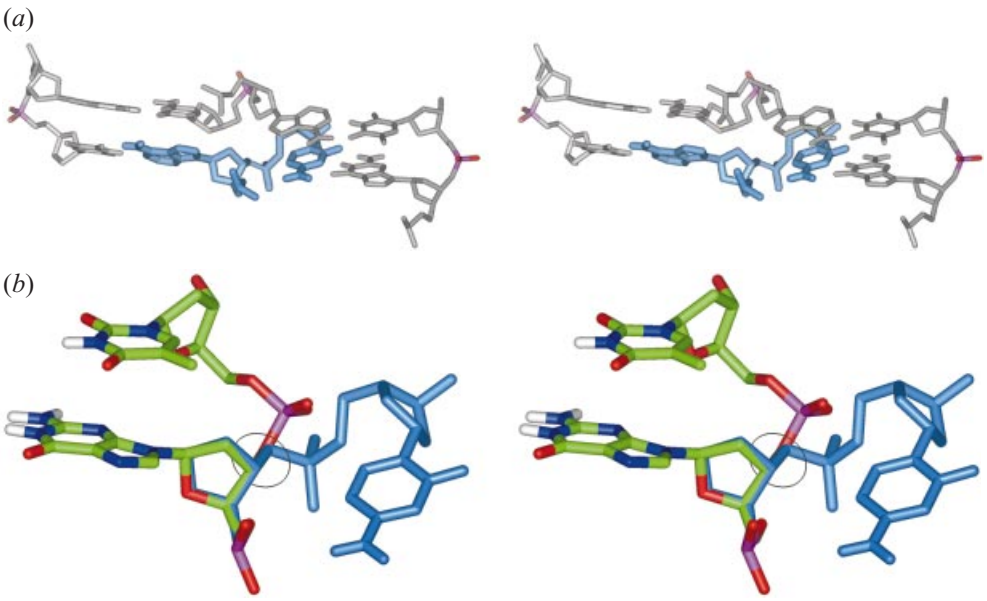


Fig. 8. The geometry of the strand crossover. (*a*) Stereoview of the four central basepairs defining the junction, taken from the structure of Ho and coworkers (Eichman *et al*. 2000). One of the two exchanging strands is highlighted in blue. (*b*) The change in stereochemistry at the point of strand exchange. Two different purine–pyrimidine base steps have been taken from this structure and superimposed by their 5′ ribose rings. One step is a GpT step taken from a regular B-form helical stem, shown in CPK colouring. The other is the ApC step of the crossover, shown in blue. This corresponds with the nucleotides highlighted in blue in (*a*). The torsion angle ϵ (about the $C_{3'}$–$O_{3'}$ bond) is encircled. In the regular helix this angle is *trans*, while at the point of strand exchange it is rotated to *gauche*$^-$, generating the change in trajectory of the backbone.

The high-resolution structures allow us to define the stereochemistry of the exchanging strands. This region of the structure is shown in detail in Fig. 8. The trajectory of the backbone required to make the exchange between helical axes is achieved largely by the rotation of a single torsion angle, i.e. the ϵ angle ($C_{3'}$–$O_{3'}$) on the 5′ side of the junction changes from *t* to g^-. This angle has been measured in the crystal to be $-74°$ (Ortiz-Lombardía *et al*. 1999) and $-89°$ (Eichman *et al*. 2000). In fact, this change in local backbone conformation was anticipated by molecular modelling (von Kitzing *et al*. 1990). The geometry of the exchanging backbones orients the central phosphate groups so that the non-bridging oxygen atoms are directed away from the centre with a measured P–P distance of 6·2 Å.

on the left contains an RNA strand, and consequently has an A-form conformation, while that on the right is B-form. Coordinates provided by Dr G. F. Joyce (Scripps Institute, La Jolla). (*b*) An all-DNA junction containing A·G mismatches (Ortiz-Lombardía *et al*. 1999). The view shown is directly onto the minor groove side. The base mismatches at the point of strand exchange result in a degree of distortion of the backbone. Coordinates provided by Dr M. Coll (CSIC, Barcelona). (*c*) A symmetrical DNA junction with complete Watson–Crick basepairing (Eichman *et al*. 2000). View of the major groove side of the junction. Note the smooth trajectory of the backbone in the absence of mismatched basepairs. Coordinates provided by Dr P. S. Ho (University of Oregon State).

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4. Role of metal ions in the folding of the four-way DNA junction

4.1 An extended structure of the four-way junction at low salt concentrations

Metal ions play an important role in determining the conformation of the four-way DNA junction. In the absence of added metal ions, the DNA junction (but not its RNA equivalent – see below) is fully extended, with no coaxial stacking between component helices. Under these conditions the bases at the point of strand exchange are accessible to attack by chemical agents such as osmium tetroxide (Duckett *et al*. 1988, 1990). The extended character suggests that metal ions are required to provide charge neutralisation in the stacked X-structure, in the absence of which the folded conformation is destabilised with respect to the extended structure. The overall shape of the low-salt form would be expected to be square (Fig. 9), as this provides the maximal extension around the centre, and would therefore probably minimise electrostatic repulsion. A square arrangement provides the maximum distance between four points connected by tethers of fixed length; of course four points on a sphere would be maximally extended in a tetrahedral distribution, but this is not possible with constant tether length. While the square conformation is predicted to be most stable under the low-salt conditions, this need not necessarily be planar. In fact, given that the two sides of the extended structure are inequivalent (there are major and minor groove faces) this is unlikely, and the structure is probably pyramidal to some degree.

Comparative gel electrophoretic experiments showed that the junction adopts a structure with approximately square symmetry in the absence of metal ions (Duckett *et al*. 1988, 1990), and clearly excluded a tetrahedral structure. This conclusion was confirmed by FRET experiments using a sensitive donor-titration approach (Clegg *et al*. 1994). A variety of ions were shown to bring about the folding (Duckett *et al*. 1990). Group II metals such as magnesium and calcium induce folding at concentrations greater than about 100 μM, while complex ions and polyamines are more efficient; 2 μM $[Co(NH_3)_6]$ (III) or 25 μM spermine are sufficient to promote folding. Group I metal ions, such as sodium or potassium, bring about at least a partial folding of the junction (Clegg *et al*. 1992), but very high concentrations are required and bases around the centre of the junction remain accessible to addition by osmium tetroxide (Duckett *et al*. 1990).

4.2 Structural interconversion between the extended and stacked X-structures

The ion-induced folding of the four-way DNA junction into the stacked X-structure suggests a balance between opposing forces of electrostatic repulsion (tending to keep the junction in the extended form) and base stacking (tending to fold the junction into the stacked X-structure). The position of equilibrium will be determined by the algebraic sum of the free energies corresponding to these two terms. At low salt concentrations the electrostatics are evidently dominant, and the junction is unable to fold, but as the electrostatic repulsion is reduced by counterion associations the stacking interactions become more significant until the stacked X-structure is the major form present. Titration with divalent metal ions, where the folding is followed by FRET efficiency between fluorophores attached to helix termini, indicated that the process could be described by a simple two-state model (Clegg *et al*. 1992):

$$J_{\mathrm{open}} + n\,M^{2+} \overset{K_{\mathrm{A}}^{\mathrm{app}}}{\longleftrightarrow} J_{\mathrm{fold}} \cdot \mathrm{M}_n^{2+} \qquad (1)$$

where J_{open} and J_{fold} are the extended and folded conformations of the junction, and $K_{\mathrm{A}}^{\mathrm{app}}$

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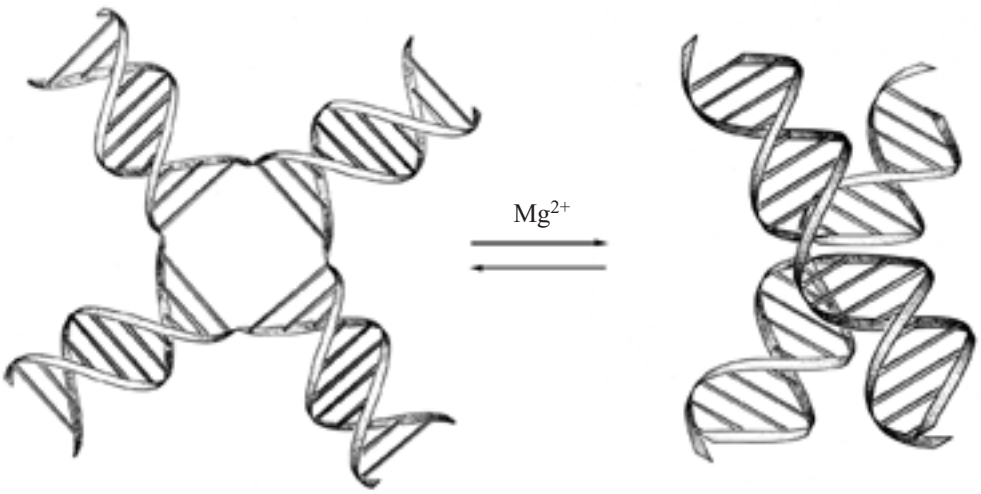


Fig. 9. Illustration of the ion-dependent folding equilibrium of the four-way DNA junction. At low salt concentrations the junction exists in an extended, unstacked conformation. On addition of divalent metal ions, such as $\geqslant 100$ μM magnesium ions, the junction folds into the stacked X-structure.

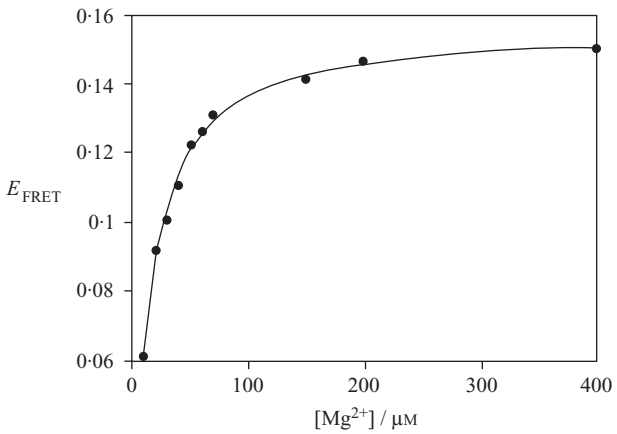


Fig. 10. Folding of the four-way DNA junction; FRET titration with magnesium ions. Junction 3 was prepared with donor and acceptor fluorophores attached to the termini of two arms (B and H) that define one of the acute angles of the folded junction. The efficiency of fluorescence resonance energy transfer (E_{FRET}) was measured as a function of magnesium ion concentration. The experimental data are the plotted points. These were fitted to a simple two-state model for folding induced by ion binding, such that the proportion of folded junction (α) is given by $\alpha = K_A^{app}[Mg^{2+}]^n/(1 + K_A^{app}[Mg^{2+})^n$ where K_A^{app} is the apparent association constant for magnesium ion binding and n is the Hill coefficient. The best fit (the line shown) gives a value of $n = 1{\cdot}1 \pm 0{\cdot}2$. The simplest interpretation is that folding is induced by the non-cooperative binding of a single magnesium ion. Original data from J. M. Fogg (University of Dundee).

represents the apparent association constant for the binding of the metal ion(s) that induce the folding. Typical data for the magnesium ion-induced folding of a junction are shown in Fig. 10. The experimental data can be fitted with a K_A^{app} in the region of 10^5 M^{-1} for divalent ions, and a value of the Hill coefficient of $n = 1$. This is consistent with a model in which folding is induced by the non-cooperative binding of one metal ion.

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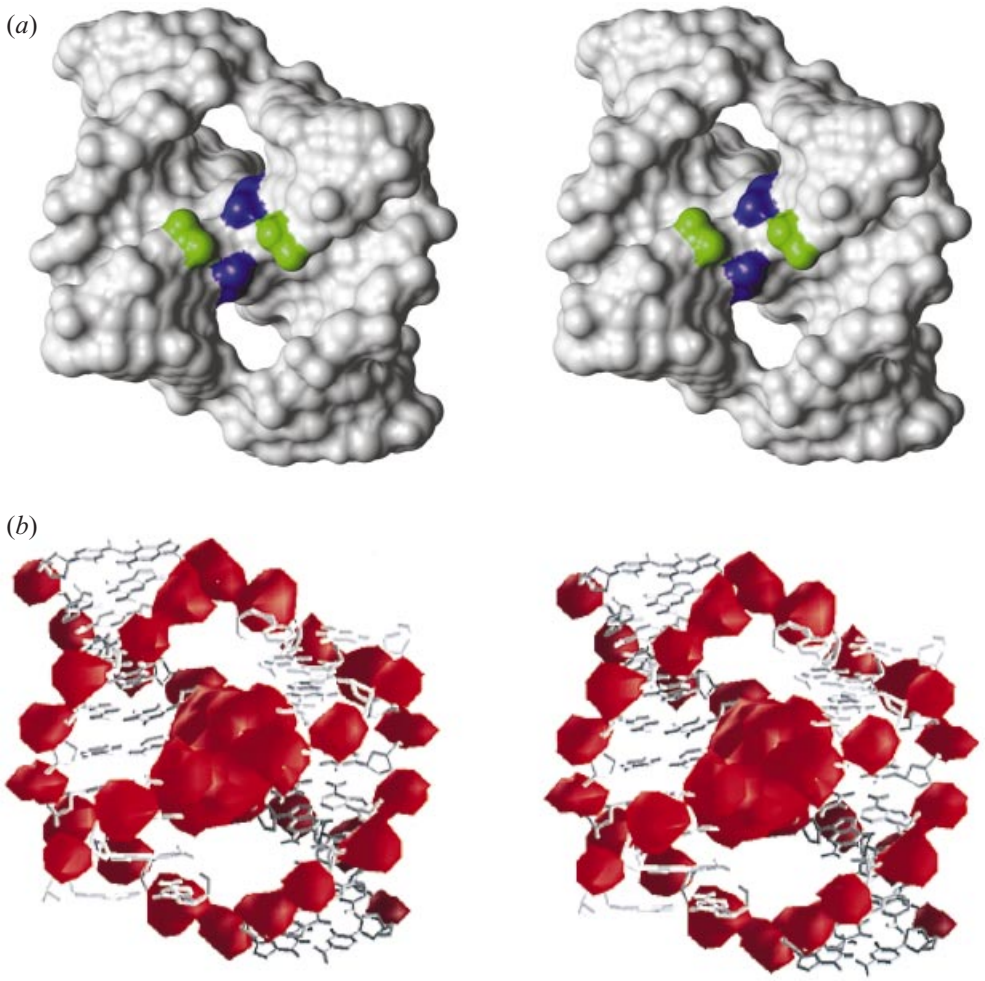


Fig. 11. Electrostatic potential at the four-way DNA junction. (*a*) Location of phosphates around the point of strand exchange. The solvent accessible surface of a junction (using the coordinates of Ho and coworkers (Eichman *et al*. 2000)) seen from the minor groove side. The phosphate groups at the point of strand exchange and those to the 3′ side are coloured purple and green respectively – note that they form a charged box on this face of the junction. (*b*) Calculation of electrostatic potential around the DNA junction. The structure of the junction (Eichman *et al*. 2000) was used to calculate electrostatic potential which is superimposed on the minor groove side contoured as 25 kT, using the program GRASP (Nichols *et al*. 1991). Note the significant region of high potential around the centre of the junction. These calculations were performed by Dr D. G. Norman (University of Dundee).

4.3 Location of structural metal ions in the four-way junction

Charge neutralisation could be the result either of site binding by ions, or by diffuse atmospheric associations of metal ions. There is a considerable clustering of phosphate groups around the point of strand exchange. In the crystal structure of the symmetrical junction (Eichman *et al*. 2000), where the backbone is undistorted by base mismatches, it can be seen that there is a box of four phosphates on the minor groove side, comprising the phosphates of the exchanging strands at the point of strand exchange and those immediately to the 3′ side (Fig. 11(*a*)). On the major groove side the exchanging strand phosphates 5′ to

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the exchange point are also relatively close to each other (7·1 Å). This clustering of charged phosphate groups results in a high electrostatic potential around the point of strand exchange (Fig. 11(*b*)). Calculation of counterion associations using simplified models of DNA junctions suggest that there will be an accumulation of counterions in the folded form (Olmsted & Hagerman, 1994; Fenley *et al*. 1998), leading to a stabilisation of the latter.

The ability of monovalent ions to achieve something like the correct folded geometry overall suggests that site-specific binding may not be essential for these processes. However, there are experimental data suggesting that divalent and more complex ions may exhibit site binding in the central region of the junction. Uranyl-induced photocleavage experiments indicated the presence of a specific ion binding site near the point of strand exchange in the folded junction (Møllegaard *et al*. 1994). The geometry of exchanging strands observed in the crystal structures (Eichman *et al*. 2000) show that the exchanging phosphate groups are close (P–P = 6·2 Å), and thus electrostatic repulsion will be large if these charges remain unscreened. Replacing charged phosphate groups with methyl phosphonates in two strands resulted in the formation of the stacking conformer that placed these electrically neutralised phosphates on the exchanging strands (Duckett *et al*. 1990), overcoming the stacking preferences of the unmodified junction if necessary. This shows that electrostatic repulsion between these phosphates can be dominant. However, the interplay of electrostatic and other forces must be subtle, because in an NMR study of a different junction Altona and coworkers (Pikkemaat *et al*. 1996) found that the normal stacking conformer was preserved despite placing uncharged methylene-acetal groups in place of the central phosphate groups on the continuous strands. The hexammine cobalt(III) ion has the highest known apparent affinity for the junction, and has even been footprinted at the junction centre (Møllegaard *et al*. 1994). In the crystal structure of the DNAzyme, a hexammine cobalt(III) ion has been found bound close to the exchanging strands (Nowakowski *et al*. 1999). The existence of this site in solution has also been indicated by NMR studies. Wijmenga and colleagues (van Buuren *et al*. 2000) located a hexammine cobalt(III) ion at an equivalent position in a different four-way junction by means of NOE connectivities between the ammine protons and those of adjacent nucleotides. Electron density consistent with a sodium ion was seen in the crystal structure of the symmetrical junction, at a distance of $< 2{\cdot}5$ Å from the oxygen atoms of phosphate groups 5′ to the centre, on the major groove side (Eichman *et al*. 2000).

It is probable that charge screening by either mechanism can induce the folding of the stacked X-structure of the junction. At sufficiently high concentrations, monovalent ions achieve sufficient reduction in electrostatic repulsion to promote at least a partial folding. However, ions such as magnesium or hexammine cobalt(III) that bind with relatively high affinity are probably bound in the central region with exchange rates that would categorise them as site-bound ions.

5. Conformational variation in the four-way junction

5.1 Formation of alternative stacking conformers and sequence-dependent bias

5.1.1 Demonstration of alternative stacking conformers

There are two conformers possible for any junction (illustrated in Fig. 4), that differ in the choice of stacking partners. It would be expected that the relative stability of the two conformers would be a function of the local sequence, and this has been shown to be the case.

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If we restrict our attention to the central four basepairs surrounding the point of strand exchange (i.e. ignoring any sequences further removed along the arms), we can write down 70 different four-way (4H) junction sequences, of which 36 are incapable of branch migration. Altona has suggested a set of rules for classifying junctions according to the distribution of purine and pyrimidine bases (Altona, 1996), and a tabulation of the complete set of 4H junctions is presented in Table 1. It is perhaps surprising how few of these have been studied experimentally.

Most sequences studied have shown a bias towards one or other of the two possible stacking conformers. For example, in this laboratory we originally studied (Duckett *et al*. 1988) a sequence called junction 1 (classified as 4–2, i.e. CGTA according to the Altona rules (Altona, 1996)). This appeared to fold into one predominant stacking conformer judged by comparative gel electrophoresis and FRET (Duckett *et al*. 1988; Murchie *et al*. 1989). However, when we reversed two or four basepairs at the point of strand exchange, to generate junction 2 (6–1, CCTT) or junction 3 (4–1, CATG) respectively, the choice of stacking partners was changed such that the alternative conformer was dominant (Duckett *et al*. 1988; Clegg *et al*. 1992). All three junctions appeared to be strongly biased towards a single stacking conformer, although the electrophoretic method would not have been sensitive to a small proportion of the other form. Similarly, junction J1 of Seeman and Kallenbach exhibited a strong bias towards one stacked form, as judged by the results of hydroxyl radical cleavage (Churchill *et al*. 1988) and transient electric birefringence (Cooper & Hagerman, 1989). Using NMR, Carlström & Chazin (1996) observed a change in stacking conformers resulting from a cyclic permutation of their junction J2 (3–1, CCAA), suggesting an important role for local DNA sequence around the point of strand exchange. Switching between stacking conformers was observed upon releasing a junction that had been constrained within a double crossover construct in the less stable form (Li *et al*. 1997).

The relative stabilities of alternative stacking conformers could be determined by differences in stacking free energies. However, these differences are generally estimated to be relatively small in DNA duplexes, of the order of 200 cal mol^{-1} (Doktycz *et al*. 1992). It is therefore likely that other interactions play a role in determining the relative stabilities of stacking conformers. Sequence-dependent contacts have been observed in the crystal structures of the junctions. For example, in both the Coll (Ortiz-Lombardía *et al*. 1999) and Ho (Eichman *et al*. 2000) structures, hydrogen bonds are found between the phosphates of the exchange points and the N_4 amine of cytosine located one nucleotide to the 3′ side of the nucleotides that define the point of strand exchange (Fig. 12). While a similar interaction might be made if an adenine were located in this position, replacement by either guanine or thymine would evidently prevent the contact. It is interesting that both crystallised sequences have the triplet ApCpC at the junction, with the phosphate between the A and C defining the exchange point. Perhaps this sequence confers a special stability to the junction, helping to explain why branch migration in the Ho sequence is frozen at this point.

5.1.2 Simultaneous presence of both stacking conformers

While some junctions exhibited a strong bias towards one stacking conformer, this is not true for all sequences. Using comparative gel electrophoresis we found that junction 7 (3–4, CCGG) comprised an approximately equimolar distribution of both conformers (Grainger *et al*. 1998). Millar, Chazin and coworkers studied the distribution of stacking conformers in

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Table 1. *Table of junction sequences*

Fixed	1-step	2-step
Class 1: RRRR		
1–1 AAGG (a)	1A–1 AAAG	1B–1 AAAA
	1A–2 AGGG	1B–2 AGAG (b)
		1B–3 GGGG
Class 2: YRRR		
2–1 CAAG	2A–1 CAAA	—
2–2 CAGG	2A–2 CAGA	
2–3 CGAA	2A–3 CGAG	
2–4 CGGA	2A–4 CGGG	
2–5 TAAG	2A–5 TAAA (c)	
2–6 TAGG	2A–6 TAGA	
2–7 TGAA	2A–7 TGAG	
2–8 TGGA	2A–8 TGGG	
Class 3: YYRR		
3–1 CCAA (d and e)	—	—
3–2 CCAG		
3–3 CCGA		
3–4 CCGG (f and g)		
3–5 CTAA		
3–6 CTAG		
3–7 CTGA		
3–8 CTGG		
3–9 TCAA		
3–10 TCAG (h)		
3–11 TCGA		
3–12 TCGG		
3–13 TTAA		
3–14 TTAG		
3–15 TTGA		
3–16 TTGG (i)		
Class 4: YRYR		
4–1 CATG (j)	4A–1 CACG	4B–1 CACA
4–2 CGTA (k)	4A–2 CATA	4B–2 CGCG
	4A–3 CGTG	4B–3 TATA (l)
	4A–4 TATG	4B–4 TGTG
Class 5: YYYR		
5–1 CCTA	5A–1 CCCA	—
5–2 CCTG	5A–2 CCCG	
5–3 CTTA	5A–3 CTCA	
5–4 CTTG	5A–4 CTCG	
5–5 TCCA	5A–5 TCTA	
5–6 TCCG	5A–6 TCTG	
5–7 TTCA	5A–7 TTTA	
5–8 TTCG	5A–8 TTTG	
Class 6: YYYY		
6–1 CCTT (m)	6A–1 CCCT	6B–1 CCCC
	6A–2 CTTT	6B–2 CTCT
		6B–3 TTTT

This table shows all possible junction sequences where only the inner set of basepairs is considered. Some of these (left column) cannot branch migrate, while others can undergo one (centre column) or two (right column) steps of branch migration. Junctions capable of branch migration could appear more than once in the table, but have only been recorded for one form. Examples of DNA junctions that have been studied experimentally are indicated; in some cases there are multiple forms, differing in sequence outside the central basepairs. The tabulation follows the scheme of Altona (1996), according to a set of

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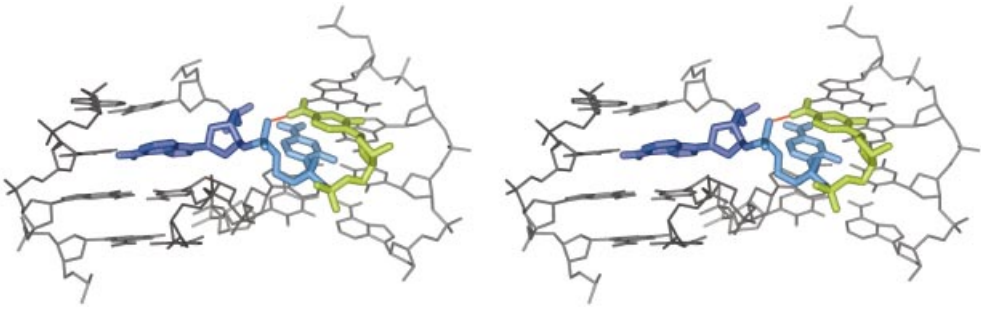


Fig. 12. An illustration of sequence dependence in the structure of a DNA junction. The stereo image shows a detail in the crystal structure of the symmetrical four-way junction (Eichman *et al*. 2000), around the point of strand exchange. The sequence A–CC (where the point of strand exchange is denoted by the dash) is highlighted dark-blue, light-blue, green. Note the hydrogen bond between the exocyclic amine of the 3′ cytosine base and the non-bridging oxygen of the phosphate at the exchange point.

three different four-way junctions using the chemical shift of ^{15}N-labelled thymine and distance distributions calculated from time-resolved FRET experiments (Miick *et al*. 1997). This showed that the distribution of stacking conformers varied considerably with junction sequence; while junction J1 (3–10, TCAG) was heavily biased (> 95 %) towards a single conformer, J2P1 (3–1, CCAA) contained around 20 % of a minor conformer while J2 (also 3–1, see below) comprised a population containing almost equal amounts of both conformers. Using *Mbo*II cleavage across the junction, we found that the minor conformer could even be detected in the heavily biased junction 1 (4–1, CGTA) (Murchie *et al*. 1991). Using a double-crossover construct to trap junctions at a fixed point, Zhang & Seeman (1994) compared the relative stabilities between conformers for a series of symmetrical sequences. They concluded that for these sequences the differences were relatively small, typically less than RT.

5.1.3 Exchange between stacking conformers

The simultaneous existence of both stacking conformers suggests that conformational equilibria probably exist, with interconversion between the two forms. Early studies of junction 1 (4–1, CGTA) using *Mbo*II cleavage indicated that interconversion between the major and minor forms must occur (Murchie *et al*. 1991), and a similar conclusion was reached for junction 7 (3–4, CCGG) (Grainger *et al*. 1998). NMR has been particularly informative in this area. While NOE connectivities in junction 2 (3–1, CCAA) were observed indicating the presence of the minor stacking conformer, the existence of a single set of resonant frequencies showed that these were in rapid exchange on the NMR timescale (Carlström &

rules for arranging and classifying the junctions. The four-letter code for each junction denotes the circular arrangement of 5′-nucleotides surrounding the junction centre. The structures of the following DNA junctions have been studied; (a) NMR structure, Wijmenga J6 (van Buuren *et al*. 2000); (b) Ho crystal structure, with junction located at this point (Eichman *et al*. 2000); (c) Seeman and Kallenbach J1SMA (Chen *et al*. 1988); (d) NMR structure, Chazin J2 (Chen *et al*. 1991); (e) NMR structure, Chazin J2P1 (Carlström & Chazin, 1996); (f) Lilley Junction 7 (Grainger *et al*. 1998); (g) NMR structure, Altona J9 (Overmars & Altona, 1997); (h) Seeman and Kallenbach J1 (Churchill *et al*. 1988); (i) NMR structure, Altona J4 (Pikkemaat *et al*. 1994); (j) Lilley Junction 3 (Duckett *et al*., 1988); (k) Lilley Junction 1 (Duckett *et al*. 1988); (l) Seeman and Kallenbach K24 (Lu *et al*. 1990a, b); (m) Lilley Junction 2 (Duckett *et al*. 1988).

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Chazin, 1996). However, NMR studies of another junction (junction 9a, 3–4 CCGG) found that a distribution of stacking conformers was in slow exchange (Overmars & Altona, 1997). From these data, lifetimes of the two conformers of 430 and 180 ms were calculated, together with an activation barrier for interconversion of $\Delta G^{\ddagger}_{298} = 16{\cdot}2$ kcal mol^{-1}.

5.1.4 Longer-range sequence dependence

While discussion of sequence effects on DNA junction structure has largely focussed on the basepairs immediately surrounding the point of strand exchange, it is clear that sequences further removed from the junction can exert a significant conformational influence. Junctions J2 and J2P1 differ by a cyclic permutation of the inner four basepairs, and thus the central sequence is CCAA (3–1) in both cases (Miick *et al*. 1997). Yet the stacking conformer bias is quite different in the two cases, proving that there is an effect of the adjacent sequences. In another example, junction 7 analysed in this laboratory, and junction J9a of Altona share the same central sequence (3–4, CCGG), yet exhibit significantly different conformational properties in terms of their stacking preferences. Junction 7 exhibits an approximately equal distribution of the two stacking conformers (Grainger *et al*. 1998), while J9a displays a 2.5:1 bias towards one stacking conformer (Overmars & Altona, 1997). The effect may be felt over longer distances. Junction 7 and junction 7rot are related by a cyclic rotation of the two inner basepairs on each arm, but in contrast to junction 7 the latter is strongly biased towards one conformer (Grainger *et al*. 1998). Since the two junctions are identical over the two inner basepairs of each arm, the conformational influence must be felt over at least three basepairs. Another precedent for such long-range effects has been noted in the conformation of three-way junctions (Welch *et al*. 1995).

There are 70 different central sequences possible at the four-way junction. Once we are required to consider the second and third basepairs out from the junction (and perhaps beyond), the combinatorial possibilities rapidly become enormous. The conformational problem is therefore likely to remain incompletely resolved for the foreseeable future. This also means that the categorisation of junctions presented in Table 1 is necessarily an over-simplification and that two junctions having the same central sequence (and hence the same designation) can have very different conformational properties.

5.2 Variability in the interhelical angle

5.2.1 The interhelical angle

There is general agreement that the natural structure of the unconstrained four-way DNA junction is a right-handed, antiparallel cross. Molecular modelling suggested that the alignment between strands and grooves would be optimal for an interhelical angle of 60° (von Kitzing *et al*. 1990). Indeed, when two arms were artificially reversed by the introduction of central 5′–5′ and 3′–3′ linkages (Sha *et al*. 1999), the resulting junction adopted a parallel conformation in the presence of magnesium ions. Angles in the region of 60° were estimated experimentally in solution by means of neutron scattering and magnetically induced birefringence measurements (J. Torbet and D. M. J. L., unpublished data; and Torbet (1992)) and on a surface by means of atomic force microscopy of an array self-assembled from four-way DNA junctions (Mao *et al*. 1999). An angle of 55° was found in the crystal structure of

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the junctions of the DNAzyme (Nowakowski *et al*. 1999, while angles close to 40° were observed in the other two crystallised junctions (Ortiz-Lombardía *et al*. 1999; Eichman *et al*. 2000). Wijmenga and colleagues (S. S. Wijmenga and B. N. M. van Buuren, personal communication) have deduced an interhelical angle of 70° for another four-way junction in solution, measured using dipolar ^{13}C–^{1}H couplings. Thus in general the observed angles distribute around the original estimate of 60°.

5.2.2 Variation, flexibility and malleability of the interhelical angle

There is considerable evidence of flexibility in the interhelical angle in the four-way DNA junction. Srinivasan & Olson (1994) carried out a molecular modelling exercise that involved a systematic rotation around the junction, and identified a range of possible angles. It would be anticipated that the equilibrium angle would be dependent on ionic conditions, since it would reflect a balance between electrostatic repulsion and other forces. This is indicated by the 'flattening' of the patterns observed in comparative gel electrophoresis experiments as the ionic strength is raised, consistent with a rotation of the junction to a narrower angle (D. M. J. L., unpublished data). Eis & Millar (1993) analysed the distribution of end-to-end distances in a four-way DNA junction using time-resolved FRET measurements, concluding that there was a natural variation in the interhelical angle.

The variability in the interhelical angle is also manifested in malleability. The variation in angles measured in the crystal suggests that packing forces might distort the global structure of the junction. This is supported by the recent observation of DNAzyme junctions in a left-handed conformation when crystallised in an orthorhombic form (Nowakowski *et al*. 2000). Experimentally, the junction has been manipulated by constraining helical termini with tethers to force the angle to adopt non-equilibrium values (Kimball *et al*. 1990). Using this approach it was found for one junction that the free energy difference between anti-parallel and parallel forms was only 1·6 kcal mol^{-1} in the presence of 5 mM magnesium ions (Lu *et al*. 1991b). However, when parallel forms were constrained more rigidly in double-crossover species they were found to be unstable, unlike their antiparallel counterparts (Fu & Seeman, 1993). Nevertheless, it is clear that DNA junctions are reasonably flexible structures, and in their natural context they would be subject to manipulation by proteins. This occurs upon binding of many junction-selective enzymes, as discussed below.

5.3 Perturbation of the junction structure

The global structure of the four-way DNA junction may also be perturbed by local changes. Breaking the continuity of one of the strands at the exchange point (but not anywhere else) results in a change to the overall structure, such that the interhelical angle rotates to an approximately perpendicular orientation (Pöhler *et al*. 1994). The nick locates itself on an exchanging strand, even if this requires the junction to undergo re-stacking to the opposite conformer. The nicked junction behaves as if one helix disengages from the other on releasing the constraint of one strand, resulting in the observed rotation.

Recombination between DNA sequences that are less than 100% identical will result in base mismatches forming at the four-way junction. These can also have structural consequences. The ion-induced folding of junctions containing a series of single-base mismatches was analysed by comparative gel electrophoresis (Duckett & Lilley, 1991). It was

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found that some were readily tolerated without disruption of the stacked X-structure, while others almost completely prevented folding; there was an approximate correlation between the thermal stability of a given mismatch and its effect on the structure of the junction. In a number of cases, the presence of a mismatch resulted in an elevation of the magnesium ion concentration required to achieve folding. Evidently the destabilisation of the stacked X-structure due to the presence of certain mismatches changes the position of equilibrium between interhelical stacking and electrostatic repulsion. This shows once more how delicate this balance is. One curious junction was obtained, containing a C·A mismatch. In this case the relative stability of stacking conformers was pH dependent (Duckett & Lilley, 1991), such that the difference between the protonated and unprotonated mispair was sufficient to tip the balance of stacking bias.

6. Branch migration

6.1 Strand exchange between homologous sequences, and branch migration

In homologous genetic recombination the four-way junction forms within homologous DNA sequences, and branch migration can therefore occur, as shown schematically in Fig. 2. The branchpoint is therefore mobile, and can move many kilobases *in vivo*. Branch migration is usually considered a step-wise random walk process; normally this would be an unbiased process, and thus the junction could move in either direction at any step. This process would be characterised by the step time (τ), and the time taken to the junction to migrate over a region of N bp would rise as τN^2. A single step of branch migration requires the breakage of two basepairs at the point of strand exchange located on diametrically opposite arms, rotation around, and pairing to form the terminal basepairs on the remaining two arms. This would be expected to require some local disruption at the junction, and thus it is not too surprising to learn that the process is intimately related to the structure of the junction. Hsieh & Panyutin (1995) have written an excellent earlier review of this topic.

6.2 The rate of branch migration

The rate of branch migration in three-stranded systems provides a baseline for the process, giving step times of 12 μs (Radding *et al*. 1977) and 20 μs (Green & Tibbetts, 1981). This is expected to be an upper limit on the rate, since relatively little structural disruption would be required to facilitate the exchange of pairing. An early estimate of the rate in four-way junctions involving the rotation of parallel DNA duplexes provided a step time of 2×10^{-5} s (Meselson, 1972). However, the history of this area has seen a constant reduction in our estimated rate for this process, which turns out to be extremely slow.

The experimental measurement of the rate of branch migration in four-way junctions was pioneered by Warner and colleagues using electron microscopy to follow the loss of chi structures formed by restriction cleavage of phage G4 figure-8 structures (Thompson *et al*. 1976a). They observed rates of branch migration of 6 kbp s^{-1}, corresponding to step rates of 170 μs. These were later revised to 100 μs (Warner *et al*. 1979). However, these rates were inconsistent with the speed of cruciform absorption (Courey & Wang, 1983; Gellert *et al*. 1983; Sinden & Pettijohn, 1984), which indicated a rate far slower than this. The most systematic study of branch migration rates has come from the work of Hsieh and colleagues (Panyutin & Hsieh, 1993, 1994; Panyutin *et al*. 1995). They devised a substrate that provides

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a known point at which the branch migration is initiated by hybridisation of two duplexes with homologous frayed ends (Panyutin & Hsieh, 1994). At the beginning of the reaction the junction can only migrate in one direction; this region therefore represents a 'reflecting barrier'. The reaction is continued until the junction runs off the other end, which constitutes an 'absorbing barrier', and this endpoint is detected electrophoretically. Computer simulation of a random walk process can then be used to extract the average step time for the process. Using this approach Panyutin & Hsieh (1994) measured a step time of 270–300 ms in the presence of 10 mM magnesium ions. This rate is orders of magnitude slower than those previously estimated, and shows that the reaction can be extremely slow.

6.3 The effect of magnesium ions on branch migration rates

One of the most interesting observations of Panyutin & Hsieh (1994) was that the measured rate of branch migration is extremely sensitive to the presence of divalent metal ions. When the experiments were repeated in the absence of these ions, step times of 300–400 μs were obtained. These values were closer to the original measurements of Warner and colleagues (Thompson *et al*. 1976a), which were made in the presence of EDTA, and recent measurements from that laboratory (Mulrooney *et al*. 1996) in the presence of magnesium ions gave very much slower rates that are more consistent with those of Panyutin & Hsieh under similar conditions. Johnston & Symington (1993) also found that rates of branch migration measured by the dissociation of chi structures were also very much faster in the absence of magnesium ions. Another illuminating difference in the process with or without magnesium ions present was revealed by the sensitivity to single-base mismatches (Panyutin & Hsieh, 1993). While these had relatively small effects on the rate of branch migration in the absence of the divalent ions, in their presence the process was completely blocked.

These results show that rates of branch migration are three orders of magnitude faster when the process takes place in the absence of magnesium ions. Of course, this corresponds to the formation of the open form of the four-way junction, and suggests that the rate is severely reduced when the junction is folded into the stacked X-structure. Panyutin *et al*. (1995) showed that the slower rate correlated with the formation of the stacked X-structure. The change in rate was steepest in the range up to 300 μM magnesium (where most junctions fold stably into the stacked X-structure (Duckett *et al*. 1990)), the same range where protection of central bases against attack by osmium tetroxide and diethyl pyrocarbonate was obtained.

6.4 Branch migration and the structure of the DNA junction

It is clear that there is a correlation between the rate of branch migration and the formation of the open form of the four-way DNA junction. Some disruption of the stacked X-structure would probably be a prerequisite for the required exchange of basepairing – it is very hard to see how this could occur in the context of the folded structure. By contrast it is easy to imagine the exchange occurring relatively easily when the junction is unstacked in the low-salt form. Modelling the kinetics of branch migration indicates that the process proceeds by a series of steps where a single pair of exchanges is made. This is also consistent with the sensitivity to a single-base mismatch, which indicates that the step size is small (Biswas *et al*. 1998), and effects of local sequence (Sun *et al*. 1998). Despite the difference in rates with and without magnesium ions present, the measured enthalpy of activation is very similar in both

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cases (36 and 38 kcal mol^{-1}) (Panyutin & Hsieh, 1994), suggesting that the same transition state is traversed. Taken together these observations suggest that the process of branch migration begins by an opening into the extended form. One step of basepairing exchange may then occur, stochastically in one direction or the other, followed by closure to reform the stacked X-structure. Thus the junction spends most of its time folded into the stacked X-conformation, with occasional 'hops' into an adjacent branch migrated form. Since this could have altered stacking preferences, this may require exchange of stacking partners and thus formation of a different stacking conformer.

An homologous junction should therefore appear little different from a non-homologous one on average; both will be well represented by the stacked X-structure. This is demonstrated by the crystal structure of the four-way junction of Ho and colleagues (Eichman *et al*. 2000), where a completely homologous junction adopts a standard stacked X-structure.

7. Three-way DNA junctions

Three-way helical junctions can be perfectly paired 3H junctions, or they can have additional unpaired bases in one ($3HS_n$) or more ($2HS_nHS_m$ or $HS_nHS_mHS_p$) strands at the point of strand exchange. These could be significant elements within the structure of single-stranded DNA species, although they would be expected to have much greater general importance in RNA structure. While at present there has been more systematic study in DNA, the overall stereochemical properties are expected to be similar for RNA. The arrangements of possible sequences around the strand exchange point has been classified by Altona (1996).

7.1 The perfectly basepaired three-way junction

In many respects the three-way junction provides a test of the generality of the stereochemical principles established with the four-way junction. One of the folding principles to emerge from the study of the four-way junction is the strong tendency for pairwise coaxial stacking of helical arms. In principle it should be possible to stack two of the three arms of a three-way junction. However, simple model building indicates that it should not be possible to construct a perfect three-way junction with a pair of coaxially stacked helices without breaking one or more basepairs. If we attempt to construct a three-way junction by fusing an additional helix to a broken phosphodiester linkage in one strand of a duplex, we must insert at least the width of the minor groove into the space previously occupied by just a single phosphate group. Comparative gel electrophoretic experiments (Duckett & Lilley, 1990) indicated that the three angles between the arms of a series of perfect three-way DNA (3H) junctions were much closer to being equal than were the six angles relating the arms of the four-way junction. This was confirmed by FRET experiments, where the three end-to-end distances of a three-way junction were found to be closely similar (Stühmeier *et al*. 1997). Cyclisation experiments on a perfect three-way junction indicated that the inter-helical angles were very similar, in the range of 60–90° (Shlyakhtenko *et al*. 1994a), and recent images using atomic force microscopy show a high proportion of symmetrical molecules (Oussatcheva *et al*. 1999). In addition it was found that thymine bases at the point of strand exchange remained reactive to osmium tetroxide even at high magnesium concentrations (Duckett & Lilley, 1990), and to diethyl pyrocarbonate (Shlyakhtenko *et al*. 1994b). This contrasts with the four-

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way junction, where folding into the stacked X-structure results in protection of thymine bases at the junction.

All of this indicates that the perfect three-way junction does not generally fold by pairwise coaxial stacking of helices. This implies a flexible structure, which is consistent with early studies indicating that the interhelical angles were sufficiently flexible to allow cyclisation into a variety of products (Ma *et al*. 1986). However, some 3H junctions are nevertheless quite asymmetrical in their global structure (Lu *et al*. 1991a), indicating that the simple unstacked Y-structure may be an oversimplification for some sequences. An extended three-way junction would have inequivalent major and minor groove sides, and so the arms are unlikely to be exactly coplanar.

7.2 The effect of unpaired bases; the bulged three-way junction

The perfect 3H three-way junction appears to break the general folding principle, i.e. that helical junctions tend to undergo coaxial helical stacking and ion-dependent folding. The rigid framework of the fully paired three-way junction evidently makes such folding very difficult. However, this restraint might be relaxed if some additional conformational flexibility were provided by the addition of a single-stranded region between the helical arms, creating a $3HS_n$ junction. Leontis (Leontis *et al*. 1991) first showed that such bulged three-way junctions had increased stability, based on gel electrophoresis studies. Using comparative gel electrophoresis we demonstrated (Welch *et al*. 1993) that such junctions undergo an ion-induced conformational change. In the absence of added ions the junctions were unstacked and extended, but underwent a folding transition upon addition of magnesium or other multivalent ions. These results were interpreted in terms of the formation of a structure in which two arms became coaxially stacked, while the third subtended an angle that was dependent upon the number of unpaired bases (Fig. 13(*a*)).

This global structure is consistent with FRET studies (Stühmeier *et al*. 1997), in which the end-to-end distance between two helices was reduced as the number of unpaired bases increased. Time-resolved FRET measurements (Yang & Millar, 1996) also revealed changes in interhelix end-to-end lengths in three-way junctions with the introduction of unpaired bases. The conformational effect of the extra bases was also consistent with lowered rates of cyclisation of DNA containing a bulged junction, compared to those carrying a perfectly paired junction (Shlyakhtenko *et al*. 1994a). Once the structural restraints imposed by the perfect three-way junction are relaxed, three- and four-way junctions exhibit the same general principles of folding. If electrostatic repulsion and steric factors can be reduced sufficiently, then coaxial helix–helix stacking will drive the folding process resulting in a stacked conformation.

7.3 Two inequivalent stacking conformers

A $3HS_n$ junction will have one continuous strand, and this would be expected to be one of the two non-bulge-containing strands. However, that leaves two possible conformers that might be adopted depending upon the choice of stacking partner, as illustrated in Fig. 13(*b*). In contrast to the four-way junction, these are not stereochemically equivalent structures, and are therefore unlikely to be equally stable. In one structure the polarity of the bulge sequence is 3′ to 5′ as it leaves the stacked helices (conformer I), while in the other it is 5′ to 3′ (conformer II).

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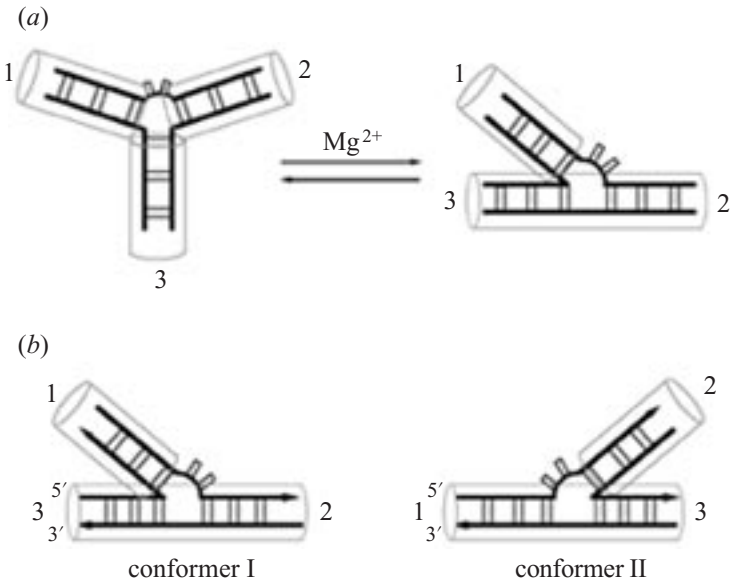


Fig. 13. Schematic of bulged three-way DNA junctions. (*a*) Ion-induced folding of a $3HS_2$ junction. At low salt concentration the junction is extended, with no stacking of helices. On addition of magnesium ions the junction can fold by coaxial stacking of two helices. The third helix is directed away at an angle that depends on the number of unpaired bases (i.e. *n* in a $3HS_n$ junction). (*b*) Alternative stacking conformers of a $3HS_n$ junction. The $3HS_2$ junction illustrated can in principle adopt one or other of two stacking conformers. Conformer I is obtained if helices 2 and 3 undergo coaxial stacking, while 1 on 3 stacking generates conformer II. These structures are not stereochemically equivalent – note that the polarity of the backbone of the bulged nucleotides is different between the two conformers.

Three-way DNA junctions containing two unpaired bases ($3HS_2$ junctions) have been the subject of three independent NMR studies, involving different sequences (Leontis *et al*. 1993; Rosen & Patel, 1993a, b; Ouporov & Leontis, 1995; Overmars *et al*. 1996). All three groups found structures based upon coaxial stacking of two helices, with the third helix unstacked and extended away from the point of strand exchange. Interestingly, the NMR structures include examples of both possible stacking conformers. The structure solved by Leontis and coworkers (Leontis *et al*. 1993; Ouporov & Leontis, 1995; Thiviyanathan *et al*. 1999) is an example of conformer I, while the junctions analysed by Rosen & Patel (1993a, b) and Overmars *et al*. (1996) are folded into conformer II.

We compared the folding of a number of different $3HS_2$ junction sequences by comparative gel electrophoresis and FRET (Welch *et al*. 1995). The majority of these folded by formation of conformer I on addition of magnesium ions. Nevertheless, when we studied a junction having the same central sequence as that of Rosen & Patel we found that this adopted the alternative stacking conformer, in complete agreement with the NMR analysis.

Despite the stereochemical inequivalence of the two structures, both can be adopted, and the relative stability is determined by local sequence. In our experience the formation of conformer II is relatively rare. Yet curiously, the Rosen–Patel sequence is the most stable three-way junction that we have examined. This junction is characterised by a higher melting temperature compared to other sequences, and folding is induced by monovalent ions alone (30 mM sodium ions is sufficient).

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7.4 The stereochemistry of the bulged three-way junction

In view of the fundamental structural difference between the possible conformers of the bulged three-way junction we would expect the local stereochemistry to be different. This seems to be confirmed by NMR structures of the examples of each form. The path of the backbone of the bulged section of the Leontis structure (conformer I) (Leontis *et al*. 1993) is relatively looped, while in the Rosen–Patel structure (conformer II) (Rosen & Patel, 1993b) the backbone passes smoothly from the stacked helices into the unstacked arm (Fig. 14). The unstacked helix of the Leontis structure is largely coplanar, and is approximately perpendicular to the stacked helices (Thiviyanathan *et al*. 1999), although global features like this angle are probably not well determined by the available NMR data in any of the structures. In the Rosen–Patel structure, the third arm is less coplanar and in addition it is bent back at an acute angle, just as our electrophoretic data would indicate. The overall folding of this junction is remarkably similar to that which would be derived by the removal of one helical arm from the right-handed stacked X-structure of the four-way DNA junction (Murchie *et al*. 1989; von Kitzing *et al*. 1990). The backbone of stem II is located in the major groove of stem III in the Rosen–Patel junction (Rosen & Patel, 1993b). This feature of the structure is very similar to the juxtaposition of the backbone of one stacked helix in the major groove of the other in the four-way junction.

In summary, bulged three-way junctions undergo ion-dependent folding by pairwise coaxial stacking of the helices into one of two alternative conformers determined by local sequence. Thus we find that the three-way junction displays many of the same folding properties exhibited by the four-way junction, given a little extra conformational flexibility.

8. Helical junctions in RNA

Helical junctions are common elements in the secondary structure of RNA, where they would be expected to play an important architectural role, organising the tertiary structure of the molecule. We might anticipate some features, such as coaxial helical stacking, to be shared with DNA junctions, but given the rather different geometry of the A-form helix adopted by RNA we would also expect conformational differences.

8.1 The four-way junction in RNA

We have studied a number of perfect four-way (4H) junctions in RNA by comparative gel electrophoresis (Duckett *et al*. 1995b) and FRET (Walter *et al*. 1998b, c). As expected, these studies revealed both similarities and differences from the DNA equivalents. The RNA junctions appear to fold by coaxial helical stacking, and even seem to exhibit the same choice of stacking partners as the same sequences in DNA in the few cases where a comparison is possible. However, the global structure can be different, and responds to changes in ionic conditions in a different way. The general structure of the RNA junction in the presence of moderate (e.g. 1 mM) magnesium ion concentrations is a 90° cross of helical stacks, i.e. a structure that is neither parallel nor antiparallel; under the same conditions the four-way DNA junction would be rotated into the antiparallel form. But the greatest difference between RNA and DNA junctions is found at low salt concentrations. In contrast to DNA, the RNA species do not suffer loss of coaxial stacking in the absence of added metal ions, but tend to rotate into a parallel-stranded form. The parallel distortion is somewhat sequence-

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dependent, but this represents the first example of a parallel orientation for any nucleic acid four-way junction. By contrast, when placed in 0·5 mM calcium ions, or elevated concentrations of magnesium ions, RNA junctions (with one notable exception) rotate in the opposite direction to adopt an antiparallel structure. The transition into the antiparallel form can be followed by the change in FRET efficiency of fluorophores attached to helical termini, and analysed in terms of a simple ion binding model (equation 1) with a value of $n = 1$ and an apparent association constant in the range of 10^2–10^3 (Walter *et al.* 1998b) (Fig. 15). This is significantly lower than the K_A^{app} observed for the folding of DNA junctions (see section 4.2), reflecting a reduced stability of the folded form of the RNA junction. The K_A^{app} will be determined by $\exp(-\Delta G_{fold}/RT)$, where ΔG_{fold} is the net free energy of forming the folded structure and binding the metal ion(s) to it. Thus if the stability of the folded form is reduced this will lower the K_A^{app}, and therefore a higher concentration of ions will be required to induce the transition.

Some of the differences between RNA and DNA junctions are likely to derive from the formation of an A-form helix by RNA, where the similarity in the widths of the major and minor grooves suggests that backbone-groove juxtaposition will be less favourable. Removal of the thermodynamic advantage of strand-groove alignment could alter the balance with other steric and electrostatic factors, resulting in a new global conformational minimum free energy. This appears to be the case for the RNA junction in the presence of 1 mM magnesium ions. The absence of a transition to an unstacked extended structure in the absence of added ions contrasts strongly with the behaviour of DNA junctions, and suggests that overall electrostatic repulsion in the RNA junction is lower. In a sense, the DNAzyme junctions for which a crystal structure is available (Nowakowski *et al.* 1999) may represent an intermediate situation. Each junction in this structure has a single RNA strand, and thus the hybrid RNA–DNA helix of the junction is clearly A-form, while the other is B-form. The resulting structure is very much the right-handed, antiparallel stacked X-structure under the crystallisation conditions, with an angle between the two axes of 55°. Evidently an A-form helix still permits strand exchange to occur with a rather similar molecular geometry. However, in a different crystal form this junction adopts a left-handed, antiparallel conformation (Nowakowski *et al.* 2000).

8.2 Some important four-way junctions in functional RNA species

There are many examples of RNA four-way junctions where they appear to play an important functional role. Two examples will be examined in greater detail.

8.2.1 The U1 snRNA junction

The U1 snRNA, that is involved in splicing of mRNA, is organised around a prominent four-way helical junction. The junction sequence is conserved in mammalian, avian and amphibian sequences (Branlant *et al.* 1981; Guthrie & Patterson, 1988), and is perfectly basepaired for at least three basepairs in each arm, except for the single G·A mismatch located at the point of strand exchange. Comparative gel electrophoresis (Duckett *et al.* 1995b) and FRET experiments (Walter *et al.* 1998a) showed that the U1 junction folded by means of coaxial helical stacking, in the conformer in which the adenine base of the G·A mismatch was located on the continuous strand (A_e stacking conformer). This was in good agreement with the earlier results of Krol *et al.* (1990), based on differences in sensitivity to ribonuclease V1. The

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G·A mismatch did not appear to influence the global structure adopted significantly, since its replacement by either G·C or T·A basepairs did not change the global conformation adopted. While the G·A mismatch is conserved in the sequences of many U1 snRNA species, it is replaced by an A·U basepair in the U1 snRNA of *Drosophila melanogaster*. We analysed the global structure of a junction based on the *Drosophila* sequence, and found that the junction folded in the same way as the mammalian sequence. Once again the structure was based on coaxial stacking of arms in the A_e stacking conformer. This suggests that there is conservation of three-dimensional structure by the different U1 snRNA species that can accommodate changes in base sequence. Perhaps the most significant difference between the U1 junction and other four-way RNA junctions is found at higher divalent ion concentrations. Where other junctions rotate into the antiparallel form under these conditions, the U1 junction remains resolutely in the 90° form (i.e. with perpendicular helical axes). This clearly shows the interplay of base sequence and tertiary structure in the four-way RNA junction.

8.2.2 The four-way junction of the hairpin ribozyme

Another example of a four-way RNA junction can be found in the hairpin ribozyme of the tobacco ringspot virus (Feldstein *et al*. 1989; Hampel & Tritz, 1989). This is based on a 4H junction that is completely Watson–Crick basepaired. Two consecutive arms (A and B) of this junction contain internal loops that form the essential core of the ribozyme, and the ion-induced cleavage occurs within the smaller of these loops (the A loop). In the absence of the loops the junction folds as a completely typical four-way RNA junction, into the stacking conformer that places the potentially loop-containing arms on different stacked pairs of helices (Walter *et al*. 1998b). Restoration of the loops preserves the stacking conformer, and on addition of magnesium ions there is a rotation into the antiparallel form, bringing the two loops into close proximity (Murchie *et al*. 1998; Walter *et al*. 1999). The characteristics of this ion-induced folding are quite different from those of the pure junction; fitting to FRET data indicate a folding induced by the cooperative binding of magnesium (or other divalent) ions with $n = 2$, and the apparent affinity is extremely high with folding occurring in the 0–150 μM magnesium ion range (Walter *et al*. 1998c). The resulting structure is less symmetrical than the perfect junction, consistent with a distortion due to a tight association between the two loops. This suggests that the junction is relatively malleable.

8.3 Three-way helical junctions in RNA

There are countless cases of three-way junctions occurring in natural RNA species; prominent examples include junctions derived from 5S rRNA (Shen & Hagerman, 1994), a ribosomal protein binding site in 16S rRNA (Serganov *et al*. 1996; Orr *et al*. 1998), the hammerhead ribozyme (Forster & Symons, 1987) and the P4–P6 domain of the group I intron ribozyme (Cate *et al*. 1996). However, there has been little systematic study of the conformation of three-way junctions in RNA. Preliminary studies suggest that the overall folding properties are likely to be similar to those of DNA junctions (D. M. J. L., unpublished data). It would be anticipated that folding of perfect three-way (3H) junctions would be difficult if basepairing is completely preserved, but that the addition of extra bases at the point of strand exchange (the simplest case being $3HS_n$ junctions) would increase conformational flexibility. Examination of the predicted secondary structures of a number of large RNA

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molecules shows that while three-way junction are quite common, they are seldom perfect 3H junctions. In our experience it is quite common for such junctions to undergo folding that involves the coaxial stacking of two arms, induced by the binding of divalent metal ions (D. M. J. L., unpublished data). An example of such behaviour is found in the $2HS_3HS_2$ junction found in 16S rRNA, that serves as the binding site for the ribosomal protein S15 (Serganov *et al*. 1996; Batey & Williamson, 1996). Williamson and coworkers (Orr *et al*. 1998) found that in the absence of added metal ions the junction was extended with no pairwise coaxial stacking of helical arms. Upon addition of 1·5 mM magnesium ions the junction underwent a folding transition in which two of the arms became coaxially stacked, while the third made an acute angle with the resulting axis. A model of the folded junction has been presented (Serganov *et al*. 1996). The ion-induced folding transition has recently been studied in single molecules attached to a surface using FRET between fluorophores attached to different helical termini (Ha *et al*. 1999).

One of the most studied examples of an elaborated three-way junction in RNA is the hammerhead ribozyme of the plant viroids (Forster & Symons, 1987; Hazeloff & Gerlach, 1988). The ribozyme is formed from three double-helical arms joined by single-stranded segments, forming a $HS_1HS_7HS_3$ junction; cleavage occurs in the shortest single-stranded section. The structure of the ribozyme in the crystal (Pley *et al*. 1994; Scott *et al*. 1995) is based on coaxial alignment of stems II and III, with stem I subtending an acute angle to stem II. An important element in this structure is the formation of consecutive A·G mismatches (domain 2) that leads to the alignment between stems II and III. The global structure seen in the crystal is in excellent agreement with solution studies employing FRET (Tuschl *et al*. 1994; Bassi *et al*. 1997), transient electric birefringence (Amiri & Hagerman, 1994) and comparative gel electrophoresis (Bassi *et al*. 1995). Like other junctions, the hammerhead ribozyme undergoes a well-defined folding process in response to the addition of magnesium and other ions. This comprises two sequential ($n = 1$) conformational transitions (Bassi *et al*. 1995, 1997), corresponding to the formation of the domain 2 structure followed by the folding of the catalytic core. Folding can be blocked at different stages by the introduction of specific point mutations (Bassi *et al*. 1996, 1999).

9. Recognition and distortion of four-way DNA junctions by proteins

Four-way DNA junctions are the substrates for a number of enzymes in cells, and can be specifically recognised by proteins. In homologous recombination the junction is acted upon by proteins that accelerate and direct branch migration, and by nucleases that achieve resolution. These proteins can exhibit binding that is essentially structure selective. However, in most cases they also distort the structure of the junctions. The subject of proteins that interact with DNA junctions is very large, and the discussion here is restricted to those aspects that specifically involve the DNA conformation.

9.1 Junction-resolving enzymes

One of the best studied class of junction-binding enzymes is that of the junction-resolving enzymes (Table 2). These are nucleases that are specific for the structure of the four-way DNA junction. This is a large topic in itself, and cannot be fully reviewed here; the interested reader is directed towards a more comprehensive earlier review on this subject (White *et al*. 1997).

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9.1.1 Occurrence of junction-resolving enzymes

The first junction-resolving enzymes were isolated from phage-infected eubacteria; they are relatively small proteins, comprising < 160 amino acids per monomer. T4 was found to encode a 157 amino acid protein endonuclease VII that was required to resolve multiply branched newly synthesised phage DNA (Kemper & Janz, 1976); DNA from phage mutated in gene *49* that encoded endonuclease VII had the form of a branched network that could not be packaged into phage heads (Kemper & Brown, 1976). Phage T7 encodes a functionally similar enzyme called endonuclease I (149 amino acids, encoded by gene *3*) (Center & Richardson, 1970a, b; Sadowski, 1971). Both endonucleases VII and I will cleave a variety of branched DNA structures including three- and four-way junctions and bulge-containing duplexes. RusA is a smaller junction-selective endonuclease, encoded by a lambdoid prophage DLP12 of *Escherichia coli* (Mahdi *et al*. 1996).

Junction-resolving enzymes have been isolated and cloned from eubacteria. The major junction-resolving activity of *E. coli* is RuvC (Connolly *et al*. 1991; Dunderdale *et al*. 1991; Iwasaki *et al*. 1991; Sharples & Lloyd, 1991; Takahagi *et al*. 1991), a 19 kDa enzyme encoded by the *ruvC* gene (Dunderdale *et al*. 1994).

Enzymes with similar activities to RuvC have also been isolated from yeast. The *CCE1* (*MGT1*) gene was identified by screening mutants of *Saccharomyces cerevisiae* unable to cleave DNA junctions (Kleff *et al*. 1992). It encodes a 41 kDa protein CCE1, which has been overexpressed in *E. coli* (Kupfer & Kemper, 1996; White & Lilley, 1996). Nuclear recombination is unaffected in *cce1* mutants (Kleff *et al*. 1992), but the mitochondrial DNA of these cells contain large numbers of unresolved three and four-way junctions, suggesting a role for CCE1 in resolving recombining mitochondrial genomes (Lockshon *et al*. 1995). A functionally closely similar enzyme called YDC2 has been isolated from the fission yeast *Schizosaccharomyces pombe* (Whitby & Dixon, 1997; White & Lilley, 1997a; Oram *et al*. 1998). Recently, junction-resolving enzymes have been found in *Pyrococcus* (Komori *et al*. 1999) and *Sulfolobus* (Kvaratskhelia & White, 2000), and thus these examples of these enzymes have now been isolated from bacterial, eukaryal and archaeal sources.

It is tempting to suppose that junction-resolving enzymes should be ubiquitous, but at present this cannot be stated with certainty. Some eubacteria appear to have no *ruvC* homologue. Residual cruciform-cutting activity has been detected in *cce1* mutants of *S. cerevisiae*, suggesting the presence of at least one other enzyme (Kleff *et al*. 1992), but to date no *bona fide* nuclear enzyme has been identified in yeast. Lastly, while junction-cleaving activities have been identified in mammalian cells, none has been taken to the stage of purity or cloning (Hyde *et al*. 1994). A junction-resolving activity has also been detected in extracts of cells infected with vaccinia virus (Stuart *et al*. 1992), and very recently this has been shown to be the A22R protein (Garcia *et al*. 2000). The resolving enzymes can be placed into different superfamilies (Lilley & White, 2000).

The crystal structures of three junction-resolving enzymes have been solved. These are RuvC (Ariyoshi *et al*. 1994), T4 endonuclease VII (Raaijmakers *et al*. 1999) and most recently T7 endonuclease I (Hadden *et al*. 2000). Structures of complexes with four-way DNA junctions are eagerly awaited.

9.1.2 Cleavage of DNA junctions by resolving enzymes

Having been targeted to a junction, the junction-resolving enzymes cleave their DNA

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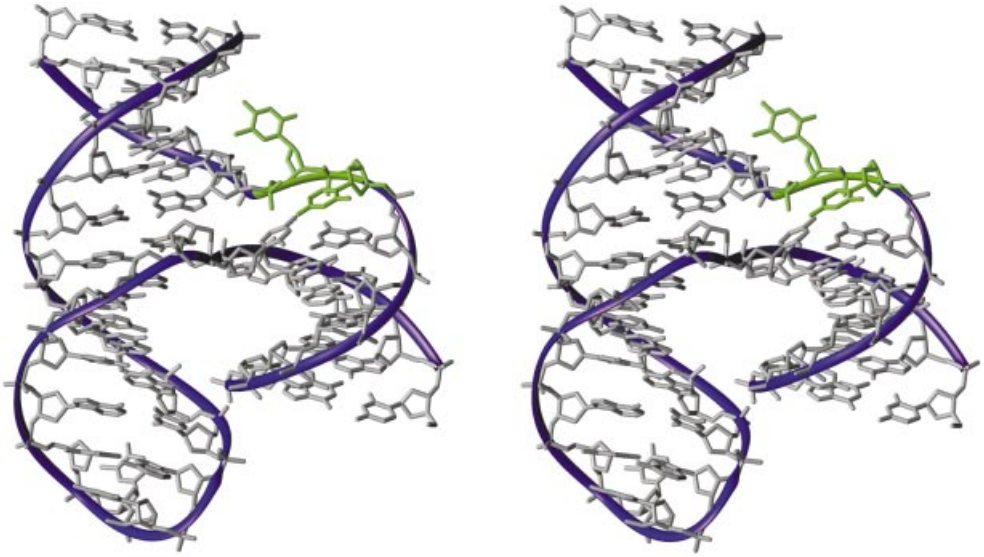


Fig. 14. Structure of a three-way DNA junction. Stereoview of the minor groove face of a $3HS_2$ junction solved by NMR by Rosen & Patel (1993a, b). Two helices are clearly coaxially stacked, and the structure is an example of conformer II. The two additional cytosine nucleotides that constitute the bulge are highlighted in green. The 3′ cytosine is stacked onto the top of the non-stacked helix, while the 5′ cytosine is directed upwards and is located in the minor groove of the top helix. Coordinates provided by Professor D. Patel (Memorial Sloan–Kettering Cancer Center, New York).

Table 2. *A summary of the properties of junction-resolving enzymes*

Source	Enzyme	Size aa	pI calc.	K_D nM	Sequence specificity	Quatern. structure	Exchange rate ($t_{1/2}$)	Crystal structure	Ref.
T4	endo VII	157	8·8	40	low	dimer	< 2 min	+	a
T7	endo I	149	9·5	2	low	dimer	> 16 h	+	b
DLP12 (λ)	RusA	120	9·6	5	⇓ CC	dimer	4 min		c
E. coli	RuvC	172	9·6	2	T/ATT ⇓ C	dimer		+	d
S. cerev.	CCE1	352	9·4	2	ACT ⇓ A	dimer	7 min		e
S. pombe	YDC2	258	9·4	1	CT ⇓	dimer	13 min		f
Sulfolobus	Hjc	143	9·8	2	low	dimer			g

a, Kemper & Janz (1976); b, Center & Richardson (1970a, b); Sadowski (1971); c, Mahdi *et al.* (1996); d, Connolly *et al.* (1991); Dunderdale *et al.* (1991); Iwasaki *et al.* (1991); Sharples & Lloyd (1991); e, Kleff *et al.* (1992); Kupfer & Kemper (1996); White & Lilley (1996); f, Oram *et al.* (1998); Whitby & Dixon (1997); White & Lilley (1997a, b); g, Kvaratskhelia & White (2000).

substrates by hydrolysis of a phosphodiester bond. As in many other nucleases (Fothergill *et al.* 1995), the hydrolytic water is probably held in the coordination sphere of a magnesium ion, which is itself held by acidic residues. Essential aspartate and glutamate residues have been found in all the resolving enzymes including T7 endonuclease I (Duckett *et al.* 1995a; Parkinson *et al.* 1999), T4 endonuclease VII (Giraud-Panis & Lilley, 1996; Pöhler *et al.* 1996)) and RuvC (Saito *et al.* 1995), and four acidic side chains are found clustered in the structure of RuvC (Ariyoshi *et al.* 1994).

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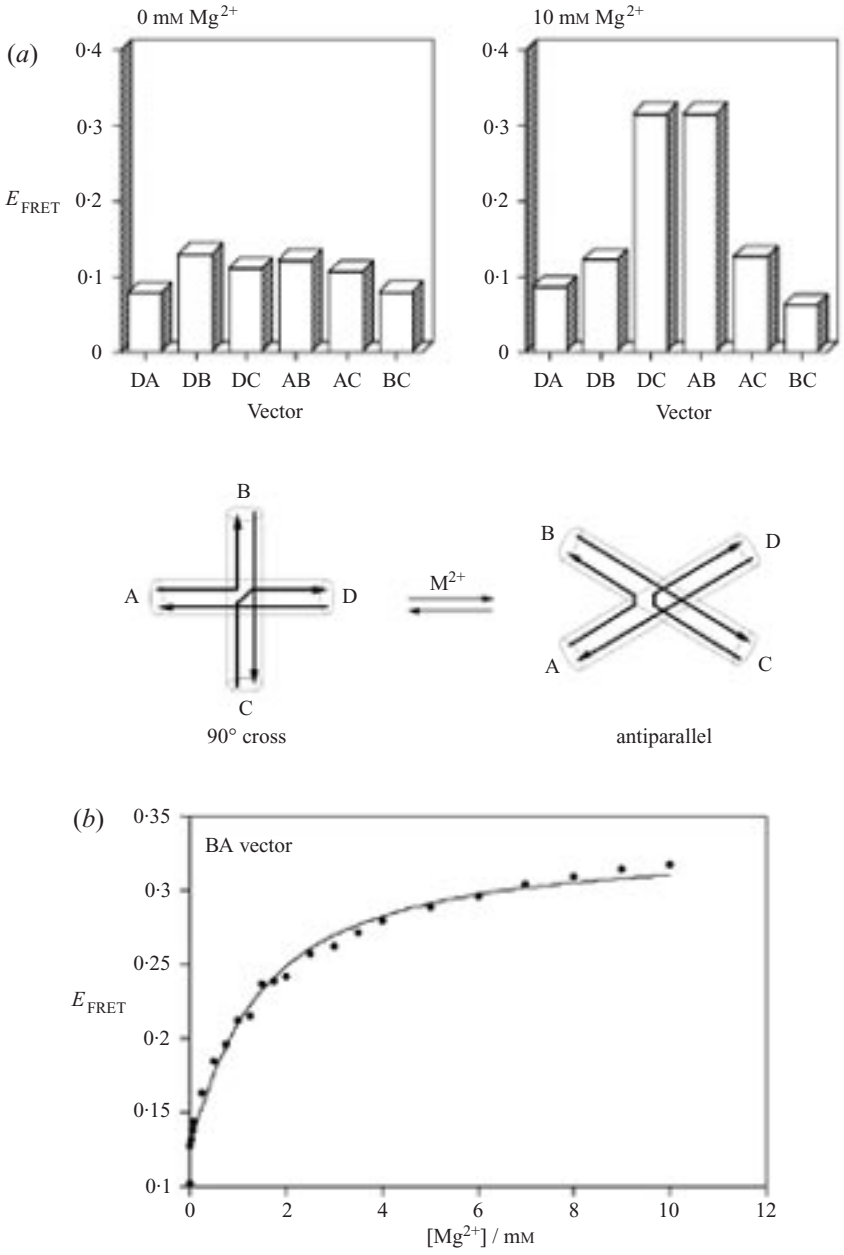


Fig. 15. Folding of a four-way RNA junction. Analysis of the ion-induced structural transition of a 4H RNA junction using FRET. The arms are named A, B, C and D in a circular manner around the junction. (*a*) The histograms show the efficiency of energy transfer (E_{FRET}) between fluorophores attached to the 5′-termini of the indicated arms (e.g. the vector AB represents a junction labelled with donor fluorophore on the A arms and with acceptor fluorophore on the B arm). In the absence of magnesium ions the pattern of efficiencies is consistent with a global structure based on coaxial stacking of A on D and B on C arms, with the two axes at 90°. On addition of magnesium ions the pattern indicates that the axes are rotated to generate an antiparallel structure. (*b*) Shortening of the B–A distance induced on addition of magnesium ions followed by the increase in FRET efficiency. This is fitted to a simple two-state model for ion-induced folding.

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While all the junction-resolving enzymes will bind to four-way DNA junctions of any sequence with equal affinity, some exhibit strong sequence specificity at the level of DNA cleavage. T4 endonuclease VII and T7 endonuclease I have very little sequence specificity, and will cleave almost any junction. By contrast, most of the remaining enzymes have pronounced preferences for cleavage at specific sequences, exemplified by RuvC (optimal cleavage sequence (T ∼ A)TT ⇓ (C > A ∼ G) (Fogg *et al*. 1999)) and CCE1 (optimal cleavage sequence ACT ⇓ A (Schofield *et al*. 1998)). In most cases the greatest discrimination occurs at the two central bases, and thus the sequence specificity is not quite at the level found in restriction enzymes.

9.1.3 Structure-selective binding of resolving enzymes to four-way junctions

The junction-resolving enzymes bind four-way DNA junctions with dissociation constants in the range K_D = 1–50 nM (Giraud-Panis & Lilley, 1996, 1998; White & Lilley, 1997b). All of these enzymes bind in dimeric form (Pöhler *et al*. 1996; White & Lilley, 1996; Parkinson & Lilley, 1997; Giraud-Panis & Lilley, 1998), consistent with the pair of cleavages required for resolution of the junction. Dimeric forms are also seen in the crystal structures of RuvC (Ariyoshi *et al*. 1994), T4 endonuclease VII (Raaijmakers *et al*. 1999) and T7 endonuclease I (J. Hadden, M Convery, A.-C. Déclais, D. M. J. L. and S. E. V. Phillips, unpublished data). Hydrodynamic (Kosak & Kemper, 1990; Iwasaki *et al*. 1991; Parkinson & Lilley, 1997) and chemical crosslinking (Pöhler *et al*. 1996; Parkinson & Lilley, 1997) evidence indicates that the junction-resolving enzymes also tend to exist in dimeric form in free solution. However, subunit exchange rates vary widely. While T4 endonuclease VII, yeast CCE1 and RusA have half-times for subunit exchange under normal solution conditions of < 5, 7 and 4 min respectively (Pöhler *et al*. 1996; White & Lilley, 1996; Giraud-Panis & Lilley, 1998), exchange cannot be detected in T7 endonuclease I under the same conditions (Parkinson & Lilley, 1997).

Binding is fundamentally structure selective – in fact, these enzymes could serve as a paradigm for structure-selective binding. Thus the complex of a dimer of any junction-resolving enzyme and a four-way DNA junction is not displaced by a 1000-fold excess of duplex DNA of the same sequence (Pöhler *et al*. 1996; White & Lilley, 1996, 1997b; Giraud-Panis & Lilley, 1998). Despite the considerable efforts of several laboratories, we still do not know the origin of this specificity. It is known that the four-way junction binds intercalators with high affinity (Guo *et al*. 1989, 1990; Lu *et al*. 1990b), and thus a plausible mechanism might involve intercalation of an aromatic side chain at the point of strand exchange. However, this is probably not consistent with the degree of structural distortion imposed by the enzymes, see below. Another earlier idea was that the enzymes might recognise the angles subtended between different arms in the junction (Bhattacharyya *et al*. 1991), but this is complicated by the same observations. Resolution of this problem will probably require the crystallographic solution of complexes of the enzymes bound to DNA junctions.

9.1.4 Distortion of the structure of junctions by resolving enzymes

All the junction-resolving enzymes distort the structure of the four-way DNA junction. In view of the impressive level of structural specificity exhibited by these enzymes, this came as something of a surprise, since they alter the very aspect that they seem to recognise. This has

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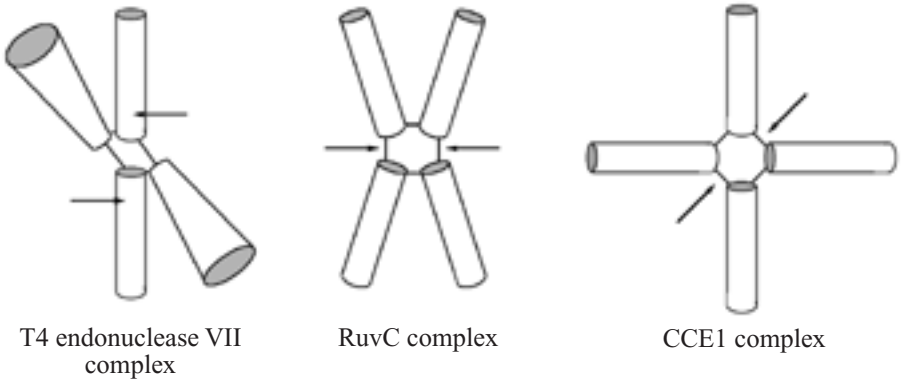


Fig. 16. Global geometry of DNA junctions bound by resolving enzymes. Schematic showing the conformations of four-way junctions bound by T4 endonuclease VII, RuvC and CCE1, determined by comparative gel electrophoresis. All enzymes open the structure of the junction to one extent or another, the most extreme case of which is CCE1.

been demonstrated for T7 endonuclease I (Duckett *et al*. 1995a), T4 endonuclease VII (Pöhler *et al*. 1996), RusA (Giraud-Panis & Lilley, 1998), RuvC (Bennett & West, 1995), CCE (White & Lilley, 1997b) and YDC2 (White & Lilley, 1998). The manipulation of the structure of the junction occurs on a large scale, and some examples are illustrated in Fig. 16. Although distortion is common to all the enzymes, the structure formed is different in almost every case, to one degree or another. The most extreme example is CCE1, where the disposition of the arms is essentially square, just as in the free junction at low ionic concentration (White & Lilley, 1997b); a closely similar structure is also induced by YDC2 (White & Lilley, 1998), and indeed by the branch migration protein RuvA (Parsons *et al*. 1995) discussed below. CCE1 also distorts the local, as well as global structure, breaking all four basepairs surrounding the point of strand exchange (Déclais & Lilley, 2000). RuvC also induces the formation of an open global structure (Bennett & West, 1995), although there is no evidence for unpairing of central bases. The structure of the DNA in the complex with T4 endonuclease VII appears more folded, and, as with the stacked X-structure of the free junction, there are two equivalent forms depending upon the local sequence at the centre of the junction (Pöhler *et al*. 1996).

9.1.5 Relationship between distortion and cleavage of DNA junctions

The functional reason for the distortion of the structure of the four-way DNA junction by resolving enzymes is not known. One possibility is that the cellular substrate for these enzymes would include additional proteins that also exert structural effects. It has often been suggested, for example, that RuvC would act in concert within the branch migration complex including RuvAB, and evidence for such an assembly has been obtained (Zerbib *et al*. 1998). Thus the preferred DNA conformation for a resolving enzyme could be that occurring in a complex with other proteins; in the absence of these proteins the enzyme might tend to distort the junction into a similar global conformation.

Opening of the junction may also be required for the cleavage mechanism, perhaps for access. The resolving enzymes all bind in dimeric form, making the two cleavages required

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for resolution. The use of cruciform substrates has shown that both cleavages occur within the lifetime of the enzyme-junction complex for T4 endonuclease VII (Giraud-Panis & Lilley, 1997), T7 endonuclease I (Parkinson & Lilley, 1997) and CCE1 (Fogg *et al*. 2000). This might indicate a requirement for simultaneous cleavage by the two subunits. However, a heterodimeric enzyme comprising one active and one inactive mutant introduces unilateral cleavage, so coordinated cleavages by the two protomers is not essential. In fact, analysis of cleavage products at short time revealed the existence of a transient nicked species in cleavage by fully active CCE1 or endonuclease I (Fogg *et al*. 2000), and thus the mechanism must be based on sequential unilateral cleavage events. For a cruciform substrate this requires that the enzyme stabilises the structure of the junction after the first cleavage, preventing reabsorption of the cruciform structure. Analysis of the kinetics of cleavage showed that the rate of the second cleavage event was accelerated by a factor of 5–10 relative to the first cleavage for CCE1 (Fogg *et al*. 2000), and for RuvC the factor is considerably greater. This suggests that the initial binding to the junction may result in a strained DNA conformation in which cleavage is not optimal. Upon cleavage of either site the structure becomes relaxed, such that cleavage at the second site is accelerated.

Thus it is likely that structural recognition, distortion and cleavage of the junction are all intimately related, and that to understand one aspect we need to understand them all.

9.2 Branch migration proteins

Branch migration of DNA junctions is accelerated by the combined action of the RuvA and RuvB proteins in eubacteria (Iwasaki *et al*. 1992; Tsaneva *et al*. 1992; Muller *et al*. 1993a, b). This has been best characterised in *E. coli*, where RuvA is a junction-selective protein that acts in concert with RuvB to facilitate branch migration. This topic is reviewed in greater depth elsewhere (Lilley, 1996; West, 1997).

The RuvAB complex is targeted to the junction by the tetrameric RuvA protein (Mitchell & West, 1996). RuvA distorts the global structure of the DNA into something very close to an extended square structure (Parsons *et al*. 1995). This would be expected to facilitate the process of branch migration, as discussed in section 6.4, since the exchange of basepairing proceeds orders of magnitude faster in the context of an open junction (Panyutin & Hsieh, 1994). RuvB is a helicase (Tsaneva *et al*. 1993; Adams & West, 1995) that forms hexameric rings around the DNA (Stasiak *et al*. 1994). The DNA is effectively 'pumped' by RuvB, thereby accelerating the process by conferring directionality.

Crystal structures of RuvA proteins have been solved both in the presence and absence of DNA junctions (Rafferty *et al*. 1996; Hargreaves *et al*. 1998; Roe *et al*. 1998). In *E. coli* RuvA, the four monomers are intimately linked together with four-fold rotational symmetry, forming a flat object of dimensions $80 \times 80 \times 45$ Å. The DNA binding face is strongly positively charged. The surface is divided by four electropositive grooves that lie between the subunits. Four 'pins', comprising glutamate and aspartate side chains lie in the middle of these grooves near the centre, forming negatively charged turrets surrounding the four-fold axis. Two types of complex have been analysed crystallographically. One comprises a single *E. coli* RuvA tetramer bound to one face of the extended form of the junction (Hargreaves *et al*. 1998). The other structure comprises to *Mycobacterium leprae* tetramers bound on either face of the junction (Roe *et al*. 1998). The conformation of the DNA in these complexes is

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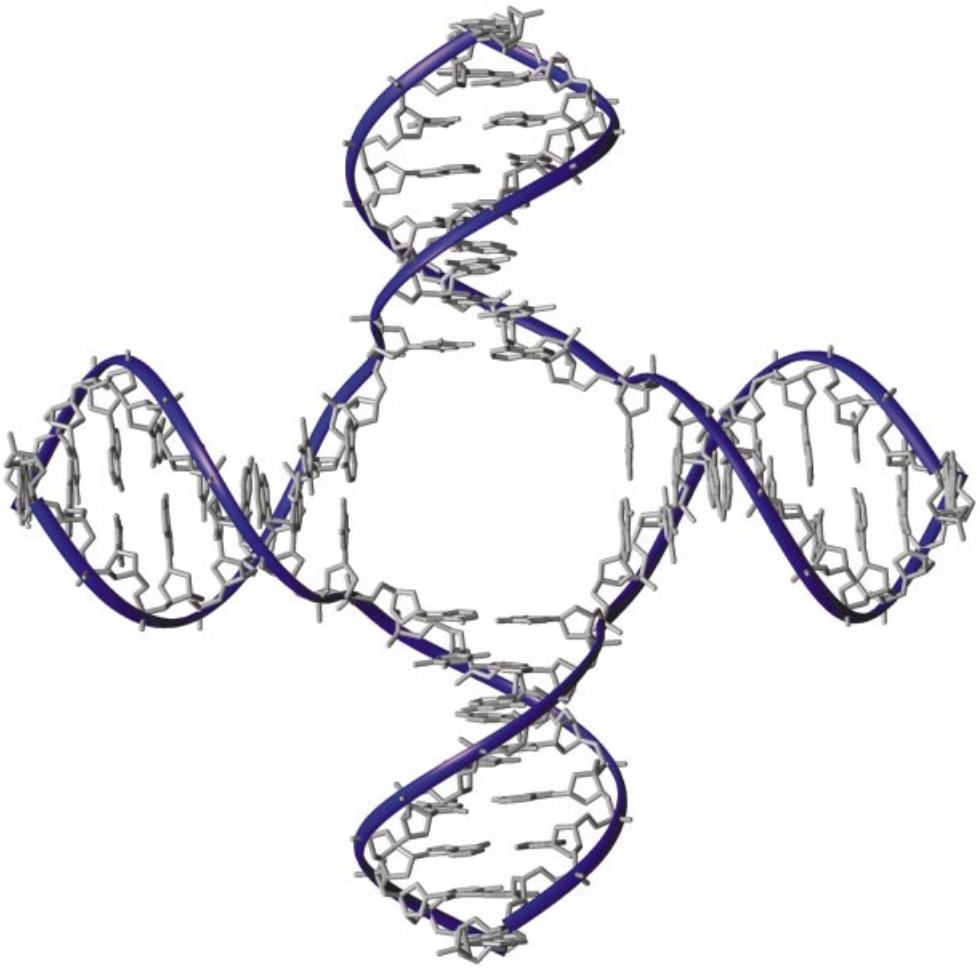


Fig. 17. The crystal structure of a DNA junction bound by RuvA. The global structure of a four-way DNA junction as it exists when complexed by a tetramer of RuvA. The protein has been removed from this image to leave DNA alone. The view shown is of the major groove side. The global structure is four-fold symmetrical, and is closely similar to that proposed for the free junction in the absence of divalent metal ions. Coordinates provided by Professor D. Rice (University of Sheffield).

consistent with an extended open-square form (Fig. 17). However, RuvA alone does not appear to induce basepair opening judged from spectroscopic data (A.-C. Déclais and D. M. J. L., unpublished data). Thus it is likely that RuvA carries out its function by operating on the global structure of the junction alone, thereby facilitating the exchange of basepairing. In a free junction this would occur as a random walk process, but the RuvB helicase action ensures that migration proceeds in one direction only.

9.3 Site-specific recombinases

The integrase family of site-specific recombination systems involve a mechanism that requires two sequential strand exchange reactions, with a four-way junction as the intervening intermediate (Argos *et al*. 1986; Nunes-Düby *et al*. 1998; Sherratt & Wigley, 1998). These

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reactions are brought about by the tyrosine recombinases, a group of enzymes that includes lambda integrase (Pargellis *et al*. 1988; Nash & Robertson, 1989), Cre (Hoess & Abremski, 1984), Flp (Jayaram *et al*. 1988) and XerCD (Blakely *et al*. 1990). The sites at which these recombinases act comprise inverted repeats separated by a sequence of 6–8 bp in which the recombination takes place. A dimer (or heterodimer in the case of XerCD) binds to the inverted repeat, and interacts with an equivalently bound recombining site by protein–protein contact. Phosphodiester linkages in one strand of each DNA molecule are broken by the nucleophilic attack of active site tyrosine side chains, strands are exchanged and new phosphodiester linkages made to create a four-way junction. Attack of the second set of protomers on the remaining two strands then resolves the junction into recombinant duplex species. A major question of interest is how a directionality is imposed on this system, so that recombinant products are generated at the second stage of the process rather than reverting to the parental form. Recent studies show that the structure of the DNA junction plays an important role in generating the required bias.

Most progress in the definition of the mechanism at the molecular level has been made in the Cre system, where crystallographic structures have been obtained for various complexes between Cre protein and its *lox*P DNA binding site at different stages along the pathway of recombination by van Duyne and coworkers (Guo *et al*. 1997; Gopaul *et al*. 1998; Guo *et al*. 1999). This has been reviewed in greater depth elsewhere (Gopaul & van Duyne, 1999; Lilley, 1997). Guo *et al*. (1997) first obtained the structure of a dimer of Cre bound as a covalent complex to a symmetrised duplex *lox*P site. Each DNA duplex had undergone a single transesterification reaction by a Cre subunit, but continuation of the process was blocked by a strategically placed gap. The DNA was severely bent by the protein, and interacted with a second such duplex to create a structure that closely resembled a planar four-way junction. This was locked together by cyclical protein–protein interactions that included the donation of one helix to the next protomer around the four Cre subunits. Such intimate protein–protein interaction appears to be a relatively common feature in enzymes that interact with DNA junctions, and may reflect a requirement for a rigid protein assembly in order to recognise and manipulate DNA structures on a large scale.

Encouraged by this success, van Duyne and coworkers (Gopaul *et al*. 1998) went on to crystallise a complex of four Cre subunits with two complete *lox*P sites in the form of a four-way junction. This was achieved twice using different DNA junctions, one of which could undergo some branch migration in principle. The Cre-bound junction is in an open, unstacked conformation (Fig. 18(*a*)). It is close to planar, with a slightly concave major groove face on which the cleavage and ligation reactions occur. The junction has two-fold rather than four-fold symmetry, with interhelical angles of 76 and 101°, quite similar in fact to the proposed global structure for a DNA junction bound by the junction-resolving enzyme RuvC (Bennett & West, 1995). The phosphate groups orient in different ways on the different diagonals (Fig. 18(*b*)). At the wider interhelical angle the ϵ torsion angle is $-87°$, and the phosphate is oriented so that the non-bridging oxygen atoms are directed away from the junction centre. This is similar to the structure found at the point of strand exchange in the protein-free stacked X-structure (von Kitzing *et al*. 1990; Ortiz-Lombardía *et al*. 1999; Eichman *et al*. 2000), and indeed the helices that are connected by these phosphates have a slightly right-handed relationship that is reminiscent of the stacked X-structure. By contrast, at the narrower angle, the two torsion angles about the phosphorus are distorted; $P{-}O_{3'}$ (ζ) = $+44°$ and $P{-}O_{5'}$ (α) = $+58°$ compared to their normal -90 and $-60°$ conformations

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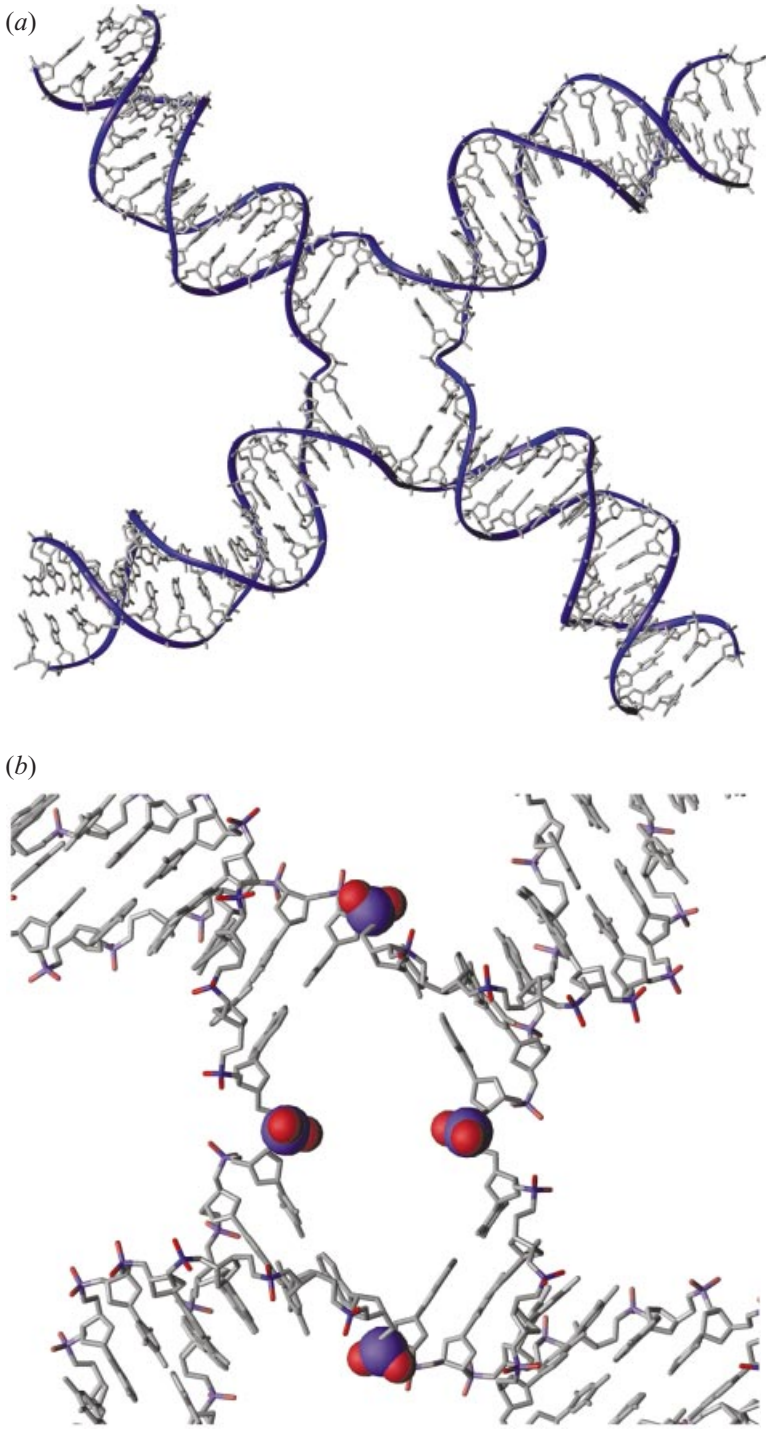


Fig. 18. The crystal structure of a DNA junction bound by Cre. (*a*) The global structure of a four-way DNA junction as it exists when complexed by two dimers of Cre protein. The protein has been removed from this image to leave DNA alone. The view shown is of the minor groove side. Note that the global structure of the Cre-bound junction is significantly distorted away from four-fold symmetry, with two diametrically opposed interhelical angles of 76° and the remaining two of 101°. (*b*) Close-up view of the

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respectively, whereas angle ϵ adopts a more conventional *trans* conformation. This leads to a 180° reorientation of the phosphate so that the oxygen atoms point towards the centre of the junction, causing the backbone of these two strands to kink sharply. Van Duyne and coworkers suggest that the key event that leads to the required bias in outcome of the recombination reaction is a rearrangement that exchanges the conformation of the strands, with the four central phosphate groups each undergoing a swivel-like reorientation. In Xer recombination the two sets of strand exchanges are catalysed by different proteins, i.e. XerC and Xer D (Blakely & Sherratt, 1994), which must be active at different stages of the overall process. Mutational analysis of these proteins has led Sherratt and coworkers (Hallet *et al*. 1999) to propose a mechanism in which interactions between the C-terminal regions cause changes in the conformation of the protein-bound junction that generate the required activation and inhibition of the Xer proteins.

9.4 Other proteins

A significant number of proteins without any immediate connection to recombination processes have been found to exhibit binding specificity to four-way DNA junctions. These include the HMG-box family of proteins (Bianchi *et al*. 1989), the HU protein of *E. coli* (Pontiggia *et al*. 1993), histone H1 (Varga-Weisz *et al*. 1993), MSH2 (Alani *et al*. 1997), human papilloma virus E6 protein (Ristriani *et al*. 2000) and other proteins of unknown function (Pearson *et al*. 1995). However, in most cases this binding is unlikely to indicate a cellular interaction with DNA junctions, and some caution is required in interpreting such results. For example, we have shown that the HMG-box proteins bind to the junction in its open, extended form, not the folded stacked X-structure that would exist in the presence of metal ions (Pöhler *et al*. 1998). It is probable that the natural binding of such proteins to DNA does involve distortion of the structure, and that some aspect of the structure of the four-way junction resembles that of the normal substrate. A strong candidate for the feature that is recognised in the case of the HMG-box proteins would be the minor groove that is effectively widened where the strands splay apart at the point of strand exchange in the open form of the junction.

10. Summary and conclusions

Our knowledge of the architectural principles of nucleic acid junctions has seen significant recent advances. The conformation of DNA junctions is now well understood, and this provides a new basis for the analysis of important structural elements in RNA. The most significant new data have come from X-ray crystallography of four-way DNA junctions; incidentally showing the great importance of serendipity in science, since none of the three groups had deliberately set out to crystallise a junction. Fortunately the results confirm, and of course extend, the earlier conformational studies of DNA junctions in almost every detail.

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point of strand exchange of the Cre-bound DNA junction (minor groove side), with the four phosphates at the point of strand exchange rendered spacefilling. The phosphate non-bridging oxygen atoms point outwards at the smaller interhelical angles, but inwards at the larger vertices. The latter create the distinct kinking of the backbone evident in (*a*). Coordinates provided by Dr G. van Duyne (University of Pennsylvania, Philadelphia).

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This is important, because it means that these methods can be applied with greater confidence to new systems, especially in RNA. Methods like FRET, chemical probing and even the humble polyacrylamide gel can be rapid and very powerful, allowing the examination of a large number of sequence variants relatively quickly. Molecular modelling in conjunction with experiments is also a very important component of the general approach. Ultimately crystallography provides the gold standard for structural analysis, but the other, simple approaches have considerable value along the way.

At the beginning of this review I suggested two simple folding principles for branched nucleic acids, and it is instructive to review these in the light of recent data. In brief, these were the tendency for pairwise coaxial stacking of helical arms, and the importance of metal ion interactions in the induction of folding. We see that both are important in a wide range of systems, both in DNA and RNA. The premier example is the four-way DNA junction, which undergoes metal ion-induced folding into the stacked X-structure that is based on coaxial stacking of arms. As in many systems, there are two alternative ways to achieve this depending on the choice of stacking partners. Recent data reveal that both forms often exist in a dynamic equilibrium, and that the relative stability of the two conformers depends upon base sequence extending a significant distance from the junction.

The three-way junction has provided a good test of the folding principles. Perfect three-way (3H) DNA junctions seem to defy these principles in that they appear reluctant to undergo coaxial stacking of arms, and exhibit little change in conformation with addition of metal ions. Modelling suggests that such a junction is stereochemically constrained in an extended conformation. However, upon inclusion of a few additional base pairs at the centre (to create a $3HS_2$ junction for example) the additional stereochemical flexibility allows two arms to undergo coaxial stacking. Such a junction exhibits all the properties consistent with the general folding principles, with ion-induced folding into a form based on pairwise coaxial stacking of arms in one of two different conformers. The three-way junction is therefore very much the exception that proves the rule.

It is instructive to compare the folding of corresponding species in DNA and RNA, where we find both similarities and differences. The RNA four-way junction can adopt a structure that is globally similar to the stacked X-structure (Duckett *et al*. 1995a), and the crystal structure of the DNAzyme shows that the stacked X-conformation can include one helical pair in the A-conformation (Nowakowski *et al*. 1999). However, modelling suggests that the juxtaposition of strands and grooves will be less satisfactory in RNA, and the higher magnesium ion concentration required to fold the RNA junction indicates a lower stability of the antiparallel form. Perhaps the biggest difference between the properties of the DNA and RNA four-way junctions is the lack of an unstacked structure at low salt concentrations for the RNA species. This clearly reflects a major difference in the electrostatic interactions in the RNA junction. In general the folding of branched DNA provides some good indications on the likely folding of the corresponding RNA species, but caution is required in making the extrapolation because the two polymers are significantly different.

A number of studies point to the flexibility and malleability of branched nucleic acids, and this turns out to have particular significance in their interactions with proteins. Proteins such as the DNA junction-resolving enzymes exhibit considerable selectivity for the structure of their substrates, which is still not understood at a molecular level. Despite this, it appears to be universally true that these proteins distort the global, and in some cases at least the local, structure of the junctions. The somewhat perplexing result is that the proteins appear to

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distort the very property that they recognise. In general it seems that four-way DNA junctions are opened to one extent or another by interaction with proteins. In the case of branch migration proteins like RuvA this can be readily understood in terms of the requirements for exchange of basepairing. For the junction-resolving enzymes and tyrosine recombinases this is less clear. It may reflect a requirement for simple access, or it could be a consequence of structures imposed by other proteins.

Experiments in a number of systems show that there is a deep connection between the action of junction-specific enzymes and the conformation of the DNA, and thus the recognition and manipulation of junction conformation and enzymatic activity are all intimately related. We need to grasp all these features simultaneously to gain a full understanding.

The structure of the nucleic acid junctions plays a central role in their biological function. This is the real importance of these studies.

11. Acknowledgements

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