Volume 14 Number 24 1986 Nucleic Acids Research

Three-arm nucleic acid junctions are flexible

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Received 12 September 1986; Revised and Accepted 13 November 1986

Abstract

Nucleic acid junctions are stable analogs of branched DNA structures which occur transiently in living systems. We show here that junctions which contain three double helical arms can be enzymatically oligomerized, using conventional sticky-ended ligation procedures, to create larger complexes. The products consist of a series of linked junctions separated by 20 base pairs. Junction dimers are formed that have free termini only, whereas trimers and larger species are found to be both unclosed and cyclized. The formation of a series of macrocyclic products which, surprisingly, begins with trimers and tetramers indicates that this junction is flexible about a bending axis, and perhaps twist-wise as well. We have obtained the same results from three different 3-arm junctions, two in which the junction is flanked by a 3 Watson-Crick base pairs, and one in which a G-G base pair flanks the junction.

It is possible to design oligonucleotides which will preferentially associate to form the arms of stable branched nucleic acid structures (called immobile junctions) via Watson-Crick base pairing[1-3]. We have recently demonstrated that these imobile junctions can be formed from oligonucleotides in solution[4,5]. It has been suggested[1,2,6,7] that immobile nucleic acid junctions can serve as macromolecular valence clusters, capable of linkage by the enzymatic ligation procedures[8] commonly used with sticky-ended linear duplex DNA. These building blocks could then be used to build up geometrical stick figures, on the 10-10000 A scale. The vertices of these figures are formed by the junctions, while their "edges" consist of double helical nucleic acids. We have tested this suggestion with junctions containing three arms, two of which terminate in complementary sticky ends (Figure 1). Here we report the enzymatic formation of a series of covalently joined structures containing two or more junction components. Those structures containing three or more junctions are able to form closed macrocyclic figures. This behavior suggests that the three-arm junction behaves as a flexible structure, rather than as a rigid body.

The three strands comprising the junction shown in Figure 1 (designated Jy), contain 18, 20 and 22 nucleosides. This junction was designed so that joining two sticky-ended arms would yield a junction-to-junction separation of 20 nucleotide pairs, approximately two complete double helical turns. A second junction (designated Js), composed of 3 20-mers also yields a 20 nucleotide pair separation upon ligation. The double helical structure of DNA makes the torsion angle between two ligated junctions a function of their separation[6]; the separation of twenty

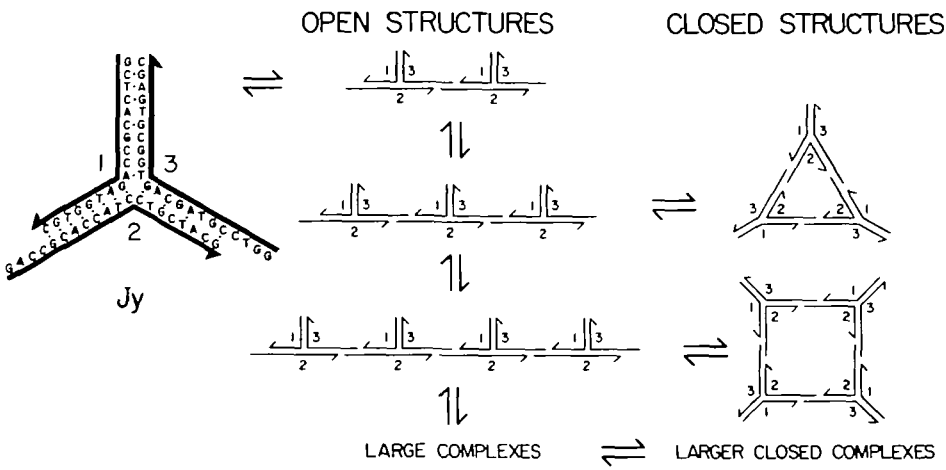

Figure 1. The Ligation of Nucleic Acid Junctions. On the left, this figure shows the sequence of the 3-arm junction, Jy, and its main cohesive-ended oligomerization products. The half-arrows indicate the 5′–>3′ direction of the nucleotide chain. The individual components are maintained throughout the diagram, for clarity, but ligation would seal the gaps between successive strands. In the center the smallest open products are illustrated, schematically, while the closed products are at the right. Note that following the fate of the ligation of strand 2 is equivalent to following the entire junction.

residues was chosen to permit the formation of planar closed structures if the junction is reasonably loose, and was not expected to impose much twist on the structure.

Previously, we have constructed junctions with four arms, and have determined their stoichiometry and frictional properties using electrophoresis on polyacrylamide gels[4,5]. In our current experiments, the products have also been characterized on polyacrylamide gels. Figure 2a summarizes the electrophoretic behavior of a 1:1:1 stoichiometric mixture of Jy strands 1, 2 and 3, in the absence of any ligation enzyme. The appearance of a "ladder" of species of progressively decreasing electrophoretic mobility, in this stained native gel, is consistent with sticky-ended cohesion of the unitary complex.Figure 2b shows the electrophoretic behavior of the strands that comprise Jy. Note that the extensive ladder pattern is only observed when all three strands are present in the complex. In the absence of the sticky ends, the three 16-mers which form the core of Js run as a single unitary complex, with no evidence for a "ladder" (gel not shown). Thus, in the presence of high Mg^{2+} and low temperature (4° C .), native polyacrylamide gels reveal cohesive association of sticky-ended complexes.

Having shown that Jy oligomerizes in the stoichiometric mixture, it is important to observe its behavior in ligation experiments. In each of the ligation experiments reported here:

- strand 1 was unphosphorylated at the 5′ end,
- strand 2 was labelled with 5′ ^{32}P phosphate,
- strand 3 was kinased with non-radioactive 5′ phosphate.

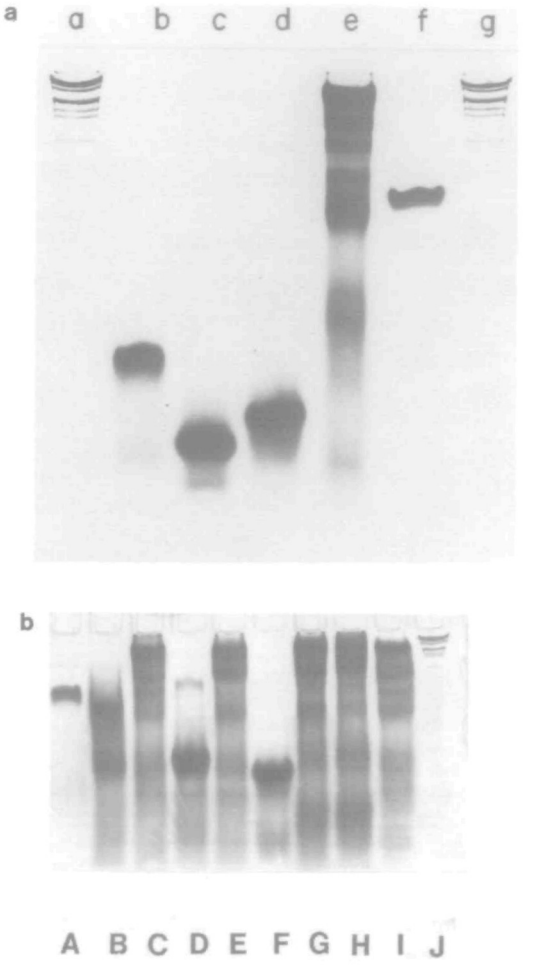

Figure 2. Native Stained Gels Showing the Cohesion of 3-arm Junctions. The native gels shown here are 20% polyacrylamide, run in a buffer containing 40 mM Tris-acetate, 2 mM EDTA, pH8, (TAE buffer), to which 12.5 mM magnesium acetate is added.
(a). Lanes a and g are reference Hae III restriction endonuclease digests of ØX 174-RF DNA; the lowest band is 72 base pairs long, and the next is 118 base pairs long. Lanes b, c and d are respectively individual Jy strands 1 (18-mer), 2 (20-mer) and 3 (22-mer). Lane e is an equimolar mixture of the three strands. Lane f is a 4-arm junction (J1) composed of hexadecamers[4], which lacks sticky ends. The most prominent feature of this gel is that the junction formed in lane e oligomerizes because of its sticky ends. ATP-driven ligation changes this distribution towards the higher-molecular weight species (not shown). Note that the reduced mobility of strand 1 indicates that it pairs with itself in the absence of the other strands; this band is absent in their presence.
(b). Equimolar mixtures of : lane a, the four strands of J1; lane b, strands 2+3 of Jy; lane c, strands 1p+2+3 of Jy; lane d, strands 1+3 of Jy; lane e, strands 1+2p+3; lane f, strands 1+2: lane g, strands 1+2+3p; lane h, strands 1+2p+3p; lane i, strands 1+2+3. A p following the strand number above indicates that the strand is phosphorylated at the 5' end. Lane j contains a Hae III digest of OX 174 -RF DNA, the lowest visible band of which contains 118 b.p.

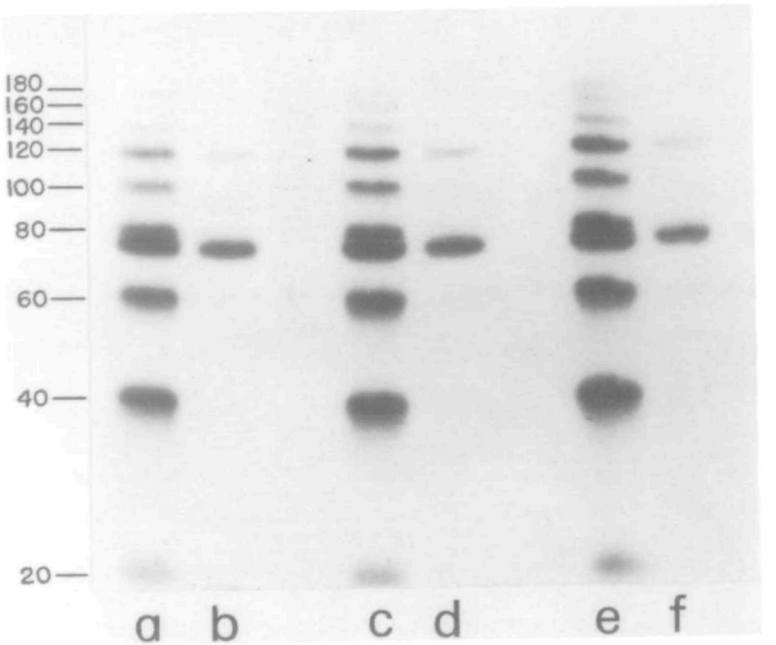

Figure 3. A Triplicate Oligomerizing Experiment. Lanes a, c and e all represent autoradiograms of the electrophoresed products of an oligomerization reaction of Jy, catalyzed by T4 DNA Ligase, on a denaturing 10% polyacrylamide gel (TAE buffer,7M urea, 55 °C) . The lowest band corresponds to a single 20-mer, unligated strand 2. As indicated, the bands above it represent strands containing 40, 60, 80, 100, 120, 140, 160 and 180 residues. Note the thickening of the bands at 80 and 120, corresponding to tetramers and hexamers of the junction. When the products thus obtained are treated with Exonuclease III, the patterns seen in lanes b, d and f result. These bands correspond to closed structures without 3′ ends. Circular strands with mobilities corresponding those of linear single strands containing about 60 and 80 residues are the major products shown here, although larger closures through 120-mers have been detected. Nicking and sizing these bands shows that these circles are indeed 60 and 80 residues long.

This phosphorylation scheme prevents ligation involving the blunt-ended arm, yet permits the sticky-ended ligation of the other two arms (see Figure 1). This figure also shows that the progress of the reaction can be monitored by following the oligomerization of strand 2; since only this strand is radioactively labelled, monitoring may be done conveniently by autoradiography.

The non-covalent cohesion noted in Figure 2 suggests that these junctions should be readily ligated into oligomers. This is confirmed by the experiment shown in Figure 3 (lanes a, c and e), which demonstrates covalent joining of the strands by DNA ligase. However, the pattern in Figure 3 suggests that the size distribution of the concatenates is not a monotonically decreasing one, as would be expected from a simple linear polymerization reaction at equilibrium[9]. To test whether the concatenated chains represent open, linearly extended structures or closed macrocyclical ones, we carried out the exonuclease digestion experiment summarized in Figure 3, lanes b, d and f.

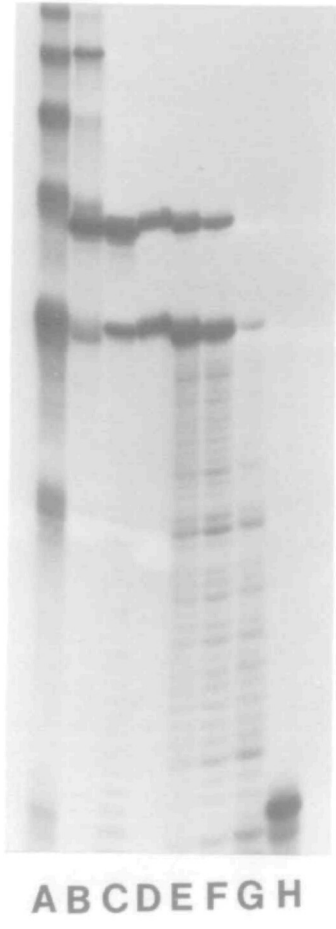

Figure 4. Characterization of the Exonuclease III Resistant Bands. This is an autoradiogram of samples with strand 2 kinased with 32 P phosphate at the 5' end, and electrophoresed in a 10% polyacrylamide gel, in 7M urea. Lane a: a ligated complex of strands 2 and 3; lane b: an exonuclease III digested complex of strands 1+2+3 following ligation; lane c: excised exonuclease III-resistant bands, after limited acid hydrolysis (pH 5, 3 hours, 70 ^{o}C, followed by alkaline treatment); lanes d-g: the sample treated as in c, exposed to the Maxam-Gilbert reaction specific for G, for 4,10,20 and 40 minutes, respectively; lane h: strand 2 alone. Note that the monomer band corresponds to a 20-mer, and that the circle is therefore a 60-mer, which is a trimer of strand 2. The appearance of the intrinsic G-sequence pattern in the forty bands above the monomer reflects site selective nicking of the circle.

Exonuclease III of E. Coli has the ability to catalyze hydrolysis of double stranded DNA chains from an exposed 3′ terminus[10]. Exhaustive digestion of the products of the ligation reaction (lanes 3a, 3c and 3e) with Exonuclease III reveals a second logarithmic series, corresponding to nuclease resistant strands (lanes 3b, 3d and 3f). These nuclease resistant bands contain single-stranded

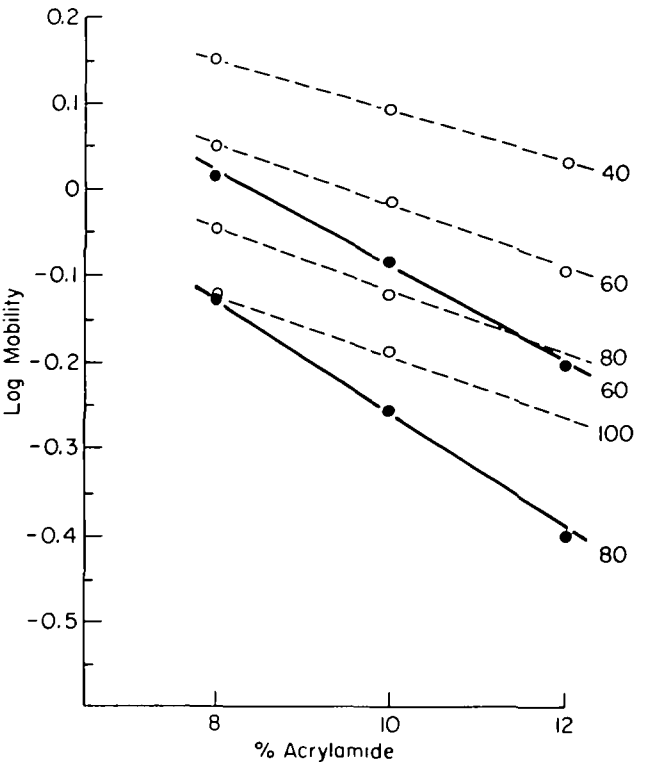

Figure 5. Ferguson Plot of the Linear and Cyclic Species Resulting from the Oligomerization of Jy. Gels were cast with different compositions of total acrylamide containing 7M urea (60°C), and the mobility of the cyclic 60-mer and 80-mer compared with that of the linear 40-mer, 60-mer, 80-mer and 100-mer. The ordinate is the logarithm of the mobility to base 10. The linear molecules are indicated by the dotted lines, while the circular molecules are represented by solid lines. Xylene Cyanol FF was used as the reference dye in these gels.

circular species. When the prominent strands are excised from the gel, nicked, and sized on a denaturing gel (Figure 4), their lengths correspond to trimers, tetramers and successively higher consecutive multiples of the unit 20-mer (strand 2), consistent with the major macrocyclized structures indicated in Figure 2.

We investigated the mobility of these cyclic species on a series of polyacrylamide gels. Figure 5 shows a Ferguson plot of the circular single-stranded 60-mers and 80-mers, compared with the linear single-stranded 60-, 80- and 100-mers. The friction constant of these cyclic species, estimated from the slope of the Ferguson plot[11] is larger than for the linear species, over the range of compositions studied.

In the ligation experiment involving the junction Jy, the junction region was flanked by potential Watson-Crick pairing partners G-C, C-G and T-A. Replacing the first G-C pair with a

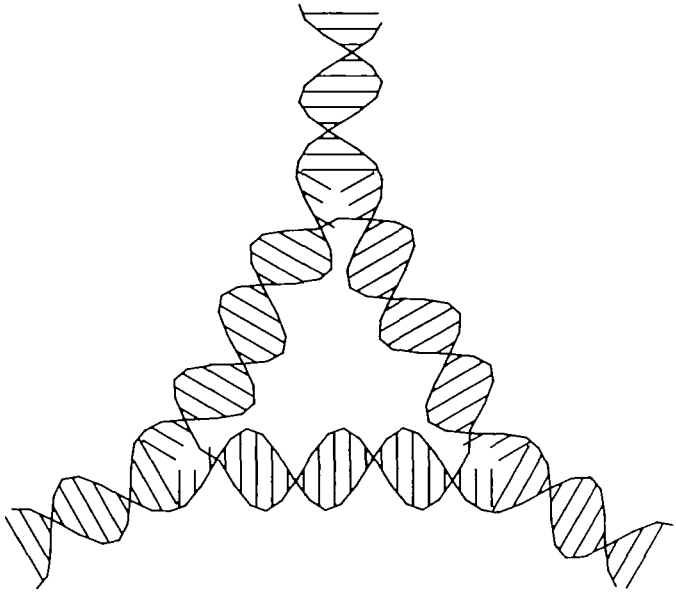

Figure 6. A Model of the Cyclic Trimeric Species of Jy. The three 3-arm junctions have been joined together, with the exocyclic double helix bisecting the exterior angle of the equilateral triangle. Note that this configuration results in some unpaired bases in the vicinity of the junction. If this model is representative, it would account for the extreme flexibility of the junction as well as its insensitivity to a base-pair mismatch.

G-G pair had no effect upon the results. Neither did substitution of the three pairs with T-A, A-T and G-C, in a parallel series of experiments involving Js (data not shown). The results of oligomerizing Js by this protocol were identical to those for Jy, but the details of those ligations will be treated elsewhere. The invariance of the oligomerization results to the sequence which flanks the junction, and its Watson-Crick pairing capability in particular, suggests that the bases at the branch site are only loosely paired. Molecular model-building of the macrocyclic trimer is consistent with this possibility, as shown in Figure 6. If the bases which flank the 3-arm junction are, indeed, not completely paired, these junctions are not necessarily immobile, regardless of sequence, as has been assumed[2,12].

The analysis of 3-arm junction dynamics is more complex than for an analogous 3-connected bonded atomic system such as the nitrate anion. Even if one treats the arms as rigid, ignoring the torsional flexibility of individual covalent bonds, there are three degrees of freedom for each of the paths through the junction. There are 3 paths through a 3-arm junction and A(A-1)/2 for a junction with A arms. The degrees of freedom are most readily treated as the bending of the two helix axes about an axis normal to the junction (as in the nitrate anion), a twisting about the helix axis as it passes through the junction on this path, and a third bending direction perpendicular to the other 2 axes.

From a single qualitative experiment, it is not possible to draw conclusions about all these degrees of freedom. However, it is clear that some combination of the two bending modes results in significant flexibility for the junction, since all macrocycles greater than two can be formed. Analogy to trigonal chemical species suggested by inter-arm phosphate-phosphate repulsion would lead to a 120° inter-arm angle for Jy. Formation of macrocycles with 3,4,5,... units indicates that this angle is variable in the junctions we have studied. Nevertheless, formation of the macrocyclic trimer was quite unexpected.

The twisting through the junction is less clearly defined by these experiments. The inter-junction separation of 20 nucleotide pairs is actually inconsistent with the currently accepted double helix geometry of about 10.5 base pairs per turn[13,14]. The twist-debt resulting from this discrepancy is about 34 degrees per arm. Using a random-walk statistical model with the known flexibility of DNA[15], simple (un-supercoiled) closure would occur at about 2.2, 2.6 and 2.85 sigma (2.9%, 1.1%, .5% of the time) for the closed trimer (twist-debt = 103°), tetramer (137°) and pentamer (171°), respectively, if the double helical dinucleotide unit appropriately represented both the torsional stiffness and the twist of the helix as it traverses the 3-arm junction. Nevertheless, it is clear from Figure 3 that macrocyclic products are present to a significant extent. Thus, either the twisting flexibility is larger for the junction than for a routine dinucleotide unit, or the trans-junctional twist itself is somewhat greater (ideally about 34° greater) than for a dinucleotide. Whether stiffness or geometry is being compensated can be determined by means of detailed experiments similar to those of Shore and Baldwin[16-18].

The closure reactions reported here indicate a surprising amount of flexibility for the 3-arm immobile nucleic acid junction. Thus,one appropriate paradigm for this "soft" junction appears to be closer to a marshmallow impaled by three sticks, rather than a rigid trigonal cluster such as the fork of a slingshot. (Other models are left to the reader's imagination.) Two further points should be noted in this discussion. First, the ligation reaction requires ATP as a cofactor, and this could influence the local conformational equilibrium. Second, the macrocyclization traps covalently joined molecules. The distribution of products corresponds to an effective picture of the reaction taken over many hours of exposure to enzyme. All conformational excursions compatible in a cyclic structure are thus accumulated, and the resulting distribution does not conform to that of instantaneous equilibrium, in the absence of ligase. Finally, it must also be borne in mind that we do not know whether or not the "average" structures formed by macrocycles are planar or whether they represent more complicated space curves. Nevertheless, the success of the ligation reactions in this oligomerization experiment suggests that junctions with specifically designated sticky ends[6] can be ligated to yield unique products.

Acknowledgements
We would like to thanks Drs. Brian Rymond and Nancy Casna for advice on ligation reactions, Dr. Richard Cunningham for his generous gift of Exonuclease III and for his advice on nicking single stranded circles, and Ms. Sissel M. Kjelstrup and Mr. John Mueller for rendering valuable

technical assistance. This work has been supported by Grants GM-29554, ES-00117 and CA-24101 from the National Institutes of Health.

+Recipient of an NIH Research Career Development Award

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