Stability and structure of three-way DNA junctions containing unpaired nucleotides

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ABSTRACT

Non-paired nucleotides stabilize the formation of three-way helical DNA junctions. Two or more unpaired nucleotides located in the junction region enable oligomers ten to fifteen nucleotides long to assemble, forming conformationally homogeneous junctions, as judged by native gel electrophoresis. The unpaired bases can be present on the same strand or on two different strands. Up to five extra bases on one strand have been tested and found to produce stable junctions. The formation of stable structures is favored by the presence of a divalent cation such as magnesium and by high monovalent salt concentration. The order-disorder transition of representative three-way junctions was monitored optically in the ultraviolet and analyzed to quantify thermodynamically the stabilization provided by unpaired bases in the junction region. We report the first measurements of the thermodynamics of adding an unpaired nucleotide to a nucleic acid three-way junction. We find that $\Delta\Delta G°(37°C) = +0.5$ kcal/mol for increasing the number of unpaired adenosines from two to three. Three-way junctions having reporter arms 40 base-pairs long were also prepared. Each of the three reporter arms contained a unique restriction site 15 base-pairs from the junction. Asymmetric complexes produced by selectively cleaving each arm were analyzed on native gels. Cleavage of the double helical arm opposite the strand having the two extra adenosines resulted in a complex that migrated more slowly than complexes produced by cleavage at either of the other two arms. It is likely that the strand containing the unpaired adenosines is kinked at an acute angle, forming a Y-shaped, rather than a T-shaped junction.

INTRODUCTION

Three-way junctions are important structural motifs in nucleic acids. Three-way junctions are relevant to the structure of single-stranded DNA molecules. Interest in three-way junctions also arises in the execution of studies aimed at dissecting the steps involved in site-specific recombination (1). Gel mobility shift experiments and chemical probing have shown that a three-way junction is likely to be symmetrical in structure, with the arms radiating to define an equilateral triangle or a trigonal pyramid, rather than an unsymmetrical structure such as a T (2). Other work, in which three-way junctions have been used as building blocks for creating three-dimensional lattices, has indicated that three-way DNA junction structures are conformationally flexible (3).

Three-way junctions are commonly found in naturally occurring RNA molecules, with 5S ribosomal RNA constituting a notable example. Unpaired nucleotides are frequently found in junction regions of RNA molecules. For example, the three-way junction of the 5S rRNA of *E. coli* contains unpaired CAGUA between helices A and B, unpaired G between helices B and D, and unpaired GAA between helices D and A (4). Nothing is yet known regarding the effects of these unpaired nucleotides on the structure or stability of the junction region. When unpaired nucleotides are found within a duplex region of a nucleic acid they are called bulge loops and are found to destabilize the double-helix (5). Here we show that two or more unpaired bases stabilize the structure of three-way DNA junctions.

Elucidation of the structure and dynamics of a three-way junction on an atomic scale requires a high-resolution technique such as x-ray crystallography or NMR spectroscopy. One goal of our work on three-way junctions is directed at identifying stable complexes small enough to study by NMR techniques. We began our study with three deca-nucleotides designed to hybridize by Watson-Crick base-pairing to form three short helices of five base pairs each. We chose a rapid screening technique for determining whether strands were associating, namely mobility shifts on native polyacrylamide electrophoresis gels. This allowed us to compare expeditiously many different combinations of oligonucleotides.

The results of these studies led us to investigate the structure of the 3-way junction containing two extra adenosines on one strand at the junction using the electrophoretic methods first developed to study the geometry of four-way DNA junctions (6) and later applied to three-way junctions (2). Mobility shifts on electrophoresis gels of junction complexes containing various combinations of long and short arms reporter are compared. The results we obtained using these methods are surprising in that they indicate that the DNA strand containing the extra bases is bent at an acute angle to form a bilaterally pseudo-symmetric complex, rather than the T-shaped complex expected on the premise of optimal purine-purine stacking.

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Finally, we report UV melting studies of representative complexes containing unpaired adenosines in the junction region. We present the first measurements of the thermodynamics of adding an unpaired nucleotide to a nucleic acid three-way junction. We find that $\Delta\Delta G°(37°C) = +0.5$ kcal/mol in going from a DNA junction having two unpaired adenosines to one having three. This value will be useful in nucleic acid structure prediction (7).

MATERIALS AND METHODS

Assembly of junctions and electrophoretic analysis

The DNA oligomers were synthesized by the phosphoramidite method on a Biotix Automatic DNA Synthesizer. After synthesis and deprotection, oligonucleotides were desalted by gel filtration on Sephadex-25, lyophilized, redissolved in deionized water, and quantitated by UV absorbance. Molar extinction coefficients for single strands were calculated using the nearest neighbor approximation (8). Samples containing the individual strands, the three-pairwise combinations, and the mixture of all three strands were heated to 70°C in a Tris/borate buffer (89 mM, pH 8.3) containing 0.1 M KCl, and 5 mM $MgCl_2$ and then were slowly cooled to allow the most stable secondary structures to form. The samples (containing 10 to 15 μg of each strand in 20 μl or about 1.5×10^{-4} M) were then analyzed by electrophoresis on native 20% polyacrylamide gels (19:1 monomer:bis ratio) containing the identical concentrations of the buffer and supporting electrolytes. The gels were run overnight with recirculation of buffer in a cold room maintained at 5°C until the xylene cyanol marker dye had migrated 1/4 to 1/3 the height of the gel (16 to 20 hours). The dimensions of the gels were 13 cm (height) × 16 cm (width) × 1 mm (thickness). The current level was set at 50 mA, which resulted in a slight steady state heating of the gels relative to the ambient temperature. The temperature of the gels was found to be 8°C at this current level. The DNA was visualized by UV shadowing using a short wavelength UV lamp and a fluorescent TLC plate, allowing the gels to be photographed without staining or radioactive labelling.

The 80-mer strands used to study the structure of a junction containing two unpaired adenosines were synthesized by Oligos Etc. Inc. using a Biotix Automatic DNA Synthesizer. The 80-mers were radio-labelled at the 5′ end using T4 polynucleotide kinase and γ^{32}P-ATP and purified by electrophoresis on 15% denaturing polyacrylamide gels containing 8M urea. Junctions formed by 80-mer strands were electrophoresed in 10% native polyacrylamide gels (19:1 monomer to bis ratio) containing 0.10 M KCl, Tris/borate buffer (89 mM, pH 8.3), and either 5 mM $MgCl_2$ or 1 mM EDTA. The gels were autoradiographed overnight.

Error analysis

Migration distances were measured from the photographs. Errors were estimated to be about 0.5 mm, based on the width of the bands. The relative migration of complex bands was obtained as the ratio of the migration distance of a three-strand complex to that of a control which was chosen to be a sample consisting of two of the strands (strands 1 and 2 in Figure 2) which form a partial duplex. The error in the relative migration was calculated by propagation of errors (9).

Size-Exclusion Chromatography

Complex formation at reduced temperature was confirmed by size-exclusion chromatography on a column of high-resolution Sephacryl S-100 obtained from Pharmacia in an aqueous buffer containing 0.10 M KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 10 mM potassium phosphate (pH 6.5). The column was packed and run according to the manufacturer's suggestions using a peristaltic pump (Model P-1). The column was 63 cm long by 1.6 cm diameter (total volume approximately 126 ml). Blue dextran was included in each sample to monitor the reproducibility of the chromatography. Two ml fractions were collected at a flow rate of 60. ml/hour using a Frac-100 fraction collector interfaced to the P-1 pump. The flow rate was consistent with the manufacturer's guidelines. The blue dextran consistently eluted between fractions #24–25.

UV melting experiments

Melting curves were recorded in 1.0 cm quartz cuvettes at 260 nm using a Cary 219 Spectrophotometer set at a spectral bandwidth of 2 nm and a pen period of 10 seconds. The temperature of the sample was controlled by a Haake A80 circulating water bath. Stoichiometric amounts (approximately 3 nanomoles) of each strand, calculated from optical density measurements, were mixed and dissolved in 1.00 ml of a buffer consisting of 1.0 M KCl, 5.0 mM $MgCl_2$, 0.1 mM EDTA, and 10 mM potassium phosphate (pH 6.4). Samples were annealed at 90°C prior to cooling to the starting temperature and allowed to equilibrate for at least 15 minutes until the absorbance stabilized. Samples were heated at a rate of approximately 0.5°C/minute and the absorbance was recorded approximately every 0.2°C. The data were transferred to a Macintosh computer and analyzed using the Kaleidograph graphical data analysis software package (v 2.1). Kaleidograph may be obtained from Synergy Software, 2457 Perkiomen Avenue, Reading, PA 19606.

UV Data Analysis

Data were analyzed using the two-state model in accordance with published guidelines (8). The equations for extracting thermodynamic data for trimer complexes were taken from published work (10). The measured absorbance data were entered into the Kaleidograph program and subjected to the smoothing routine provided in the program. Data were normalized to give absorbances of 1.0 at the lowest temperature. 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 ($A_{single\text{-}strands}$) and of the complex ($A_{complex}$), respectively. The fraction of strands in complex (f) at intermediate temperatures was calculated by assuming that the degree of hypochromicity is directly proportional to the base stacking, which is proportional to the molar fraction of complex. This is expressed by Equation 1:

$$\text{Eq. (1)} \quad A_{total} = A_{complex} \cdot f + A_{single\text{-}strands} \cdot (1-f)$$

A_{total} in Eq. (1) is the normalized, smoothed absorbance data. The association constants were calculated using Equations 2 and 3, which apply to the case of non-identical strands present in equal molar concentrations. C_t represents the total strand concentration (10):

$$\text{Eq. (2)} \quad K_{Trimer} = 9f/(C_t 2(1-f)^3)$$

$$\text{Eq. (3)} \quad K_{Dimer} = 2f/(C_t(1-f)^2)$$

The melting temperature, T_m, was obtained as the temperature corresponding to f=0.50. The data corresponding to $0.15 < f$

< 0.85 were plotted as ln(K) vs. (1/T), fitted by linear least squares, and used to calculate the van't Hoff estimates of $\Delta H°$ and $\Delta S°$ for the melting transition. The slope of the van't Hoff plot corresponds to $-\Delta H°/R$ while the intercept yields $(\Delta S°/R)$. Another estimate of $\Delta H°$ may be obtained from the slope of the df/dT curve, using Equations 4 and 5:

Eq. (4) Trimer: $\Delta H° = 8RT_m(df/dT)_{T=Tm}$

Eq. (5) Dimer: $\Delta H° = 6RT_m(df/dT)_{T=Tm}$

Agreement within 5% of $\Delta H°$ obtained by these two methods is an indication that the two-state model is valid (8). The fraction vs. Temperature data were smoothed and differentiated numerically using routines supplied by Kaleidograph to evaluate $(df/dT)_{T=Tm}$.

RESULTS AND DISCUSSION

Identification of stable complexes using native gel electrophoresis

First we prepared the three DNA decamers labelled 1, 2, and 3 in Figure 1. The parent strands contain no inserted nucleotides in the junction region. Insertions are indicated by '(X)' and '(Y)' in the figure. The following considerations guided our design of these oligonucleotides: Strand 3 was designed to be pseudo-symmetric to eliminate the possibility of its forming a hair-pin structure or the possibility that the 5′ half of Strand 1 could associate with the 3′ half of strand 2. Likewise, the 3′ half of Strand 1 was designed to minimize hairpin formation or association with the 5′ half of Strand 3, while simultaneously limiting the association of the 5′ half of Strand 2 with the 3′ half of Strand 3. The G/C content of all three strands was kept high, to optimize the free-energy of formation of the three-strand complex. AT base pairs were included in all three helical domains to provide spectroscopic markers in the NMR work that we plan to carry out in this series of molecules.

The association of strands 1, 2, and 3, assayed by native polyacrylamide gel electrophoresis, is incomplete even at reduced temperature (Figure 2). This experiment also reveals that strands associate in pairs (1+2, 1+3AA, and 2+3AA) to produce single bands of almost identical mobility. The mobilities of the half-duplexes are slightly retarded relative to those of the single strands, which, as expected, exhibit differing mobilities. The three-strand mixture 1+2+3 produces a smear on the gel, indicating multiple complex conformations.

We included extra bases in the junction, to act as 'spacers' to provide extra flexibility as well as stacking interactions at the junction. The result of including one or two extra adenosine nucleotides in Strand 3 at the position marked '(X)' in Figure 1 is also shown in Figure 2. Like 1+2+3, the 1+2+3A mixture produces a smear which is shifted to slower mobilities relative to 1+2+3. A dramatic change is observed when two extra nucleotides are included in Strand 3 (complex 1+2+3AA). A single band corresponding to the trimer complex now appears on the gel. The band is significantly retarded in mobility relative to the half-duplex molecules (1+2, 1+3AA, or 2+3AA). We have quantitated the mobility shifts by normalizing them to the mobilities of the half-duplex 1+2, used as a control in our experiments. The relative mobility shift of the 1+2+3AA complex was determined in five separate experiments. It is highly reproducible. The value of the relative mobility, expressed as the ratio of the distance of migration of the complex to that of the 1+2 control, is 0.69 ± 0.025, where the error calculated from the standard deviation of five experiments is in agreement with the error calculated by error propagation techniques based on the ability to measure the distances to within 0.5 mm.

We examined the effect of adding more nucleotides to the junction region. The results are shown in Figure 3 and are summarized in Table 1. The complexes containing 3, 4, and 5 extra adenosines in the junction migrate incrementally more slowly than the 1+2+3AA complex. There is very little

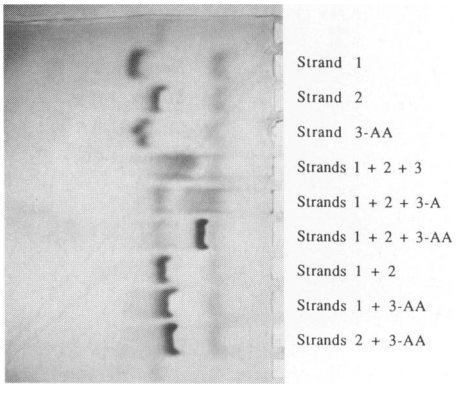

Figure 2. Native polyacrylamide gel analysis of three-way complex formation at reduced temperature (6−8°C). Gel composition: 20% polyacylamide, 19:1 monomer to bis ratio, 0.10 M KCl, 5 mM $MgCl_2$, 89 mM Tris-borate buffer, pH 8.3. The marker dye xylene cyanol is visible in each lane above the complexes.

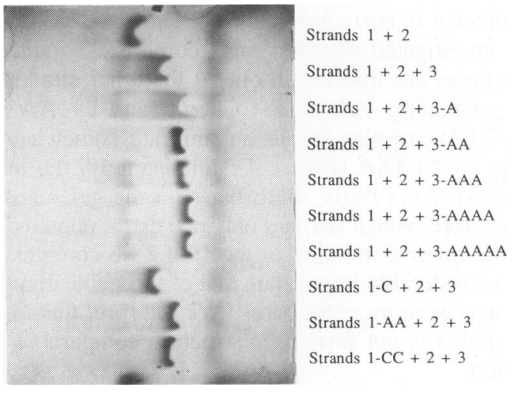

Figure 3. Native polyacrylamide gel analysis of three-way complex formation at reduced temperature (6−8°C). Conditions identical to Figure 2.

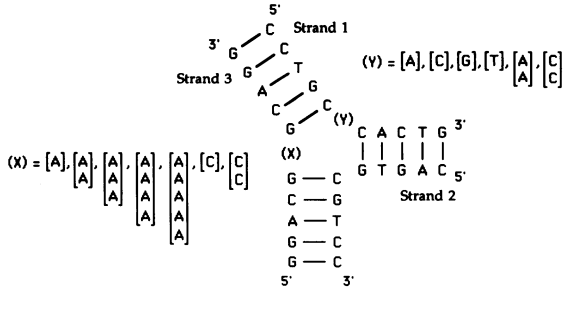

Figure 1. Three-way junctions studied in this work. '(X)' and '(Y)' indicate positions of insertions of unpaired nucleotides used to stabilize junction formation.

Table 1. Relative gel mobilities of complexes run at 5°C. Relative mobilities were calculated by taking ratios of distances migrated by three-strand complexes to the duplex 1+2. Errors in relative mobilities calculated by error propagation techniques equal approximately 0.025.

Complex	Relative Mobility	Standard Deviation	Number of Experiments
1+2+3AA	0.69	0.025	5
1+2+3AAA	0.68	0.015	4
1+2+3AAAA	0.67	–	1
1+2+3AAAAA	0.67	–	1
1+2+3CC	0.81	0.015	2
1A+2+3	1.00	–	1
1C+2+3	0.88	0.030	5
1G+2+3	1.10	–	1
1T+2+3	1.00	–	1
1A+2+3A	0.94	–	1
1C+2+3A	0.78	0.052	4
1G+2+3A	0.85	–	1
1T+2+3A	0.92	–	1
1C+2+3AA	0.67	0.017	2
1AA+2+3	0.76	–	1
1CC+2+3	0.73	0.049	2
1AA+2+3AA	0.61	0.003	2
1CC+2+3AA	0.63	0.029	3

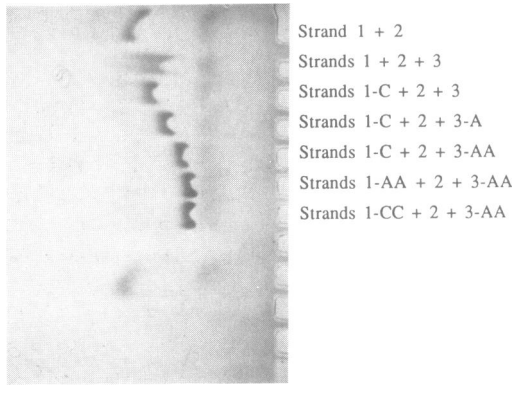

Figure 4. Native polyacrylamide gel analysis of three-way complex formation at reduced temperature (6−8°C). Conditions identical to Figure 2.

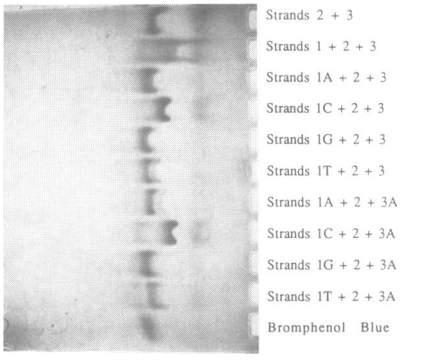

Figure 5. Native polyacrylamide gel analysis of three-way complex formation at reduced temperature (6−8°C). Conditions identical to Figure 2.

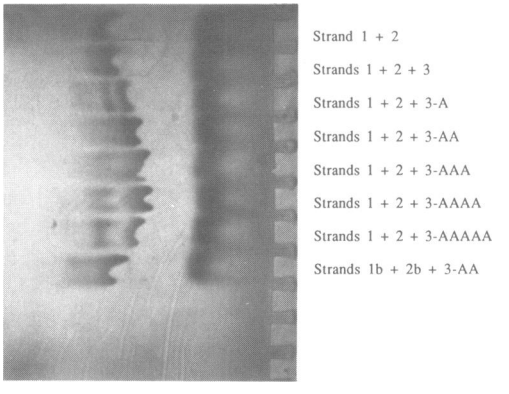

Figure 6. Native polyacrylamide gel analysis of three-way complex formation in EDTA at reduced temperature (6−8°C). Conditions identical to Figure 2 except for substitution of 1 mM EDTA for 5mM $MgCl_2$.

difference in the mobilities of the complexes containing 3, 4, and 5 extra nucleotides in the junction. A small excess of the 1+2 duplex is visible in some of the lanes. This does not appear when the stoichiometry is corrected (data not shown).

Next we investigated whether stable complexes would result when extra bases are inserted in one of the other strands. The complexes containing -AA- and -CC- in strand 1 (1AA+2+3 and 1CC+2+3) are also stable but migrate somewhat more rapidly than 1+2+3AA (Figure 4). Interestingly, the mixture 1C+2+3 produces a fairly sharp band on the gel, unlike the 1+2+3A mixture, which also has only one extra, unpaired base. However, the relative mobility of the 1C+2+3 complex, 0.88 ± 0.03, is considerably larger than that of the stable complexes containing two or more extra bases. We interpret this to mean that this complex is not forming a structure comparable to the other junctions.

Other combinations of unpaired nucleotides were tested (Figure 4). The 1AA+2+3AA and the 1CC+2+3AA, which contain four extra nucleotides in the junction, exhibit the slowest relative mobilities of all the complexes examined. The 1C+2+3A junction exhibits a mobility somewhat greater than those of junctions having two unpaired bases on the same strand. It too is likely to assume a somewhat different conformation. Its mobility is, however, significantly slower than that of 1C+2+3.

The complexes 1A+2+3 and 1G+2+3 are probably not forming. The mobilities of the species observed on the gel are identical to that of the control, 2+3 (Figure 5). A small amount of complex 1T+2+3 is evident. As noted above, complex 1C+2+3-A forms stoichiometrically, however, only a small amounts of 1G+2+3A, 1A+2+3A or 1T+2+3A seem to form.

We examined the effect of removing magnesium by annealing the strands in a buffer containing 1mM EDTA in place of magnesium. The samples were run on a gel containing the same buffer in the cold. The results are shown in Figure 6 and are summarized in Table 2. In EDTA, the mobilities of all complexes are increased and bands which appear sharp in the presence of magnesium are smeared. In the case of some complexes, two bands are observed, indicating an equilibrium in slow exchange. The sample 1b+2b+3-AA contains an additional GC base-pair in the helix formed by Strands 1 and 2. Even this does not significantly stabilize the complex in the absence of magnesium. The complexes containing three or more extra bases appear to be the most stable in the absence of magnesium.

Table 2. Relative gel mobilities of complexes run in EDTA at 5°C or at room temperature in 5mM $MgCl_2$, calculated as in Table 1. Data are based on one or two experiments.

Complex	Relative Mobilities	
	EDTA	Room Temperature
1+2+3	0.96	0.83
1+2+3A	0.87	
1+2+3AA	0.86	0.75
1+2+3AAA	0.80	0.72
1+2+3AAAA	0.76	0.73
1+2+3AAAAA	0.83	0.76
1+2+3CC	0.94	0.76
1b+2b+3AA	0.95	0.70
1C+2+3A		0.84
1CC+2+3AA		0.84
1AA+2+3		0.85
1CC+2+3		0.81

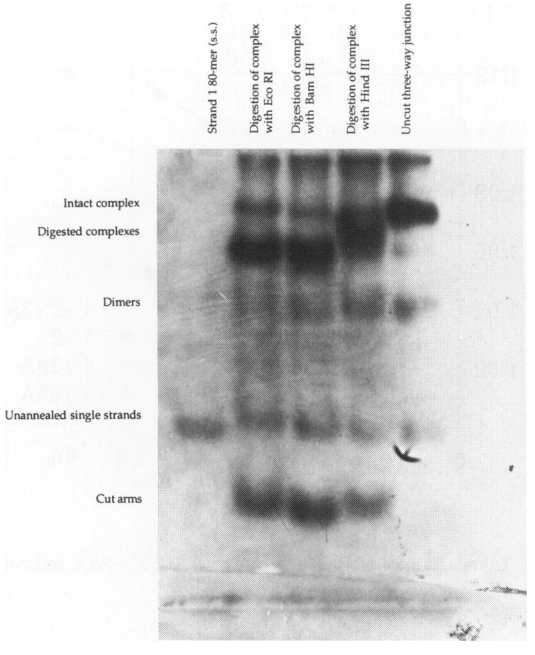

Figure 7. Autoradiogram of native gel electrophoresis of extended three-way junction (containing unpaired -AA-) run at reduced temperature (6−8°C). Gel composition: 10% polyacylamide, 19:1 monomer: bis ratio, 0.10 M KCl, 5 mM $MgCl_2$, 89 mM Tris-borate buffer, pH 8.3. The position of dimers was confirmed independently.

Running the samples at room temperature, even in the presence of magnesium, results in smeared bands and increased relative mobilities for the trimers. Again, the complexes containing three or more extra bases appear to be the most stable, but it is unlikely that single conformations are forming (data not shown). Results are summarized in Table 2.

Three-way complex formation at 20°C and 5°C (0.1M KCl) was compared using Size Exclusion Chromatography (gel filtration) on a high resolution Sephacryl S-100 column at 5°C. The complex studied, 1b+2b+3AAA, elutes with a peak at fraction #39 (62% of the column volume), whereas the single strands 1b, 2b, and 3AAA elute with peaks at fraction #45 (71% of column volume). At room temperature, both samples elute at fraction #45.

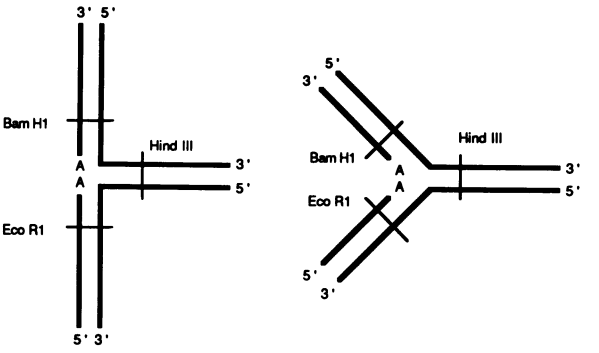

Figure 8. Schematic drawing of two alternative conformations for the extended three-way junction containing two unpaired adenosines in one strand. The right panel shows the Bam H1 and Eco R1 arms subtending an acute angle, the model favored by the native gel electrophoresis data (Figure 7).

Structure of the Three-way junction containing two extra adenosines on one strand

Junction molecules having various combinations of long and short arms were electrophoresed on native polyacrylamide gels to obtain qualitative information regarding the angles subtended by the arms of the three-way junction having two unpaired adenosines. We synthesized three 80-mer DNA strands designed to contain the 1+2+3 sequences at the core of the junction (no unpaired nucleotides) and an 82-mer corresponding to strand 3AA, having two unpaired adenosines. The terminal 22 base pairs of each arm of the trimer complexes (1+2+3 and 1+2+3AA) are identical to each other and to the arms used by Lilley's group in their three-way junction, which also did not contain any unpaired nucleotides ((2)). Each arm contains a unique restriction site 15 nucleotides from the junction, and is referred to below according to the restriction site it contains (H for Hind III, R for EcoRI, and B for BamH1). Each 80-mer strand is referred to by the two restriction sites it encompasses (HR, HB, and BR). The sequences are:

RB 80-mer:
5′-CGC AAG CGA CAG GAA CCT CGA GGA ATT CAA CCA CCG GAC G GCA GGC TAG GAC GGA TCC CTC GAG GTT CCT GTC GCT TGC G-3′

RB 82-mer:
5′-CGC AAG CGA CAG GAA CCT CGA GGA ATT CAA CCA CCG GAC GAA GCA GGC TAG GAC GGA TCC CTC GAG GTT CCT GTC GCT TGC G-3′

HR 80-mer:
5′-CGC AAG CGA CAG GAA CCT CGA GAA GCT TCC GGT AGC AGT GCG TCC GGT GGT TGA ATT CCT CGA GGT TCC TGT CGC TTG CG-3′

BH 80-mer:
5′-CGC AAG CGA CAG GAA CCT CGA GGG ATC CGT CCT AGC CTG CCA CTG CTA CCG GAA GCT TCT CGA GGT TCC TGT CGC TTG CG-3′

The radiolabelled, gel-purified H, R, and B strands were annealed by heating to 80°C and slowly cooling. Aliquots of the annealed sample were cleaved with the three restriction enzymes and analyzed on 10% native polyacrylamide gels (19:1) made

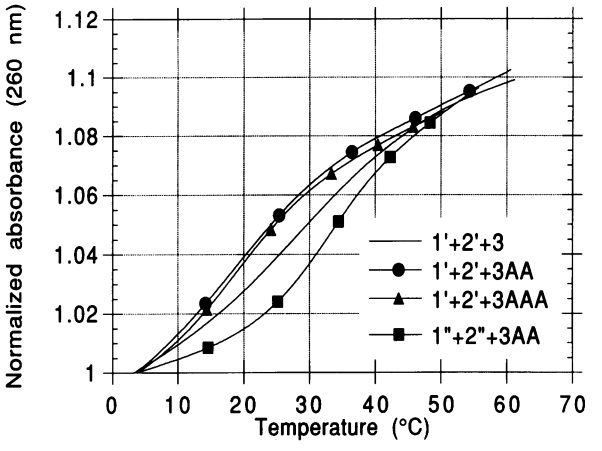

Figure 9. UV melting curves of representative three-way complexes obtained as described in Methods.

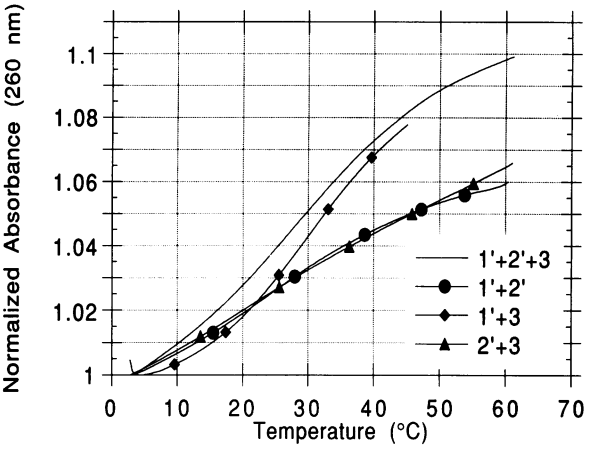

Figure 10. Comparison of UV melting curves of 1'+2'+3 and component duplexes.

up in the same buffer used for the small complexes. The DNA was electrophoresed as described above in the cold room.

The autoradiogram in Figure 7 shows that the three-way complex RB(82-mer)+HR+BH (which contains unpaired -AA-) does form and that it exhibits a much reduced mobility relative to the unannealed single strands. Cleavage of this complex in the R or the B arm results in trimer complexes that migrate identically, whereas cleavage of the H arm produces a trimer that migrates more slowly. Further experiments (data not shown) reveal that cleavage of the trimer junction containing no unpaired nucleotides (complex RB{80-mer}+HR+BH) with each of the restriction enzymes produced complexes of identical electrophoretic mobility in agreement with previous work (2). Furthermore, in the absence of magnesium ion, the gel mobilities of the three complexes produced by cleavage of the 1+2+3AA junction are also identical.

The strand containing the two extra adenosines includes the Eco RI and Bam H1 sites, so cleavage at these sites was expected to produce pseudo-symmetrically related complexes having nearly identical mobility shifts, as was in fact observed. We further expected that this strand would exhibit continuous stacking, given the all-purine GAAG sequence at the junction, in which case the junction would be expected to assume a T-shaped structure, as shown in the left panel of Figure 8. If this were the case we would expect that cleavage of the H arm would produce an elongated complex in which the two reporter arms, R and B subtend an angle approaching 180°. Such a complex would be expected to migrate more rapidly than the complexes formed by cleavages of either the R or B arms, which would result in molecules having the two reporter arms at right angles to each other. We observe instead that the Hind III cleaved complex exhibits a *reduced* mobility. This result favors a geometry in which arms B and R subtend an acute angle, as shown in the right panel of Figure 8. A divalent cation such as magnesium is required to stabilize this structure. In the absence of magnesium, the junction appears to loosen up insofar as the arms appear to subtend equal angles on average.

UV Melting Studies

The order-disorder transitions of nucleic acids can be conviently monitored using absorption spectrophotometry at 260 nm. We

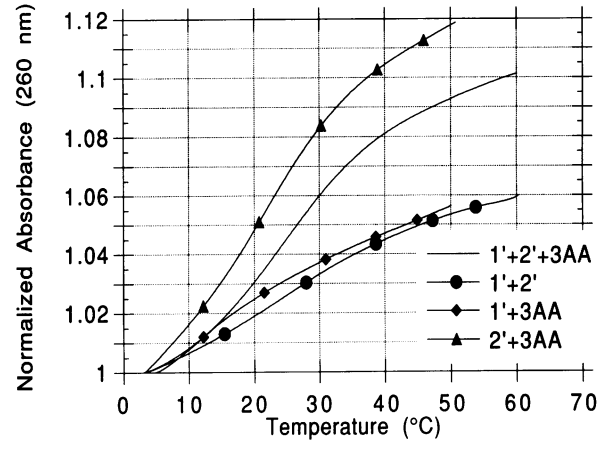

Figure 11. Comparison of UV melting curves of 1'+2'+3AA and component duplexes.

undertook UV melting studies of selected three-way junctions to address the following questions:

(1) Is it possible to quantify thermodynamically the apparent stabilization of the junction structure provided by the presence of unpaired nucleotides?

(2) What is the relative stability of otherwise identical junctions containing varying numbers of unpaired nucleotides?

(3) By how much can we stabilize the junction by increasing the length of the base-paired regions from 5 to 6 base-pairs? Is this stabilization consistent with that predicted by nearest neighbor parameters derived for B-DNA? Such a comparison serves to indicate how well the helices near a three-way junction conform to B form DNA.

We sought to answer the first question by comparing the melting curves of a three-way junction lacking unpaired nucleotides with junctions containing two or three unpaired adenosines in strand 3. For these studies, we changed the AT base pair at the end of the helix formed by strands 1+2 to GC to create a more stable junction. The sequence of the modified strand 1, designated 1', is 5'CCT GCC ACC G. The corresponding strand 2' is 5'CGG TGC GTC C. We also extended each arm in the three-way junction by one GC base-

Table 3. Thermodynamic parameters derived by van't Hoff analysis of UV melting data of representative junction molecules. Melting experiments were carried out at approximately 3×10^{-6} M concentrations in aqueous buffer consisting of 1.0 M KCl, 5.0 mM $MgCl_2$, 10 mM potassium phosphate (pH 6.4) and 0.1 mM EDTA.

Sample	From slope of ln(K) vs. 1/T $-\Delta H°$ (Kcal/mol)	$-\Delta S°$ (Cal/K−mol)	Calculated $-\Delta G°(37°C)$ (Kcal/mol)	Experimental T_m(°C)	From (df/dT) $-\Delta H°$ (Kcal/mol)
1′+2′+3 (dimer)	41.11	105.8	8.31	33.3	38.9
1′+2′+3 (trimer)	54.71	120.1	17.45	33.3	51.9
1′+2′+3AA	66.36	165.7	14.96	24.8	63.2
1′+2′+3AAA	70.99	182.2	14.48	22.2	68.6
1″+2″+3AA″	77.60	198.7	15.97	29.9	73.6
2′+3AA	55.28	156.1	6.88	26.4	51.2
1′+3	48.07	134.5	6.34	23.0	46
1′+2′	38.98	99.76	8.04	31.7	36

pair by preparing the following oligonucleotides: Strand 1″, 5′GCC TGC CAC CGC; Strand 2″, 5′GCG GTG CGT CCG; and Strand 3AA″, 5′CGG ACG AAG CAG GC.

It is desirable to obtain melting curves with well-defined baselines above and below the transition to carry out a thermodynamic analysis of UV melting data. This was achieved by raising the monovalent salt concentration to 1.0 M. This choice of solvent conditions also permits direct comparison of our data to studies of duplex DNA melting. The thermodynamic parameters for the ten Watson-Crick nearest neighbor interactions were recently reported (11) based on measurements made in aqueous buffer containing 1.0 M NaCl, 10 mM phosphate, and 1 mM EDTA. We carried out our melting studies in aqueous buffer containing 1.0 M KCl and 5mM $MgCl_2$. KCl was chosen in preference to NaCl to minimize the possibility of tetrameric self-association by our G-rich strand 3 (12). Magnesium was included to favor the formation of the structures we observed electrophoretically.

The normalized melting curves of the complexes 1′+2′+3, 1′+2′+3AA, 1′+2′+3AAA, and 1″+2″+3AA are compared in Figure 9. The data were analyzed as described above to give the melting parameters compiled in Table 3. A significantly higher melting temperature (T_m) is obtained for the complex 1′+2′+3 than the complexes 1′+2′+3AA or 1′+2′+3AAA. We investigated this further by measuring melting curves for the half duplexes 1′+2′, 1′+3, 1′+3AA, 2′+3, and 2′+3AA. Melting curves for the trimer 1′+2′+3 and for the duplexes 1′+2′, 1′+3, and 2′+3 are compared in Figure 10. The duplexes 1′+2′ and 1′+3 are amenable to two-state analysis whereas the melting of 2′+3 is not.

The melting behavior of the trimer 1′+2′+3AA is compared to that of its component half duplexes in Figure 11. The duplex 2′+3AA is amenable to two-state analysis, whereas the melting of 1′+3AA is not because the low-temperature baseline could not be established. The melting of 1′+3AA occurs below 20°C. The thermodynamic parameters derived for all the complexes which could be analyzed as described above are tabulated in Table 3.

The melting behavior of the bulge-containing complexes 1′+2′+3AA, 1′+2′+3AAA, 1″+2″+3AA″ appears to be cooperative. This conclusion is supported by the measured $\Delta H°$ values of these complexes, which are all significantly larger than for any of the duplex pairs which could be analyzed, and also larger than obtained for 1′+2′+3, analyzed as a trimer. The electrophoretic and gel filtration experiments also provide justification for treating the melting data for these complexes using the formulas derived for trimer complexes. Finally, it should be noted that the T_m's of 1′+2′+3AA and 1′+2′+3AAA are significantly lower than that of the most stable duplex pair, 1′+2′.

The melting of complex 1′+2′+3 was analyzed both as a trimer and as a dimer. Which analysis is more valid? We contend that the melting of 1′+2′+3, in contrast to the complexes containing two or more unpaired adenosines, occurs in two-stages. First, strand 3 dissociates, and then strands 1′ and 2′ separate. This conclusion is supported by the thermodynamic parameters for 1′+2′+3, analyzed as a dimer, which are identical, within experimental error, to those obtained for the most stable dimer pair, 1′+2′. Adding weight to this conclusion are the gel electrophoresis experiments which indicate that even at reduced temperatures, 1′+2′+3 does not form a stable complex.

The melting parameters of complexes 1′+2′+3AA and 1′+2′+3AAA are very similar, but 1′+2′+3AAA does appear to be less stable, within experimental error. The T_m's calculated from the thermodynamic parameters for fixed $C_{total} = 3\times10^{-6}$ M are 23.95°C for 1′+2′+3AA and 22.82°C for 1′+2′+3AAA. In Table 3 we report $\Delta G°(37°C)$ for each complex allowing $\Delta\Delta G°(37°C) = +0.48$ kcal/mol to be calculated for the addition of one unpaired A to the three-way junction 1′+2′+3AA. The melting curves for these two complexes were analyzed identically insofar as the same temperature ranges were used to calculate lower (3°−8°C) and upper (45°−50°C) baselines. Because the lower baseline is not well determined for these complexes, the derived parameters were very sensitive to the choice of temperature range. This was not the case for the upper baseline.

The trimer complex 1″+2″+3AA″ is more stable than the complex 1′+2′+3AA, as expected from the fact that each arm contains 1 more GC basepair. Is the stabilization achieved by the presence of the extra base pairs that which would be predicted from the published parameters derived by nearest neighbor analysis for B-DNA helices (11)? The stabilization calculated from these parameters is $\Delta\Delta H° = 34.1$ kcal/mol, $\Delta\Delta S° = 81.2$ cal/K-mol, and $\Delta\Delta G(25°C) = 9.8$ kcal/mol. From our data for these two complexes we calculate $\Delta\Delta H° = 11.2$ kcal/mol, $\Delta\Delta S° = 33$ cal/K-mol, and $\Delta\Delta G(25°C) = 1.4$ kcal/mol. The discrepancy between the calculated and experimental values for these quantities is large enough to be significant. It suggests that the three-way junction perturbs the structure of the component helices to the fifth and sixth base-pairs such that thermodynamic parameters derived for B-DNA do not apply. By contrast, a thermodynamic study of a four-way junction consisting of eight base-pairs per arm showed that the junction behaved thermodynamically as the sum of its component helices (13).

CONCLUSIONS

The reproducability of relative gel mobilities is quite remarkable and allows one to make quantitative comparisons between different complexes. To demonstrate the reproducability we present the ratio of mobility of the complex 1+2+3AA to the mobility of the 1+2 combination (run in the cold room, 20% native gel containing 10 mM Magnesium−our standard conditions) for six different experiments: 0.66 ± .02, 0.73 ± .02, 0.71 ± .03, 0.69 ± .02, 0.71 ± .02. This results in an average of 0.70 with a standard deviation of .026, a result in agreement with the error analysis.

Removing magnesium ion by adding the chelating agent EDTA results in substantial increase in relative mobility for all species as well as instability of the complexes as judged by the greater degree of smearing of the bands on electrophoresis gels. The requirement for magnesium is further evident in the results obtained with the junctions having elongated reporter arms. A mobility shift is observed for the three-way complex containing unpaired -AA- when it is cleaved in the H arm *only in the presence of magnesium*. In the presence of EDTA, all three cleaved complexes show identical mobilities, indicating that the average angles subtended by the arms are approximately equal or that there is rapid equilibrium between conformationally equivalent structures.

Native gel electrophoresis of trimer complexes at 20°C results in increased relative mobility and smearing of bands corresponding to complexes. The most stable complex at room temperature appears by gel electrophoresis to be the 1+2+3AAA complex. However, the formation of a complex containing three unpaired adenosines (1b+2b+3AAA) could not be observed by size exclusion chromatography at room temperature at 0.1M KCl. UV melting experiments indicate that the 1′+2′+3AAA complex is slightly less stable than the 1′+2′+3AA complex. By comparing the thermodynamic parameters obtained from our UV melting experiments of these complexes we are able to present the first measurement of ΔΔG°(37°C) for the addition of one unpaired nucleotide to a three-way nucleic acid junction. The value we measured (ΔΔG°{37°C} =0.48 kcal/mol) will be verified using the more stable complexes 1″+2″+3AA″ and 1″+2″+3AAA″ for which much better determined lower baselines can be obtained.

The only complex containing a single extra base that gives a relatively sharp band on gels in the cold is the 1C+2+3 complex. However the relative mobility of this complex is significantly greater than that of the complexes containing 2 or more extra bases (0.87 ± 0.026 vs. 0.70 ± 0.023) and it was not affected by running at room temperature. We conclude that 1C+2+3 does not form a structure comparable to the three-way junctions containing two or more unpaired bases.

The complex 1C+2+3A, containing two extra bases on different strands gives a sharp band on gels in the cold with a relative mobility slightly greater than that of 1+2+3AA (0.76 ± .03 vs. 0.70 ± .023), indicating that it is forming a comparable, stable three-way junction. The complexes 1A+2+3A and 1G+2+3A are not as stable, perhaps because they involve two purine nucleotides, whereas the 1C+2+3A complex contains a purine and a pyrimidine. It is unclear why 1T+2+3A appears to be less stable than 1C+2+3A.

The junction containing two extra adenosines on one strand appears to form a bilaterally symmetrical structure in which the strand containing the extra bases is kinked at an acute angle (left panel of Figure 8). This result is at variance with simple-minded ideas about base-stacking, but could be rationalized as follows: The unpaired bases provide the conformational freedom to form a kink in one strand which allows maximal base-pairing and stacking along the other two strands. NMR and computer modelling studies are underway to develop more detailed models of the structures of three-way nucleic acid junctions containing unpaired nucleotides.

It is not possible at this point to quantify the stabilization to a three-way junction provided by including one or two unpaired nucleotides because the reference complex, 1′+2′+3, did not melt as a trimer. These data will be forthcoming using complexes containing six or seven base pairs in each arm, such as 1″+2″+3″. The addition of a third base (complex 1′+2′+3AAA) does not greatly perturb the thermodynamic stability of the junction relative to the complex 1′+2′+3AA. This is consistent with the notion that the unpaired bases are looped out of the junction, allowing the bulge-containing strand to kink. Thus our thermodynamic data also supports the structural model obtained from electrophoresis experiments.

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