Structures of bulged three-way DNA junctions

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ABSTRACT

We have studied a series of three-way DNA junctions containing unpaired bases on one strand at the branch-point of the junctions. The global conformation of the arms of the junctions has been analysed by means of polyacrylamide gel electrophoresis, as a function of conditions. We find that in the absence of added metal ions, all the results for all the junctions can be accounted for by extended structures, with the largest angle being that between the arms defined by the strand containing the extra bases. Upon addition of magnesium (II) or hexammine cobalt (III) ions, the electrophoretic patterns change markedly, indicative of ion-dependent folding transitions for some of the junctions. For the junction lacking the unpaired bases, the three inter-arm angles appear to be quite similar, suggesting an extended structure. However, the addition of unpaired bases permits the three-way junction to adopt a significantly different structure, in which one angle becomes smaller than the other two. These species also exhibit marked protection against osmium addition to thymine bases at the point of strand exchange. These results are consistent with a model in which two of the helical arms undergo coaxial stacking in the presence of magnesium ions, with the third arm defining an angle that depends upon the number of unpaired bases.

INTRODUCTION

Helical junctions, or branchpoints, are important elements of nucleic acid structure. The potential for the formation of three- and four-way junctions is found in the sequences of many nucleic acids, and they are therefore significant in the architecture of certain DNA species (single-stranded DNA viruses for example), and RNA. Important examples of three-way junctions in RNA molecules include 5S RNA (1), snU4/U6 RNA (2) and the hammerhead ribozyme (3, 4). Helical junctions are also of importance as intermediates in processes leading to DNA rearrangements, including recombination (5–9), integration and site-specific recombination (10–14) and replication (15).

Extensive studies of the four-way junction in DNA (16–27) have established a set of well defined folding principles (reviewed in (28, 29)). The conformation is profoundly ion-dependent (19, 26). In the absence of added metal ions the four arms are extended

in a square arrangement, with no coaxial stacking. However, in the presence of sufficient metal ions the four-way junction undergoes a folding transition based on pairwise coaxial stacking of helical arms. The two stacked pairs of helices that result are rotated in a right-handed sense, to generate an X-shaped structure in which the alignment of the continuous strands (the strands with undeviated helical axes) is antiparallel (22). The principle that appears to govern the formation of the antiparallel, right-handed stacked X-structure is the favourable interaction between the strands of one helix with the major grooves of the other. The transition between unfolded and folded conformations of the four-way junction illustrates the interplay between two important interactions in nucleic acids, notably electrostatic repulsion between phosphates and stacking interactions between basepairs. At low salt concentrations the former interactions are dominant and the structure remains extended and unfolded. When ionic interactions reduce electrostatic repulsion to a critical level the stacking interactions may then dominate, and the junction folds into the compact stacked X-structure. Some aspects of the folding of the four-way junction are probably of general significance. The tendency of basepairs to undergo stacking interactions is a major force for the stabilisation of nucleic acids, and coaxial helix-helix interaction is seen in RNA species such as tRNA (30–33) and pseudoknots (34, 35). Yet the polyelectrolyte character of nucleic acids means that electrostatic repulsion must always be reduced before folding into compact structures is possible, and thus interactions with ions are critical to the folding process.

There have been a number of conformational studies of the three-way DNA junction in solution (36–40). We made an electrophoretic and chemical probing study of a series of three-way junctions (36), and concluded that they remain extended without coaxial helical stacking, even in the presence of magnesium ions. This appears to conflict with the tendency to undergo ion-dependent folding based on coaxial stacking noted above. The reason for the failure of the three-way junction to fold in a manner akin to the four-way junction seems to reside in the stereochemistry of the structure – if all the basepairs remain unbroken it is not physically possible to construct a model of the three-way junction that includes coaxial stacking of two helices. This suggests that the three-way junction is left in a rather strained condition, unable to satisfy the tendency to maximise stacking interactions. However, Leontis et al (38) showed that if additional unpaired bases were introduced onto one strand of

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the three-way junction there was an increase in stability. Model building suggested to us that the inclusion of extra bases would release the stereochemical constraint holding the junction in an extended conformation, and could allow folding into a structure in which there was coaxial helix-helix stacking.

We have therefore carried out an analysis of the global conformation of a series of three-way junctions in which extra adenine or thymine bases have been introduced onto one strand at the point of connection between two helices. Electrophoretic analysis and chemical probing studies indicate that the inclusion of these extra bases permits the three-way junction to undergo ion-dependent folding, and we suggest that this is based on pairwise coaxial stacking between helices.

MATERIALS AND METHODS

Synthesis of oligonucleotides

Oligonucleotides were synthesised by β -cyanoethyl phosphoramidite chemistry (41, 42) implemented on an Applied Biosystems 394 DNA synthesiser. After purification by gel electrophoresis, they were radioactively labelled using T4 polynucleotide kinase (Amersham) and [γ - 32 P] ATP (43).

Construction of three-way junctions

The following oligonucleotides were synthesised:

h strand:

5'-CGCAAGCGACAGGAACCTCGAGAAGCTTCCGGTAGCAGCGAG-
AGCGGTGGTTGAATTCCTCGAGGTTCTGTGCGTTGCG-3'

r strand:

5'-CGCAAGCGACAGGAACCTCGAGGAATTCACCACCGCTCTX_n-
TCTCAACTGCAGTCTAGACTCGAGGTTCTGTGCGTTGCG-3'

x strand:

5'-CGCAAGCGACAGGAACCTCGAGTCTAGACTGCAGTTGAGACGC-
TGCTACCGGAAGCTTCTCGAGGTTCTGTGCGTTGCG-3'

Eight versions of the r strand were synthesised, in which X_n stands for no base, A, AA, AAA, AAAAA, T, TTT or TTTTT. Restriction sites incorporated into the oligonucleotides are underlined. In each strand, the sequence between the restriction sites is unique, but beyond this point the sequence of each arm is identical. The unique centre allowed correct assembly to occur under stringent hybridisation conditions, without competition by hairpin forms. Annealing reactions were carried out by incubating stoichiometric amounts of the appropriate oligonucleotides (one of which was radioactively labelled) for 15 minutes at 90°C in 50 mM Tris.HCl, pH 7.5, 100 mM MgCl₂, 1 mM spermidine, 50 mM DTT, followed by slow cooling. Junction DNA was purified by gel electrophoresis in 8% polyacrylamide, and recovered by band excision and electroelution.

Restriction enzyme cleavage

Junction DNA was cleaved with restriction enzymes (Amersham) using conditions recommended by the manufacturer.

Chemical modification

Osmium tetroxide was dissolved in distilled water as a 20 mM stock solution. Purified junction DNA was incubated with 1 mM osmium tetroxide (19, 44) and 1% pyridine in 10 mM Tris.HCl, pH 7.5, 1 mM MgCl₂, in a 50 μ l final volume on ice for 5 min.

Purified junction DNA was incubated with 1.5% diethyl pyrocarbonate (45, 46) in 50 mM sodium cacodylate, pH 7.1 in a final volume of 200 μ l at room temperature for 20 min. The reaction mixture was vortexed every 3 minutes.

Both osmium and diethyl pyrocarbonate adducts were cleaved by incubation with 1 M piperidine at 90°C for 30 min., followed by extensive lyophilisation.

Sequencing reactions

A+G (purine) and C+T (pyrimidine) sequencing ladders were generated from junction with [5' 32 P]-labelled r strand, using piperidine-formate and hydrazine reactions respectively (43). The modified DNA was incubated with 1 M piperidine at 90°C for 30 min., followed by extensive lyophilisation.

Gel electrophoresis

Purified junction DNA was digested with the appropriate restriction enzymes and loaded directly on to 8% polyacrylamide gels (29:1 monomer/bis ratio) and electrophoresed at room temperature for 20 h at 100 V. The buffer system contained either 90 mM Tris-borate, pH 8.3, 10 mM EDTA (TBE), or 90 mM Tris-borate, pH 8.3 (TB) with added salts (50 μ M–1 mM MgCl₂, 30 mM NaCl or 2–10 μ M [Co(NH₃)₆]Cl₃) and was recirculated at > 1 l/h. Piperidine-cleaved junctions were analysed on 0.4 mm 12% polyacrylamide gels in TBE containing 7 M urea, that were run hot to the touch. All polyacrylamide gels containing radioactively labelled DNA were dried on to Whatman 3 MM paper and autoradiographed at –70°C using Konica X-ray film with Ilford fast-tungstate intensifier screens.

RESULTS

A series of three-way junctions containing single-stranded bulges

The three-way DNA junctions employed in these studies were based on sequences originally studied as four-way junctions (19), and subsequently as three-way junctions (36). The central sequences are shown in Figure 1A. These junctions were derived from our original junction 1 (19), effectively by removal of the B arm. Thus the basis of these studies was the three-way junction that we previously termed HRX. It comprised three strands, each of 80 nucleotides in length, called h, r and x', which hybridised together to generate a three-way junction with arms called H, R and X, each of which was 40 bp in length. Each arm contained a unique restriction site (*Hind*III, *Eco*RI and *Xba*I in arms H, R and X respectively) by cleavage at which its length could be reduced by a factor of almost four. In the present investigation we have constructed two new series of three-way junctions containing an additional single-stranded bulge at the point of intersection between the R and X arms. This was achieved by synthesis of modified sequences for the r strand, in which additional numbers (between one and five) of either adenine or thymine bases were placed at the point of strand exchange in the junction.

Electrophoretic analysis of the structure of three-way junctions

Gel electrophoresis has been very powerful in the analysis of the global structures of DNA junctions (16, 18, 19, 26, 36, 38, 47). The first observation of anomalous electrophoretic mobility in polyacrylamide due to a junction was deduced to be a consequence of the angular configuration of the helical arms (16), and the basic method of the electrophoretic analysis of junction structures was first applied by Cooper and Hagerman to a four-way DNA junction (18). The electrophoretic analysis of a three-

Figure 1. Construction and gel electrophoretic analysis of the structure of bulged three way DNA junctions. **A.** Central sequence of the series of three-way junctions. Each component strand of the basic junction (h, r and x') comprises 80 nt. (full sequences are given in Materials and Methods), and thus the three arms are each 40 bp in length. Each arm (H, R and X) contains a unique site for a restriction site (*HindIII*, *EcoRI* and *XbaI*) respectively, allowing the arm to be selectively shortened by cleavage. **B.** Schematic to illustrate the analysis of three-way junction structure by polyacrylamide gel electrophoresis. The junction is subjected to cleavage of one arm by each of *HindIII*, *EcoRI* and *XbaI* in separate incubations, and we compare the electrophoretic mobilities of the resulting species with two long and one short arms. These species are designated by the names of the two long arms, eg HR has been cleaved with *XbaI*.

way junction is shown schematically in Figure 1B. Three separate restriction digests of the junction are made, such that in each case a different arm is substantially reduced in length, leaving two arms of 40 bp. The electrophoretic mobility in polyacrylamide of these three species is compared, with the underlying assumption that the mobility will reflect the angle subtended between the long arms. Thus if the junction were Y-shaped with very similar angles between all three arms, it would be anticipated that the three digested species should exhibit closely similar mobility.

The three-way junctions adopt an extended structure in the absence of added metal ions

We initially chose to examine the series of three-way junctions in the absence of added metal ions, in 90 mM Tris.borate, pH 8.3, 1 mM EDTA (TBE). Under these conditions the four-way

Figure 2. Gel electrophoretic analysis of three-way junctions in the absence of added metal ions. In this and subsequent autoradiographs, the tracks are grouped in series of three, with each triplet of tracks being derived from the three digests of a different junction. The three species with one permuted shortened arm for each of two series of junctions were analysed by electrophoresis in 8% polyacrylamide at room temperature in 90 mM Tris-borate, pH 8.3, 10 mM EDTA. The junctions were radioactively [³²P] labelled on one strand, and the gel subjected to autoradiography. **A.** The series of three-way junctions containing 0, 1, 2, 3 or 5 extra adenine bases on the r strand. **B.** The series of three-way junctions containing 1, 3 or 5 extra thymine bases on the r strand. **C.** An explanation for the electrophoretic data. We propose that in the absence of added ions the junction adopts an extended structure. In the junction lacking unpaired bases the three inter-arm angles are approximately equal, but on addition of bases on the r strand the angle subtended between the R and X arms progressively widens.

junction remains unfolded and fully extended with four-fold symmetry (19, 26). The results for the series of three-way junctions as a function of added adenine bases is shown in Figure 2A.

First it can be seen that the mobilities of the three species with one shortened arm (ie with two long arms) are closely similar

for the junction lacking added adenine bases. This agrees with our earlier studies of a series of perfect three-way junctions (36), confirmed by Lu et al (39), suggesting that the junction has a Y-shape with very similar angles subtended between all the arms.

Upon addition of extra adenine bases on the r strand opposite the H arm, one species (that with the shortened H arm) acquires significantly faster mobility in the gel, while the other two species have approximately equal, slower mobility. The difference in mobility between the faster and the two slower species becomes greater as the number of extra adenine bases in the bulge increases. This pattern of mobilities does not appear to depend on the nature of the added bases. We repeated the electrophoretic experiments using the series of three-way junctions with added thymine bases (Figure 2B). The patterns of relative mobility are very similar to those obtained using the adenine bulged junctions. This suggests that the important feature is probably the added length of phosphodiester backbone at the bulge.

The changes of mobility pattern on addition of adenine or thymine bases indicates a lowering of the apparent three-fold symmetry of the junction in the absence of added metal ions. The increased mobility of the species with a shortened H arm suggests that the angle subtended between the R and X arms (ie the angle defined by $\angle$ R-centre-X) becomes greater as the extra bases are added, shown schematically in Figure 2C. Small differences in the mobilities of the two slow species for different bulges indicates that the two-fold symmetry of the extended junctions is not perfect, but the approximation is probably close.

Bulge-dependent folding of the three-way junction in the presence of added magnesium ions

The gel electrophoretic experiments using the junctions with permuted shortened arms were repeated in 90 mM Tris.borate, pH 8.3 (TB) with added salts. In the presence of magnesium ions the patterns shown in Figure 3 A, B and C were obtained. The mobilities of the three species derived from the unbulged junction exhibit small differences in relative mobility compared to those in the absence of added metal ions. However, these differences become much greater as unpaired bases (either adenines or thymines) are added to the r strand. This suggests that the

Figure 3. Gel electrophoretic analysis of three-way junctions in the presence of added magnesium ions. The three species with one permuted shortened arm for each of two series of junctions were analysed by electrophoresis in 8% polyacrylamide at room temperature in 90 mM Tris-borate, pH 8.3 with added $MgCl_2$. **A.** The series of three-way junctions containing 0, 1, 2, 3 or 5 extra adenine bases on the r strand, electrophoresed in the presence of 200 μ M magnesium ions. Note the difference in the relative mobilities compared to the equivalent experiment in the absence of added magnesium ions (Figure 2A). **B.** The series of three-way junctions containing 1, 2, 3 or 5 extra adenine bases on the r strand, electrophoresed in the presence of 1 mM magnesium ions. **C.** The series of three-way junctions containing 1, 3 or 5 extra thymine bases on the r strand, electrophoresed in the presence of 1 mM magnesium ions. The relative mobilities can be compared to the equivalent experiment in the absence of added magnesium ions (Figure 2B). **D.** A model that is consistent with the electrophoretic data. In the absence of unpaired bases the mobilities of the three two-long-arm species are quite similar, indicating a structure in which the three inter-arm angles are similar. Upon addition of extra bases on the r strand the mobilities of the two-long-arm junctions become quite different, with the fastest being species HX. It seems probable that this results from coaxial stacking of the H and X arms. The mobility of the RX species depends strongly on the number of added unpaired bases; this is consistent with the relative angles subtended between the R arm and the coaxial HX arms varying with the bulge size.

symmetry of the perfect three-way junction becomes slightly lowered in the presence of magnesium, but that the departure from three-fold symmetry is much greater in the bulged molecules. Once again the patterns are very similar for the adenine and thymine bulges, suggesting that the most significant factor is the additional backbone.

The patterns of relative two-long-arm species mobilities for the bulged junctions are quite different in the presence of added magnesium from in its absence. This suggests that the metal ions are inducing a folding of the bulged junctions, perhaps akin to the folding of the four-way junctions in response to added cations (19, 26). The concentration of magnesium clearly influences the structures adopted by the bulged junctions. When we repeated the electrophoretic analysis in TB plus 50 μ M magnesium chloride we observed marked differences in relative mobilities, particularly for the junction with the five adenine bulge. This suggests that there is a critical magnesium ion concentration (between 50 and 200 μ M) required to fold the bulged three-way DNA junction.

Figure 4. Chemical probing of bases in and around the bulges of the three-way junctions. The three-way junction series with added adenines were prepared with radioactively [$5'$ - 32 P] labelled r strands. These were subjected to chemical modification by either osmium tetroxide or diethylpyrocarbonate, followed by cleavage at chemical adducts by hot piperidine. The DNA fragments were separated by sequencing gel electrophoresis and visualised by autoradiography. The results of osmium tetroxide modification of the junction with 0, 1, 2, 3 or 5 extra adenine bases are shown in tracks 2 to 6. Note the strong reactivity of junction thymine bases in the unbulged junction, and slightly lower reactivity for the junction with a single-adenine bulge, but almost no reactivity for the junctions with larger bulges. The results of diethylpyrocarbonate modification of the junction with 0, 1, 2, 3 or 5 extra adenine bases are shown in tracks 8 to 12. Note that all the added adenine bases are reactive to this probe. Tracks 1 and 7 contain A+G and C+T sequencing markers respectively, derived from chemical sequencing of the unbulged r strand. The location of the point of strand exchange (ie the point of insertion of extra adenine bases) is indicated by the arrow shown on the right.

Moreover, the pattern of mobilities are not exactly the same for the bulged molecules in 200 μ M and 1 mM magnesium ion. For example, at the higher magnesium ion concentration the five-base (either A or T) bulged junctions exhibit a pattern characterised by the HX species having the fastest mobility, whereas at 200 μ M this has actually become slightly slower than RX. This suggests that the five base bulges are significantly more destabilising than the smaller bulges.

We may analyse the patterns of electrophoretic mobility in terms of the assumption that the mobility of a given junction reflects the size of the angle subtended between the long arms, with mobility becoming lower as the angle (ie end-to-end distance (48)) is reduced. Despite the presence of the added magnesium ions, the perfect three-way junction (ie lacking additional bases on the r strand) exhibits quite similar mobilities for the three species with one shortened arm. The structure is probably quite well described by an extended, slightly distorted Y-shape, as we have proposed in the past (36). As the bulges are introduced, the HX species (short R arm) becomes the fastest species, and the HR species (short X arm) is the slowest. This holds for all the bulged species (A_n or T_n , $n = 1$ to 5). However, the relative mobility of the RX species (short H arm) changes continuously as the size of the single-stranded bulged increases; from having equally slow mobility to the HR species for one additional adenine or thymine base, to acquiring the same mobility as the fast HX species for the addition of five extra bases. The clear ion-dependent folding undergone by the bulged three-way junctions is strongly reminiscent of the folding of the four-way junction into the stacked X-structure by metal ions, and suggests that coaxial helical stacking may be occurring. Since the fastest species is always HX (at the higher ion concentrations at least), this would indicate that helices H and X may be undergoing coaxial stacking to form a colinear quasicontinuous helix, shown schematically in Figure 3D. The change in mobility of the RX species with bulge size indicates that the angle subtended between the R and X arms changes significantly with the size of the unpaired bulge region. We suggest that the angle of the R arm relative to the HX stacked pair could change as the number of additional bases is increased, with the angle defined by $\angle$ X-centre-R (ie the angle that follows the axes of the bulged r strand) becoming larger as the bulge is increased in size. Thus for the single A or T bulge this angle is approximately 90° , so that the angles $\angle$ R-centre-H $\approx \angle$ R-centre-X $\approx 90^\circ$, and thus the mobilities of RX and HR are very similar. As the number of extra bases has increased to five, the $\angle$ X-centre-R angle has become rather more obtuse, so that the mobilities of HX and RX are now similar, and the slowest species, HR, now includes a relatively small angle between long arms.

The lower mobility of the HX species for the A_5 -bulged junction in 200 μ M magnesium ion is consistent with a lower stability of this species. This would suggest that there is an equilibrium (fast relative to gel equilibration) between the folded and unfolded conformations, with a significant population of the unfolded species under these conditions for the A_5 -bulged junction. Thus folding of this species requires a higher magnesium concentration than those with smaller bulges. By contrast to magnesium, sodium ions do not appear to facilitate the folding of the bulged three-way junctions. When the gel electrophoresis experiments were repeated in TB plus 30 mM sodium chloride patterns of mobility that were closely similar those seen in TBE were obtained (data not shown).

Figure 5. Gel electrophoretic analysis of three-way junctions in the presence of added hexammine cobalt (III) ions. The three species with one permuted shortened arm for the series of junctions containing 0, 1, 2, 3 or 5 extra adenine bases on the r strand were analysed by electrophoresis in 8% polyacrylamide at room temperature in 90 mM Tris-borate, pH 8.3, 2 μ M hexammine cobalt chloride. Note the similarity in patterns to those obtained in the presence of magnesium ions (Figure 3A).

Folding is accompanied by protection of junction thymine bases against attack by osmium tetroxide

We have found that selective use of chemical probing of base accessibility can be extremely informative in the study of the folding of DNA junctions (19, 26, 36). In particular, we have employed osmium tetroxide as a probe of the environment of thymine bases at the point of strand exchange in the four-way junction; these are reactive in the extended conformation, but become protected against attack as the junction undergoes cation-induced folding into the stacked X-structure. We have therefore employed this probe to the analysis of the folding of the series of three-way junctions with additional adenine bulges. In these junctions there are two thymine bases that flank the extra adenine bases on the r strand, the reactivity of which might be expected to respond to any conformational changes of the junction, particularly those that involve coaxial helical stacking. We therefore prepared the A-bulge series of three-way junctions from r strands that were [5'- 32 P] radioactively labelled and carried out chemical modification reactions in 1 mM osmium tetroxide, 1% pyridine at 0°C in the presence of 1 mM MgCl_2 . The extent of osmium cis-diester adduct formation at individual thymine bases on the r strand was assessed by base cleavage with piperidine, sequencing gel electrophoresis and autoradiography (Figure 4). The result is striking—despite the presence of the magnesium ions, the critical thymine bases of the unbulged three-way junction are strongly reactive to addition by osmium tetroxide, as we have observed previously (36). However, this reactivity is very strongly suppressed as additional bases are added to the r strand, consistent with a folding of the structure of the bulged junctions, although some protection was observed even in the absence of added cations.

In contrast to the behaviour of the junction thymine bases, the extra adenine bases are strongly reactive to diethyl pyrocarbonate (Figure 4), indicating that the bases of the bulge are accessible

Figure 6. Bulged three-way DNA junctions undergo an ion-dependent transition to a stacked structure. Schematic to show the transition to the folded structure of the junctions with unpaired bases induced by the addition of magnesium or hexammine cobalt (III) cations.

to small-molecule probes. The extra thymine bases of the thymine-bulge series were similarly found to be reactive to osmium tetroxide (data not shown).

Hexammine cobalt (III) is very efficient for folding the bulged three-way junctions

The hexammine cobalt (III) cation is extremely effective in promoting the folding of the four-way DNA junction (26), and we wondered if it might be similarly efficient in folding the three-way DNA junction. The results of a gel electrophoretic analysis of the adenine-series of bulged three-way junctions in TB plus 2 μ M $[\text{Co}(\text{NH}_3)_6]^{3+}$ is shown in Figure 5. The patterns of mobilities for the species with two long arms are very similar to those observed in the presence of magnesium ions, indicating that low concentrations of this complex ion can fold the bulged three-way junctions. There is some indication however that this concentration may not be sufficient for the complete folding of the A₅-bulged junction, as the RX species is migrating a little faster than the HX species suggesting an equilibrium with the unfolded form for this junction. A similar pattern of mobilities is observed at limiting magnesium concentrations (see above); this effect is not observed in the presence of 10 μ M $[\text{Co}(\text{NH}_3)_6]^{3+}$, where HX and RX have equal mobilities for the A₅-bulged junction (data not shown).

DISCUSSION

These studies have shown that the conformation adopted by three-way junctions are determined by two factors:

- The presence of additional unpaired bases at the point of connection between two helices, and,
- The presence of metal ions in the solution.

In the absence of added metal cations all the junctions adopt essentially extended structures, that are broadly Y-shaped conformations without helical stacking. Upon addition of metal ions, such as magnesium, the junctions may undergo a folding process, if the presence of extra bases permits it.

In the absence of the extra bases the junction remains approximately Y-shaped, with strongly reactive thymine bases, even in the presence of added cations. This indicates that the perfect three-way junction does not undergo a substantial folding process, and remains essentially extended. Such a structure might be expected to exhibit a degree of flexibility, in agreement with an early experimental study (49). The similarity in mobility of the three two-long-arm species in the presence of magnesium could be taken to indicate a fast dynamic exchange between a series of folded species, but the strong reactivity of the thymine bases suggests that an open conformation is more probable. This is in agreement with our earlier analysis of a series of perfect three-way junctions (36), although the conclusion was challenged

by Kallenbach and coworkers (37, 39). The pattern of mobilities of species with two long arms derived from the perfect three-way junction indicates some deviation from perfect three-fold symmetry in the presence of magnesium, but the differences are quite small in comparison to those between the different species derived from either bulged three-way junctions (see Figure 3), or indeed four-way junctions (36). Moreover, we would not expect the perfect three-way junction to be able to fold unless basepairing is broken, because the structure provides a rather rigid framework that does not lend itself to coaxial stacking. If we try to build a model of a stacked three-way junction by fusing the end of a DNA helix to a nick in another DNA molecule, it is impossible to achieve this without breaking basepairing. This is because the minimum distance between the two phosphodiester backbones of the incoming helix is the width of a minor groove, and the 3' and 5' termini of the new helix cannot be inserted into the nick without considerable distortion. The simplest way to accommodate the new helix is by bending the nicked duplex at the site of insertion in the direction of tilt, effectively to open the nick; this is closely similar to the kinking that occurs to accommodate a base bulge in duplex DNA (50, 51, 52) or RNA (53, 54, 55). Our data do not distinguish between planar and non-planar structures. However, we would not expect complete planarity, because the two sides of the three-way junction are not equivalent; the connectivity of the three-way junction generates opposite sides with major and minor groove characteristics (analogous to those of the four-way junction (25)). For this reason some pyramidal distortion of the structure is probable.

The addition of unpaired bases into the three-way junction introduces considerable extra conformational flexibility, and folding may now occur in the presence of added metal ions that is consistent with coaxial stacking (Figure 6). The gel electrophoretic patterns at limiting ion concentrations suggest that the two and three-base additions generate the most stable folded conformations. This is consistent with the earlier results of Leontis *et al* (38), although we are proposing a somewhat different structure for the folded conformation. The probable formation of stacked structures by the bulged three-way junctions in the presence of metal ions is rather analogous to the ion-induced folding of the four-way DNA junction (19).

Thus the folding of the bulged three-way junctions illustrate some general tendencies of branched DNA structures. Folding is influenced by two main types of interactions, the stacking forces between basepairs and the electrostatic repulsion between phosphate groups of the backbone. At low ionic concentrations the latter forces appear to dominate, and neither four-way nor bulged three-way junctions fold into stacked conformations under these conditions. As the concentration of cations is raised the electrostatic repulsions may be reduced to the point where an increase in favourable stacking interactions predominates, and the structure folds into a more stacked conformation. This appears to be prevented by steric restrictions in the case of the perfect three-way junction, but the addition of extra bases on one strand allows the extra stacking potential to be realised. The folding of the bulged three-way junctions is facilitated by magnesium and hexammine cobalt (III), at concentrations that are similar to those that induce folding of the four-way junction (36). When the three-way junction is destabilised by inclusion of the larger A5 bulge, the electrostatic repulsion must be further reduced (by increased ion concentration) to offset this destabilisation of the folded conformation. Monovalent ions appear not to promote

folding of the bulged three-way junctions up to the concentrations studied; sodium and potassium apparently induce a partial folding of the four-way junction at very high concentrations (36, 56).

In broad terms these folding principles are probably applicable to all nucleic acids, including RNA species. When natural RNA sequences are found to have the potential to form three-way junctions, these are seldom perfectly basepaired (eg 5S (1) and sn U4/U6 RNAs (2)), and it is likely that the resulting structures will therefore undergo folding under physiological ionic conditions. These structures will undoubtedly be important in the biological function of these molecules, both in terms of the conformation imparted and its interaction with metal ions.

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