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The role of metal ions in the conformation of the four-way DNA junction

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Metal ions fold DNA junctions into a compact conformation that confers protection of all thymine bases to modification by osmium tetroxide. In the absence of the cation the arms of the junction are fully extended in an approximately square-planar configuration. Group IIa cations are effective in achieving a folded conformation of the junction at 80 – 100 μM, and there is an excellent agreement between the ionic concentrations that fold the junctions as deduced from gel electrophoretic experiments, and those that prevent osmium tetroxide reaction at the junction. Hexamminecobalt(III) achieves full folding at 2 μM, while spermine and spermidine are effective at 25 μM. Some transition metal ions such as Ni(II) may replace the group IIA cations. Monovalent ions of group IA are only partially effective in folding the junctions. Very much higher concentrations are necessary, gel electrophoretic mobilities suggest that a less symmetrical conformation is adopted and thymine bases at the junction remain reactive to osmium tetroxide. Charge – charge interactions at the centre of the junction are structurally extremely important. Substitution of junction phosphate groups by uncharged methyl phosphonates severely perturbs the structure of the junction. If just two phosphates are substituted, diametrically facing across the junction, the structure always folds in order to place the electrically neutral phosphate on the exchanging strands. We suggest that folding of the junction into the stacked X-structure generates electronegative clefts that can selectively bind metal ions, depending on the chemistry, size and charge of the ion. Moreover, occupation of these cavities is essential for junction folding, in order to reduce electrostatic repulsion. These results demonstrate that metal ions are an integral component of the Holliday junction.

Key words: DNA junction/Holliday structure/junction folding/metal ions/osmium tetroxide

Introduction

The importance of metal ions in determining the conformation of nucleic acids may be insufficiently appreciated. Specific cation binding is important in tertiary structured RNA molecules, of which the best studied is perhaps transfer RNA (Jack *et al.*, 1977; Holbrook *et al.*, 1978; Quigley *et al.*, 1978). Such co-ordinated metal ions can act as central participants in chemical reactions, such as the lead-induced cleavage of tRNA (Brown *et al.*, 1983), cleavage by RNaseP (Guerrier-Takada *et al.*, 1983) and reactions carried out by catalytic RNA molecules (Buzayan *et al.*, 1986; Hutchins *et al.*, 1986 Uhlenbeck, 1987). In DNA the role of metal ions in promoting structural transitions between different helical conformations, including A – B and B – Z interconversions, has been appreciated for a number of years. Specific ion-binding effects have been demonstrated for transitions involving gross changes in conformation, including the formation of cruciform structures (McClellan and Lilley, 1987; Sullivan and Lilley, 1987) and H-triplexes (Kohwi and Kohwi-Shigematsu, 1988).

The four-way helical junction, or Holliday junction, is the central intermediate of homologous genetic recombination (Holliday, 1964; Broker and Lehman, 1971; Sobell, 1972, 1974; Messelson and Radding, 1975; Orr-Weaver *et al.*, 1981), as well as some kinds of site-specific recombination events such as the integration of bacteriophage λ (Kitts and Nash, 1987; Nunes-Düby *et al.*, 1987) and Cre-*lox* (Hoess *et al.*, 1987) and FLP (Jayaram *et al.*, 1988) recombination. The structural basis of genetic recombination requires an understanding of the three-dimensional structure of the four-way junction, and its mode of interaction with the proteins mediating the recombination process, notably the resolvases that are required to cleave the junctions thereby re-creating unconnected duplexes.

We have recently proposed a general structure for the four-way helical junction in DNA (Duckett *et al.*, 1988), that we term the stacked X-structure. This is based on evidence from two independent sources. We have employed a gel electrophoretic method related to that of Cooper and Hagerman (1987) to estimate the relative angles subtended between the different arms of a junction (Duckett *et al.*, 1988), and fluorescence energy transfer to estimate relative end-to-end distances for the arms (Murchie *et al.*, 1989). The most significant feature of the stacked X-structure is co-axial pairwise stacking between adjacent arms, generating two quasi-continuous helices. There are two ways in which this pairing may occur, and we have shown that the local sequence at the centre of the junction determines which of the two possible isomers is more stable. The X-structure proposed is also fully consistent with the results of hydroxyl radical probing of small synthetic junctions (Churchill *et al.*, 1988). The two quasi-continuous helices subtend an angle estimated to be ~60°, the X-structure generated is right-handed, and the sequences aligned in an anti-parallel manner. The cleavage of the junctions by the resolvase T4 endonuclease VII was sensitive to the isomeric structure adopted, indicating that the outcome of a recombination event might be influenced by the sequence and structure of the junction formed (Duckett *et al.*, 1988; Mueller *et al.*, 1988).

A number of studies have suggested that the folding of the four-way junction is associated with ion binding.

We showed (Gough and Lilley, 1985) that a junction-containing fragment migrated anomalously slowly in polyacrylamide, and that the electrophoretic mobility was strongly influenced by the presence of metal ions (Diekmann and Lilley, 1987). Electrophoretic studies of synthetic junctions (Duckett *et al.*, 1988) revealed that the structure was radically different if magnesium was omitted. No matter what sequence was chosen, the pattern of doubly cleaved junctions (i.e. the six species with two long and two short arms that can be generated from a junction) consisted of four slow and two fast species (4:2 pattern), indicative of a square-planar structure. This was in marked contrast with the two fast, two intermediate and two slow species (2:2:2 pattern) found in the presence of magnesium, which is consistent with an X-structure. These results implied that in the absence of the cation, the four arms of the junctions were unstacked and maximally extended, presumably as a consequence of electrostatic repulsion. Further weight was given to these conclusions by the observation that under these conditions, thymine bases located at the junction could be chemically modified by osmium tetroxide. This probe attacks the unsaturated 5,6 bond of thymine, and such electrophilic attack is necessarily out-of-plane, and thus prevented by normal base stacking. The observation of thymine reactivity in the junction therefore implies that helix unstacking occurs in the absence of magnesium ions.

These studies have indicated a major structural role for magnesium ions in the four-way helical junction. In this paper we define the structural requirements for cation binding in terms of the chemistry of the ion, and the chemical nature of phosphate groups at the junction. The results reinforce the view of the critical role of cation binding in the formation of the correct structure.

Results

Junction folding by multivalent metal cations

We have previously indicated the importance of magnesium ions in folding four-way helical junctions. In the absence of ions the junction adopts a square-planar configuration, in which thymine bases located immediately at the junction are reactive to osmium tetroxide. Figure 1 shows the thymine reactivity in junction 3 as a function of added magnesium concentration. The thymine remains reactive up to 70 μM Mg^{2+}, but the reactivity becomes largely suppressed beyond this point. In order to see if these concentrations of magnesium were adequate to fold the junction into the X-shape, we examined them by gel electrophoretic methods.

In these experiments, the six possible pairwise double restriction digests of the junction are compared by electrophoresis in polyacrylamide. As the junctions are assembled from four 80mer oligonucleotides, restriction cleavage reduces the length of any given arm from 40 bp to ~12 bp, and thus pairwise cleavage gives a junction with two long and two short arms. It is the relative mobility of the six such possible species that reveals the configuration of the arms at the junction. In the absence of added ions, junction 3 shows a pattern of four slow and two fast species (4:2 pattern), characteristic of the square-planar configuration in which the four arms are maximally extended towards the corners of a square. These data are not presented at this point, but the interested reader may refer ahead to Figure 5. Addition of 80 μM $MgCl_2$ to the gel buffer changes the pattern completely, as shown in Figure 1(C). In place of the 4:2 pattern we see the two slow, two intermediate and two fast species pattern (2:2:2 pattern) that is characteristic of the junction folded in to the X-configuration. Junction 4,

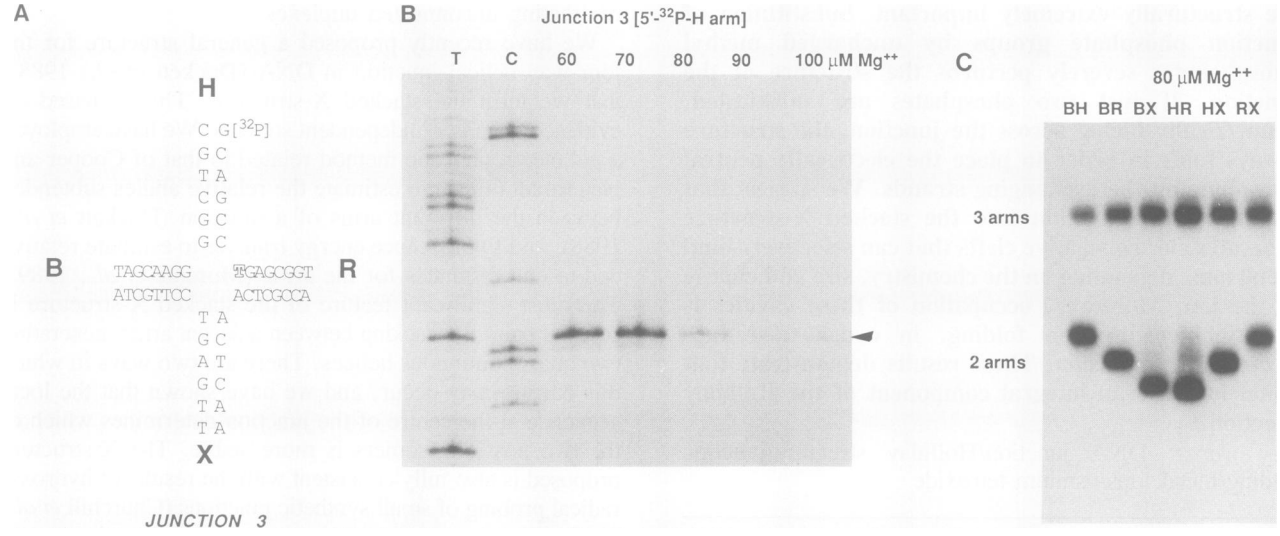

Fig. 1. Folding of a junction as a function of magnesium ion concentration—comparison of gel mobility and osmium tetroxide reactivity. (**A**) The sequence of the central part of junction 3, with the reactive thymine denoted in open type. The complete sequence of this and other junctions can be found in Duckett *et al.* (1988). (**B**) Osmium tetroxide reactivity of junction 3 5'-^{32}P-labelled in the H strand, as a function of added magnesium concentration. Radioactively labelled junction 3 was reacted with 1 mM osmium tetroxide, 3% pyridine in the presence of the indicated concentration of $MgCl_2$, followed by piperidine cleavage, electrophoresis and autoradiography. Tracks T and C contain thymine- and cystosine-specific sequencing reactions of the same radioactively labelled oligonucleotide used in the construction of the junction. The position of the centrally located thymine is indicated by the arrow to the right of the autoradiograph. (**C**) Electrophoretic mobility in the presence of 80 μM magnesium junction 3 that had been subjected to restriction cleavage in two arms. The six possible pairwise digests were electrophoresed in consecutive wells in a polyacrylamide gel in the presence of 80 μM $MgCl_2$. The tracks are each labelled in this and subsequent figures with two letters, that denote the long, i.e. uncleaved, arms. Thus BH indicates a junction that has been digested with *Eco*RI and *Xba*I, thereby shortening the R and X arms. The digests are normally partial, and the slower species are those that have been shortened in one arm only.

which has the opposite stacking pattern (i.e. B on H, as opposed to B on X for junction 3), was also folded under these ionic conditions (data not shown). Thus the concentration sufficient to confer protection against attack by osmium tetroxide is associated with the folded X-shaped configuration of the helical arms.

Magnesium may be replaced by certain other metal cations, summarized in Table I. Other group IIA cations behave in a manner indistinguishable from magnesium. A level of 100 μM Ca^{2+} generates the stacked X-configuration of junction 3, and this concentration of calcium also prevents attack by osmium tetroxide (data not shown). This is typical of all the group IIA cations.

Some multivalent ions are rather more efficient in their ability to fold four-way junctions than the group IIA metals. Hexamminecobalt(III) is particularly effective. Figure 2(A) shows the effect of 2 μM $[Co(NH_3)_6]^{3+}$ on the relative gel mobility of junction 3. The 2:2:2 pattern is identical to that induced by 80 μM Mg^{2+}, but is seen at one-fortieth the concentration of ion. Protection of junction thymine against attack by osmium tetroxide occurs at a corresponding lower concentration (see Table I). Polyamines are very effective in folding the four-way junction. Figure 2(B) demonstrates the folding of junction 3 by 25 μM spermine. The junction was efficiently folded by 25 μM spermine, as was junction 4, of opposite isomeric conformation. A similar concentration of spermidine was also effective, and these concentrations of polyamines confer resistance to attack by osmium tetroxide at the junction thymines.

Some transition metal ions can fold the junctions to give gel patterns indistinguishable from those due to magnesium and other ions. For example, inclusion of 1 mM Ni(II) in the gel buffer gave a pattern typical of the folded junction, indicating that this ion can successfully generate the X-configuration of the junction arms. Other transition metals, such as Cu(II), may also substitute in this way. However, although these ions appear to fold the junctions correctly, their binding constants appear to be relatively low compared to the divalent cations of group IIA, although competition with buffer components will influence this.

Table I. Summary of the effects of different cations on the folding of four-way junctions

Ion	Charge	Concentration (μM)	Folding	Thymine reactivity to OsO_4
Mg	+2	80	complete	full protection
Ca	+2	100	complete	full protection
Ba	+2	100*	ND	full protection
Sr	+2	100*	ND	full protection
$[Co(NH_3)_6]$	+3	2	complete	full protection
spermine	+4	25	complete	full protection
spermidine	+3	25	complete	full protection
Na	+1	35 mM	partial	reactive junction thymines
K	+1	50 mM	partial	reactive junction thymines
$N(CH_3)_4$	+1		ND	reactive junction thymines

The concentrations are the lowest observed to cause junction folding, judged from electrophoretic mobility experiments (or osmium tetroxide protection, where denoted by *), and the extent of folding is estimated by this technique. Where experiments have not been performed this is indicated by ND.

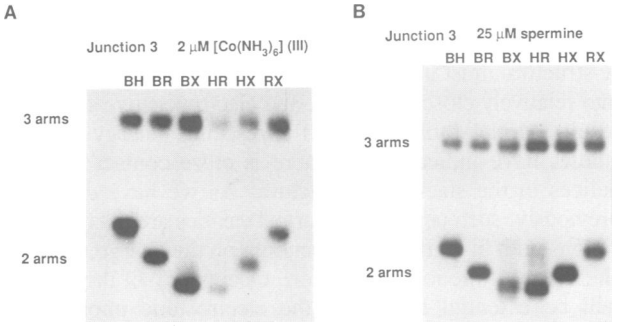

Fig. 2. Folding of a junction by a complex ion and polyamines. Electrophoretic mobility of the six double restriction digests of junction 3 in the presence of (**A**) 2 μM hexamminecobalt(III) and (**B**) 25 μM spermine.

Group IA metal ions fold junctions incompletely and inefficiently

We have examined the effect of sodium on the configuration of the four-way junction. Figure 3(A) shows the gel mobility pattern of junction 3 in the presence of 50 mM Na^+. Despite this high concentration of sodium ions, the pattern produced is significantly different from those where divalent ions have been used. Although the pattern is clearly related to a 2:2:2 pattern, the twofold symmetry has been disrupted. The two fast species are no longer equivalent, for example, where the BX species (that with uncleaved B and X arms) migrated in a position intermediate between HR and HX. This change in the pattern was also seen at the other concentrations of sodium, with potassium in place of sodium (Figure 3B), and with other junctions, and must reflect an incompletely folded conformation that lacks the full twofold symmetry. This incomplete folding is also reflected by sensitivity to attack by osmium tetroxide. Figure 4 shows that osmium tetroxide can readily modify junction thymine bases in the presence of 50 mM Na^+. However, addition of a further 1 mM Mg^{2+} strongly suppresses this reactivity implying that the group IIA ion completes the folding process. We have repeated this experiment using different buffer systems, with identical results.

Methyl phosphonate substitution at the junction can result in major structural perturbation

In addition to examining the effects of the chemical nature of the cation on the structure of the junction, we may choose an alternative experimental strategy, i.e. changing the potential target of the ion, the phosphates at the junction.

Our initial substitutions involved the introduction of methyl phosphonate at the central phosphodiester linkage in each of the four component strands of junction 2. In the methyl phosphonate group one of the non-esterified oxygen atoms is replaced by a methyl group, and the resulting phosphonate is electrically neutral. The result of these substitutions on the relative mobilities of the cleaved forms of junction 2, both in the presence and absence of 1 mM Mg^{2+}, is shown in Figure 5. In the absence of metal ions, the species adopt the characteristic 4:2 pattern, indicating the unfolded square-planar configuration. However, in the presence of Mg^{2+} ions, the modified junction fails to fold to give the normal 2:2:2 pattern, resulting in extensively smeared tracks in the place of the relatively sharp bands seen for normal junctions. Clearly the substitution has had a major structural effect, preventing the normal ion-induced folding of the junction.

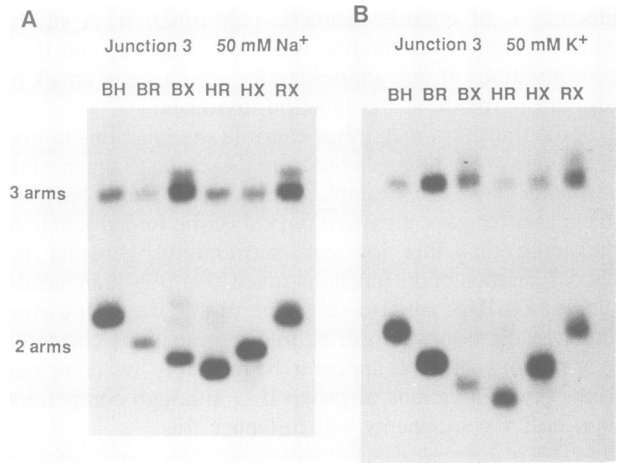

Fig. 3. Folding of a junction by group IA metal ions. Electrophoretic mobility in the presence of (**A**) 50 mM sodium and (**B**) 50 mM potassium of the six double restriction digests of junction 3. Note the different relative mobilities of the two fast species in each case.

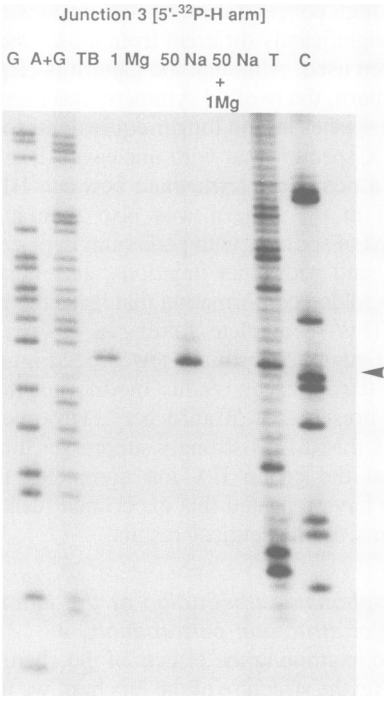

Fig. 4. Sodium fails to protect junction thymine bases from modification by osmium tetroxide. Osmium tetroxide reactivity of junction 3 5'-^{32}P-labelled in the H strand, as a function of added ions. Radioactively labelled junction 3 was reacted with 1 mM osmium tetroxide, 3% pyridine in the presence of Tris–borate buffer either alone (track TB), or supplemented with 1 mM $MgCl_2$ (track 1 Mg), 50 mM NaCl (track 50 Na), or 50 mM NaCl plus 1 mM $MgCl_2$ (track 50 Na + 1 Mg). This was followed by piperidine cleavage, electrophoresis and autoradiography. Tracks G, A + G, T and C contain base-specific sequencing reactions of the same radioactively labelled oligonucleotide used in the construction of the junction. The position of the centrally located thymine is indicated by the arrow to the right of the autoradiograph.

In view of the strong twofold symmetry of the normally folded junction, we decided to replace first two phosphates at a time, rather than all four, and to do this on the diagonals. Once again, in the absence of ions, the junction gave the 4:2 pattern characteristic of the unfolded junction (data not shown). The result in the presence of magnesium is shown in Figure 6. Although the bands are somewhat more diffuse than is normal for an unsubstituted junction, the patterns are now recognizable as 2:2:2 patterns, indicating folded junctions. The striking result emerges from a comparison of the two alternative diagonal substitutions—which indicate the formation of opposite junction conformations, i.e. different helix stacking partners. Thus with the methyl phosphonates substituted on the H and X strands, we see that BX and HR are the slow species ('moustache' pattern), revealing B on H stacking (i.e. the opposite configuration compared to the unsubstituted junction). By contrast, B and R substitution leads to a pattern of bands in which BH and RX are the slow species ('smile' pattern), indicating that the isomer formed has B on X stacking. However substituted, the junction folds to present the neutral methyl phosphonate group on the exchanging strand, indicating that these phosphates are close together in the folded junction. This illustrates the importance of charge–charge interactions in controlling junction structure, and once again suggests that specific ion–phosphate interactions occur in the folding of the junction.

Phosphorothioate substitution leads to no detectable structural change

An alternative way in which to modify phosphate groups is to replace a non-esterified oxygen atom by sulphur, generating a phosphorothioate group. Unlike the substitution above, this does not alter the charge on the phosphate. This was carried out for junction 2, with the result that (data not shown) the gel electrophoretic patterns were indistinguishable from those of unsubstituted junctions. We conclude that central phosphorothioate substitution allows the formation of a normally folded junction configuration in the presence of magnesium ion.

Discussion

The results presented above emphasize the importance of ion binding in the generation of the fully folded conformation of the four-way junction in DNA. Cations are essential for the folding of the DNA into the X-shaped structure, as judged by the conformation in gel electrophoresis and by the reactivity of thymine bases at the junction. In the absence of metal ions the junctions adopt a structure in which the four helical arms are unstacked and maximally extended into a square-planar configuration.

The process whereby the junction folds into the compact X-structure must involve forcing charged phosphate groups into relatively close proximity, and electronegative clefts will result. Model building and fluorescence energy transfer studies have indicated that there is close contact between helices in the stacked X-structure. As we have discussed previously, such negatively charged cavities are likely to bind cations with high affinity, depending on the charge, size and chemistry of the ion in question. Occupancy of these clefts will be essential to reduce the electrostatic phosphate–phosphate repulsion, and thereby permit the junction to complete the folding process. The ion-binding properties of such charged clefts will be quite different from normal DNA. The former can be considered in terms of specific and selective ion binding (Tam and Williams, 1985; Sullivan and Lilley, 1987), as opposed to general charge screening effects

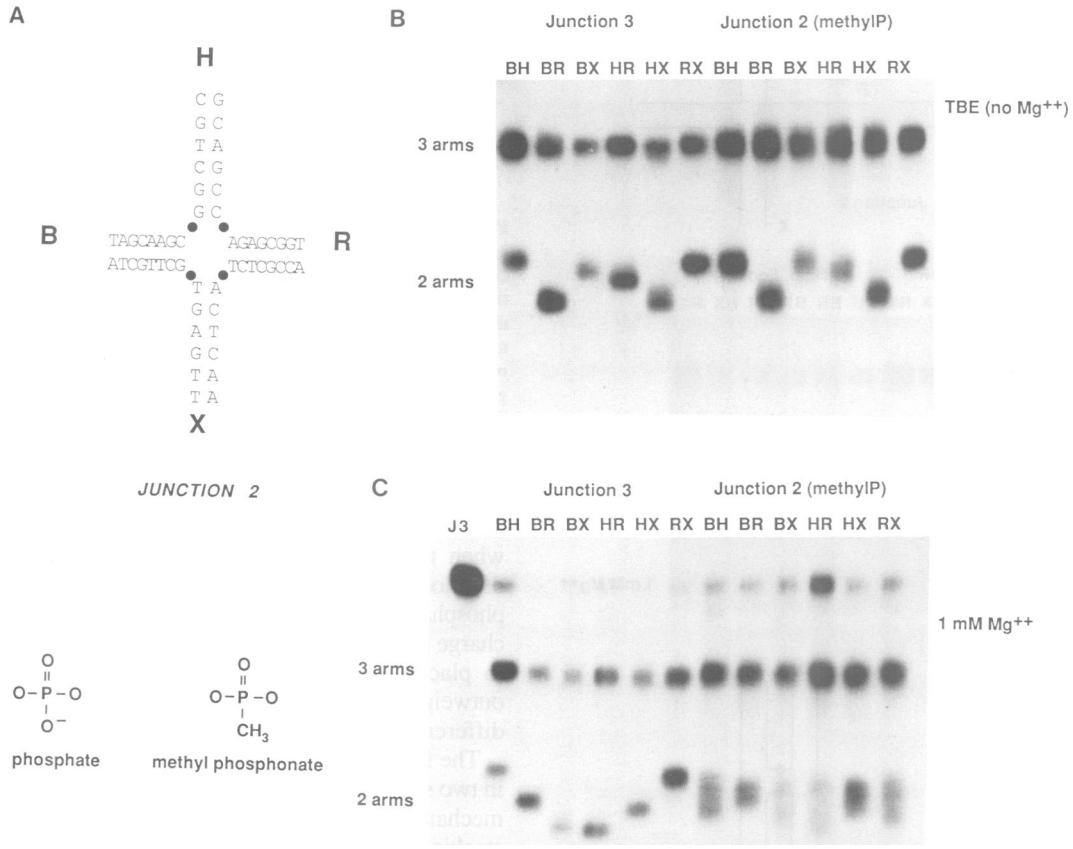

Fig. 5. Disruption of junction structure by methyl phosphonate substitution at the junction. (**A**) Central sequence of junction 2 used for methyl phosphonate substitution experiments. The substituted phosphate groups are indicated by the filled circles. The chemical structures of phosphates and methyl phosphonates are compared below. (**B**) Comparison of the electrophoretic mobilities in the absence of cations of a normal junction 3 and a methyl phosphonate substituted junction 2, that have been subjected to restriction cleavage in two arms. The junctions give rise to closely similar 4:2 patterns typical of the square-planar shape found in the absence of metal cations. (**C**) Comparison of the electrophoretic mobilities of the same species in the presence of 1 mM magnesium. The unsubstituted junction 3 gives a normal pattern, while the methyl phosphonate substitution leads to smeared bands (junction 2 gives patterns indistinguishable from that of junction 3 in the absence of phosphate modification; Duckett *et al.*, 1988). The left-most track (J3) of this gel contains uncleaved junction 3.

of ion clouds around B DNA (Manning, 1979; Zimm and Le Bret, 1983), where specific ion pairing is avoided.

We have demonstrated that ionic charge is one determinant of the efficiency of junction folding, as estimated by the minimum concentration of cation required. Thus divalent ions of periodic group IIA fold the junctions at 100 μM, while monovalent ions require much higher concentrations, and even then folding is not complete. Multivalent ions such as hexamminecobalt(III) and the polyamines are capable of folding the junctions at still lower concentrations. $[Co(NH_3)_6]^{3+}$ can cause junction folding at concentrations <2 μM—almost two orders of magnitude lower than the group IIA metal ions. This difference is too large to be due to charge alone, and is probably a complex function of charge, size and the ability of the NH_3 ligands to donate hydrogen bonds. For the same reasons this ion is also extremely effective in promoting B−Z transition in DNA (Behe and Felsenfeld, 1981; Gessner *et al.*, 1985). In general the transition metal divalent ions are not very efficient in folding the junctions. For example, although Ni(II) permits the junction to adopt the X conformation, as judged from gel electrophoresis experiments, the concentration required is higher than for the group IIA cations. This inefficiency stems from the chemical nature of these ions, which are relatively 'soft', and thus interact best with softer anions, such as nitrogen and sulphur groups (Irving and Williams, 1948). The differences between the various classes of ions reveals that ion binding of a rather specific nature is required to permit the junction to fold.

The group IA metal ions are extremely inefficient in folding the junctions. Very high concentrations are required —more than two orders of magnitude greater than for magnesium, for example. Even then, the structure is not completely folded into the X-structure, since the normal 2:2:2 gel pattern is distorted, and the junction thymines remain reactive to osmium tetroxide. The latter is also true for a number of transition metal ions, such as Cu(II). For the group IA cations we believe the character of interaction with the DNA is different, being less akin to specific binding. However, it is important to note that physiological concentrations of magnesium can readily out-compete sodium, generating a fully folded junction.

The importance of direct interaction with the phosphate groups is demonstrated by the experiments in which the

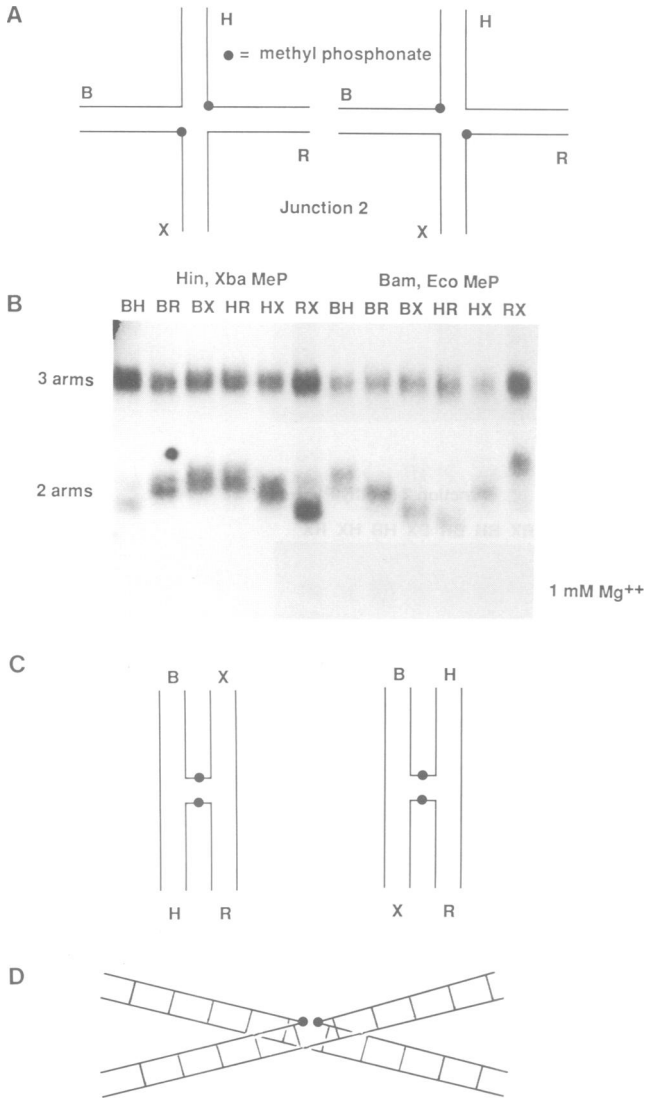

Fig. 6. Junction 2 isomerization in response to diagonal methyl phosphonate substitution. (**A**) Junctions were assembled from two oligonucleotides containing centrally located methyl phosphonate groups, and two unsubstituted oligonucleotides, such that the electrically neutral methyl phosphonates (filled circles) were on the diagonals as indicated in the schematics. (**B**) Comparison of the electrophoretic mobilities in the presence of 1 mM magnesium of the six double restriction digests arising from the two substituted junctions. Left six tracks, junction 2 substituted on the H and X arms; right six tracks, junction 2 substituted on the B and R arms. These correspond to the schematics directly above. (**C**) Interpretation of the relative electrophoretic mobilities. In both cases the results are interpreted in terms of a helix−helix stacking that places the methyl phosphonates on the exchanging strands. After rotation of these stacked structures, both have the form indicated in (**D**).

phosphates at the junction were chemically substituted. Although each substituted phosphate group was a mixture of diastereomeric species, clear-cut results were obtained. The more pronounced effects were due to phosphate charge, because junctions containing phosphorothioates, with unaltered charge, were conformationally indistinguishable from the unsubstituted junctions. By contrast, the electrically neutral junctions containing methyl phosphonates exhibited significant structural differences. Substitution of just two phosphates was sufficient to isomerize the junction

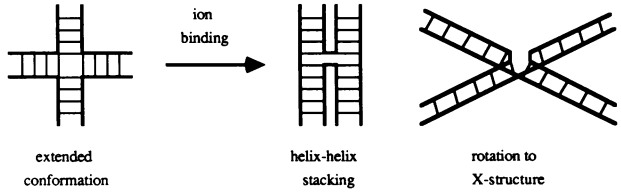

Fig. 7. Schematic of junction folding. In the absence of metal ions the junction is maximally extended in an approximately square-planar configuration. Binding of metal ions reduces phosphate repulsions to the point at which helix−helix stacking may occur. The parallel alignment of the quasi-continuous helices is not stable, presumably due to electrostatic repulsion along the length of the helices, resulting in a rotation into the X-structure. The tertiary folding of the junction generates electronegative clefts which bind cations with high affinity. This scheme is *not* intended to convey a kinetic mechanism for the folding process.

completely. These junctions adopted a structure which placed the neutral phosphate groups on the exchanging strand, even when this isomer was clearly less stable in the absence of phosphate modification. This implies that the central phosphates on the exchanging strands experience a very high charge potential, such that it is energetically rather favourable to place the neutral phosphate at this position, and this outweighs other thermodynamic considerations, such as differences in stacking energies.

The folding of the junction may be visualized as occurring in two stages, although we do not imply that this is the actual mechanism of the process. These steps are the helix−helix stacking, which creates the quasi-continuous helices, followed by a rotation of these parallel helices to generate the X-structure observed by gel electrophoresis experiments, and by fluorescence energy transfer experiments (Murchie *et al.*, 1989). This is illustrated schematically in Figure 7.

Nucleic acid folding brought about by specific metal ion binding may be general, with important biological consequences. Transfer RNA shares a number of features in common with the DNA junctions. Both can be drawn as four connected helices that fold by helix−helix stacking. tRNA is complicated by the presence of unpaired bases, non-Watson−Crick basepairing and modified bases, but nevertheless this represents a related folded nucleic acid structure in which charged clefts are generated. This results in the generation of specific high-affinity binding sites for magnesium ions and for polyamines, which have been located by crystallography (Jack *et al.*, 1977; Holbrook *et al.*, 1978; Quigley *et al.*, 1978). Mg^{2+} induces a conformational change in tRNA (Crothers and Cole, 1978), and this ion, together with spermine appear to be essential for crystallization (Dock *et al.*, 1984). Cruciform structures in supercoiled DNA (Gellert *et al.*, 1979; Lilley, 1980; Panayotatos and Wells, 1981) are essentially semi-homologous Holliday junctions, and are recognized by enzymes that cleave four-way DNA junctions (Mizuuchi *et al.*, 1982; Lilley and Kemper, 1984; de Massey *et al.*, 1984; Symington and Kolodner, 1985; West and Korner, 1985). The importance of ion binding in cruciform structure has been demonstrated both kinetically (Sullivan and Lilley, 1987) and thermodynamically (McClellan and Lilley, 1987). Kohwi and Kohwi-Shigematsu (1988) have recently demonstrated structural changes in a DNA triplex structure formed from a cloned oligo(dG)·oligo(dC) tract, in response to changes in magnesium ion concentration. Site-specific

recombination *in vitro* has been shown (Castell *et al.*, 1986) to be dependent on metal ions. Highly charged nucleic acid folds are energetically unstable because of electrostatic repulsion, but create electronegative clefts that can bind ions with considerable selectivity. Specific nucleic acid–ion interaction is extremely biologically important—it lies at the heart of ribozyme function, for example (Uhlenbeck, 1987). The interior of the cell contains millimolar concentrations of magnesium ion, together with some calcium and polyamines, all of which bind and stabilize the four-way helical junction. Cations should be regarded as an integral structural component of the Holliday junction.

Materials and methods

Synthesis of oligonucleotides

Oligonucleotides were synthesized using β-cyanoethyl phosphoramidite chemistry (Beaucage and Caruthers, 1981; Sinha *et al.*, 1984) implemented on an Applied Biosystems 381A DNA synthesizer. Fully deprotected oligonucleotides were purified by gel electrophoresis in 12% polyacrylamide containing 7 M urea, the bands excised and DNA electroeluted on to DEAE cellulose (Whatman DE52), eluted in 2 M NaCl and recovered by ethanol precipitation.

Methyl phosphonates (Miller *et al.*, 1986) (Applied Biosystems) were incorporated into the central position of the oligonucleotide using methyl phosphonamidites (Applied Biosystems) and standard synthesis cycles. Deprotection was effected by published procedures.

Phosphorothioate substitution was achieved by oxidation of the phosphate in the central position by means of a manual column wash step, using a solution of 50 mg/ml elemental sulphur in carbon disulphide/pyridine/triethylamine (12:12:1) (Aldrich) for 15 min at room temperature (Connolly *et al.*, 1984). This replaced the standard iodine oxidation in the synthesizer cycle.

For each synthesis involving phosphate group modification, no resolution steps were possible, and the products were chiral mixtures in each case.

Construction of four-way junctions

For each junction, four 80 base oligonucleotides were synthesized, with the sequences given in Duckett *et al.* (1988). For example, the 5′ B strand of junction 3 had the sequence 5′CGCAAGCGACAGGAACCTCGAG-**GGATCC**GTCCTAGCAAGGGGCTGCTACCGG**AAGCTT**CTCGA-GGTTCCTGTCGCTTGCG3′ (*Bam*HI and *Hin*dIII sites shown in bold). In each strand, the sequence between the restriction sites was unique, but beyond this point the sequence of each arm was identical. The unique centre allowed correct assembly to occur, without detectable competition by hairpin forms. Annealing reactions were carried out by incubating stoichiometric amounts of the appropriate DNA fragments for 2 h at 65°C in 20 μl of 10 mM Tris–HCl (pH 8.0) and then allowed to cool slowly. In each annealing reaction only one of the four oligonucleotides was radioactively labelled, giving four different species for each junction. Junction DNA was purified by gel electrophoresis in 5% polyacrylamide, and recovered by band excision and electroelution.

Enzymes and chemicals

Junction DNA was cleaved with restriction enzymes (Bethesda Research Laboratories and Boehringer) using conditions recommended by the manufacturers. Oligonucleotides were labelled at their 5′ termini using [γ-^{32}P]ATP and T4 polynucleotide kinase (Amersham). The highest available grades of metal salts were obtained from Aldrich Chemical Co., and osmium tetroxide was purchased from Sigma.

Osmium tetroxide modification reactions

Osmium tetroxide was dissolved in distilled water as an 8 mM stock solution. Purified junction DNA was incubated with 1 mM osmium tetroxide and 3% pyridine in 90 mM Tris–borate (pH 8.3), together with the concentrations of metal salt solutions indicated in the text, in 50 μl final volume at 20°C for 15 min (Lilley and Palecek, 1984; McClellan and Lilley, 1987; Duckett *et al.*, 1988).

Sequencing reactions

Chemical sequencing reactions were performed on 5′-^{32}P-labelled oligonucleotides bound to Hybond M and G paper (Amersham), using dimethyl sulphate (G), formic acid (A + G), potassium permanganate (T > C) and hydroxylamine (C) reactions (Rosenthal *et al.*, 1985).

Gel electrophoresis

Purified junction DNA was digested with the appropriate enzymes and loaded directly on to 5% polyacrylamide gels (29:1 monomer/bis ratio) and electrophoresed at 20°C for 16 h at 90 V. Jacketed electrophoresis plates with circulated water ensured that the temperature of the gel remained at 20 ± 0.1°C throughout the electrophoresis experiment. The buffer system contained either 90 mM Tris–borate (pH 8.3), together with concentrations of metal salt solutions indicated in the text, or 90 mM Tris–borate (pH 8.0), 10 mM EDTA (TBE). Electrophoresis buffers were continuously recirculated at 1 l/h. Junctions reacted with osmium tetroxide were analysed on 0.4 mm thick 10% polyacrylamide gels in TBE containing 7 M urea, which were run hot to the touch. Polyacrylamide gels containing radioactively labelled DNA were dried on to Whatman 3MM paper and autoradiographed at −70°C using Fuji RX X-ray film with Ilford fast tungstate intensifier screens.

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