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Nucleic acid structure and recognition [1]

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Abstract

We review the global structures adopted by branched nucleic acids, including three- and four-way helical junctions in DNA and RNA. We find that some general folding principles emerge. First, all the structures exhibit a tendency to undergo pairwise coaxial helical stacking when permitted by the local stereochemistry of strand exchange. Second, metal ions generally play an important role in facilitating folding of branched nucleic acids. These principles can be applied to functionally important branched nucleic acids, such as the Holliday DNA junction of genetic recombination, and the hammerhead ribozyme in RNA. © 1997 Elsevier Science B.V.

1. Helical junctions in nucleic acids

Junctions are created in nucleic acids where double helical segments intersect with axial discontinuities, such that strands are exchanged between helical sections. These can be perfect junctions, where every base is paired with its Watson–Crick complement, or they can contain mismatches or unpaired bases. A systematic nomenclature has been devised for the unambiguous definition of different junctions [1].

Branched helical species are of major biological significance as intermediates in DNA rearrangements of various kinds, such as recombination. The four-way junction has been proposed to be the central intermediate in homologous genetic recombination [2–8], and there is good evidence for a four-way junction intermediate in certain site-specific recombination events [9–13]. DNA junctions are substrates for proteins involved in the latter stages of genetic recombination. These recognise their DNA substrates at the level of tertiary structure.

Helical junctions are quite common features of the secondary structures of natural RNA species. A number of functional catalytic RNA molecules contain helical junctions, such as the hairpin ribozyme [14] which is a four-way junction, and the hammerhead ribozyme [15,16] which can be regarded as a kind of three-way junction.

2. The four-way DNA junction

The four-way (4H) junction can exist in a number of different conformers, and undergoes ion-dependent folding transitions (Fig. 1). In the absence of added cations the structure is unfolded; the arms

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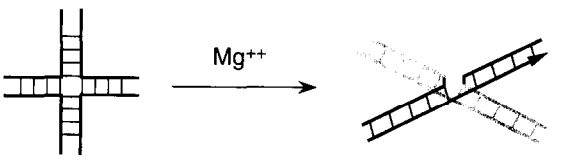

Fig. 1. Ion-dependent folding of the four-way DNA junction into the stacked X-structure. In the absence of added metal ions the four-way junction in DNA exists as an open, extended structure. Upon addition of ions (such as 100 μM magnesium ions) the junction undergoes a folding process that involves the pairwise coaxial stacking of helical arms [18]. The folding creates two different kinds of strand; the continuous strands turn about the helical axis of the stacked helices, while the exchanging strands pass are exchanged between helices in different coaxial stacks. In the antiparallel structure the continuous strands run in opposite directions (their chemical polarity is indicated by the arrows). Two alternative conformers of this structure can exist, reflecting the possible choices of stacking partners.

remain unstacked and fully extended in a square configuration [17]. Upon addition of metal ions (such as 100 μM magnesium ions) the four-way DNA junction undergoes a precise folding, to generate the stacked X-structure. The essential features of this structure are:

- The arms of the junction associate in pairs by helix–helix stacking. Two stereochemically equivalent conformers are possible [18], differing in their choice of stacking partners. Relative stability of the alternative stacking conformers depends on local sequence.
- The two pairs of stacked helices are rotated, in the manner of opening a pair of scissors. This minimises electrostatic repulsion without perturbing the helix–helix stacking.
- The two-fold symmetry of the structure generates two sets of inequivalent strands in the structure. One pair (the continuous strands) are related by an unbroken helix axis that passes through the point of strand exchange. The other pair (the exchanging strands) exchange between two non-coaxial helices.
- The structure is approximately antiparallel [18–20]. The exchanging strands are disposed about the smaller angle of the X-structure, and do not cross. The two coaxial helical stacks lie across each other with a right-handed sense [19], allowing a favourable juxtaposition between DNA strands and grooves (see Fig. 2); the alignment is best for a small angle of about 60°. If the backbone of one of the exchanging strands of the four-way junction is interrupted by a covalent discontinuity (nick), the helical pairs appear to disengage (while remaining stacked) and take up a new angle of crossing of about 90° [21].
- The structure has two sides of different character. On one side of the junction the point of strand exchange has major groove characteristics, while the other side has minor groove characteristics.
- The structure can accommodate single-base mismatches without extensive disruption to the global structure [22]. Some mismatches do not appear to destabilise the structure significantly, while others elevate the concentration of ions required to permit folding into the stacked X-structure.

The global structure is consistent with all available experimental evidence, including comparative gel electrophoretic experiments [17,18,22–24], fluorescence resonance energy transfer [19,25,26], enzyme and chemical probing [27–31] and other physical

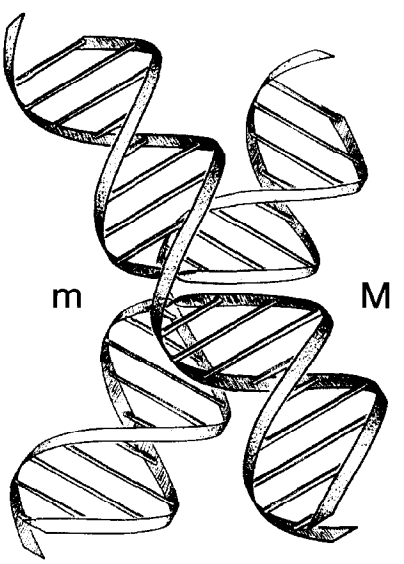

Fig. 2. The stacked X-structure of the four-way DNA junction. The ribbon indicates the path of the backbones in the right-handed, antiparallel stacked X-structure [19]. The two sides of the structure are not equivalent. One side (m) presents minor groove edges of the basepairs at the point of strand exchange, while at the other (M) the major groove edges are presented.

measurements [32]. There have been a number of attempts to model the geometry of the exchange point of the four-way junction [20,33,34]. Recently, significant progress with understanding the local stereochemistry of the junction has been made by ^{1}H NMR [35–38]. While full structural determination has not yet been achieved, clear evidence has been obtained for a number of aspects of the structure. Most significantly, evidence for base–base stacking across the exchange point has been obtained for several junctions [36,38], and sequence-dependent stacking isomer bias has been observed [37].

Metal ions play a critical role in the structure of the four-way DNA junction. In the absence of added cations the junction is unable to undergo folding to form the stacked X-structure, but remains in an extended conformation with no coaxial stacking of helical arms. Comparative gel electrophoretic experiments show that the junction adopts a structure with approximately square symmetry in the absence of metal ions [17,18], and this is confirmed by FRET experiments [26]. Thymine bases are reactive to addition by osmium tetroxide in the extended structure of the junction under low-salt conditions [18]. Uranyl-induced photocleavage experiments indicate the presence of a specific ion binding site near the point of strand exchange in the folded junction [39]. Experiments in which selected phosphate groups were electrically neutralised by replacement with methyl phosphonates [17] revealed that repulsion between phosphates at the point of strand exchange was very significant, as might be expected. Folding the junction probably generates an electronegative cleft that binds divalent ions with increased affinity, whereupon the central bases become inaccessible to osmium tetroxide.

3. The three-way DNA junction

The first three-way junctions studied in DNA were constructed such that three helices were connected without the intervention of unpaired bases (3H junctions). Simple model building suggests that such junctions will be unable to undergo coaxial helical stacking, unless basepairing is sacrificed. If we attempt to construct a model of 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. This is confirmed by experiment. Comparative gel electrophoretic experiments [40] indicated that the three angles between the arms of such perfect three-way DNA junction were much closer to being equal than were the six angles relating the arms of the four-way junction. This was supported by FRET experiments, where the three end-to-end distances of a three-way junction were found to be closely similar [41]. This was consistent with the permanent reactivity of thymine bases even at high magnesium concentrations [40], suggesting that no coaxial stacking could occur in these junctions.

The stereochemical restraint of the perfect 3H junction could 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. Using both comparative gel electrophoresis [42] and FRET [41], we demonstrated that such junctions undergo a magnesium-dependent conformational change whereby the angles between arms become markedly different. These results can be interpreted in terms of the formation of a structure in which two arms are now coaxially stacked, while the third subtends an angle that is set by the number of unpaired bases (Fig. 3). Changes in helix–helix lengths in three-way junctions with the introduction of unpaired bases were also observed by time-resolved FRET measurements [43]. Thus we find that once the structural restraints imposed by the perfect three-way junction are removed, 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.

In principle, we can write down two possible isomeric forms of the folded $3HS_n$ junction (Fig. 3). However, in marked 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 (isomer I), while in the other it is 5′ to 3′ (isomer II). Three-way DNA junctions containing two unpaired bases ($3HS_2$

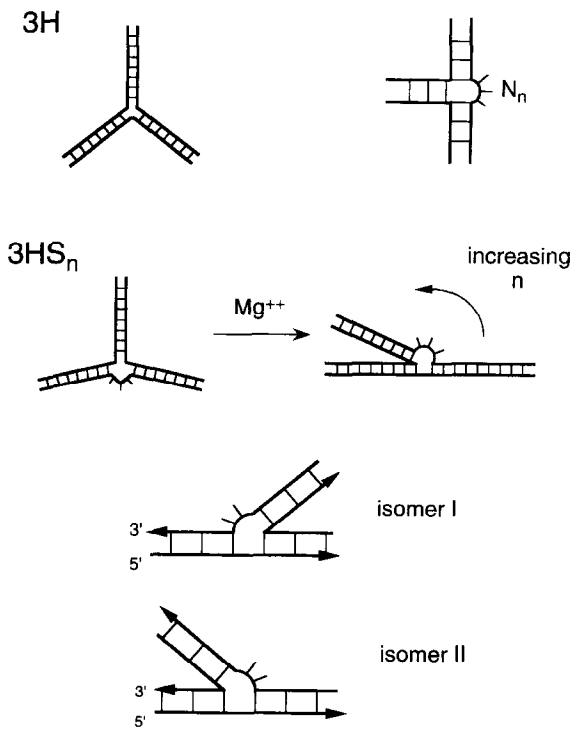

Fig. 3. The global structure of the three-way DNA junction. The perfectly basepaired three-way junction (3H) adopts an extended structure that lacks coaxial helical stacking under all conditions [40]. Inclusion of extra unpaired bases ($3HS_n$) provides additional conformational flexibility, allowing the junction to undergo an ion-induced folding process [42]. This is based on coaxial stacking of two arms; the angle that includes the unpaired bases becomes larger as the number of extra bases increases. There are two possible isomers of the $3HS_n$ junction, that are stereochemically inequivalent [49]. In isomer I the polarity of the bulge is 3′ to 5′ as it passes from the stacked to the unstacked arm, while in isomer II this becomes 5′ to 3′. Despite their chemical differences, examples of both isomeric forms have been found for different DNA sequences [45,46,48].

junctions) have been the subject of two studies by nuclear magnetic resonance (NMR), and interestingly both isomers have been observed using different sequences. Thus a three-way junction studied by Leontis and colleagues [44,45] adopted isomer I, while another studied in Patel's laboratory [46,47] formed isomer II. In our hands a greater number of sequences fold into the isomer I structure, yet a more recent NMR study of two further $3HS_2$ junctions by Altona and coworkers [48] revealed additional examples of isomer II structures. We have compared the folding of a number of different three-way junction sequences by comparative gel electrophoresis, and have found that they fold into isomer I on addition of magnesium ions. Nevertheless, when we studied the same sequence as that investigated by Rosen and Patel [46,47] we found that this adopted the alternative stacking isomer [49], in complete agreement with the NMR analysis. Thus despite the stereochemical differences between the two structures, both can be adopted, and the relative stability is clearly governed by local DNA sequence.

4. Helical junctions in RNA molecules

Helical junctions are quite common in RNA species. If we examine the secondary structure of a rRNA species for example, we will find examples of three- and four-way junctions. They are rarely 100% basepaired however, and one or more single-stranded bases are quite often found at the point of strand exchange.

We have examined the global structure of a number of 4H RNA junctions of different central sequence [50], using the comparative gel electrophoresis technique modified for the analysis of RNA. We found both similarities and differences from the DNA equivalents. The RNA junctions appear to fold by coaxial helical stacking, and even exhibit the same choice of stacking partners as corresponding sequences in DNA in the limited number of cases examined. However, the global structure of RNA junctions is different, and responds to changes in ionic conditions in a very different way from DNA. The general structure of the RNA junction in the presence of 1 mM magnesium ion is a 90° cross of helical stacks (Fig. 4). In the absence of added metal ions, the RNA species did not suffer loss of coaxial stacking (in strong contrast to DNA junctions) but tended to rotate into a parallel-stranded form, with some sequence dependence. When the junction was placed in 0.5 mM calcium ions, or elevated concentrations of magnesium ions, the junctions rotated in the opposite direction to adopt an antiparallel structure.

Thus the conversion from DNA to RNA has significant consequences for the global folding of the four-way junction. Some of the differences are likely

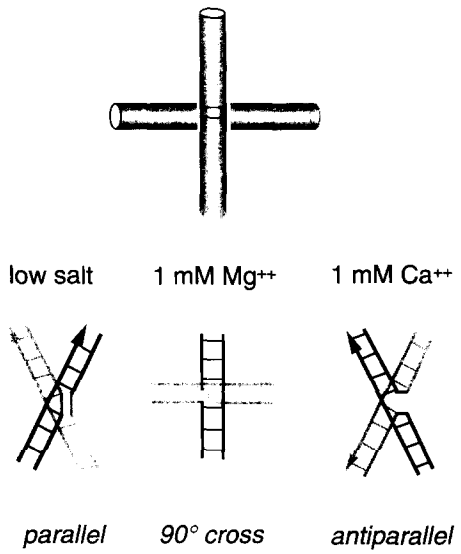

Fig. 4. The global structure of the four-way RNA junction. Unlike its DNA counterpart, the RNA four-way junction appears to be folded by pairwise coaxial stacking of helical arms under all conditions [50]. Two conformers of the structure are possible, that differ in stacking partner choice. In the presence of 1 mM magnesium ions, all four-way RNA junctions studied adopt a structure in which the angle between helical axis is approximately perpendicular (centre, and illustrated above). At higher magnesium ion concentrations, or in the presence of ≥ 500 μM calcium ions, the RNA junctions undergo a rotation towards a structure in which the continuous strands are approximately antiparallel (right). At low concentrations of added divalent ions, the junction may rotate towards a structure in which the continuous strands are now parallel to each another (left); however this behaviour appears to be strongly sequence-dependent. The handedness of these structures is unknown, and the depiction is purely arbitrary.

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 interaction will be less favourable. If the thermodynamic advantage of backbone–groove alignment is reduced, then the balance of other factors may result in a new global conformational minimum free energy. 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.

An example of a natural four-way junction is found in the U1 snRNA, that is involved in splicing of mRNA. The junction sequence is conserved in mammalian, avian and amphibian sequences [51,52], and is perfectly basepaired for at least three basepairs in each arm, apart from a single G · A mismatch located at the point of strand exchange. We analysed the global structure of a junction in which the central RNA core was based upon the U1 sequence, including the G · A mismatch [50]. We found that this adopted a folded structure based on coaxial helical stacking, adopting in the isomer in which the adenine base of the G · A mismatch was located on the continuous strand (A_c stacking isomer). We found that the two stacks subtended an angle of 90° under all ionic conditions tested. Interestingly, the G · A mismatch did not appear to destabilise the structure, nor did it influence the overall structure adopted, since it could be replaced with either G · C or T · A basepairs without changing the global conformation.

Another four-way RNA junction can be found in the hairpin ribozyme in its natural context of the tobacco ringspot virus [14,53]. This ribozyme has been almost exclusively studied in the form of a nicked duplex containing two bulged regions, one of which contains the scissile phosphodiester bond [54]. The essential sequences are largely located in the two bulges [55,56], and evidence suggests that the molecule must hinge about the nick to bring these two regions together to generate the active site for self-cleavage [57]. In the natural viral sequence, the secondary structure places the two bulges on successive arms of the four-way junction, suggesting that the junction should fold in such a way that the two bulges would be brought together. We have analysed the global structure of the tobacco ringspot virus junction, and found that is naturally adopts the stacking isomer that places the would-be bulge-containing arms on opposite stacks (AIHM, F Walter and DMJL in preparation). Moreover, as the magnesium ion concentration was raised, the junction adopted a progressively more antiparallel conformation, whereby the potential bulges would be brought closer together. Thus the junction has exactly the structural propensity required to bring the bulges together to generate the active ribozyme.

The hammerhead ribozyme [15,16] may be considered as a special case of a three-way RNA junction. The core of this self-cleaving RNA species is a $HS_1HS_7HS_3$ junction, and the folded structure has

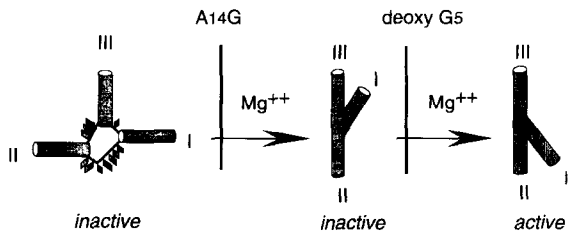

Fig. 5. Ion-dependent folding of the hammerhead ribozyme. The core of the hammerhead ribozyme is a kind of three-way RNA junction, that undergoes an ion-induced folding process to generate the active species [60,61]. In the absence of added divalent metal ions, the hammerhead is extended in something like the conventional depiction such that arms I and II subtend the largest angle. Upon addition of 800 μM magnesium ions there is a conformational change that involves an interaction between arms II and III, probably via a coaxial stacking through the GAAA oligopurine region. At this stage the hammerhead is still not an active ribozyme, and the catalytic core remains unfolded. This initial stage of folding is blocked by replacement of A14 (in the oligopurine sequence) by G. On elevation of magnesium ion concentration beyond 1 mM, the hammerhead undergoes a further folding process that results in a rotation of helix I to lie in the same quadrant as helix II. This second folding stage is likely to involve the folding of the catalytic core into its active form, and covers the same magnesium ion range as that giving maximal ribozyme activity. This stage is blocked by removing the 2′-hydroxyl from the G5 ribose.

been determined in two crystallographic studies [58,59]. We have found that the structure is highly dependent on the ions present (Fig. 5). In the absence of added metal ions the hammerhead core is unfolded with extended helical arms, and upon addition of divalent metal ions it undergoes a two-stage folding process [60,61]. The first step (0–800 μM magnesium ions) involves the coaxial alignment of two of the helical arms, leaving the rest of the core relatively unstructured. In the second stage (1–15 mM magnesium ions), the probable catalytic core folds, causing a rotation of the remaining helical arm in space. The range of magnesium ion concentration over which this folding occurs is the same as that leading to the activation of the ribozyme [62]. The folding must therefore generate a conformation that facilitates the trajectory into the transition state of the S_N2 cleavage reaction. This would ultimately require colinear alignment of the attacking 2′ oxygen atom, the phosphorus atom and the leaving oxygen atom. There is metal ion participation in the cleavage reaction [62,63], and the folding would be expected to generate some kind of electronegative binding site for one or more metal ions. We have detected a high-affinity metal ion binding site within the proposed catalytic core, by means of uranyl-induced photocleavage [60].

5. Interaction between DNA junctions and proteins

The geometry of the four-way DNA junction can be recognised by a set of structure-selective proteins important in genetic recombination and repair processes. Such proteins may accelerate branch migration, or resolve the junction back to duplex species. Junction-selective nucleases, or resolving enzymes, have been isolated from a wide variety of sources, including bacteriophage-infected eubacteria [64,65], eubacteria [66–68], yeast [69,70], mammals [71,72] and their viruses [73].

Endonuclease VII from phage T4 [64] cleaves isolated four-way junctions of various sequence in vitro [18,74], as well as supercoil-stabilised cruciform structures [75,76]. We have expressed endonuclease VII from a synthetic gene, and constructed a number of mutants [77]. The protein appears to be constructed along modular lines. The N-terminal section contains four cysteine residues that coordinate a single zinc ion [77]. In the centre of this 39 amino acid autonomously-folding region lies a cluster of histidine and acidic residues, a number of which appear to play a role in the catalysis of DNA cleavage [78]. At the C-terminus is a section that is 47% identical to a region of the T4 pyrimidine-dimer glycosylase endonuclease V. The structure of the latter enzyme is known, and the region of similarity is a helix and turn [79]. When the sequence from endonuclease V was used to replace the corresponding section of endonuclease VII, the resulting chimaeric enzyme was unchanged in terms of its selectivity for the cleavage of DNA junctions [77]. Located between these two sections is one with lower similarity to T7 endonuclease I. We have isolated one mutant in this region (E86A) that lacks catalytic activity but retains the full selectivity for binding to DNA junctions [80]. We have also isolated a number of functionally equivalent mutants in the correspond-

ing region of T7 endonuclease I, that are catalytically-deficient while retaining their structural selectivity for binding to DNA junctions [81].

The resolving enzymes cleave DNA junctions with considerable precision. For example, T4 endonuclease VII will cleave at just two phosphodiester bonds within a given four-way junction in general. These enzymes bind DNA junctions in the form of dimers [80,82] and the junction–protein complexes migrate as discrete retarded species in polyacrylamide electrophoresis (Fig. 6). A number of nuclease-defective mutants of T7 endonuclease I [81], T4 endonuclease VII [80] and yeast CCE1 [MF White and DMJL unpublished data] retain their selectivity of binding to DNA junctions, demonstrating that the binding and catalytic functions are divisible. In general protein–junction complexes cannot be displaced by 1000-fold excesses of duplex DNA of the same sequence [80–83], showing that the interaction is fundamentally structure-selective. However, the subsequent cleavage of the junctions can exhibit sequence selectivity in the case of some of the enzymes. While this is a relatively weak preference for the phage enzymes, the sequence selectivity is considerably stronger for RuvC of *E. coli* [84] and for CCE1 of yeast [82].

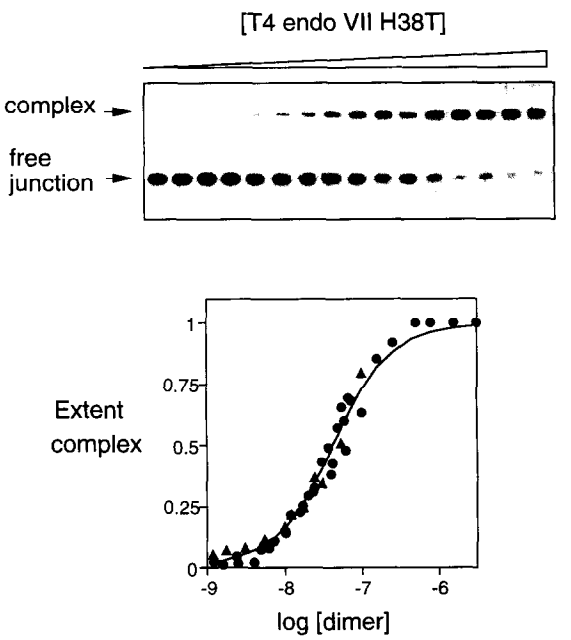

Fig. 6. Binding of T4 endonuclease VII to a four-way DNA junction. Increasing concentrations (1.2 to 72.7 nM) of endonuclease VII H38T (as an N-terminal protein A fusion) were incubated with a radioactive four-way DNA junction (24.2 nM), and free junction and DNA–protein complexes were separated by electrophoresis in polyacrylamide (upper) [78]. The enzyme binds as a dimeric species to give well-defined retarded species. This cannot be displaced with a thousand-fold excess of duplex DNA of the same sequence, demonstrating that the protein is highly selective for the structure of the junction. From these and related data a binding isotherm was generated (lower); three independently measured sets of data are plotted, differentiated by the use of three different plotting symbols. The data were fitted to a model for the binding process, from which an association constant $K_A = 3.29 \times 10^7\ M^{-1}$ was calculated.

In addition to recognising the structure of DNA junctions, the resolving enzymes also generally distort that structure. This has been demonstrated for T7 endonuclease I [81], T4 endonuclease VII [80], RuvC [85] and CCE1 [86]. The resulting global structure of the complex is different for each protein. The most extreme is probably that of CCE1, where the resulting global structure imposed on the DNA junction is very close to that of the extended square conformation [86], just like that of the free junction in the absence of added ions. However the CCE1–junction complex exists in this extended structure with or without added metal ions. The open centre of the CCE1–junction complex can be demonstrated by the accessibility of thymine bases at the point of strand exchange to attack by potassium permanganate.

6. Folding of branched nucleic acids — some general principles?

Can we establish some general folding principles for helical branchpoints in nucleic acids? We find two themes recur:

- *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. Coaxial stacking can create alternative conformers, the relative stability of which is usually dependent on local sequence.
- *Ion-dependent folding*. Nucleic acids are highly charged polyelectrolytes, and thus their folding

will be very different from that of proteins. Phosphate–phosphate repulsion will tend to keep the structure extended in the absence of charge neutralisation, and thus metal ions will play an important role in the folding. The folding may in turn create specific ion-binding pockets, and such site-bound ions can themselves be very important in the function of the nucleic acid, particularly in ribozyme catalysis.

In general, branched nucleic acids undergo metal ion-induced folding transitions, driven by the reduction in electrostatic repulsion. The importance of electrostatic interactions is clearly seen in the four-way DNA junction (4H), where selective phosphate neutralisation can switch the folding between alternative conformations [17]. In the absence of added metal ions the four-way DNA junction is completely unfolded. Perhaps surprisingly, however, this is not the case for the corresponding RNA junction, which remains folded even under very low salt conditions. Nevertheless, the global conformation of the four-way RNA junction is responsive to the nature of the metal ions present, and can change between parallel and antiparallel forms. Divalent ions such as magnesium are much more efficient than monovalent ions like sodium in promoting junction folding, and specific ion binding sites have been revealed in the four-way DNA junction by uranyl-induced photocleavage reactions [39]. However, partial folding can be induced by monovalent ions, for which site binding can probably be excluded in these systems. Thus a combination of site binding and more general overall charge neutralisation is probably important for the folding process.

Coaxial stacking of pairs of helices is seen to be a very common feature of the folding of branched nucleic acids. Folding of four-way junctions in both DNA and RNA is based on pairwise coaxial stacking, and in each case this generates alternative conformers based on the two possible choices of stacking partners. This choice seems largely determined by the bases flanking the point of strand exchange. Usually, but not always, one form is thermodynamically favoured over the other.

The three-way DNA junction is an interesting case that challenges these general folding principles. The perfect three-way junction (3H) does not change conformation upon addition of metal ions, nor does it undergo coaxial helical stacking. This is a result of the constrained framework of the backbone, which would require loss of basepairing before helix stacking could occur. However, the addition of unpaired bases ($3HS_n$ junctions) generates extra conformational freedom that allows the junctions to undergo metal ion-induced folding via pairwise coaxial stacking. Once again two (in this case, stereochemically inequivalent) stacking isomers can be formed, the relative stability of which depends on local sequence. Thus, given a little extra flexibility, the three-way junction conforms to the general folding principles established —very much the exception that proves the rule.

The folding principles established in DNA and RNA junctions appear to have general validity, and can therefore usefully be applied to natural and functional nucleic acids.

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