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Relative stabilities of DNA three-way, four-way and five-way junctions (multi-helix junction loops): unpaired nucleotides can be stabilizing or destabilizing

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ABSTRACT

Competition binding and UV melting studies of a DNA model system consisting of three, four or five mutually complementary oligonucleotides demonstrate that unpaired bases at the branch point stabilize three- and five-way junction loops but destabilize four-way junctions. The inclusion of unpaired nucleotides permits the assembly of five-way DNA junction complexes (5WJ) having as few as seven basepairs per arm from five mutually complementary oligonucleotides. Previous work showed that 5WJ, having eight basepairs per arm but lacking unpaired bases, could not be assembled [Wang, Y.L., Mueller, J.E., Kemper, B. and Seeman, N.C. (1991) *Biochemistry*, 30, 5667–5674]. Competition binding experiments demonstrate that four-way junctions (4WJ) are more stable than three-way junctions (3WJ), when no unpaired bases are included at the branch point, but less stable when unpaired bases are present at the junction. 5WJ complexes are in all cases less stable than 4WJ or 3WJ complexes. UV melting curves confirm the relative stabilities of these junctions. These results provide qualitative guidelines for improving the way in which multi-helix junction loops are handled in secondary structure prediction programs, especially for single-stranded nucleic acids having primary sequences that can form alternative structures comprising different types of junctions.

INTRODUCTION

The convergence of three or more mutually complementary nucleic acid strands results in the formation of multi-helix junctions (junction loops). The simplest are the three-way junctions (3WJ), which for example are found in 5S ribosomal RNA (2), the hammerhead ribozymes (3) and in the terminal repeats of the single-stranded DNA comprising the genome of the adeno-associated virus (AAV) (4). Four-way nucleic acid junc-

tions occur transiently in DNA during recombination and are termed Holliday intermediates (5). Immobile four-way junctions (4WJ) have been studied extensively as models of Holliday intermediates. Physical and biochemical studies generally agree that such structures assume a preferred conformation that exhibits pair-wise stacking of adjacent helical arms to form an anti-parallel Chi (X) shaped structure (6–8). Transfer RNAs are also 4WJ (9). Large ribosomal RNAs (rRNA) contain junction structures ranging from three-way to seven-way junctions, most of which include unpaired bases at the branch point (10). The peptidyl transferase center in 23S rRNA is an RNA five-way junction containing unpaired bases.

Unpaired (bulged) nucleotides destabilize duplex DNA and RNA (11,12). However, unpaired nucleotides stabilize nucleic acid 3WJ; DNA 3WJ were assembled with only 5 bp per arm by including two unpaired bases at the junction site (13). On the other hand, 3WJ with 5 or 6 bp per arm lacking unpaired bases were found to be unstable in gel electrophoresis and optical melting studies. NMR spectroscopy, applied by two independent groups to DNA 3WJ complexes, has demonstrated that 3WJ having two unpaired pyrimidines form structurally homogeneous complexes that exhibit unique basestacking interactions between two of the three helices that meet at the junction (14–16). Three-dimensional models refined using the NMR data of a 3WJ containing unpaired 5'-TC-3' in one strand indicate that the unpaired bases provide the necessary covalent bridge to allow stacking to take place across the junction (17). NMR studies carried out on immobile DNA 4WJ (having no unpaired bases) demonstrate that base stacking interactions play an important stabilizing role and that optimal basestacking can be achieved without the need for bridging unpaired nucleotides, as in 3WJ (18). Structural evidence obtained by NMR and by chemical probing is consistent with a model for the 4WJ in which all four helices are involved in pairwise stacking interactions (19). Four-way junctions containing unpaired bases, however, have not been studied previously to our knowledge.

The assembly of DNA five-way (5WJ) and six-way (6WJ) junctions was demonstrated by Seeman and co-workers using oligonucleotides forming 16 bp per double helical arm (1). The

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complexes studied by these workers contained no unpaired bases. They found that stable 5WJ or 6WJ complexes containing no unpaired bases could not be assembled from 16mer oligonucleotides designed to form 8 bp per arm. In the present study, we demonstrate that 5WJ complexes can be assembled from five mutually complementary strands forming only 7 bp per arm, provided unpaired nucleotides are included in the junction.

The prediction of nucleic acid secondary structure from sequence information requires estimates of the thermodynamic parameters for forming various structures, so that the most stable among a set of likely choices can be determined. The correct treatment of multi-stem loops is a significant unsolved problem in this regard (20,21). Presently used programs assign a small, additive penalty to all unpaired bases found in multi-branch loops. The experiments described in this report indicate that this may not be appropriate for multi-branch loops containing an odd number of helices (3WJ and 5WJ). These experiments also provide qualitative information regarding the relative stabilities of three-, four-, and five-way DNA junctions containing various numbers of unpaired nucleotides. The methods employed can be extended to RNA.

METHODS AND MATERIALS

Design of sequences

The sequence of the strands comprising the junctions was designed by following published guidelines (22). The sequences comply with these stipulations: (i) no series of four nucleotides is repeated in the sequence to avoid the mispairing of strands. (ii) All arms terminate in G-C base pairs to minimize fraying of the ends. (iii) No more than three consecutive guanines are employed, to prevent wobble self-pairing. The oligonucleotide strands are numbered as shown in Figure 1. For example, strand 1 is as shown in the figure, whereas strand 1AA includes two adenosines at the position marked '[1]'. For competition binding experiments, oligonucleotides forming 8 bp per arm were used to assemble 5WJ complexes, whereas the 3WJ and 4WJ contain only 7 bp per arm. The longer strands are designated by the prime (') symbol. For example, strand 1' contains two more nucleotides than strand 1, one at each end. 'M' designates modified strands. Strand 3M is modified to pair with strands 1 and 2 to form 3WJ, whereas strand 3 is designed to pair with strands 1, 2 and 4M to form a 4WJ. Strands 3, 4 and 5, are designed to assemble with strands 1 and 2 to form 5WJ. The DNA oligomers, prepared using phosphoramidite chemistry, were obtained from Oligos Etc. Inc. (Wilsonville, OR 97070). Oligonucleotides were dissolved in deionized water and the concentrations were determined by UV absorbance at 260 nm using molar extinction coefficients calculated from published nearest neighbor parameters (23). More exact stoichiometric ratios of strands were determined by titration on native electrophoresis gels.

Assembly experiments

The samples were analyzed by electrophoresis on 15% polyacrylamide gels (19:1 monomer:bis ratio) in buffered solutions consisting of 89 mM Tris(hydroxymethyl)amino methane, 89 mM borate (pH 8.3), 2 mM EDTA and 10 mM MgCl₂. The gels were run at 5°C with constant recirculation of the buffer for ~5

h and were autoradiographed overnight. Migration distances were measured from the autoradiograms.

Competition experiments

Oligonucleotides were 5'-end labelled using T4 polynucleotide kinase and [γ -³²P]ATP and purified on 15% polyacrylamide gels containing 8 M urea. Samples containing DNA samples were heated to 90°C in buffer composed of 89 mM Tris(hydroxymethyl)amino methane, 89 mM borate (pH 8.3), 2 mM EDTA and 10 mM MgCl₂ and allowed to cool slowly to 5°C. The samples were allowed to equilibrate at this temperature for \geq 48 h. Non-competing and control strands were added at 40 μ M concentration, competing non-labelled strands at 80 μ M, and the labelled strand at 8 μ M, in total volumes of 10–20 μ l.

UV melting curves

Melting curves were recorded in 1.0 cm quartz cuvettes at 280 nm using a Cary 219 Spectrophotometer (spectral bandwidth 2 nm, pen period of 2.5 s). The temperature of the sample was controlled by a Haake A80 circulating water bath. Stoichiometric amounts (1–3 nmol) of component oligomers, calculated from optical density measurements, were mixed and dissolved in 1 ml of a buffer consisting of 1.0 M KCl, 5.0 mM MgCl₂, 0.5 mM EDTA and 10 mM potassium phosphate (pH 7) or 1.0 M NaCl, 0.5 mM EDTA, 10 mM potassium phosphate (pH 7). Samples were annealed by heating to 90°C followed by slow cooling to the starting temperature and allowed to equilibrate for \geq 15 min until the absorbance stabilized. Samples were heated at a rate of \sim 0.5°C/min and the absorbance was recorded approximately every 0.2°C. The data were logged using a personal computer (IBM compatible) and a home-built interface. The data were then transferred to a Macintosh computer using the software package Access PC (version 1.0, Insignia Solutions Inc., 254 San Geronimo Way, Sunnyvale, CA 94086). Data were analyzed with Kaleidograph (version 2.1, Synergy Software, 2457 Perkiomen Avenue, Reading, PA 19606).

UV melting data were analyzed with the two-state model according to published guidelines (23). Absorbance data were transferred to the Kaleidograph program and subjected to the smoothing routine provided in the program. Data were normalized to give absorbances of 1.0 at 80°C. The linear regions above and below the melting transition were fitted by linear least squares to approximate the temperature dependences of the absorbances of the single strands, $A_{\text{single-strands}}(T)$, and of the complex, $A_{\text{complex}}(T)$, as functions of temperature, T . The fraction of strands in complex, $f(T)$, was calculated at intermediate temperatures using the formula:

$$A(T) = A_{\text{single-strands}}(T) - f(T) \cdot \{A_{\text{single-strands}}(T) - A_{\text{complex}}(T)\}$$

where $A(T)$ is the smoothed absorbance data. This equation is based on the assumption that the extent of a melting transition in a nucleic acid is a linear function of the hypochromism, $A_{\text{single-strands}}(T) - A_{\text{complex}}(T)$. This assumption is justified by theoretical work showing that the hypochromism is approximately a linear function of the number of stacked bases (24) combined with the idea that single-strands at elevated temperatures have very little base-stacking. The melting temperature (T_m) is defined as the temperature at which $f(T)$ is equal to one half.

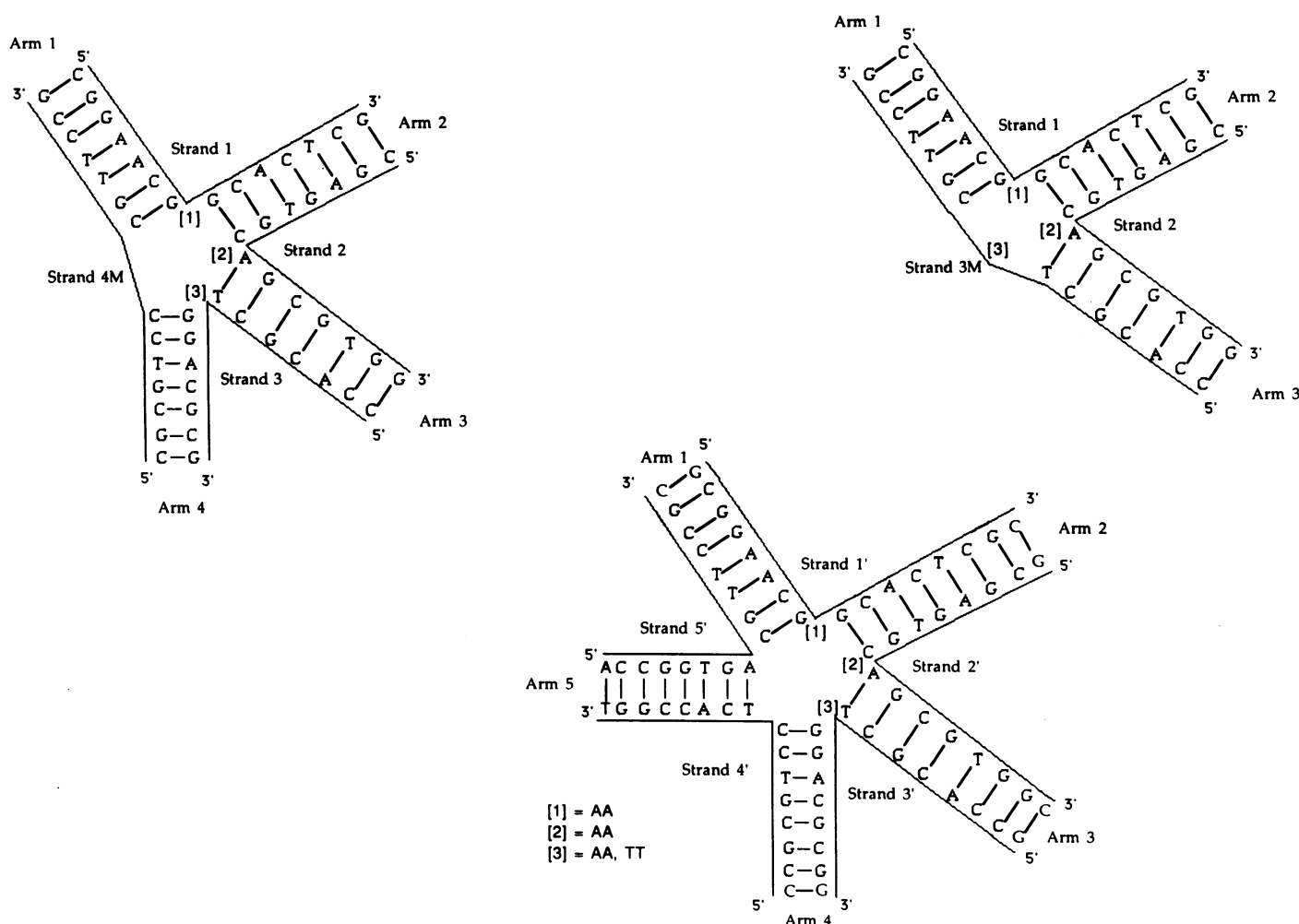


Figure 1. The sequences of the oligonucleotides used in this study to form 3WJ, 4WJ and 5WJ complexes. The points at which unpaired bases were inserted are indicated with [1], [2] and [3]. Note that the 5WJ used in competition studies contains one extra base pair on each arm, as shown. Also note that strands 3M and 4M are designed to basepair on their 3' halves to strand 1.

RESULTS

Assembly of junctions

Electrophoretic behavior of monomers and two-strand combinations. The single strands 1, 1AA, 2, 2AA, 3, 3M and 4M ran on native gels at 5°C as monomers; the single strands 3AA, 4 and 5 ran with mobilities intermediate between the other single strands and dimers, indicating some self-association. The adjacent strand pairs 1+2, 1AA+2, 1+2AA, 1AA+2AA, 2+3, 2+3M, 2AA+3, 2+3AA, 3+4, 3AA+4, 4+5 and 5+1AA all ran on electrophoresis gels as single bands with reduced mobilities relative to the single strands, indicating the expected dimeric association. The non-adjacent pairs, including 1+4, 2+4, 2+5 and 3+5, did not associate with each other (two bands of nearly identical mobility were evident on the gels corresponding to the mobilities of the individual strands).

Three-way junctions. As mentioned above, 3WJ having short arms (no more than 6 bp per arm) and lacking unpaired bases are unstable on native electrophoresis gels, even at 5°C. By

extending the length of the arms to 7 bp, the 3WJ complex (1:2:3M) lacking unpaired bases was stabilized sufficiently to assemble at 5°C, as shown in Figure 2, lane III and Figure 7, lane III. Various strand combinations were tested using gel electrophoresis to confirm assembly of 3WJ complexes. All the following strand combinations were found to form stable 3WJ by gel electrophoresis (5°C): 1:2:3M (no unpaired bases), 1AA:2:3M, 1:2AA:3M, 1:2:3MTT, 1AA:2:3MTT, 1:2AA:3MTT, 1AA:2AA:3M, 1AA:2AA:3MTT. However, 1:2:3M was unstable on gels run at room temperature; all 3WJ having unpaired bases that were tested were stable at room temperature.

Four-way junctions. 4WJ with and without unpaired bases are stable on electrophoresis gels run at 5°C (for examples, see Fig. 3, lanes III–V). However the following 4WJ containing unpaired bases were found to be unstable on gels run at room temperature: 1AA:2:3:4M, 1AA:2:3AA:4M, 1AA:2:3TT:4M. The following 4WJ are stable on gels run at room temperature: 1:2:3:4M, 1:2AA:3:4M, 1:2:3AA:4M, 1:2:3TT:4M, 1AA:2AA:3:4M, 1:2AA:3AA:4M, 1:2AA:3TT:4M, 1AA:2AA:3AA:4M and 1AA:2AA:3TT:4M. Evidently the number as well as the position

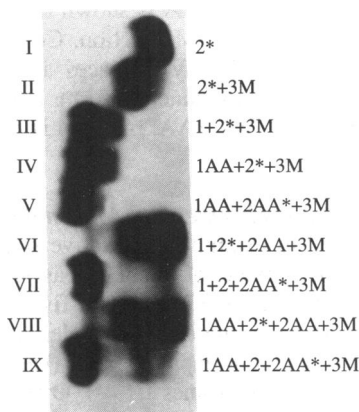


Figure 2. Autoradiogram of electrophoresis gel showing equilibrium competition experiments involving 3WJ containing either 0, 2 or 4 unpaired bases at the branch point. Lanes I–V are the various controls and lanes VI–IX analyze the products of competition reactions as indicated on the figure. The asterisk (*) indicates the (³²P) labelled strand used in each lane.

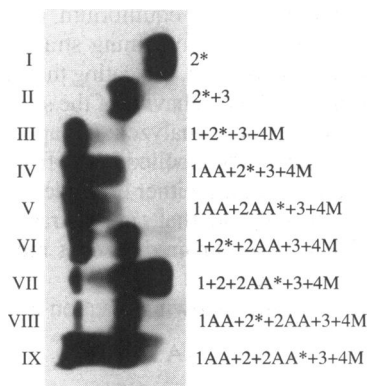


Figure 3. Autoradiogram of electrophoresis gel showing equilibrium distribution of 4WJ containing either 0, 2 or 4 unpaired bases at the branch point. Lanes I–V are the controls and lanes VI–IX are the competition reactions.

of unpaired bases are factors in determining the relative stability of different 4WJ. Representative UV melting studies confirming the destabilizing effect of unpaired bases in 4WJ are discussed below.

Five-way junctions. The assembly of 5WJ comprising 7 bp per arm was demonstrated on native electrophoresis gels by annealing together the five component oligonucleotides in stoichiometric amounts. On gels run at 5°C the following samples formed complexes exhibiting the expected retarded gel mobilities: 1AA:2:3:4:5, 1AA:2AA:3:4:5, 1AA:2:3TT:4:5, 1AA:2AA:3AA:4:5 and 1AA:2AA:3TT:4:5. To verify the assembly of 5WJ, the electrophoretic mobilities of a putative 5WJ complexes were compared to the mobilities of the single-stranded sample 1AA, the duplex 1:2, and the corresponding 3WJ and 4WJ complexes. The graph of mobilities as a function of the logarithm of the number of nucleotides is shown in Figure 4. All complexes fall on a straight line, providing evidence for the formation of the expected complexes (25). Under identical conditions, the combination of strands 1+2+3+4+5 exhibited the same mobility as

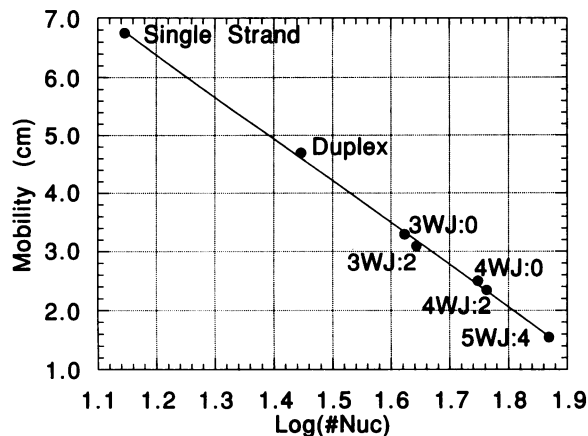


Figure 4. Graph of the relative mobilities of a series of DNA samples versus the log₁₀ of the number of nucleotides present in each sample. 3WJ:0, three-way junction with no unpaired bases; 3WJ:2, three-way junction with two unpaired bases; 4WJ:0, four-way junction with no unpaired bases; 4WJ:2, four-way junction with two unpaired bases; 5WJ:4, five-way junction with four unpaired bases.

duplex DNA, indicating that it fails to form a stable 5WJ. With the exception of the complex 1AA:2:3:4:5, samples containing only two unpaired nucleotides, failed to form stable 5WJ. Even the one exception, 1AA:2:3:4:5, showed two faster running species on the gel, indicating that this 5WJ complex is in equilibrium with complexes containing fewer strands. By contrast, the samples containing four or six unpaired nucleotides consistently formed stable 5WJ complexes. However, most combinations that included strand 3AA failed to form the five stranded complex because this strand is partly self-associating and duplex formation was favored.

Competition binding experiments

The competition method employed in this work is similar to that described by Kallenbach and co-workers to estimate the stability of 4WJ relative to the corresponding double helices (26). The strands needed to form a particular junction were mixed with the competing strand(s) and the complexes were allowed to reach equilibrium before analysis by gel electrophoresis. In some experiments the competing strands differ from the radioactively labelled strand only in the number of unpaired bases, for example strands 2 and 2AA. In other experiments the competing strands are related as strands 3M, 3 and 4M are (Fig. 1). The experiment is repeated with samples in which the labelled and competing strands are reversed. Examples of typical experiments are shown schematically in Figure 5. The upper panel shows (intra-junction) competition of labelled strand 2 and unlabelled 2AA for binding to strands 1 and 3M to form 3WJ complexes with and without unpaired bases. The lower panel shows (inter-junction) competition between 3M and 3:4M for binding to strands 1 and 2 to form either a 3WJ (1:2:3M) or a 4WJ (1:2:3:4M) neither of which contains unpaired bases. The results of such experiments are discussed below.

Effects of unpaired nucleotides on 3WJ stability. Experiments comparing the relative stabilities of 3WJ complexes with and without unpaired bases are shown in Figure 2. In these

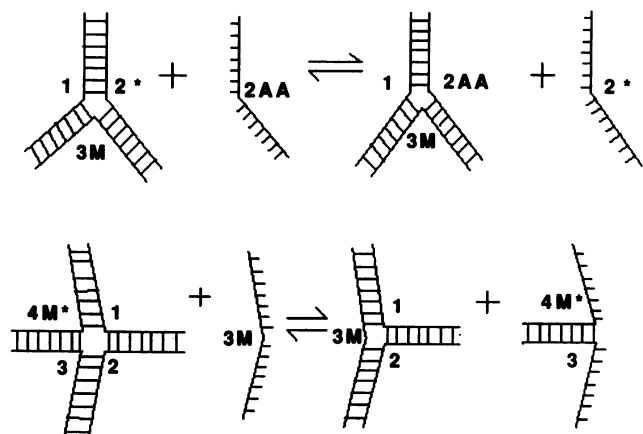
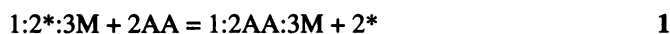


Figure 5. A schematic drawing of representative competition reactions: the upper panel shows schematically the competition reaction carried out in Figure 2, lane VI. The lower panel shows schematically the competition reaction carried out in Figure 6, lane VIII.

experiments, either strand 2 or strand 2AA was radiolabelled. Strand 2 pairs with strands 1 and 3M to form the 3WJ 1:2:3M containing no unpaired bases. Strand 2AA pairs with strands 1 and 3M to form the 3WJ 1:2AA:3M which contains two unpaired adenosines at the junction. Lanes I–V are controls: lane I contains only strand 2*. The asterisk indicates that strand 2 is radiolabelled. Lane II shows the dimer formed by base-pairing between strand 2* and 3M. Lanes III–V demonstrate the formation of the 3WJ 1:2:3M, 1AA:2:3M and 1AA:2AA:3M.

To determine which 3WJ is more stable we performed competition studies as described above. In lanes VI and VII, the stability of 1:2:3M is compared to that of 1:2AA:3M. In lane VI this is done by adding excess unlabelled strand 2AA to the junction complex 1:2*:3M, where strand 2 is radiolabelled. The reaction is:



Lane VI shows that after equilibration, very little of the labelled strand 2* remains in the 3WJ form. It is primarily single stranded, with some dimer (which could be 1:2 or 3M:2). Equation 1 is shifted far to the right. Other equilibria involving dimer species are evidently also involved, so equation 1 is not a complete description of the system.

In lane VII, excess unlabelled strand 2 was added in an attempt to displace radiolabelled strand 2AA* from the junction. The reaction is:



All the labelled strand 2AA* is found in the 3WJ form in lane VII with the exception of a very small amount in single-stranded form. Evidently the equilibrium in equation 2 is shifted largely to the left indicating again that 1:2AA:3M is more stable than 1:2:3M. The results of lanes VI and VII are consistent, an indication that sufficient time was allowed for equilibrium to be achieved in these experiments.

In lanes VIII and IX, the following reaction is analyzed:



In lane VIII excess strand 2AA is shown to displace radiolabelled strand 2 from 1AA:2:3M junction. Consistent with this result, in lane IX strand 2 only displaces a small amount of labelled strand 2AA from the junction form. Lanes VIII and IX therefore show that the 3WJ, 1AA:2AA:3M is more stable than 1AA:2:3M.

Effects of unpaired nucleotides on 4WJ stability. The same procedure was used to examine the relative stabilities of 4WJ containing different numbers of unpaired bases. Representative results are shown in Figure 3. There are five control lanes: a single-stranded 2 (lane I), the dimer complex 2:3 (lane II) and 4WJ with zero, two and four unpaired bases (lanes III–V). All of the 4WJ samples assemble (lanes III–V). Overexposure of the autoradiogram reveals some smearing in lane IV, indicating that a small amount of oligonucleotide participates in dynamic equilibrium with other complexes. Lanes VI and VII involve the reaction:



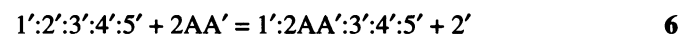
The addition in lane VI of excess strand 2AA partly displaces strand 2 from the 4WJ containing no unpaired bases, 1:2:3:4M. There is a roughly equimolar equilibrium between the 4WJ 1:2*:3:4M and dimer species containing strand 2*. No single-stranded 2* is observed however, indicating that equation 4 is not a complete description of the behavior of the system. In lane VII the complementary reaction is analyzed; it is apparent that excess strand 2 largely displaces radiolabelled 2AA* from the 4WJ form. Almost all the 2AA* is present either in single stranded or dimer form. These results indicate that, in contrast with the 3WJ situation, the 4WJ with no unpaired bases is more stable than a 4WJ having two unpaired bases.

In lanes VIII and IX the following reaction is analyzed:



In lane VIII it is apparent that excess strand 2AA largely displaces radiolabelled 2* from 1AA:2*:3:4M, again giving rise to species exhibiting the mobilities of dimers. However excess strand 2 is less effective at displacing radiolabelled strand 2AA* from 1AA:2AA*:3:4M (lane IX). This indicates that 1AA:2AA:3:4M is somewhat more stable than 1AA:2:3:4M.

Effects of unpaired nucleotides on 5WJ stability. Competition experiments were carried out to test the relative stabilities of 5WJ containing varying numbers of unpaired bases. Note that for these experiments, the longer strands, designated with primes ('), which form helical arms having 8 bp were used because 5WJ with only 7 bp disproportionated in competition experiments. Representative results are shown in Figure 6. The first three control lanes establish the mobilities of single stranded (2'), dimer (1':2AA') and 5WJ forms (1AA':2AA':3':4':5'). In lanes IV and V of Figure 6 the following reaction is analyzed:



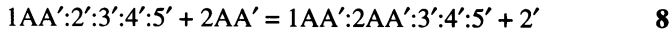
Lane IV shows that in the presence of excess 2AA', the radiolabelled 2'* strand is only found in dimer and single-stranded species. No 5WJ (1':2'*:3':4':5') is observed, consistent with assembly experiments that show that this complex is unstable. In lane V most of radiolabelled 2AA'* persists in the 5WJ form (1':2AA'*:3':4':5'), even in the presence of excess 2'. Only a small amount of dimer species form.

Lanes VI and VII concern the reaction:



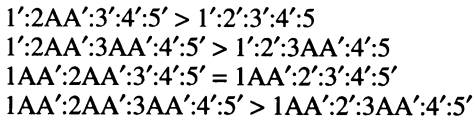
In lane VI excess 2AA' completely displaces 2'* from 1':2'*:3AA':4':5' while 1':2AA':3AA':4':5' is stable in the presence of excess 2'. Thus the 5WJ complex 1':2AA':3AA':4':5' is more stable than 1':2':3AA':4':5'.

The reaction in equation 8 is shown in lanes VIII and IX, and equation 9 shows the reaction in lanes X and XI:



It is apparent that 1AA':2':3':4':5' and 1AA':2AA':3':4':5' are approximately equally stable (lanes VIII and IX), whereas strand 2AA' completely displaces 2' from 1AA':2':3AA':4':5', demonstrating that 1AA':2AA':3AA':4':5' is more stable than 1AA':2':3AA':4':5' (lanes X and XI).

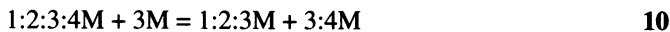
In summary:



These data indicate that in general, additional unpaired bases increase the stability of 5WJ complexes.

Relative stabilities of 3WJ and 4WJ. Competition experiments were carried out to determine the relative stabilities of 3WJ and 4WJ in which the number of unpaired bases was varied. Representative results are shown in Figure 7. Lanes I through VI are control lanes showing the assembly of 3WJ and 4WJ complexes with and without unpaired bases on the third strand. As discussed above, the 3M and 3MTT strands are designed to pair with strands 1 and 2 to form 3WJ complexes whereas strands 3 and 3AA are designed to pair with strands 1, 2 and 4M to form 4WJ (see Fig. 1). Lanes III to VI in Figure 7 show that all the junctions, 1:2:3M, 1:2:3MTT, 1:2:3:4M and 1:2:3AA:4M cleanly assemble and that the mobilities of the 3WJ differ from those of the 4WJ.

Lanes VII and VIII involve this reaction:



In lane VII, strand 3M* is radiolabelled. The radioactivity is found exclusively at the position corresponding to the single-stranded form. Therefore the equilibrium lies far to the left. The 3WJ with no unpaired bases, 1:2:3M, is much less stable than the corresponding 4WJ, 1:2:3:4M. This result is confirmed in lane VIII, in which strand 4M* is radiolabelled. All the radioactivity is found at the position corresponding to the 4WJ, 1:2:3:4M, and no discreet single stranded 4M is observed above background levels. This indicates that the 4WJ containing no unpaired bases remains intact in the presence of strand 3M, which is unable to successfully compete to form the 3WJ having no unpaired bases. Thus the 4WJ, 1:2:3:4M, is significantly more stable than the 3WJ, 1:2:3M. Clearly, other factors, which are likely to include hydration, base-stacking, and electrostatic and steric factors, contribute to the free energies of junction formation in such a way as to counterbalance the entropy of association, which favors the

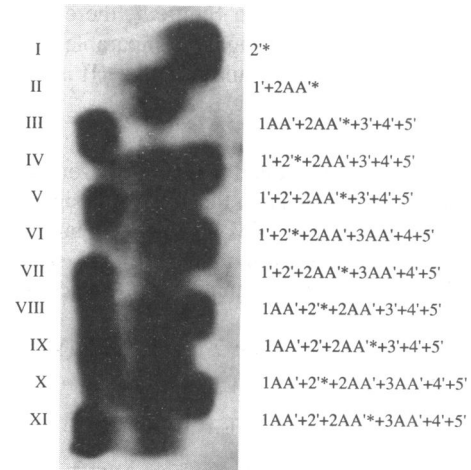


Figure 6. Autoradiogram of electrophoresis gel showing equilibrium distribution of 5WJ containing either 0, 2, 4 or 6 unpaired bases at the branch point. Lanes I-III are the controls and lanes IV-XI are the competitions.

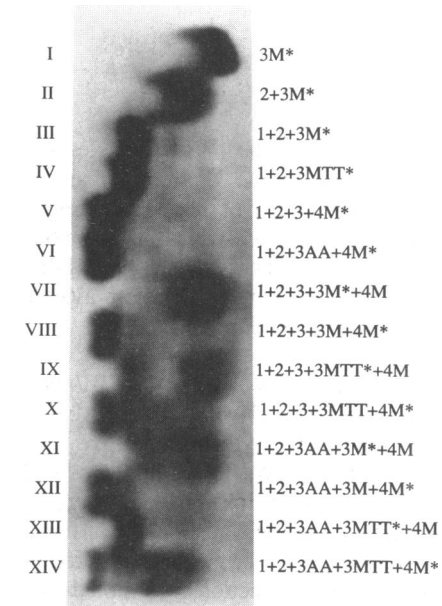
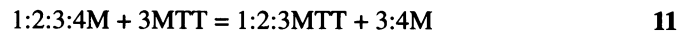


Figure 7. Autoradiogram of electrophoresis gel showing equilibrium distribution between 3WJ and 4WJ with and without unpaired bases. Lanes I-VI are the controls and lanes VII-XIV are the competitions.

assembly of three strands to form a trimer complex as opposed to the association of four strands to form a tetrameric complex.

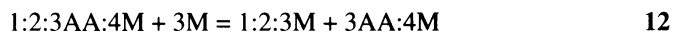
In lanes IX and X this reaction is investigated:



When radiolabelled 3MTT* is employed, a partitioning is observed between the 3WJ form and the single stranded form. A significant amount of intact 3WJ is observed. When radiolabelled strand 4M was employed to study the same reaction (lane X), a partitioning is observed between the 4WJ form, the single stranded form, and several other forms of intermediate mobility, possibly corresponding to 3:4M dimers as indicated in the

equation 11. Lanes IX and X in Figure 7, therefore show that 1:2:3MTT and 1:2:3:4M are roughly comparable in stability.

The reaction investigated in lanes XI and XII is:



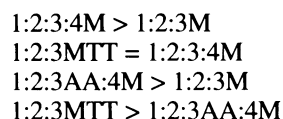
In lane XI none of the labelled strand 3M is found at mobility corresponding to 3WJ, indicating that 1:2:3M is significantly less stable than the 4WJ 1:2:3AA:4M. Likewise in lane XII none of the labelled strand 4M is found in the single stranded form, indicating that strand 3M is unable to displace it from the 1:2:3AA:4M.

In the last two lanes of Figure 7 (XIII and XIV) the reaction investigated is:



In lane XIII all the radiolabelled strand 3MTT is found in the 3WJ form and in lane XIV, most of the labelled 4M is found in the single stranded or dimer form (most likely 3AA:4M). This result clearly indicates that the 3WJ 1:2:3MTT is more stable than the 4WJ 1:2:3AA:4M.

To summarize:



The surprising result is that the 4WJ complexes are more stable or comparable in stability to the 3WJ complexes except for the case where the most stable form of the 3WJ (having two unpaired bases) was compared to the 4WJ in its least stable form (also having two unpaired bases). These data argue for the importance of the second helical stacking interaction available in 4WJ complexes that is not available in 3WJ.

Stabilities of 5WJ complexes relative to 3WJ and 4WJ. The stability of a representative 5WJ, 1AA':2AA':3':4':5', having four unpaired bases, was tested relative to the 3WJ 1AA':2AA':3M and to the 4WJ 1AA':2AA':3':4M. In Figure 8, strands 3M and 4M form only 7 bp per arm, whereas all the other strands (designated with primes) are capable of forming 8 bp per arm. The extra stability of the prime strands is required for 5WJ formation in competition reactions, as noted above. In the experiments shown in Figure 8, one of the strands 3M, 4M or 5' was radiolabelled (*). The first five lanes establish the mobilities of single strand (5'*), dimer (4':5'*), and representative 3WJ, 4WJ and 5WJ complexes.

In lane VI, excess amounts of strands 3', 4' and 5' were added to the 3WJ 1AA':2AA':3M*, in which strand 3M was radiolabelled, to determine whether strand 3M can be displaced from the 3WJ according to the following reaction:



In lane VI, almost all the radioactive 3M* strand is found at the mobility corresponding to the intact 3WJ and very little is present as free single strand. This indicates the equilibrium lies to the left. In the corresponding experiment in which excess strand 3M is added to the 5WJ 1AA':2AA':3':4':5'*, a significant amount of the radioactive strand 5'* is found in higher mobility species, indicating that strand 3M is partly able to disrupt the 5WJ, liberating strand 5' as well as strands 3' and 4', which evidently

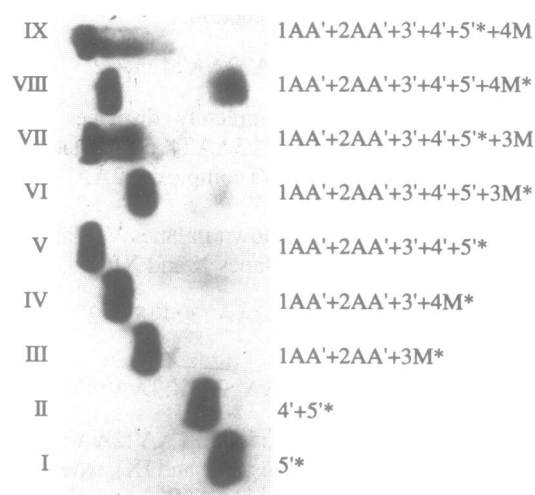


Figure 8. Autoradiogram of electrophoresis gel showing assembly of 5WJ and equilibrium distribution between 3WJ or 4WJ and 5WJ complexes. Lanes I-V are the controls and lanes VI-IX are the competitions reactions.

remain bound to each other giving species which migrate with mobilities comparable to that of the intact 3WJ. Some of the 5WJ remains intact. When the same experiment is carried out using 5WJ having only 7 bp per arm (complex 1AA:2AA:3:4:5), no 5WJ is observed.

In lanes VIII and IX of Figure 8, the reaction is:



Adding an excess of strands 4' and 5' results in partial liberation of 4M in the single stranded form, but most of the 4WJ remains intact. The complementary reaction in lane IX, shows that addition of excess 4M causes a partitioning of 5'* between intact 5WJ complexes and higher mobility forms. As was the case for strand 3M, addition of 4M to 5WJ complexes containing only 7 bp per arm resulted in complete disruption of the 5WJ.

These studies show that, as expected from the molecularity, the 5WJ is less stable than the 3WJ and 4WJ, but that small changes in the number of basepairs all the 5WJ to compete, so that the equilibria can be studied.

UV melting curves

UV melting studies were also carried out on representative complexes. Melting curves are shown in Figure 9. In panels A and B, the greater stability of 1:2:3:4M compared to 1AA:2:3:4M, 1:2:3M and 1:2:3MTT is evident. The melting temperature (T_m) of 1:2:3:4M (46.2°C) is significantly higher than that of the 3WJ with or without unpaired bases (1:2:3M, 39.5°C; 1:2:3MTT, 38.6°C) or of a 4WJ with unpaired bases, 1AA:2:3:4M (40.7°C). The melting temperature of 1AA:2:3:4M is close to that of the 3WJ samples. The slightly higher melting temperature of 1:2:3M, compared to 1:2:3MTT is discussed below. Panel C compares the melting curves of samples containing strands designed to form 5WJ. The strand combination 1+2+3+4+5 gives a melting curve with a higher apparent melting temperature (53.9°C) than does 1AA+2AA+3+4+5 (39.7°C). The gel studies reported above show that the latter forms a stable 5WJ (1AA:2AA:3:4:5), whereas the combination 1+2+3+4+5 does not. Comparison with melting curves of two strand combinations indicate that the

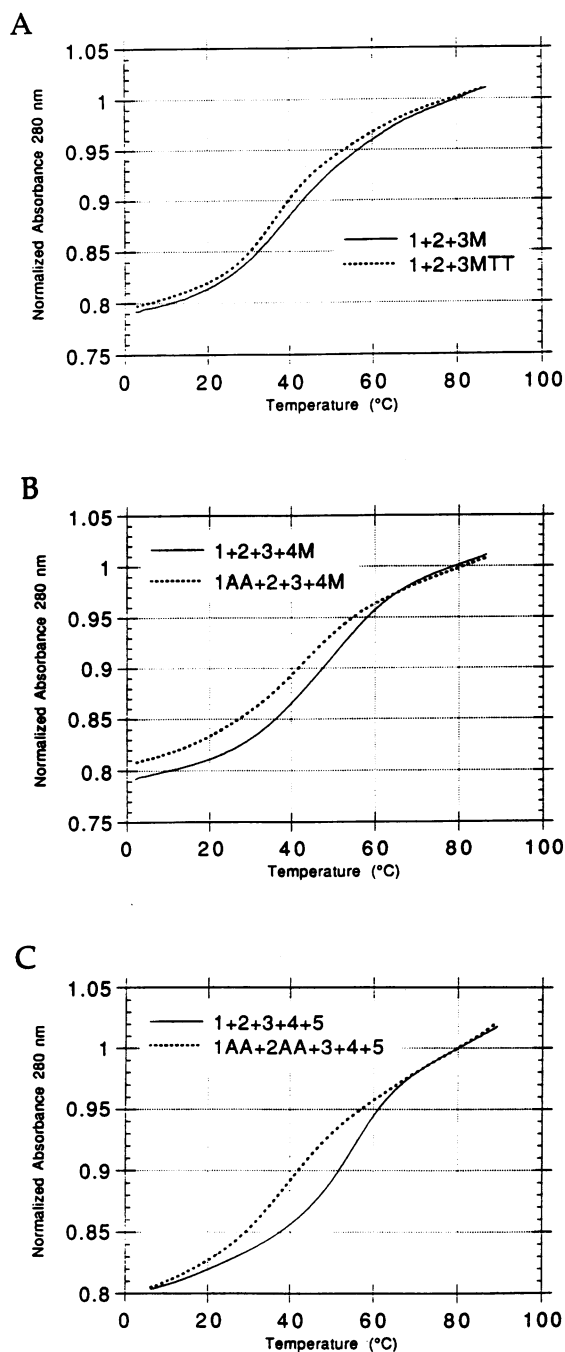


Figure 9. UV melting curves. (A) and (B) compare the stabilities of 3WJ and 4WJ with and without unpaired bases. (C) compares the melting curves of samples containing strands designed to form 5WJ.

melting curve of 1+2+3+4+5 is roughly a superposition of the melting curves of all possible adjacent two strand combinations (i.e. 1:2, 2:3, 3:4, 4:5 and 5:1).

The $f(T)$ curves derived from the melting curves of the 3WJ (Fig. 9A) are shown in Figure 10. The melting temperature (T_m) of the junction 1:2:3M is slightly higher than that of 1:2:3MTT. This seems to contradict the competition binding experiments, which clearly indicate that 3WJ containing unpaired bases are more stable, at least at the reduced temperatures at which these

experiments were carried out. Gel experiments carried out at room temperature shed some light on this point. At 25°C only 3WJ complexes having unpaired bases are stable enough to form observable complexes on gels, whereas 1:2:3M dissociates and only bands corresponding to two-strand complexes are observed. Moreover, the $f(T)$ curve of 1:2:3M is less steeply sloping for 1:2:3M than for 1AA:2:3M (Fig. 10). This indicates that smaller ΔH values are associated with the dominant melting transitions for the 1:2:3M sample, and/or that the transition is less cooperative. The melting of two-strand complexes is expected to yield a smaller enthalpy than the cooperative melting of an intact three-strand complex. An explanation that accounts for the more shallow slope and the slightly higher T_m observed for 1:2:3M is that melting of the junction itself begins at a lower temperature than for 1AA:2:3M (consistent with the room temperature gel data), and consequently the contributions of the two-strand complexes to the melting curve are dominant at higher temperature and shift the observed T_m of 1:2:3M upward. This interpretation is consistent with results obtained in previous work in which we employed gel electrophoresis and UV melting to study 3WJ complexes of unrelated sequence having only 5 bp per arm (13). When no unpaired bases were included in these junctions, no stable complex was observed by gel experiments, even at 5°C. As in the present case the sample without unpaired bases exhibited a higher melting temperature than 3WJ-forming samples having two or more unpaired bases (8° higher in this case), and the slope of the melting curve was more shallow than for the junction-forming samples. In subsequent work we found that the melting curve for the sample lacking unpaired bases corresponds exactly to a curve constructed from the sum of the melting curves of individual pairs of strands (unpublished work). It was found that two-strand partial duplexes formed by pairwise combination of the 3WJ-forming strands melt at consistently higher temperatures than do 3WJ complexes. The melting curves of 3WJ therefore consist of convolutions of melting curves of the intact junctions and of the duplexes which result when the 3WJ first dissociate. We have measured enthalpies of 3WJ formation calorimetrically for comparison (27). The enthalpies obtained from analysis of the shapes of the UV melting curves are significantly lower than those obtained calorimetrically, further indication that the UV melting behavior of three-strand 3WJ complexes does not conform to the two-state model, but likely involves significant populations of intermediates. Using a two-strand 3WJ system in which one of the helical arms terminates in a hairpin loop we have been able to obtain enthalpy values that agree with the directly measured calorimetric ones (unpublished data). The melting curves conform more closely to the two-state model and therefore the melting temperatures of junctions containing zero, one and two unpaired bases are observed in the expected order: the melting temperature of the 3WJ with two unpaired bases is about 8°C higher than that of the 3WJ having one unpaired base and ~16°C higher than that having no unpaired bases.

The UV melting behavior of the 5WJ complexes is expected to be even more complex than that of 3WJ. Multiple intermediate states are probably populated, so one should expect the melting curve of the 5WJ-forming samples to be convolutions of the melting transitions of the intact complex and of all four-strand, three-strand and two-strand intermediates that are significantly populated. In the case of the sample 1+2+3+4+5 (which has no bulged bases), the five-strand complex is not present (as indicated

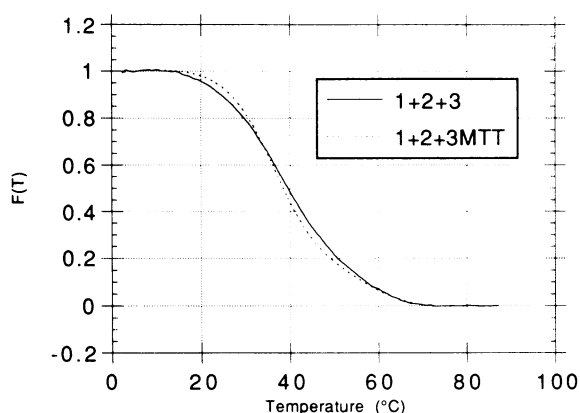


Figure 10. The fraction of complex, $f(T)$, curves derived as described in Methods for the melting curves of the 3WJ complexes 1:2:3M and 1:2:3MTT shown in Figure 9A.

by gel experiments) and does not contribute to the melting curve. The melting curve of the 5WJ-forming sample is multiphasic. It exhibits a low temperature melting transition attributable to dissociation of the intact complex (known from the gel experiments discussed above to be stable $<25^{\circ}\text{C}$), followed by dissociation of complexes of lower molecularity at higher temperature. This gives the appearance of a melting curve with lower cooperativity. A quantitative analysis of 5WJ melting curves will probably require a system in which the molecularity of the system is reduced from five separate strands to two by terminating three of the five helical arms with hairpin loops.

DISCUSSION

Our competitive binding studies, which ideally report the relative stabilities of nucleic acid complexes at a given temperature, provide data that agree with UV melting studies, when one is comparing samples in which the desired complexes are known to form. UV melting experiments can lead to erroneous conclusions regarding the stabilities of junctions, if one compares apparent melting temperatures without regard to other evidence. The five-strand mixture, 1+2+3+4+5, which does not form a 5WJ by gel electrophoresis, gives a higher apparent melting temperature than the 5WJ sample, 1AA:2AA:3:4:5, as discussed above. Thus, UV melting curves need to be interpreted with caution.

The competitive binding method corroborates previous work showing that 3WJ are stabilized by two unpaired bases (13). This method appears suitable for examining the effects of changing the number, type and position of unpaired bases in other junction complexes. The method should be applicable for examining RNA 3WJ, mixed RNA–DNA junction complexes, as well as the relative stabilities of RNA and DNA 3WJ. In light of the greater stability of RNA duplexes, RNA junction complexes having one less basepair per arm should probably be used in studies of RNA versus DNA junction stability. UV melting studies indicate that DNA 3WJ with two to five unpaired bases are roughly equal in stability and more stable than 3WJ having only one unpaired base; RNA 3WJ having one to three unpaired bases are comparable in stability, and more stable than RNA 3WJ lacking unpaired bases (unpublished data). Competition experiments offer a simple and direct method for ordering closely related

structures along a scale of relative thermodynamic stability at a fixed temperature.

The multiple equilibria obtained in the multi-strand systems studied here preclude determining equilibrium constants for the reactions of interest. Equilibrium constants for binary oligonucleotide association reactions have been obtained from gel electrophoretic data as illustrated in the work of Schepartz and co-workers on the binding of modified (tethered) RNA oligonucleotides to mRNA target sequences (28). Comparable studies should be possible in systems designed to model multi-junction loops by reducing the molecularity to two strands. For example, competition studies to quantitate the stability difference between 3WJ and 4WJ could be carried out by mixing a ‘targeting’ strand corresponding in sequence to strand 1 with two alternative ‘target’ molecules which would compete for binding to strand 1: one target molecule would consist of a single oligonucleotide identical in sequence on its 5′ end to strand 2 and on its 3′ end to strand 3M. The two halves would be linked by a short hairpin loop-forming sequence. The other target would consist of the sequences of strands 2, 3 and 4M, separated also by short hairpin loop-forming sequences. The hairpin-loop structures (one in the first and two in the second target strands) would be expected to fully form at room temperature or below.

Evidence is accumulating that naturally occurring nucleic acids exhibit equilibria between alternative conformations, characterized by alternative base-pairing arrangements. An example is the spliced leader RNA of *Leptomonas collosoma*, which is found to form two alternative secondary structures on its 5′ half; the two conformations are comparable in free energy and are able to interconvert on a fast (<1 s) time scale (29). One conformation consists of a hairpin loop and a stem interrupted by a bulge of several nucleotides that suggests an incipient 3WJ structure. The present studies indicate that nucleic acids having suitable sequences can exist in equilibrium between alternative junction structures, for example, 3WJ versus 4WJ.

NMR studies on DNA 3WJ provide structural information explaining why unpaired bases stabilize such structures (15–17,30). The 3WJ studied by NMR are completely unrelated in sequence to those studied here by competition binding methods, yet the same stability increase is seen when unpaired bases are added. This suggests that one can generalize from the structural studies that the increased stability observed in the 3WJ studied here is also due to improved inter-helical stacking between two of the helical arms, which is made possible by the presence of the unpaired bases. The present studies do not indicate which two helices are stacked in this case, or how the stacking pattern depends on the position of the unpaired bases.

The 4WJ with no unpaired bases, 1:2:3:4M was found to be more stable than the corresponding 3WJ, 1:2:3M and comparable in stability to the 3WJ, 1:2:3AA, which has two unpaired bases. This is so in spite of the fact that the 4WJ is assembled from four strands, and requires a higher entropic cost for assembly. The 4WJ with two unpaired bases, 1:2:3AA:4M is less stable than the 3WJ, 1:2:3MTT, but comparable in stability to the 3WJ, 1:2:3M. The conclusion may be drawn that 4WJ are generally more stable than 3WJ unless measures are taken to destabilize the 4WJ and to stabilize the 3WJ. The destabilizing effect of including unpaired nucleotides on one strand in a 4WJ is comparable to that observed in duplexes containing bulges (which can be considered ‘junctions of order zero’). One can hypothesize that the greater stability of 4WJ versus 3WJ is due to the presence of one more helix–helix

basepair stacking interaction. One might then infer that optimal base stacking between helical arms in 4WJ is achieved without unpaired bases, and that the addition of unpaired bases to one strand disrupts this optimal arrangement. The addition of unpaired bases on more than one strand restabilizes the 4WJ.

Immobile 4WJ serve as models for genetic recombination involving strand crossover as first proposed by Holliday. Extensive work has shown that adjacent helical arms in 4WJ (having no unpaired bases) stack pairwise to form two quasi-continuous helical domains. These have been shown to prefer an anti-parallel χ (Chi) conformation featuring unique base-stacking interactions between adjacent helices. The base sequence immediately adjacent to the junction appears to determine the preferred conformation. These results have been rationalized in terms of computer models presented for 4WJ (19). In principle, there are two energetically comparable conformations which differ as to the choice of stacking partners; it has been found that generally one arrangement dominates over the other (7,31,32). The present work shows that the insertion of unpaired bases into one of the strands of a 4WJ is destabilizing. In terms of the model, the strand containing the unpaired bases could be one of the two cross-over strands or one of the continuously stacked strands. The degree of destabilization may differ, depending on which is the case. Moreover, the introduction of unpaired nucleotides could change the conformation of the original junction: the incorporation of unpaired bases into one of the strands that is continuously stacked in the original 4WJ could destabilize that conformation, causing a switch to a conformation in which the strand with the unpaired bases becomes a crossover strand. One might therefore expect the least degree of disruption in the conformation of a 4WJ when the unpaired bases are included in one or both of the cross-over strands. Moreover, unpaired bases in the cross-over strands might even be expected to decrease electrostatic repulsions between the phosphate groups of the four converging strands at the junction by acting as molecular spacers. This might be manifested as a decreased requirement for the presence of divalent cations for optimal folding of such junctions. Based on these considerations, one might hypothesize that 4WJ would be stabilized by incorporation of equal numbers of unpaired bases in each of the two cross-over strands but would be destabilized if the unpaired bases are placed in the continuously stacked strands. In this regard it is interesting to note that we do observe small differences in stabilities of 4WJ containing two to four unpaired bases depending on which strands these are put into. Gel electrophoresis experiments at room temperature indicate, for example, that 1AA:2:3AA:4M and 1AA:2:3TT:4M are less stable than 1AA:2AA:3:4M. The latter forms a stable complex at room temperature whereas the former do not.

Most studies to date have focussed on immobile junctions, whereas the 4WJ relevant to genetic recombination are symmetrical and therefore mobile. It should therefore be mentioned that clever competition experiments were recently reported in which energy differences between alternative crossover isomers in symmetrical 4WJ were measured (33).

The conformation of 4WJ is largely determined by the base sequence immediately flanking the junction region (34,35). The particular junction sequence studied here has not been characterized structurally to determine which strands cross-over. With such information in hand, one could determine whether a greater destabilizing effect is obtained when unpaired bases are inserted into one of the continuously stacked strands or into one of the

cross-over strands. NMR studies of two immobile 4WJ that only differ in sequence by the exchange of two basepairs at the junction site have confirmed that it is the local sequence that determines the stacking conformation at the junction (18). One junction (designated by the authors 'J1') was found to exist in a unique conformation involving stacking of helix I on helix II and of helix III on helix IV, in agreement with the stacking pattern inferred by less direct methods. The NMR data in the second junction (J2), on the other hand, indicated that the molecule exists as an equilibrium mixture of the two possible stacking conformations in a ratio of 5:1. This indicates that even 4WJ having no unpaired bases, may not exist in unique conformations. The introduction of unpaired bases may be expected to further increase the range of possible conformations having accessible energies. Clearly further work is needed to understand how unpaired bases affect the structures and stabilities of 4WJ.

The importance of base stacking interactions between helical domains in nucleic acids has been recognized as a key stabilizing force in determining the tertiary structure of nucleic acids, since the first crystal structures of tRNAs appeared in the 1970s. The work of Turner and co-workers has shown that base stacking is a significant stabilizing force in RNA secondary and tertiary structure. In a recent report it was shown that coaxial stacking interactions provide significant stabilization in RNA duplexes in which a break exists in the phosphodiester backbone of one of the strands (36). The present findings are completely consistent.

The present work shows that in addition to exclusively functional roles, unpaired nucleotides may be present in naturally occurring 3WJ or 5WJ to confer additional stability to such structures. This work suggests that it may be appropriate to modify computer programs that predict secondary structure in nucleic acids with regard to the way unpaired bases in junction loops are treated. At least the first two unpaired bases in 3WJ should be assigned a stabilizing role and likewise the first four in 5WJ. The situation in 4WJ is clearly more complex.

The present work suggests that by including unpaired bases, it may be possible to design conformationally homogeneous 5WJ small enough to study by NMR. A single strand designed to form a terminal helix consisting of 6 basepairs, four hairpin loops and stems each containing 4 bp and four loop nucleotides (to form the other four helical arms of the junction), and four unpaired bases would have a total of 64 bases, bringing it within range of modern NMR spectroscopic methodologies.

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