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Construction of Circular Oligodeoxyribonucleotides on the New Structural Basis of i-Motif

Tianhu Li,* Dongsheng Liu, Jian Chen, Alex H. F. Lee, Jianying Qi, and Albert S. C. Chan*

Open Laboratory of Chirotechnology of the Institute of Molecular Technology for Drug Discovery and Synthesis and Department of Applied Biology and Chemical Technology The Hong Kong Polytechnic University Hung Hom, Kowloon, Hong Kong

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Circular oligonucleotides possess many distinctive properties, when compared to their linear counterparts, such as higher DNA-binding affinity, greater sequence selectivity, enhanced resistance to degradation by exonuclease, and an ability to serve as efficient templates for DNA and RNA polymerase.[1−3] The earliest preparation of this type of circular oligonucleotide relied on solid-phase synthesis and had the limitation that only circular sequences with less than 10 nucleotides could be made efficiently.[4] For the preparation of longer sequences of circular oligonucleotides, template-directed methodologies were subsequently developed in conjunction with enzymatic or chemical ligation to facilitate the formation of phosphodiester linkages. Double-helical complexes of linear oligonucleotides in a "dumbbell" conformation were, for example, utilized as open precursors to generate *self-paired* circular oligonucleotides of over 20 nucleotides in length.[5] Most significantly, a strategy for constructing *unpaired* circular oligonucleotides was performed by using the triple helical conformation of oligonucleotides as intermediates.[2] These assemblies do not possess strong internal structures and are therefore readily able to form complexes with their target nucleic acids.[1] These unpaired circular oligonucleotides have since played significant roles in molecular diagnostics, hybridization, and sequence-specific inhibition of gene expression.[1,6] We now report for the first time that beyond the scope of the previous duplex and triplex strategies, the *i-motif*,[7,8] a four stranded assembly, can direct the sequence-specific formation of a phosphodiester linkage and thus represents a new type of structural template for constructing circular oligonucleotides.

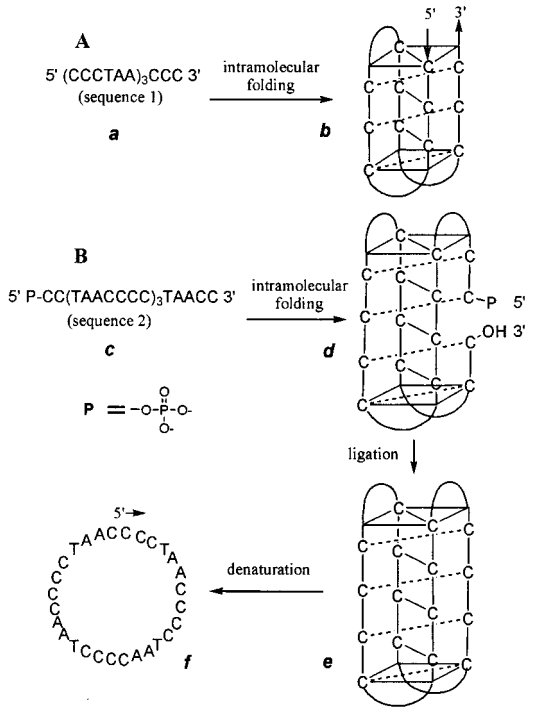

Figure 1. (A) Diagrammatic illustration of the conformation of i-motif adopted by 5′-(CCCTAA)$_3$CCC-3′ (sequence **1**), a short portion of the vertebrate telomeric end, in which the 5′- and 3′-termini are separated from each other.[8] (B) Schematic representation of our designed circularization course of linear oligodeoxyribonucleotides. The 3′-hydroxyl and 5′-phosphate termini are juxtaposed once the linear 28-mer folds up intramolecularly, the conformation vital to the circularization process.

The i-motif is a structural entity composed of two parallel-stranded duplexes zipped together in an antiparallel orientation held together by hemiprotonated C−C$^+$ base pairs. This compact feature can also be generated unimolecularly by 5′-(CCCTAA)$_3$-CCC-3′ (sequence **1**), a short portion of the vertebrate telomeric ends, under a slightly acidic condition (see ***b*** in Figure 1A).[8] On the basis of the currently available information on the physical properties of the i-motif,[7,8] sequence **2**, (5′-CC(TAACCCC)$_3$TAA-CC-3′) (Figure 1B), was designed for our studies with the expectation that the 3′-hydroxyl and 5′-phosphate ends would be proximal to each other, once the structure folds up intramolecularly (see ***d*** in Figure 1B). The juxtaposition of these two termini would be in the ideal conformation for the formation of a natural phosphodiester bond upon chemical activation.[2,9]

Sequence **2** (200 nM) in a pH 5 buffer containing 100 mM MES [2-(*N*-morpholino) ethanesulfonic acid monohydrate] and 50 mM NaCl was incubated at 4 °C for 2 h to allow the desired conformation of the i-motif to form (see ***d*** in Figure 1B). The 5′-terminal phosphate was then activated by addition of *N*-cyanoimidazole in the presence of $MnCl_2$[10] to effect the formation

* Corresponding authors. E-mail: bctli@polyu.edu.hk or bcachan@polyu.edu.hk.

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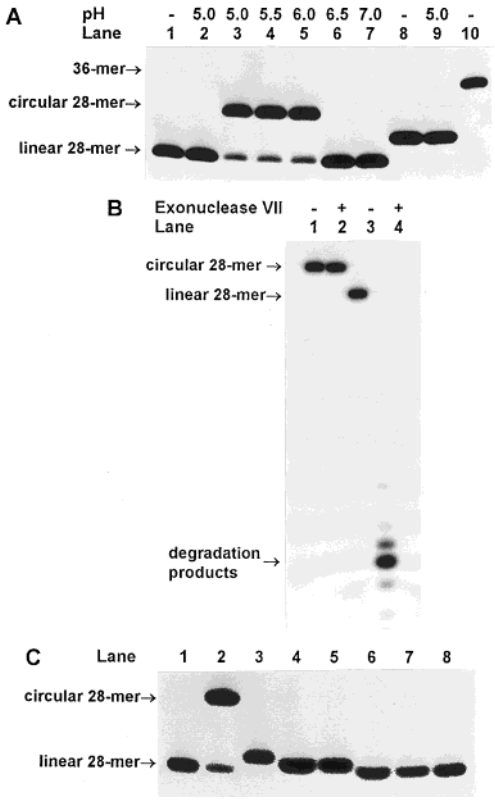

Figure 2. Circularization of linear oligodeoxyribonucleotides and product analysis. Radioactive linear strands were prepared by phosphorylation of the synthetic oligodeoxyribonucleotides at their 5′-ends with [γ-^{32}P] ATP and T4 polynucleotide kinase. Reaction products were analyzed by 20% PAGE and visualized by autoradiography. (A) Generation of circular oligodeoxyribonucleotides from their linear precursors. Lane 1: sequence **2** alone. Lanes 2−7: 200 nM sequence **2** in 100 mM MES and 50 mM NaCl was kept at 4 °C for 2 h to allow the intramolecular folding to take place. The reaction mixture consisting of 100 nM sequence **2**, 100 mM MES, 50 mM NaCl, 100 mM $MnCl_2$ and 50 mM *N*-cyanoimidazole in a total volume of 40 μL was then prepared and incubated at 4 °C for 12 h. These reactions were terminated through refrigeration and further analyzed via PAGE. In a control experiment, *N*-cyanoimidazole was omitted from the reaction mixture (lane 2). Lane 8: 5′-TT(TAATTTT)$_3$TAATT-3′ alone. Lane 9: Same reaction as the one loaded in lane 3 except for replacing sequence **2** with 5′-TT(TAATTTT)$_3$TAATT-3′. Lane 10: 36-mer [5′-CCC(TAACCCCCC)$_3$TAACCC-3′] alone. (B) Hydrolysis of the ligation products by exonuclease. Lane 1: Ligation products alone. Lane 2: Ligation product after treatment with 20 units of exonuclease VII at 37 °C for 2 h. Lane 3: sequence **2** alone. Lane 4: sequence **2** after treatment with 20 units of exonuclease VII at 37 °C for 2 h. (C) Sequence-dependency of the circularization process. Lane 1: sequence **2** alone. Lane 2: Same reaction as the one loaded in lane 3 in Figure 2A. Lanes 3−8: Same reaction as the one loaded in lane 2 except for that sequence **2** was replaced with 5′-GG(TAACCCC)$_3$TAACG-3′ (lane 3), 5′-GG-(TAACCCC)$_3$TAACC-3′ (lane 4), 5′-GC(TAACCCC)$_3$TAACG-3′ (lane 5), 5′-GC(TAACCCC)$_3$TAACC-3′ (lane 6), 5′-AC(TAACCCC)$_3$TAACC-3′ (lane 7) and 5′-TC(TAACCCC)$_3$TAACC-3′ (lane 8).

of a phosphodiester bond with the adjacent 3′ hydroxyl group (see ***e*** in Figure 1B). After being kept at 4 °C for 12 h, the reaction mixture was analyzed via denaturing polyacrylamide gel electrophoresis (PAGE). As observed on the autoradiograms (Figure 2A), a new product (lane 3) was generated from the ligation reaction with a mobility shift between sequence **2** (lane 1) and a 36-mer of molecular standard (lane 10), which was identified as the resultant circular oligonucleotide from the linear precursor (see the course from ***d*** to ***e*** in Figure 1B). The reactions yielded 83, 84, and 82% of the desired circular oligonucleotide at pH 5.0, 5.5, and 6.0 (lanes 3, 4, and 5) respectively and less than 10% at pH 6.5 and 7.0 (lanes 6 and 7). This pH dependency is in agreement with the necessity for the protonation of cytosines, a prerequisite for forming the structural feature of i-motif by the cytosine tracts.[7] Within experimental limit, no new product was observed (lane 9) when substitution of all cytosines for thymines was done within sequence **2**, demonstrating that the presence of cytosines is a prerequisite for the circularization process as it is required for constituting the structural formation of the i-motif.[8]

N-Cyanoimidazole is a highly efficient condensing reagent for promoting the formation of the phosphodiester linkage between the two termini of oligonucleotides in their proximal positions.[10] To verify that a circular structure is indeed generated upon the activation by *N*-cyanoimidazole, the product of this ligation reaction was purified by gel electrophoresis and digested with exonuclease VII (Amersham Phamercia Biotech, USA), exodeoxyribonuclease that degradates DNA from both 3′- and 5′-termini.[2,11] As shown in Figure 2B, this ligation product was completely resistant to degradation by this exonuclease (lane 2), indicating the absence of open ends within the newly formed structure. As a control experiment, the linear precursor of sequence **2** was also digested by exonuclease VII under identical conditions, which consequently gave rise to products of lower molecular-weight in near completion (lane 4, Figure 2B). The susceptibility of sequence **2** to degradation by exonuclease VII is due to the presence of an open 5′- or 3′-terminus within the linear 28-mer. Besides exonuclease VII, digestion of the ligation product was conducted using exonuclease I (an exodeoxyribonuclease degrading single-stranded DNA in the 3′- to 5′-direction, Epicentre Technologies, U.S.A.) and alkaline phosphatase (Amersham Phamercia Biotech, U.S.A.)[11] respectively (data not shown). Similarly, no product of degradation was observed, which further supported the suggestion that the newly formed structure was circular in nature.

If the structural feature of ***d*** (Figure 1B) is truly an intermediate of the circularization process, any deviation from this conformation will diminish the generation of phosphodiester linkage. To examine this conformation-dependent effect, six new sequences were designed in which one, two or three bases are noncytosine at their 5′- and 3′-termini, respectively. As shown in Figure 2C, none of the six sequences possessing "mismatched" bases exhibited any indication of the formation of a circularization product (lanes 3−8) under the same reaction condition as that designed for sequence **2** (lane 2). These experimental results suggested that an intact structural feature of the i-motif sustaining the correct proximity between the 5′- and 3′-termini was vital to the newly established circularization process.

In conclusion, similar to the double and triple helices, the structural feature of the i-motif is capable of directing the formation of circular oligonucleotides with high efficiency and high sequence-selectivity. Unlike the double and triple helical structures assembled via *complementary* base-pairings, the formation of the i-motif is at the structural level of individual bases, a *self-recognition* process. Utilization of the unique self-recognition pattern of the i-motif in the current study consequently not only represents a distinctive strategy for constructing circular oligonucleotides but also opens up a new method for the synthesis of oligonucleotide sequences which are not accessible via the double and triple helical methodologies.

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