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Structure of the four-way DNA junction and its interaction with proteins

DEREK R. DUCKETT, ALASTAIR I. H. MURCHIE, MARIE-JOSÈPHE E. GIRAUD-PANIS, J. RICHARD PÖHLER AND DAVID M. J. LILLEY*

CRC Nucleic Acid Structure Research Group, Department of Biochemistry, The University, Dundee DD1 4HN, U.K.

SUMMARY

The four-way DNA junction is an important intermediate in recombination processes; it is, the substrate for different enzyme activities. In solution, the junction adopts a right-handed, antiparallel-stacked X-structure formed by the pairwise coaxial-stacking of helical arms. The stereochemistry is determined by the juxtaposition of grooves and backbones, which is optimal when the smaller included angle is 60°. The antiparallel structure has two distinct sides with major and minor groove-characteristics, respectively. The folding process requires the binding of metal cations, in the absence of which, the junction remains extended without helix–helix stacking. The geometry of the junction can be perturbed by the presence of certain base–base mispairs or phosphodiester discontinuities located at the point of strand exchange. The four-way DNA junction is selectively cleaved by a number of resolving enzymes. In a number of cases, these appear to recognize the minor groove face of the junction and are functionally divisible into activities that recognize and bind the junction, and a catalytic activity. Some possible mechanisms for the recognition of branched DNA structure are discussed.

1. INTRODUCTION

Recombination is of immense evolutionary importance, facilitating the provision of new combinations of genetic variants from which the best combinations may arise by natural selection. Homologous genetic recombination may occur between any two sections of DNA, provided that stretches of homology exist within them. By contrast, site-specific recombination occurs between well-defined target sequences, and is the basis of specific DNA rearrangements used by transposons and bacteriophage, and by higher organisms (for events such as immunoglobulin gene maturation). Recombination events entail the physical rearrangement of DNA molecules and are brought about by proteins that must recognize and manipulate DNA structure.

To recombine two DNA molecules, it is necessary to juxtapose the helices in some way, and then to carry out chemical reactions on the phosphodiester backbones that generate a new connectivity. A final resolution step may be required to recreate independent duplex molecules that may, or may not, be recombinant. The four-way DNA junction is believed to be an intermediate in homologous genetic recombination (Holliday 1964), and there is good evidence for its role in site-specific recombination of the integrase class (Hoess *et al.* 1987; Kitts & Nash 1987; Nunes-Düby *et al.* 1987; Jayaram *et al.* 1988). Such branched DNA intermediates have rather precise three-dimensional structures and their manipulation will require the intervention of proteins that are largely structure-specific.

2. STRUCTURE OF THE FOUR-WAY DNA JUNCTION

The past five years has seen a substantial improvement in understanding of the structure of the four-way DNA junction. The junction undergoes a metal-ion-dependent conformational folding into a structure termed the 'stacked X-structure'. This structure is based on the pairwise coaxial-stacking of helices, and rotation into an overall X-shaped conformation. This structure is based upon evidence from a number of sources. Unfortunately, the four-way junction has been resistant to crystallization and is too large to be readily solved by nuclear magnetic resonance (NMR) methods, so it has been necessary to employ some relatively unconventional methods.

Gel electrophoresis has proved to be very powerful in the analysis of the structure of the branched DNA species. We showed that the creation of a four-way junction at the centre of a DNA fragment conferred abnormally low mobility in polyacrylamide (Gough & Lilley 1985), consistent with the introduction of a pronounced bend or kink at the position of the junction.

* To whom correspondence should be addressed.

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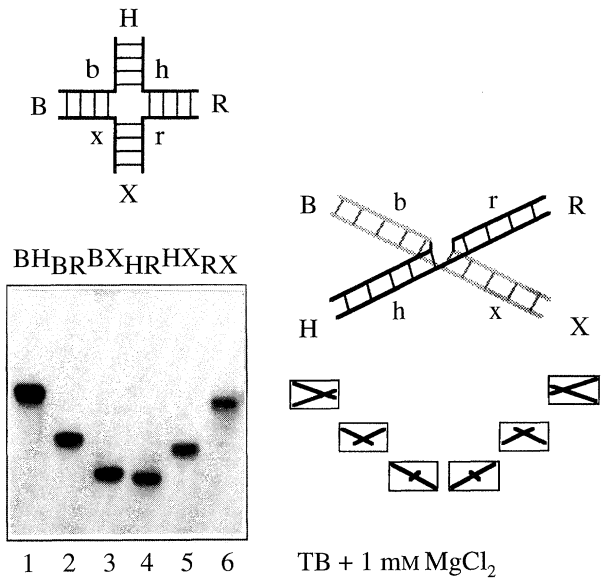

Figure 1. Gel electrophoretic analysis of the structure of a four-way DNA junction in the presence of magnesium ions. The relative configuration of the four arms was deduced by comparing the electrophoretic mobility of the six possible species in which two arms had been shortened by restriction enzyme cleavage. The junction (junction 3 from Duckett *et al.* (Duckett *et al.* 1988)) has four arms labelled B, H, R and X, and comprises four strands, labelled b, h, r and x as shown. The resulting species with two long and two short arms are indicated by their long arms, i.e. species BH has shortened R and X arms. These six long–short species have been electrophoresed on an 8% polyacrylamide gel in 90 mM Tris borate (pH 8.3) (TB buffer), 1 mM magnesium chloride. The slow-intermediate–fast-fast-intermediate-slow 2:2:2 pattern of mobilities is explained in terms of the X-shaped structure formed by coaxial stacking of B on X arms, and H on R arms as indicated. This leads to the formation of the six long–short species shown, interpreted in terms of the expectation that the mobility will be proportional to the size of the included angle between the long arms. Note that the chemical polarity of the continuous strands in this structure (i.e. strands h and x) is antiparallel, with the exchanging strands (strands b and r) turning around the small angle of the X. The relatively short distances between the ends of the B and H arms, and between the ends of the R and X arms, were further established by fluorescence resonance energy transfer experiments (Murchie *et al.* 1989; Clegg *et al.* 1992), thereby confirming the antiparallel character of the structure.

The electrophoretic mobility was found to be very dependent on the concentration and type of cation present (Diekmann & Lilley 1987), indicating a role for metal ions in the structure. Cooper & Hagerman (1987) developed a technique based on observation of the effect on the electrophoretic mobility of a four-way junction following the ligation of reporter arms. They concluded that the symmetry of the junction was lower than tetrahedral and that two of the strands were more severely bent than proposed in the model of Sigal & Alberts (1972).

We employed a related gel electrophoretic technique to compare the six isomeric junctions with two long and two short arms generated by pairwise restriction cleavage. We observed a pattern of two-fast, two-intermediate and two-slow (2:2:2 pattern) species in the presence of added cations (figure 1) (Duckett *et al.* 1988). This suggested to us that the junction is X-shaped, formed by stacking the helical arms in coaxial pairs, followed by a rotation in the manner of opening a pair of scissors.

This arrangement generates a favourable increase in basepair stacking interactions, while reducing steric and electrostatic interaction between the stacked pairs of arms. The reduction to twofold symmetry divides the four strands of the junction into two classes: two strands (continuous) have continuous axes, while the other two strands (exchanging) pass between the two helical stacks at the point of strand exchange. This distinction between strands is consistent with probing of four-way DNA junctions using hydroxyl radicals (Churchill *et al.* 1988), where it was found that two strands were more protected than the other two.

Two isomers of the stacked X-structure are possible, depending on the choice of helical stacking partners. When we altered the sequence at the point of strand exchange, the electrophoretic pattern of our long–short arm junctions changed, consistent with an exchange of stacking partners (Duckett *et al.* 1988). This isomerization changes the nature of each strand in the structure; continuous strands become exchanging strands, and vice versa. The identity of the most stable isomer will be governed by the thermodynamics of the interactions at the point of strand exchange, probably mainly by the stacking interactions.

Electrophoretic mobility of DNA that is bent by curvature or bulge-kinking is proportional to end-to-end distance. If we use this as a basis for assigning the six long–short arm junction species, we conclude that the structure is approximately antiparallel, i.e. the two continuous strands run in opposite directions. This is in contrast to the normal depiction of Holliday junctions, and to the model of Sigal & Alberts (1972). We therefore sought alternative methods to test the structure, that were independent of any assumptions about the relative mobilities of different species in polyacrylamide gels, and turned to fluorescence resonance energy transfer (FRET) (Murchie *et al.* 1989; Clegg *et al.* 1992). The two short end-to-end distances that were observed were only consistent with an antiparallel structure and were in complete agreement with the stacking isomeric forms deduced from our earlier gel electrophoresis. All experimental results using a variety of other techniques indicate that the antiparallel structure is most stable in solution and there is none consistent with the parallel structure.

Of all the possible X-shaped structures, modelling indicates that a right-handed, antiparallel structure is likely to be most stable, as it permits a favourable alignment of strands and grooves that helps avoid steric clash between backbones (figure 2). This is most effective if the small angle is 60°. The right-handed configuration is consistent with FRET measurements of the handedness (Murchie *et al.* 1989). It is clearly a very natural way for DNA molecules to interact, as similar packing has been observed in a number of crystal structures of double-stranded oligonucleotides (Timsit *et al.* 1989; Lipanov *et al.* 1993). The strand–groove alignment leads to a localized protection

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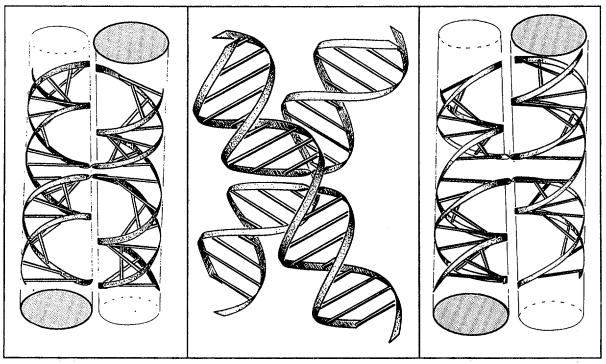

Figure 2. Ribbon model of the right-handed, antiparallel stacked X-structure of the four-way DNA junction, observed from three points of view. Note the juxtaposition of the continuous strands in the major grooves of the opposing helices, which is optimal for a small angle of 60° for the helix crossing. Centre: face view, showing the X-shape of the folded junction: the two sides of the structure are not equivalent. On one side (left) the four basepairs at the point of strand exchange all present major groove edges, while on the other side (right) the minor groove edges are presented.

against cleavage by DNase I (Lu *et al.* 1989; Murchie *et al.* 1990). Coaxial stacking of DNA helices is consistent with the results of cleavage by *Mbo*II (Murchie *et al.* 1991).

One important aspect of the stereochemistry is that the antiparallel junction presents two dissimilar sides, as seen in figure 2 (Murchie *et al.* 1989; von Kitzing *et al.* 1990). The connectivity required to generate the four-way junction creates two sides of major- and minor-groove characteristics respectively, these are preserved upon folding into an antiparallel conformation. It may be seen, for example, that on the major groove side there is a continuous major groove that runs down one arm, passes through the point of strand exchange and continues on an arm of the opposite helical stack. This has important consequences for interaction with proteins.

3. ROLE OF METAL IONS IN FOLDING THE FOUR-WAY DNA JUNCTION

Metal ions play a critical role in the structure of the four-way DNA junction. In the absence of added cations, the junction cannot fold into the stacked X-structure, and remains extended with unstacked arms. Gel electrophoretic experiments (figure 3) indicate that under low-salt conditions, the junction adopts a structure with approximately square symmetry (Duckett *et al.* 1988, 1990), which is confirmed by FRET experiments (Clegg *et al.* 1993). This suggests that the folding of the junction into the stacked X-structure creates close phosphate juxtaposition that is destabilizing unless reduced by ion screening.

Folding the four-way junction into its stacked form reflects a balance between the favourable interactions stabilizing the folded form, particularly helix–helix stacking interactions and destabilization due to electrostatic repulsion (figure 4). A variety of ions are able

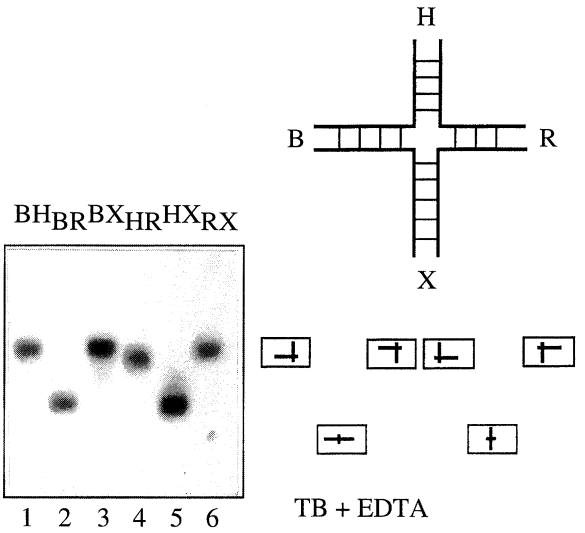

Figure 3. Gel electrophoretic analysis of the structure of a four-way junction in the absence of added metal ions. Long–short arm analysis of a junction in 90 mM Tris borate (pH 8.3), 0.1 mM EDTA (Duckett *et al.* 1988). Under these conditions, junctions of any sequence exhibit a 4: pattern of mobility in the order slow, fast, slow slow, fast, slow. This is consistent with a square configuration of helical arms, giving four species where the long arms subtend approximately 90° (slow species), and two with angles of approximately 180° (faster species). The four strands are equivalent in this structure. The extended, square configuration of arms has been confirmed by FRET measurements (Clegg *et al.* 1993), and is consistent with the reactivity of thymine bases at the point of strand exchange to osmium tetroxide at low salt concentrations (Duckett *et al.* 1988).

to bring about the folding, with differing efficiencies (Duckett *et al.* 1990). The balance between stacking and electrostatic interactions was revealed by experiments in which selected phosphate groups were replaced by electrically neutral methyl phosphonates; different stacking isomers resulted, depending on which phosphates were replaced (Duckett *et al.* 1990). A high-affinity ion-binding site at the point of strand exchange in the junction has recently been revealed using uranyl photocleavage (Møllegaard *et al.* 1994).

Thus metal ions are an integral part of the folded conformation of the DNA junction. This should not come as a surprise. DNA is a highly charged polyelectrolyte, the folding of which is likely to generate repulsive interactions that must be screened. Critical ion interactions are likely to play important roles in the folding of many functionally significant nucleic acids.

4. PERTURBATION OF THE STRUCTURE OF THE FOUR-WAY DNA JUNCTION

The right-handed, antiparallel stacked X-structure may be distorted by a variety of influences. While the angle subtended between the pairs of coaxially stacked helices is normally about 60°, this can probably be distorted relatively easily. Kimball *et al.* (1990) showed that it was possible to force a junction into a parallel configuration by restraining the distances between the ends of given arms with a molecular tether. The

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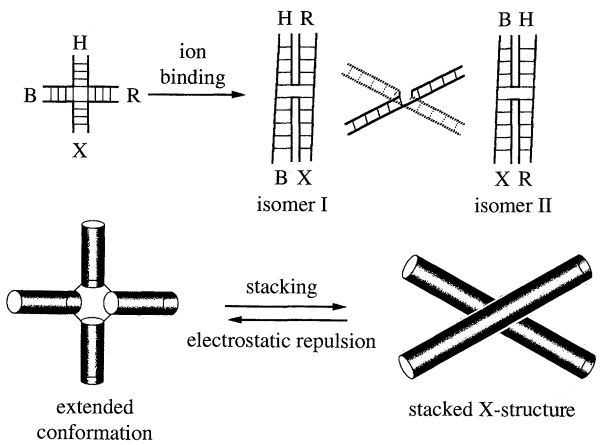

Figure 4. The cation-induced folding of the four-way DNA junction. In the absence of added cations, electrostatic repulsion outweighs the free energy available from coaxial helical stacking, and the junction remains extended. Upon addition of cations (e.g. > 80 μM Mg^{2+}), the junction folds into the stacked X-conformation. The folding generates a site of high affinity for metal ions, which is located at the point of strand exchange (Møllegaard *et al.* 1994). There are two possible stacking isomers of the folded conformation; for most sequences one isomer is significantly more stable than the other, and the relative stability of the isomeric forms is determined by the sequence at the point of strand exchange.

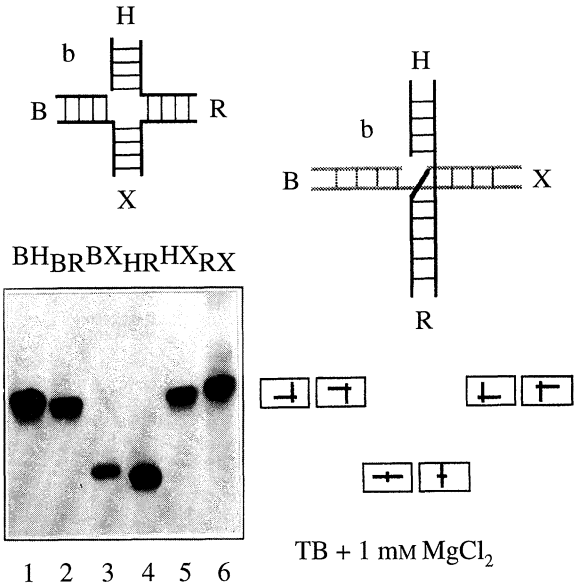

Figure 5. Effect of introducing a nick into one strand of the four-way DNA junction at the point of strand exchange. Gel electrophoretic analysis of a junction containing a nick on the b-strand, in the presence of 1 mM magnesium ions. The long–short arm analysis was carried out as before (see figure 1). The pattern of mobilities was clearly different from the 2:2:2 pattern of the intact junction in magnesium, indicating that the configuration of arms had changed. Four slow and two fast species were observed, but the pattern was slow, slow, fast, fast, slow, slow, in contrast to that observed for the intact junction in the absence of added metal cations (compare with figure 3). This suggests that coaxial pairwise helical stacking is retained, but that the angles included between the arms are close to 90° as shown. When the nick was placed on the x-strand, we observed an isomerization of the junction such that B on H stacking now occurred (Pöhler *et al.* 1994). The result of this is that the nick is once again located on an exchanging strand.

successful construction of molecules containing two four-way junctions supports the ability of a relatively flexible junction to be conformable (Fu & Seeman 1993).

Imperfections in homology between recombining sequences will lead to the introduction of non-Watson–Crick base mismatches at the four-way junction. The inclusion of such mismatches may have a very destabilizing effect on the folded structure (Duckett & Lilley 1991). In general, the effects of mismatches were dependent both on the nature of the mismatch and its context. Most junctions, even those containing severely destabilizing mismatches, could be persuaded to fold into the stacked X-structure by increasing cation concentrations, supporting the concept of a balance between stacking and electrostatic interactions.

Junctions containing covalent interruptions in the phosphodiester backbone (nicks) at a single location may be created either by a unitary strand exchange process, or by enzymic cleavage of an intact junction. We found that single nicks have a significant influence on the conformation of the junction in the presence of added metal ions (Pöhler *et al.* 1994). We obtained gel electrophoretic patterns suggesting that while coaxial helix–helix stacking was retained, the angle between the helices changes to be closer to 90° (figure 5). This suggests that the additional conformational flexibility permits the helices to disengage from the close backbone–groove juxtaposition and swing around to a perpendicular arrangement that lowers overall electrostatic repulsion. To observe the change in conformation, the nick was required to be immediately at the point of strand exchange. Interestingly, the stacking isomer was determined by the position of the strand break, in order to place the nick on the exchanging strand. Clearly, the relaxation of the structure lowered the free energy of the junction by more than the difference between stacking isomers.

5. INTERACTION WITH JUNCTION RESOLVING ENZYMES

Four-way DNA junctions are substrates for resolving enzymes, a class of structure-specific nucleases (reviewed in Duckett *et al.* 1992). Such activities have been isolated from a wide variety of sources from bacteriophage to mammals, and are probably ubiquitous enzymes for the manipulation of branched DNA. In addition, there are proteins that selectively bind DNA junctions without resulting in nucleolysis (Elborough & West 1988; Bianchi *et al.* 1989; Parsons *et al.* 1992). Some of these proteins are largely specific for certain branched DNA structures, whereas others exhibit some sequence selectivity. Some proteins interact specifically with the four-way junction, whereas others, exemplified by T4 endonuclease VII, cleave a variety of branched DNA structures.

Endonuclease VII of bacteriophage T4 (Kemper & Garabett 1981) is a well-characterized junction-resolving enzyme. The enzyme cleaves four-way DNA

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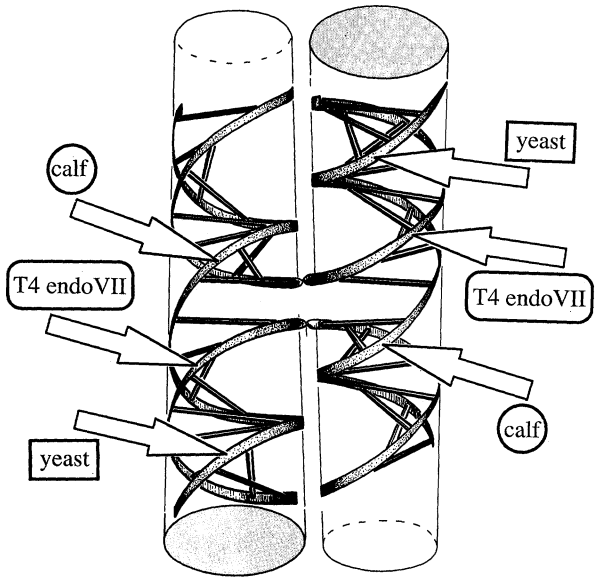

Figure 6. Cleavage sites for three different junction-resolving enzymes on the minor groove side of the four-way DNA junction. While T4 endonuclease VII cleaves the exchanging strands of a junction 3′ to the point of strand exchange, analysis of the cleavage sites of related junctions by an endo X1 from yeast (West *et al.* 1987) and an enzyme from calf thymus (Elborough & West 1990) show that these cleave the continuous strands, 5′ and 3′ respectively to the point of strand exchange. While these sets of cleavage positions appear to be quite unrelated at first sight, when they are placed onto the model of the stacked X-structure they are all found to be located on the minor groove face.

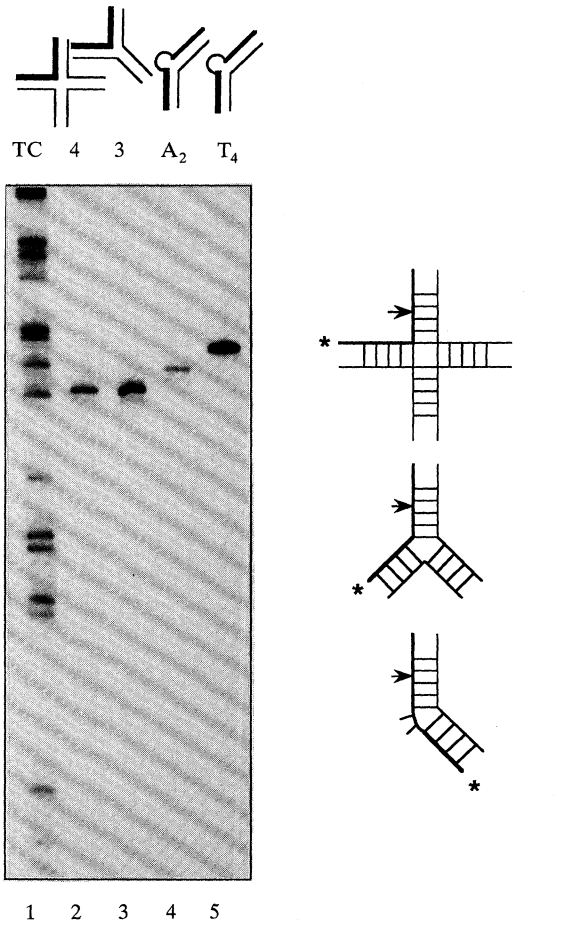

Figure 7. Cleavage of branched DNA species by T4 endonuclease VII. A four-way junction was radioactively [5′-^{32}P] labelled on one strand and incubated with T4 endonuclease VII. Cleavage at virtually a single phosphodiester bond may be seen on the sequencing gel. Three other branched DNA species of closely related sequence were examined. The same radioactive strand was incorporated into a three-way junction, and two bulged species, containing A_2 and a T_4 bulges respectively, each of which were based on the same sequence. These species were cleaved in a manner that was virtually identical to that on the four-way junction (the shift in the position of the bands on the bulged species is due to the insertion of the bulge bases).

junctions (Duckett *et al.* 1988; Mueller *et al.* 1988) and cruciform structures in supercoiled DNA (Mizuuchi *et al.* 1982; Lilley & Kemper 1984). It cleaves the exchanging strands of the junction, two or three bases 3′ to the point of strand exchange. The scissile bonds are located symmetrically on the minor-groove side of the stacked X-structure (figure 6), suggesting that the enzyme interacts selectively with this face of the junction. This is consistent with regions of the junction protected by the enzyme against hydroxyl radical cleavage (Parsons *et al.* 1990; Bhattacharyya *et al.* 1991). The minor-groove side is also the apparent target for junction resolving enzymes isolated from yeast (West *et al.* 1987) and calf thymus (Elborough & West 1990) (figure 6). This suggests that the three enzymes bind the junction in a related manner, despite their wide evolutionary separation. However, although all three enzymes appear to interact with the same face of the junction, the actual cleavage positions differ in each case. This suggests that the proteins might be functionally divisible into binding and catalytic parts, which are oriented in different ways in the various enzymes.

T4 endonuclease VII responds primarily to DNA structure. The cleavages are located primarily on the exchanging strands and upon stacking isomerization, due to local sequence changes, cleavage sites are found on the new exchanging strands (Duckett *et al.* 1988). To prove this point rigorously, a junction of constant sequence was constrained to exist in one or other stacking isomer by means of tethering (Kimball *et al.* 1990), whence cleavage by T4 endonuclease VII was restricted to the exchanging strands of each isomer (Bhattacharyya *et al.* 1991).

This raises the question of what are the critical structural features of the DNA junction that are recognized by the resolving enzyme. Two general possibilities exist:

1. *Recognition of local conformation* Clearly, the strand trajectory that generates the strand exchange in the junction leads to some unusual stereochemical features. These might be recognized by the enzyme: for example, the junction contains a high-affinity binding site for a number of intercalators (Guo *et al.* 1989). Perhaps then, an aromatic sidechain might probe the DNA structure in a similar manner to these compounds. Modelling the structure of the four-way junction indicates that local widening of grooves is required (von Kitzing *et al.* 1990), and could generate another recognizable feature.

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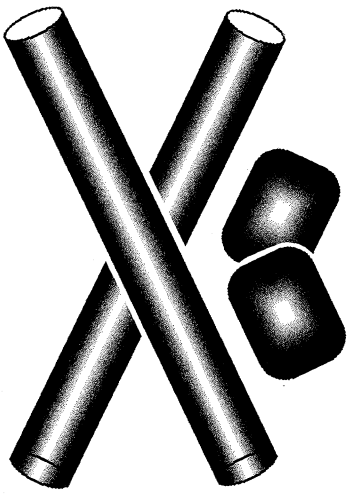

Figure 8. Schematic to show the interaction between T4 endonuclease VII and the minor groove side of the junction. It might be anticipated that the dimeric enzyme has complementarity of surface shape, such that the discrimination of the angle of helical inclination in the junction is an element in the structural selectivity of the resolving enzyme.

2. *Global recognition* The stacked X-structure provides a number of faces at which there is a precise relative inclination of the two pairs of coaxially stacked DNA helices. If two subunits of the enzyme make precise contacts with these two helices, protein–protein interactions could generate selectivity for the angle of inclination of the DNA (Bhattacharyya *et al.* 1991).

There is no completely convincing evidence for either of these models and any suggestion can only presently be regarded as a working model which may turn out to be wrong. Enzymes such as T4 endonuclease VII cleave a variety of DNA structures, including four-way and three-way junctions and bulged DNA molecules. We have shown (Bhattacharyya *et al.* 1991) that these are cleaved in a related way (figure 7). The common feature amongst these species is a mutual inclination of two DNA helices (for a discussion see Lilley & Clegg 1993), and it seems feasible that this aspect is important in the recognition process. T4 endonuclease VII exists as a dimer in solution (Kosak & Kemper 1990), and the two subunits could be oriented so as to interact most effectively with two DNA helices that are mutually inclined at 120° (shown schematically in figure 8). The nuclease activity might become activated when the included angle falls within certain limits. This idea is supported by the observation that T4 endonuclease VII cleaves bulged molecules fastest when they have particular numbers of bulged bases (e.g. two adenines) (Bhattacharyya *et al.* 1991), which might generate a degree of axial kinking that is optimal for interaction with the enzyme. Further substrates for T4 endonuclease VII are A-tract curved DNA, which becomes nicked one base to the 3′ side of each A-tract (Bhattacharyya *et al.* 1991), and DNA containing a single site of modification by *cis*-diamminedichloroplatinum (II) (Murchie & Lilley 1993), which creates a local kinking of DNA (Rice *et al.* 1988). The cleavage positions in the platinated DNA do not suggest a simple location of the enzyme on the inner face of the kink, but a kinked duplex has a rather different geometry from a stacked X-structure junction, and exact equivalence should not be expected. Some junction-resolving enzymes (such as RuvC (Connolly *et al.* 1991) perhaps) may be rather more specific for four-way junctions, when compared to the phage enzymes, although the sequence-specificity for the cleavage reaction generates greater discrimination than binding (Benson & West 1994).

6. INTERACTION OF T7 ENDONUCLEASE I WITH FOUR-WAY JUNCTIONS

T7 endonuclease I cleaves four-way DNA junctions in a manner that is different from the enzymes discussed above in that all four strands become cleaved at positions very close to the point of strand exchange (Duckett *et al.* 1988). The cleavage positions do not appear to have the same two-fold symmetric relationship that is exhibited by resolving enzymes like T4 endonuclease VII. This might suggest a different manner of junction-protein interaction. Nevertheless, like the other resolving enzymes, T7 endonuclease I is highly selective for branched DNA species and must therefore, be capable of structure-selective interaction with DNA.

To explore this further we have taken a genetic approach, based on the isolation of catalytically non-functional mutants of T7 endonuclease I (Duckett *et al.* 1995). Overexpression of T7 endonuclease I in *E. coli* leads to severe toxicity; we used this as a basis for the selection of mutant resolving enzymes. The gene for T7 endonuclease I was subjected to random chemical mutagenesis, reinserted into the expression site of a plasmid and transformed into a strain of *E. coli* lacking the LacI repressor required to repress the P_{lac} promoter. Under these conditions, the level of wild-type T7 endonuclease I produced is lethal; any surviving colonies are likely to express mutant protein. Sequencing a series of such T7 endonuclease I genes revealed in each case that the amino acid sequence of the protein had suffered one or two changes. The mutant proteins were expressed with histidine-tags for ease of manipulation and their DNA-binding properties were analysed. In each case we found that the mutant proteins bound very well to four-way DNA junctions, giving a well defined retarded species on polyacrylamide gel electrophoresis (PAGE) (figure 9*a*). Moreover, the binding to the junction could not be out-competed by a thousand-fold excess of linear DNA of the same sequence (figure 9*b*). Clearly these mutant proteins retained their full structure-selectivity for the four-way junction, whilst having lost all ability to cleave the phosphodiester backbone of the DNA. This is consistent with a functional divisibility of structure-selective binding and catalysis for this enzyme. When we analysed the positions in which the catalytically inactive proteins had suffered mutation, we found that all the amino acid changes clustered into the second quarter of the protein sequence. Interestingly, this part of the sequence shares some sequence similarity with T4 endonuclease VII, suggesting that these regions

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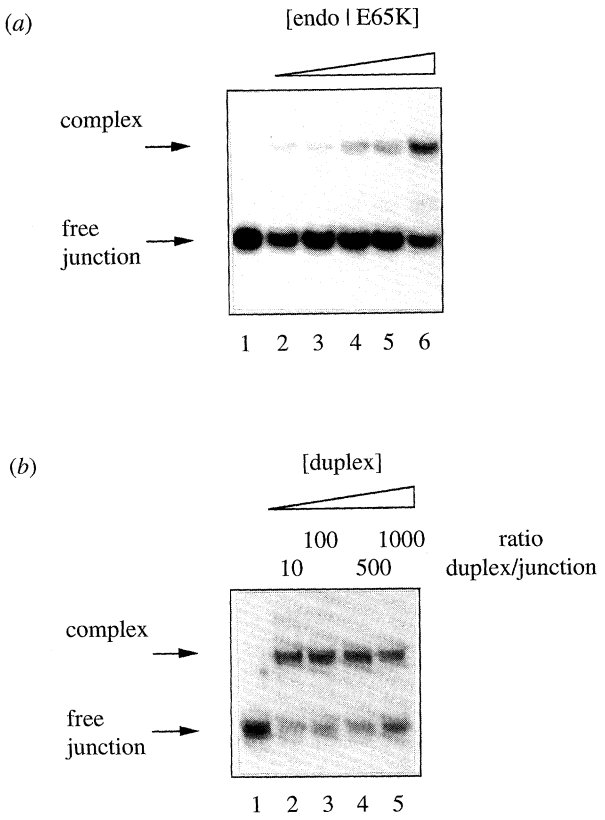

Figure 9. Structure-selective binding of T7 endonuclease I to the four-way DNA junction. (*a*) Electrophoretic retardation of the complex formed between [^{32}P] radioactively labelled DNA junction and an inactive E65K mutant of T7 endonuclease I in 90 mM Tris borate (pH 8.3), 200 μM $MgCl_2$. Track 1, junction without added protein; tracks 2–6, junction incubated with increasing concentrations of the mutant resolving enzyme. (*b*) Competition between four-way junction and duplex for binding E65K mutant T7 endonuclease I. Radioactive junction was incubated with E65K endonuclease I in 90 mM Tris borate (pH 8.3), 200 μM $MgCl_2$, either alone (track 1), or with an increasing molar ratio of unlabelled competitor duplex DNA (tracks 2–5). Note that even a 1000-fold excess of linear duplex fails to displace a significant fraction of the complex between the resolving enzyme and the four-way junction.

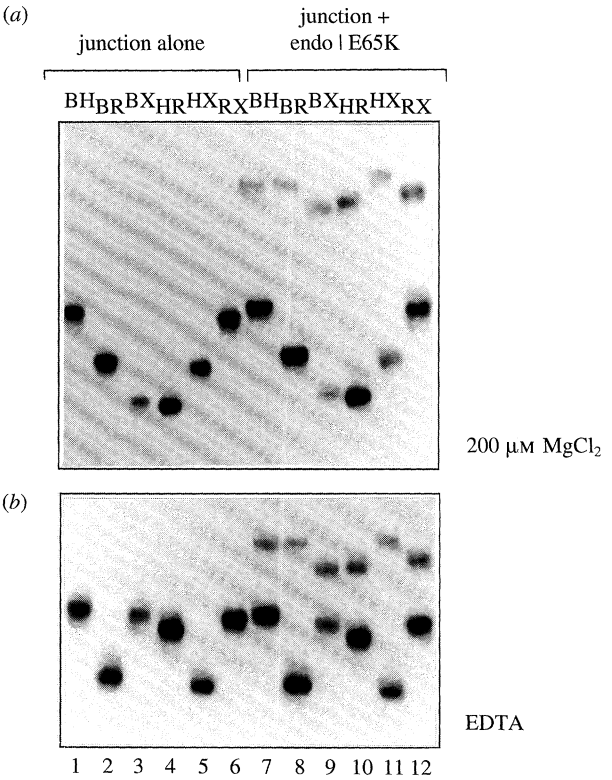

Figure 10. Gel electrophoretic analysis of the complex between a four-way junction and E65K mutant T7 endonuclease I. (*a*) The six double-restriction digests of junction 3 (see figure 1) were incubated with E65K endonuclease I in 90 mM Tris borate (pH 8.3), 200 μM $MgCl_2$. These were analysed by gel electrophoresis (tracks 6–12), in comparison with the same species in the absence of protein (tracks 1–6). Note the pattern of mobilities of the retarded species formed in the presence of the protein are different from that of the free DNA. (*b*) The equivalent experiment in 90 mM Tris borate (pH 8.3), 1 mM EDTA. Note that the pattern of retarded species is exactly the same as that found in the presence of magnesium ions.

might provide amino acids important in the active site of the resolving enzymes.

One of these catalytically inactive mutants of T7 endonuclease I was used to explore the shape of the complex bound to the four-way junction, using the gel electrophoretic analysis of junctions with the permutations of two-long and two-short arms discussed for the isolated junction above. We can observe modulation of the electrophoretic mobility of the six long–short species as a complex with the mutant T7 endonuclease I (figure 10). Although the difference in mobility between the fastest and slowest species is much less than that for the pure DNA further down the gel, it appears that the pattern of mobilities is different for the complex compared to free DNA junction. Although some caution is necessary in the interpretation of this result, due to possible effects on mobility of the presence of the protein itself, it appears that there has been a conformational change in the configuration of the arms of the junction. The pattern of the shifted species does not appear to be a fast, intermediate, slow 2:2:2 pattern, but is more similar to the slow, slow, fast, fast, slow, slow pattern of the nicked junction (Pöhler *et al.* 1994) (see above). This suggests that in the presence of the resolving enzyme, the arms might remain coaxially stacked but alter their relative juxtaposition – thereby changing the angles between arms from the 60°/120° of the free junction to something more like 90°. We observe exactly the same pattern of mobilities whether the gel is run in the presence of magnesium or EDTA, indicating that the protein can itself induce folding of the junction on binding.

7. RECOGNITION OF BRANCHED DNA STRUCTURES BY PROTEINS

DNA junctions are manipulated by proteins that recognize DNA three-dimensional structure. An ability to recognize mutually inclined DNA helices might be a general way of recognizing certain DNA structures. This facility could be important in DNA repair and T4 endonuclease VII and T7 endonuclease I are best

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classified as repair enzymes. DNA may be damaged by a variety of agents (Reardon & Sancar 1991). A fairly common feature at the site of damage may be axial kinking, as is found in the case of a cisplatin adduct, for example (Visse *et al.* 1991). This could generate a feature that is recognizable by certain proteins. We might, therefore, class together a series of proteins that recognize DNA structure and manipulate it in some way – including topoisomerases and proteins that manipulate DNA structure in a variety of cellular processes (such as IHF). The HMG1 domain (Bianchi *et al.* 1989) and HU protein (Pontiggia *et al.* 1993) selectively bind DNA junctions and might therefore be classified in the same way.

It seems probable that enzymes that are required to manipulate DNA junctions will be functionally divisible into two activities: recognition of and selective binding to the structure of the junction, and catalysis of some reaction. The binding and catalytic activities of the phage-resolving enzymes appear to be functionally divisible. These proteins might, therefore, be thought of as analogous to a missile, requiring a delivery vehicle that can locate the target and a warhead that fulfils its function when delivered to the target site. At least two different activities are required for the manipulation of the four-way DNA junction in recombination, branch migration and resolution. In *E. coli* they are catalysed respectively by RuvAB (Iwasaki *et al.* 1992; Tsaneva *et al.* 1992) and RuvC (Connolly *et al.* 1991; Iwasaki *et al.* 1991; Sharples & Lloyd, 1991). For resolution, it is clear that the catalytic activity required is a nuclease. In the case of the proteins that facilitate branch migration the catalyst is less obvious. Measured rates of spontaneous branch migration are low, especially in the presence of magnesium ions (Panyutin & Hsieh 1994), where the junction is tightly folded into the stacked X-structure. In order to catalyse the process, a protein that can destabilize the structure is required and this turns out to be a helicase. Thus in the case of RuvAB, it is the B subunit that performs catalysis, while the A subunit directs the complex to the four-way DNA junction (Tsaneva *et al.* 1993). In the case of RecG (Lloyd & Sharples 1993), the two functions are combined within one protein.

The division of recombination enzymes into binding and catalytic functions is supported by experimental data from a number of different systems: (i) comparisons of the cleavage sites for junction resolving enzymes from phage T4, yeast and calf thymus (discussed above); (ii) the demonstration that the sequence-specificity of RuvC resides at the level of catalysis, and that binding and catalysis are separable (Bennett *et al.* 1993); (iii) although yeast endo X2 exhibits some sequence-specificity, four-way junctions that could not be cleaved could nevertheless bind the enzyme and served as a competitive inhibitor of cruciform cleavage (Evans & Kolodner 1988); (iv) a fusion of T7 endonuclease I to the *lac* repressor (Panayotatos & Backman 1989) cleaved DNA adjacent to *lac* operator sites, indicating that the nuclease function of the resolving enzyme could function with a new DNA binding domain; and (v) mutants of T7 endonuclease I bind four-way junctions without cleavage (discussed above). A mutant of RuvC with equivalent properties has been reported (Sharples & Lloyd 1993). Although the recognition processes are unlikely to be identical between different enzymes, we suspect that they will be similar in principle for all these proteins whatever their source. Recombination is likely to be mediated by proteins that recognize and manipulate the structure of folded DNA, and understanding the DNA structure is an important element of understanding the overall mechanism.

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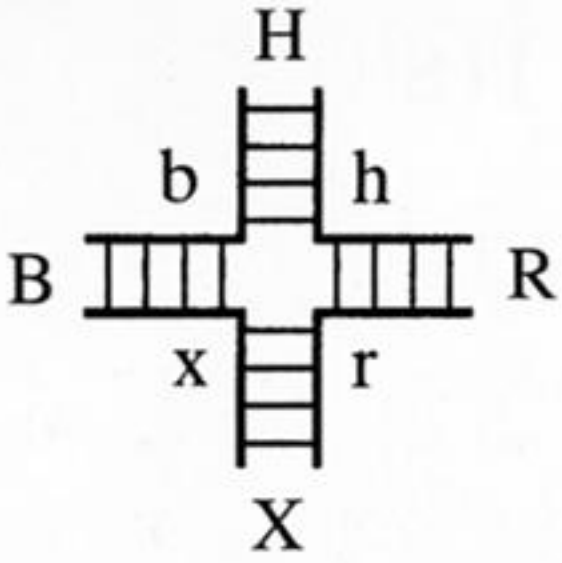
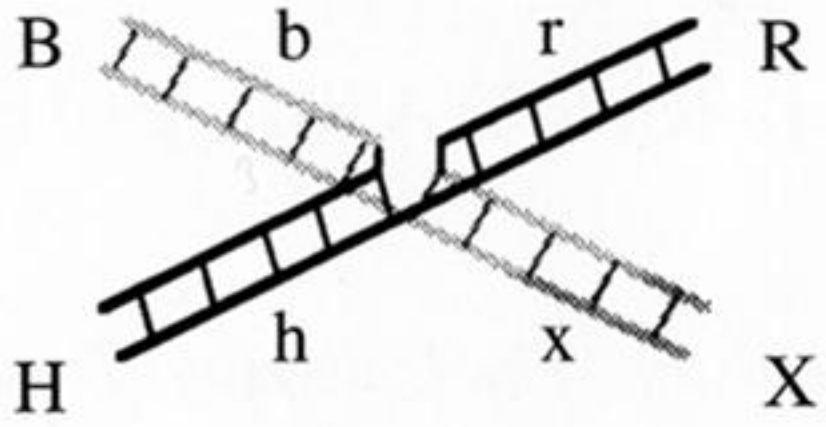
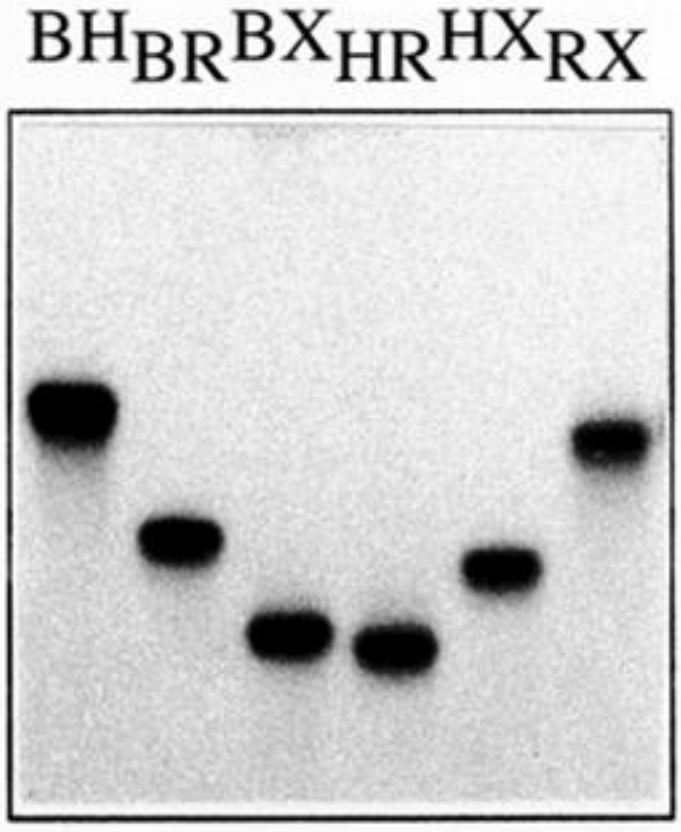






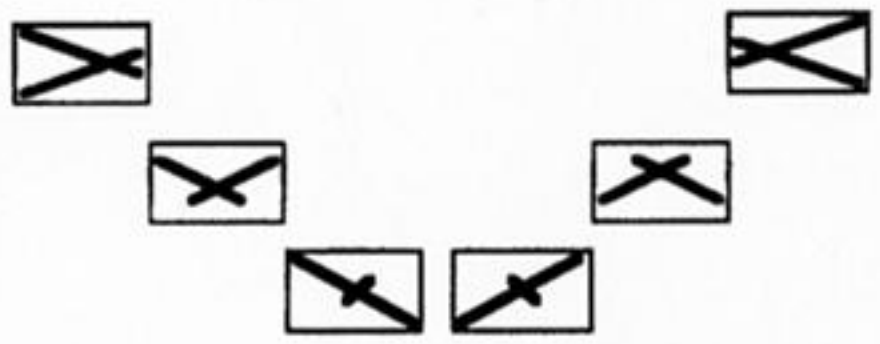
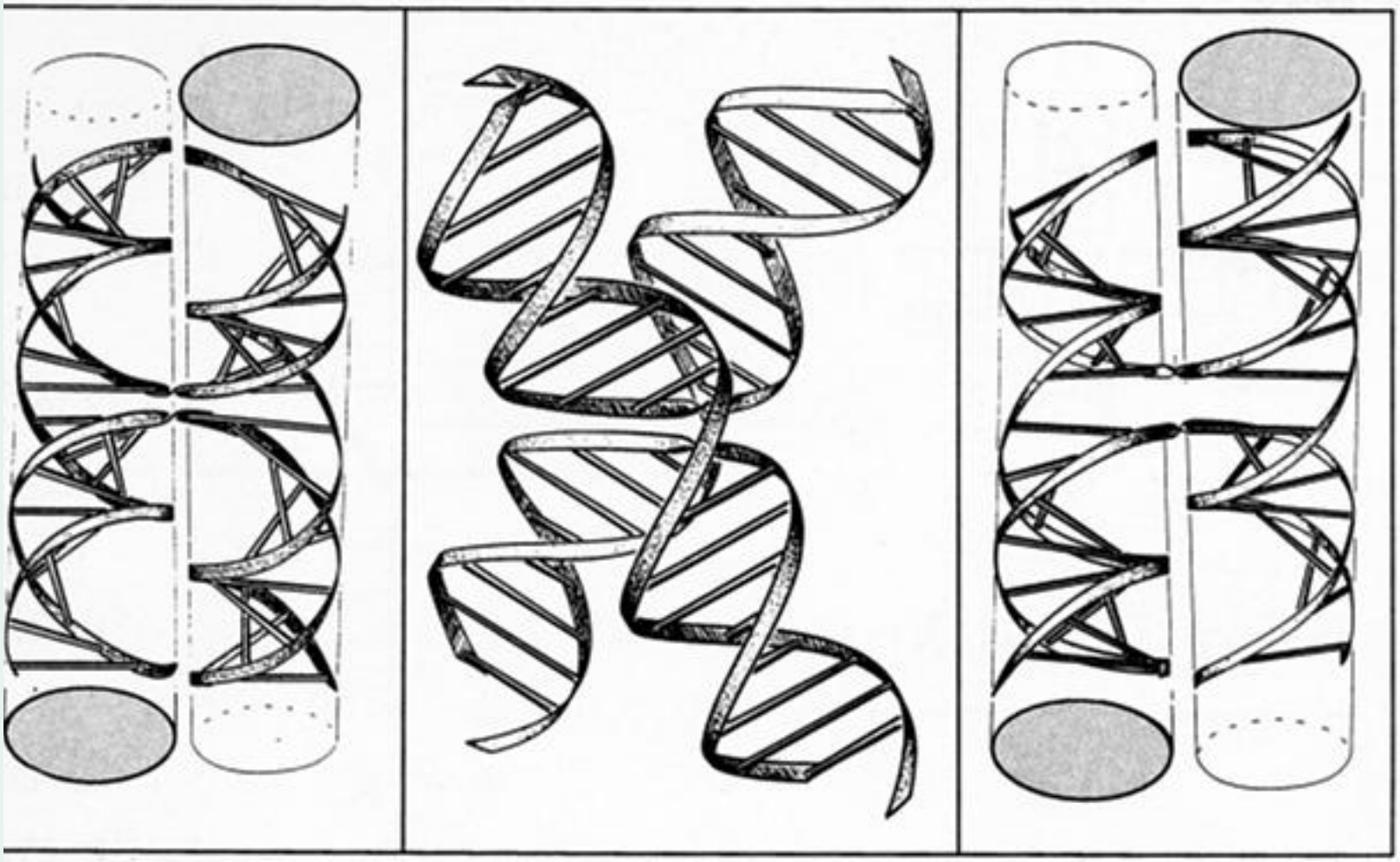


Figure 1. Gel electrophoretic analysis of the structure of a four-way DNA junction in the presence of magnesium ions. The relative configuration of the four arms was deduced by comparing the electrophoretic mobility of the six possible species in which two arms had been shortened by restriction enzyme cleavage. The junction (junction 3 from Duckett *et al.* (Duckett *et al.* 1988)) has four arms labelled B, H, R and X, and comprises four strands, labelled b, h, r and x as shown. The resulting species with two long and two short arms are indicated by their long arms, i.e. species BH has shortened R and X arms. These six long–short species have been electrophoresed on an 8% polyacrylamide gel in 90 mM Tris borate (pH 8.3) (TB buffer), 1 mM magnesium chloride. The slow-intermediate—fast-fast-intermediate-slow 2:2:2 pattern of mobilities is explained in terms of the X-shaped structure formed by coaxial stacking of B on X arms, and H on R arms as indicated. This leads to the formation of the six long–short species shown, interpreted in terms of the expectation that the mobility will be proportional to the size of the included angle between the long arms. Note that the chemical polarity of the continuous strands in this structure (i.e. strands h and x) is antiparallel, with the exchanging strands (strands b and r) turning around the small angle of the X. The relatively short distances between the ends of the B and H arms, and between the ends of the R and X arms, were further established by fluorescence resonance energy transfer experiments (Murchie *et al.* 1989; Clegg *et al.* 1992), thereby confirming the antiparallel character of the structure.



igure 2. Ribbon model of the right-handed, antiparallel acked X-structure of the four-way DNA junction, observed om three points of view. Note the juxtaposition of the ontinuous strands in the major grooves of the opposing elices, which is optimal for a small angle of 60° for the helix ossing. Centre: face view, showing the X-shape of the lded junction: the two sides of the structure are not quivalent. On one side (left) the four basepairs at the point strand exchange all present major groove edges, while on e other side (right) the minor groove edges are presented.

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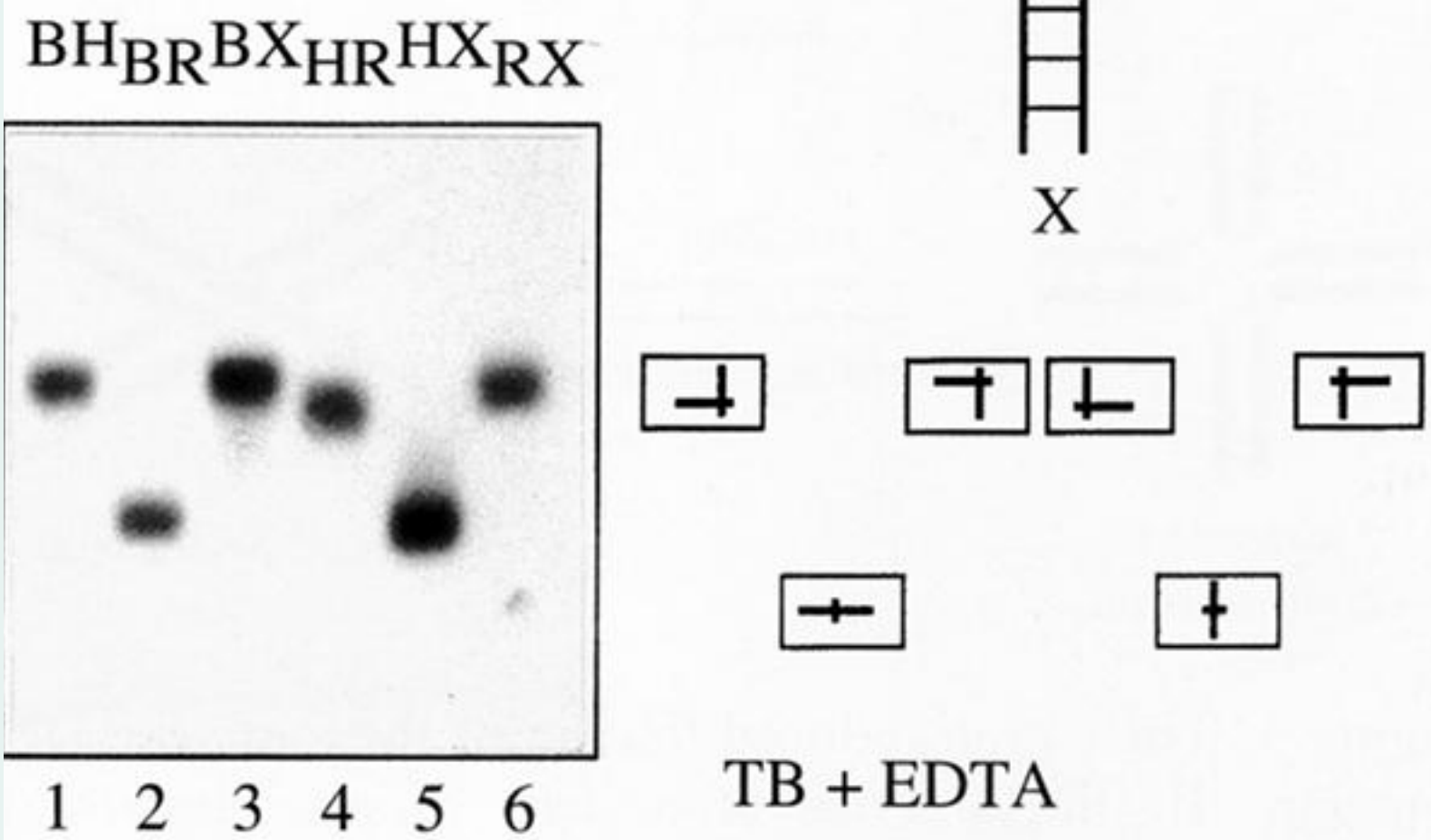
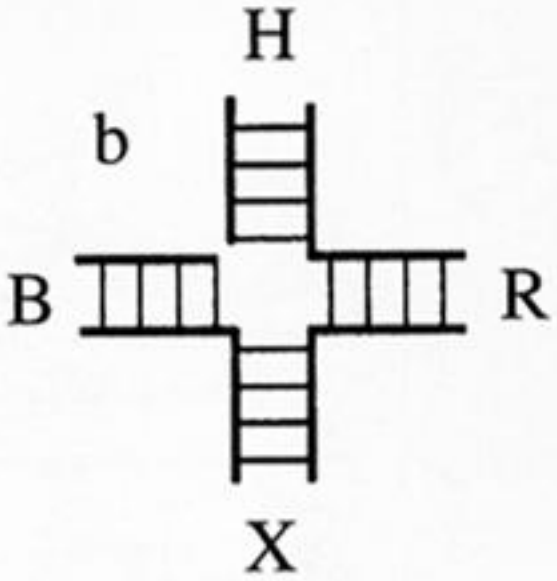
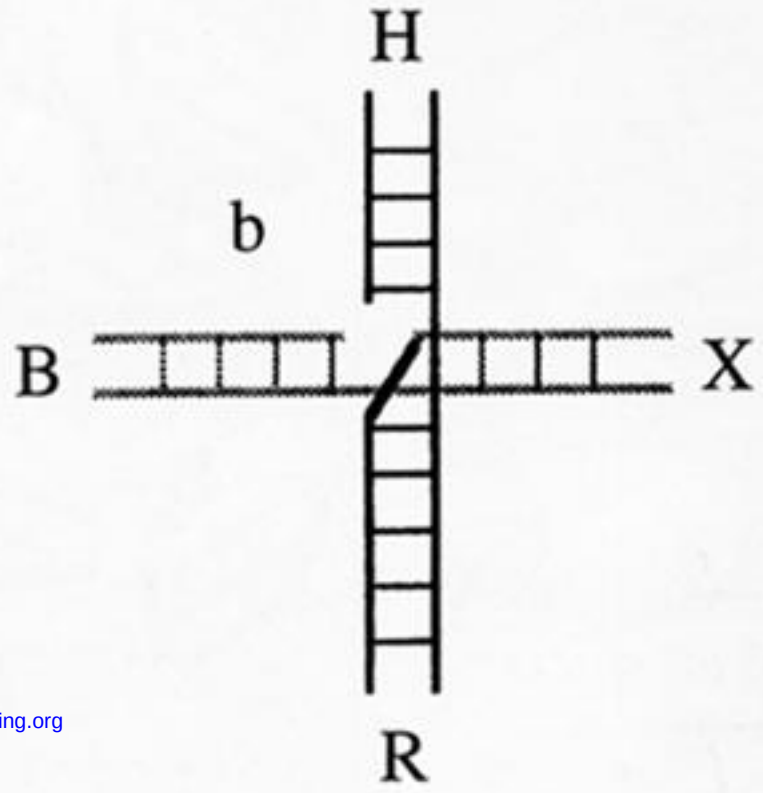
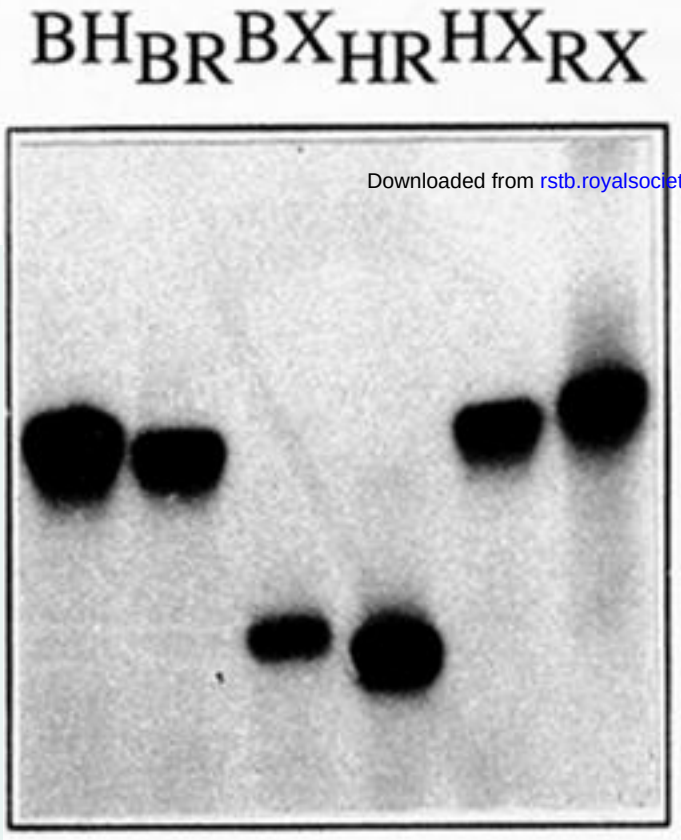
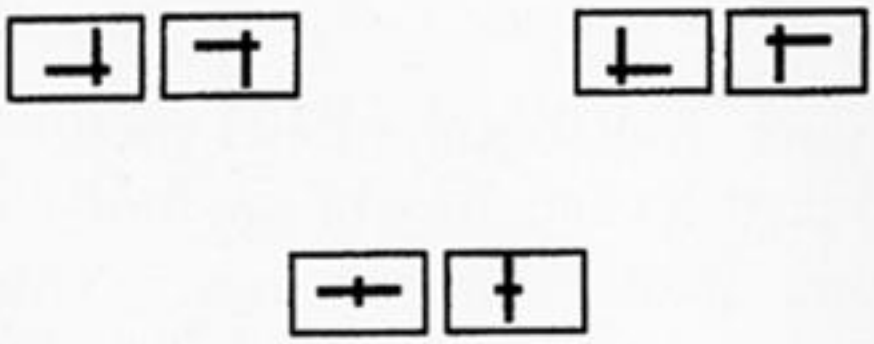


gure 3. Gel electrophoretic analysis of the structure of a ur-way junction in the absence of added metal ions. Long– ort arm analysis of a junction in 90 mM Tris borate H 8.3), 0.1 mM EDTA (Duckett *et al.* 1988). Under these nditions, junctions of any sequence exhibit a 4: pattern of obility in the order slow, fast, slow slow, fast, slow. This is nsistent with a square configuration of helical arms, giving ur species where the long arms subtend approximately 90° low species), and two with angles of approximately 180° aster species). The four strands are equivalent in this ructure. The extended, square configuration of arms has en confirmed by FRET measurements (Clegg *et al.* 1993), d is consistent with the reactivity of thymine bases at the int of strand exchange to osmium tetroxide at low salt ncentrations (Duckett *et al.* 1988).



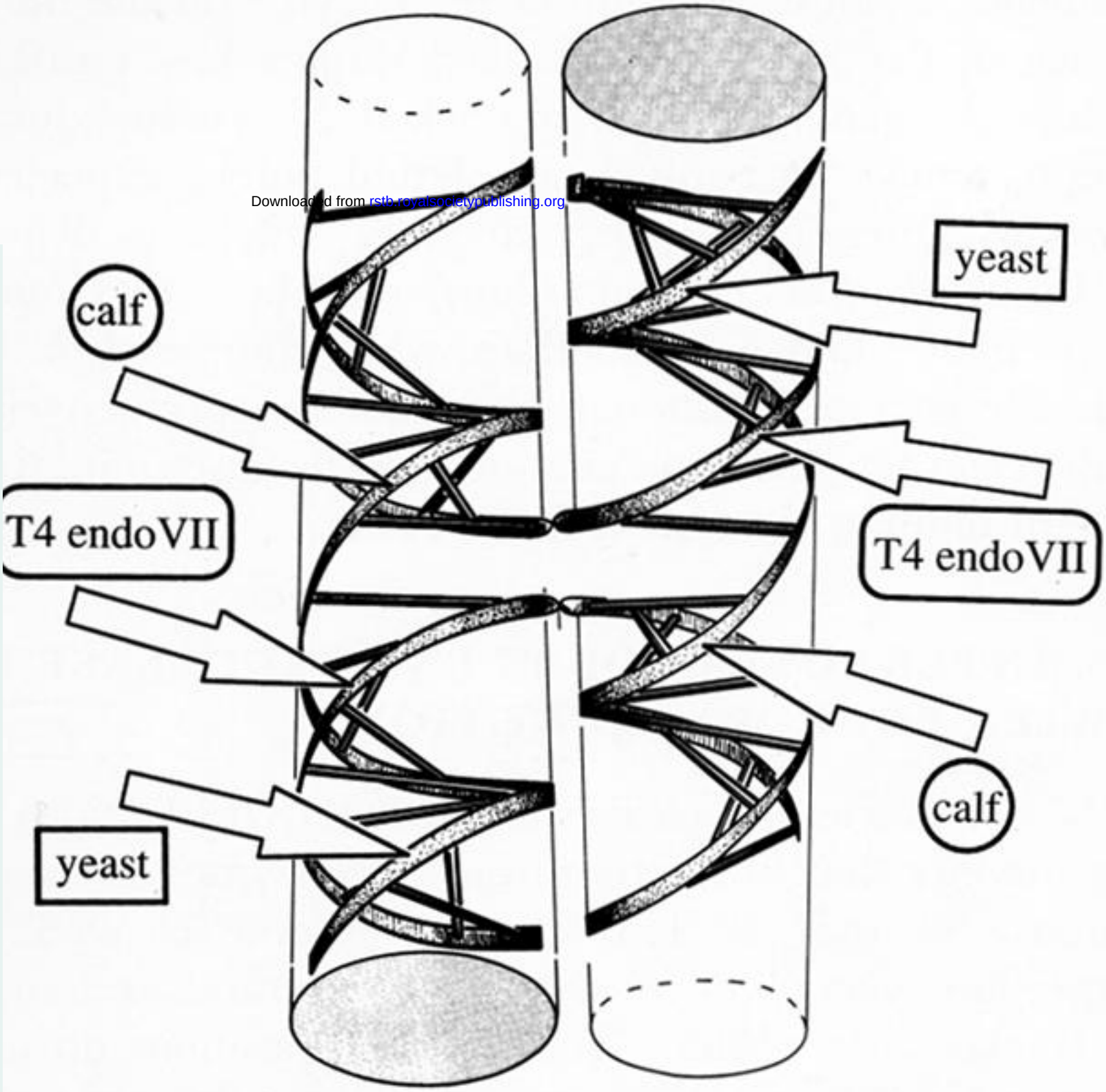




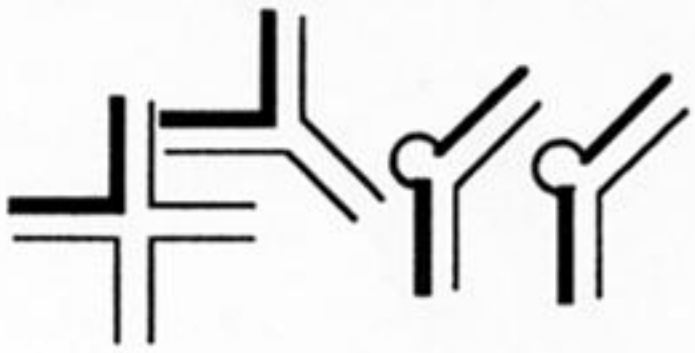


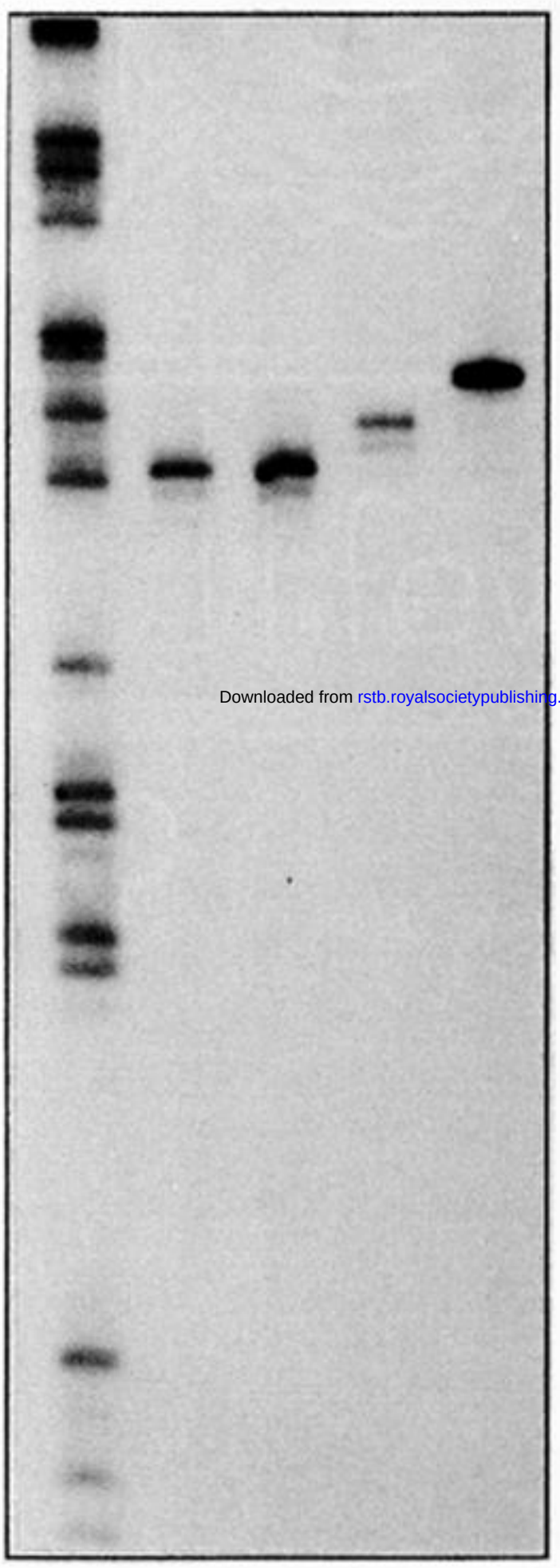
TB + 1 mM $MgCl_2$

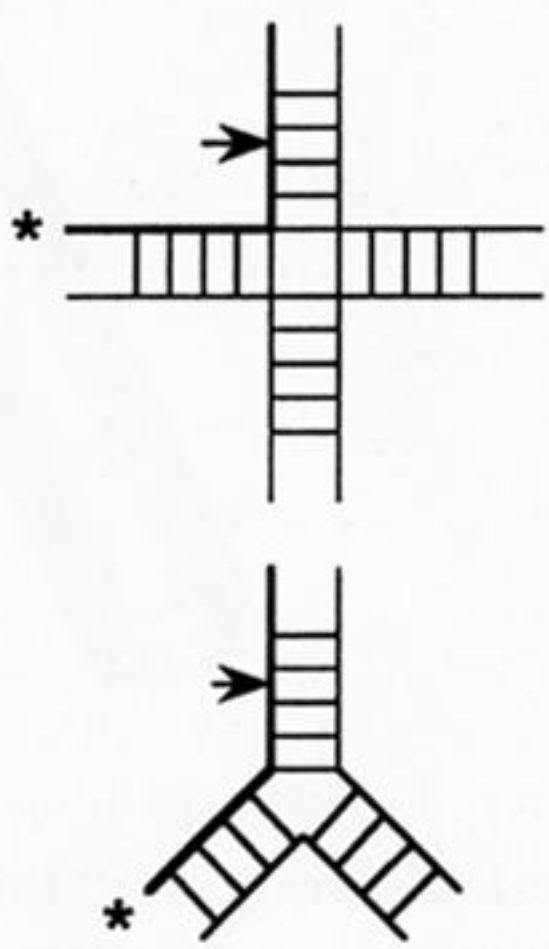
igure 5. Effect of introducing a nick into one strand of the our-way DNA junction at the point of strand exchange. Gel ectrophoretic analysis of a junction containing a nick on the -strand, in the presence of 1 mM magnesium ions. The long–hort arm analysis was carried out as before (see figure 1). he pattern of mobilities was clearly different from the 2:2:2 attern of the intact junction in magnesium, indicating that ne configuration of arms had changed. Four slow and two st species were observed, but the pattern was slow, slow, st, fast, slow, slow, in contrast to that observed for the intact nction in the absence of added metal cations (compare with gure 3). This suggests that coaxial pairwise helical stacking retained, but that the angles included between the arms are ose to 90° as shown. When the nick was placed on the x-rand, we observed an isomerization of the junction such nat B on H stacking now occurred (Pöhler *et al.* 1994). The sult of this is that the nick is once again located on an xchanging strand.



igure 6. Cleavage sites for three different junction-resolving nzymes on the minor groove side of the four-way DNA inction. While T4 endonuclease VII cleaves the exchanging rands of a junction 3′ to the point of strand exchange, nalysis of the cleavage sites of related junctions by an endo 1 from yeast (West *et al.* 1987) and an enzyme from calf ymus (Elborough & West 1990) show that these cleave the ontinuous strands, 5′ and 3′ respectively to the point of rand exchange. While these sets of cleavage positions ppear to be quite unrelated at first sight, when they are laced onto the model of the stacked X-structure they are all ound to be located on the minor groove face.







1 2 3 4 5

igure 7. Cleavage of branched DNA species by T4 idonuclease VII. A four-way junction was radioactively ′-^{32}P] labelled on one strand and incubated with T4 idonuclease VII. Cleavage at virtually a single phospho-ester bond may be seen on the sequencing gel. Three other anched DNA species of closely related sequence were amined. The same radioactive strand was incorporated to a three-way junction, and two bulged species, containing $_2$ and a T_4 bulges respectively, each of which were based on ie same sequence. These species were cleaved in a manner iat was virtually identical to that on the four-way junction he shift in the position of the bands on the bulged species is ue to the insertion of the bulge bases).

igure 8. Schematic to show the interaction between T4
ndonuclease VII and the minor groove side of the junction.
might be anticipated that the dimeric enzyme has
omplementarity of surface shape, such that the discrimi-
ation of the angle of helical inclination in the junction is an
ement in the structural selectivity of the resolving enzyme.

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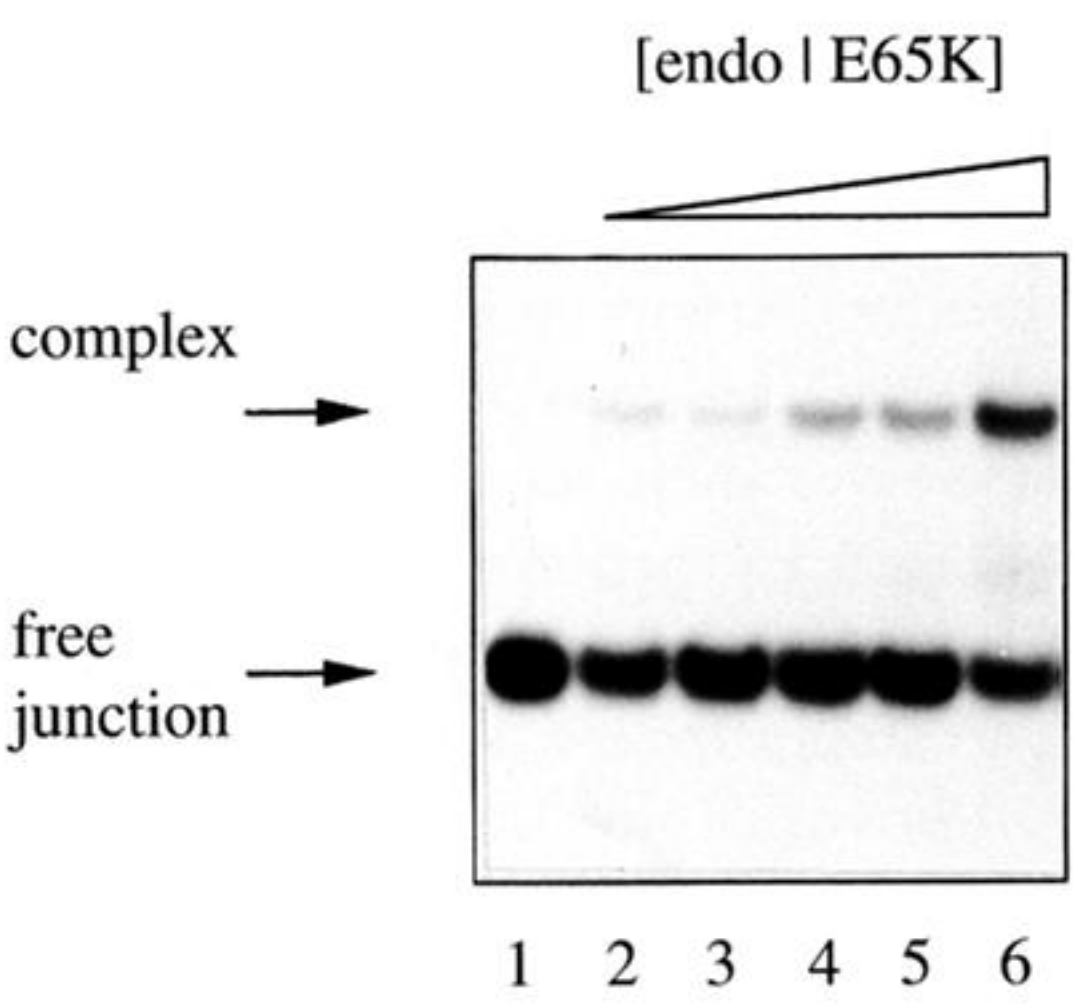
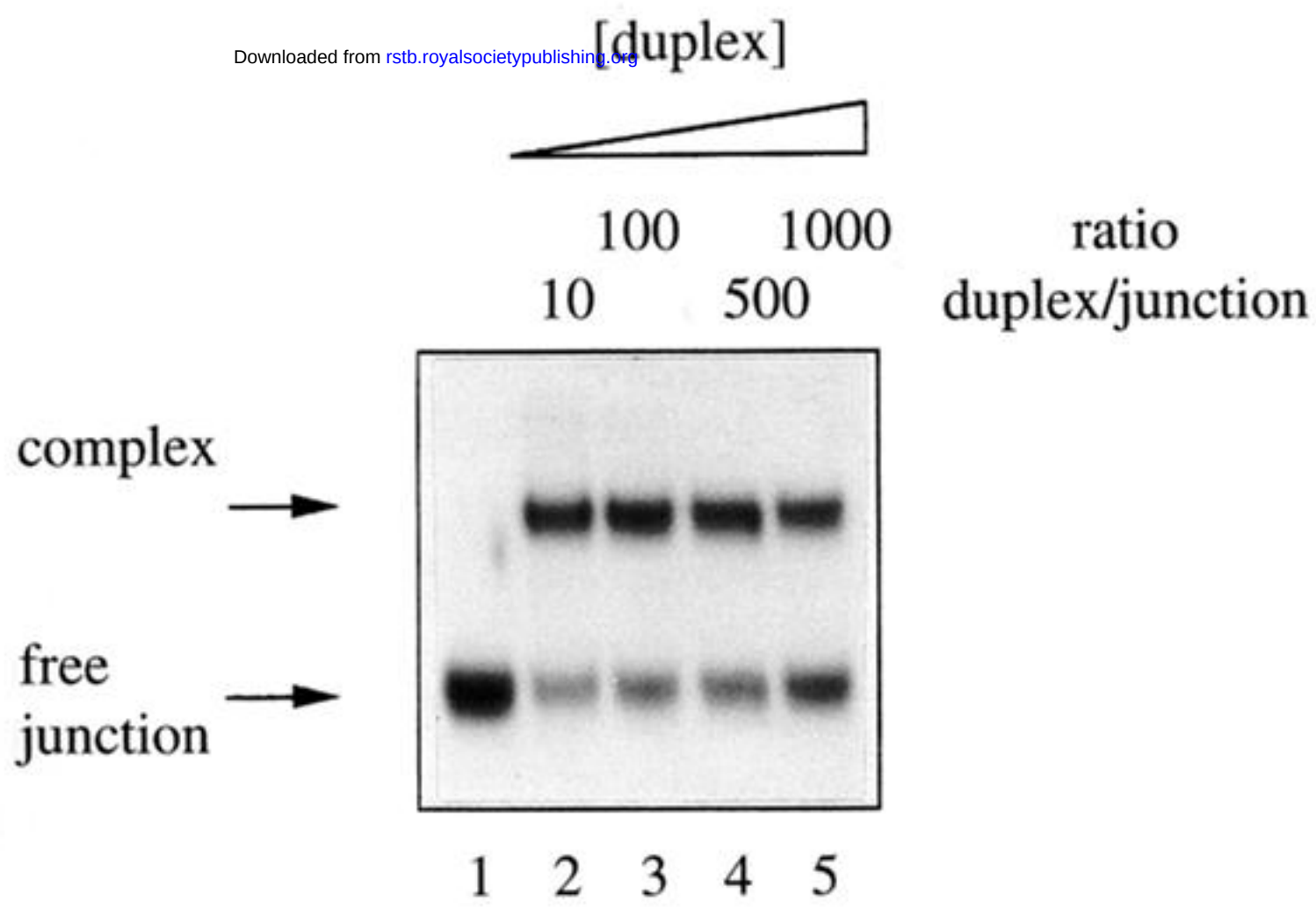
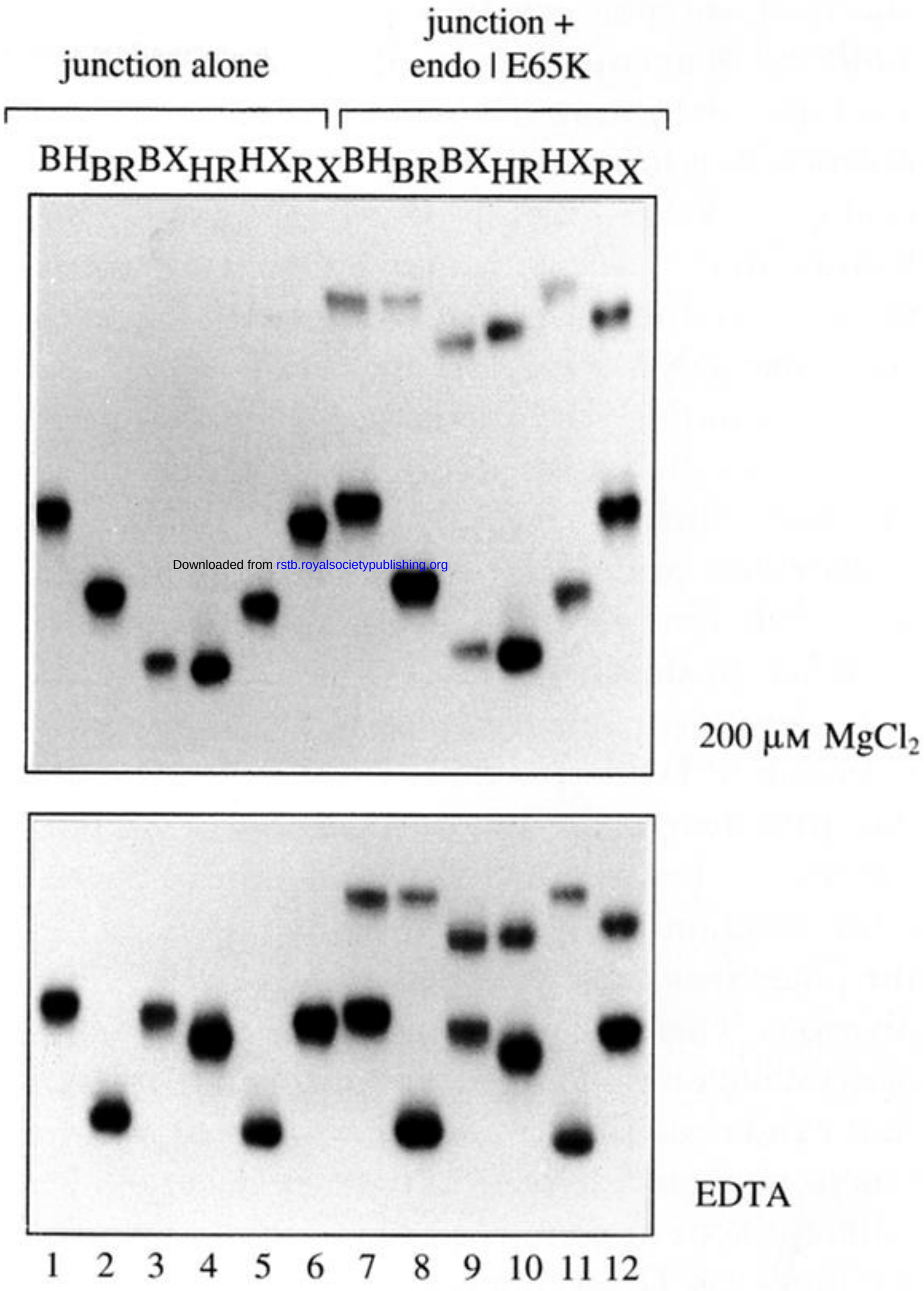




gure 9. Structure-selective binding of T7 endonuclease I to e four-way DNA junction. (*a*) Electrophoretic retardation the complex formed between [^{32}P] radioactively labelled NA junction and an inactive E65K mutant of T7 donuclease I in 90 mM Tris borate (pH 8.3), 200 μM $MgCl_2$. rack 1, junction without added protein; tracks 2–6, junction cubated with increasing concentrations of the mutant solving enzyme. (*b*) Competition between four-way junc-on and duplex for binding E65K mutant T7 endonuclease Radioactive junction was incubated with E65K endo-uclease I in 90 mM Tris borate (pH 8.3), 200 μM $MgCl_2$, ther alone (track 1), or with an increasing molar ratio of labelled competitor duplex DNA (tracks 2–5). Note that en a 1000-fold excess of linear duplex fails to displace a gnificant fraction of the complex between the resolving zyme and the four-way junction.



igure 10. Gel electrophoretic analysis of the complex etween a four-way junction and E65K mutant T7 endo-uclease I. (*a*) The six double-restriction digests of junction (see figure 1) were incubated with E65K endonuclease I in 0 mM Tris borate (pH 8.3), 200 μM $MgCl_2$. These were nalysed by gel electrophoresis (tracks 6–12), in comparison ith the same species in the absence of protein (tracks 1–6). ote the pattern of mobilities of the retarded species formed the presence of the protein are different from that of the ee DNA. (*b*) The equivalent experiment in 90 mM Tris orate (pH 8.3), 1 mM EDTA. Note that the pattern of tarded species is exactly the same as that found in the resence of magnesium ions.