Synthesis of unimolecularly circular G-quadruplexes as prospective molecular probes

Tianyan Zhou[1,4], Guoshu Chen[3], Yifan Wang[4], Qiang Zhang[1], Ming Yang[2] and Tianhu Li[4,*]

[1]Department of Pharmaceutics, School of Pharmacy, [2]National Research Laboratories of Natural and Biomimetic Drugs, Peking University, 38 Xueyuan Road, Hiandian District, Beijing 100083, The People's Republic of China, [3]Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong and [4]Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543

ABSTRACT

Synthesis of unimolecularly circular G-quadruplex has been accomplished for the first time during our investigation on the template basis of G-quadruplex through chemical ligations of guanine-rich linear sequences of oligodeoxyribonucleotides. The uniqueness of this newly designed circularization course is its self-recognition and self-templating on the scale of individual strand of oligodeoxyribonucleotide in which the same linear sequence serves both as a template and as a substrate simultaneously. The results from our exonuclease and DNAse hydrolysis studies confirm that there is indeed absence of open termini within the structure of the identified circular product. Our subsequent investigation on the loop-size effect indicates that the unimolecularly circular G-quadruplex possessing two or more thymine nucleotides within their connecting loops is readily attainable, while the linear sequence with a single thymine nucleotide between guanine tracts is not a proper precursor for our ligation reaction. In addition, conformation dependency of the circularization course as well as the effects of alkali ions, pH values and concentration of potassium ions on the circularization reaction are examined during our investigation. The implication of our current studies and possible application of the obtained unimolecularly circular G-quadruplex in certain biological processes are also discussed in this report.

INTRODUCTION

The structural feature of G-quadruplex is a self-assembling entity of DNA composed of two or more stacks of G-quartets, in which four guanines are arranged in a square planar array (1–8). The adjustable nature of strand stoichiometry (9–17) allows this self-assembling entity of G-quadruplex to be formed by the association of either one (unimolecular), two (bimolecular) or four (tetramolecular) oligomeric molecules. The well-investigated examples of the linear oligodeoxyribonucleotides that constitute unimolecular, bimolecular and tetramolecular structures of G-quadruplex (Figure 1) include $d(G_3T_2AG_3T_2AG_3T_2AG_3)$ (3.5 human telomere), $[d(G_4T_4G_4)]_2$, (Oxy-1.5) and $[d(TGGGGT)]_4$ (12,18–22), respectively. We reported previously (23) that certain bimolecular structures of G-quadruplex were capable of acting as templates for directing the formation of circular oligodeoxyribonucleotides with high efficiency and sequence specificity. One of the intriguing aspects of these bimolecular G-quadruplex entities as templates was its mutual templating fashion on the scale of individual oligodeoxyribonucleotides, in which one linear strand serves as a template for another. This mutual-templating pathway consequently led to a unique G-quadruplex entity constituted of two identical circular oligodeoxyribonucleotides (23).

The guanine-rich sequences that exhibit potential for the formation of G-quadruplex are found in a number of important DNA regions, such as those present at the ends of telomeres (24), in the promoter region of c-myc and other oncogenes (25) and the upstream of the insulin gene (26). In addition, some aptamers, such as an inhibitor of HIV integrase (27) and the thrombin-binding aptamer (28), are formed through the utilization G-quadruplex as their structural cores. Moreover, several G-quadruplex-binding proteins have been identified over the past 10 years, such as rat liver proteins uqTBP25 (29). In most of these cases, however, the structural feature of G-quadruplex formed was a unimolecular entity (24–29) rather than a bimolecular complex. It consequently becomes apparent that the unimolecular structure of G-quadruplex plays certain significant roles in various types of biological processes. On the basis of these earlier discoveries (24–29), construction of unimolecularly circular G-quadruplexes tagged with fluorescent molecules ('j' in Figure 2) as molecular probes was accordingly set up as a long-term research objective in our laboratories at an earlier time. One of the advantages in our selection of circular oligodeoxyribonucleotides as diagnostic probes is their

---
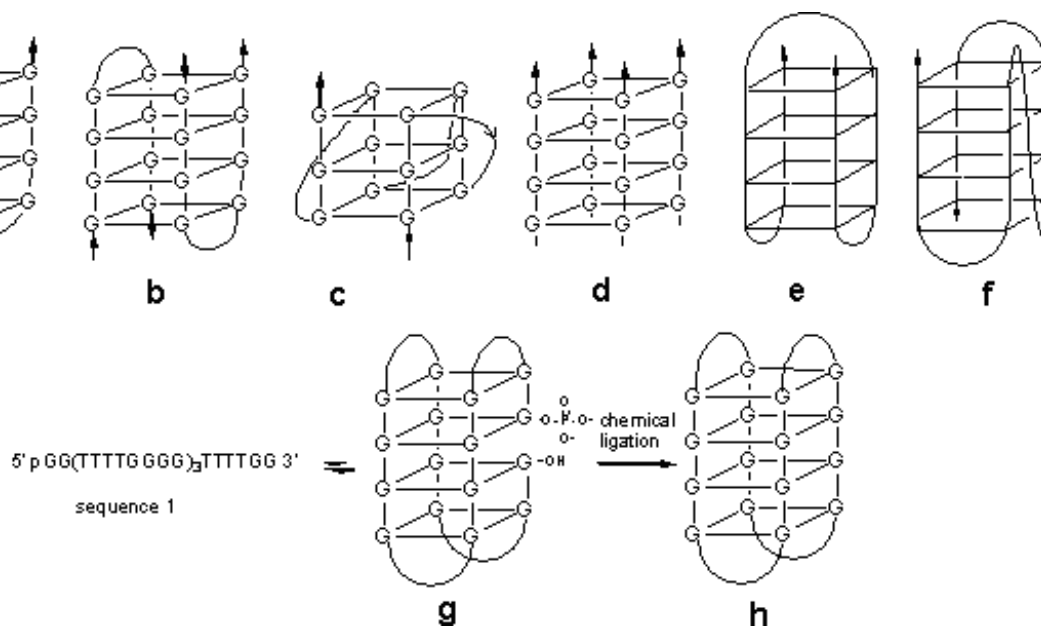
Figure 1. Schematic illustrations of certain structural features of G-quadruplex and our strategy for constructing circular G-quadruplex through chemical ligation.

complete resistance to exonuclease hydrolysis, a property resulted from the absence of open terminus within their structures (30). In addition, the structured DNA composed of circular sequence often possesses a higher melting point than those constituted of linear components, while higher stability is always preferred in the design of molecular probes (31). We hence anticipate that the fluorescence-labeled circularly unimolecular G-quadruplexes ('j' in Figure 2) could, once it has been attained, act as useful probes for identifying new types of enzymes and other functional proteins correlated with G-quadruplex *in vivo* and for elucidating the binding patterns between G-quadruplex and its corresponding binding proteins. In addition, it can be expected that the fluorescent-tagged G-quadruplex could be a useful molecular probe for tracing possible subcellular location and distribution of G-quadruplex-binding proteins inside living cells.

As the initial step, we explored the feasibility of constructing the unimolecular G-quadruplex without a fluorescence tag ('h' in Figure 1). Here, we report that certain unimolecularly circular G-quadruplexes can indeed be attained from linear precursors through chemical ligation reactions. It can be envisaged that the circular structure of G-quadruplex in the absence of fluorescent tag in its structure could be useful for interfering the regular biological function of G-quadruplex-binding proteins through physical interaction between them. In consideration of the fact that several new proteins discovered during the past few years facilitate either the formation of linear unimolecular G-quadruplex or disintegration of it (32–38), we anticipate that the unimolecularly circular G-quadruplexes attained in our current studies ('h' in Figure 1) could be a unique tool for unveiling the winding and resolving mechanisms of G-quadruplex mediated by these G-quadruplex-promoting and disintegrating proteins.

It should be pointed out that a unimolecular circular sequence tagged with fluorescence ('j' in Figure 2) rather than its bimolecular counterpart (k) was chosen as the synthetic target of our molecular probe. It is known that the concentrations of diagnostic molecules in many practical applications are very low (39,40), while bimolecular complexes (k), once dissociated into the corresponding unimolecular components (l), might not readily go back to its original state at a very low concentration due to kinetic unfavorableness. In addition, a fluorescence-tagged bimolecular G-quadruplex can in theory possess two isomeric forms (k and m), while one of them (m) may not be suitable as a molecular probe because of the presence of fluorescence tags at both ends of its columnar structure that prevent the approach of G-quadruplex-interacting proteins. This co-existence of two isomeric forms could decrease the efficiency of bimolecular G-quadruplexes as molecular probes in practical application. In contrast, unimolecular G-quadruplex ('j' in Figure 2) could, even if it is denatured occasionally, readily return to its original folding state as the result of kinetic favorableness. It consequently appears that the selection of unimolecular G-quadruplexes as molecular probes has certain advantages over the choice of their bimolecular counterparts.

MATERIALS AND METHODS

Oligodeoxyribonucleotides and reagents

All oligodeoxyribonucleotides used in these studies (Table 1) were purchased from Operon Technologies, Inc. Radioactive strands of oligodeoxyribonucleotides were prepared by phosphorylation of the synthetic sequences at their $5'$ ends with T_4 polynucleotide kinase and $[\gamma\text{-}^{32}P]ATP$ and purified through PAGE. The linear precursors in a typical ligation reaction mixture (20 µl) were composed of mainly non-radiolabeled $5'$ phosphorylated sequence purchased commercially (1 µM) and a small amount of the same sequence with a radiolabeled phosphate at its $5'$ end ($\sim$10 nM). The latter was obtained from a phosphate transfer reaction between $[\gamma\text{-}^{32}P]ATP$ and the
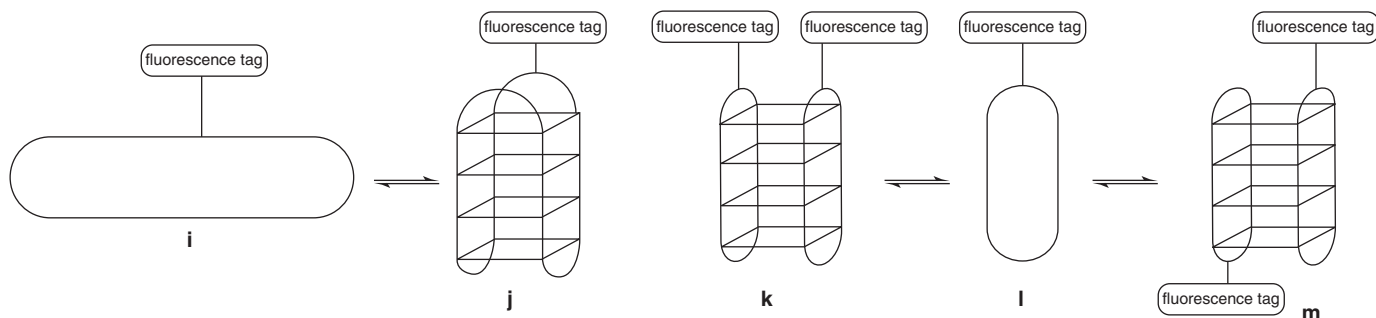
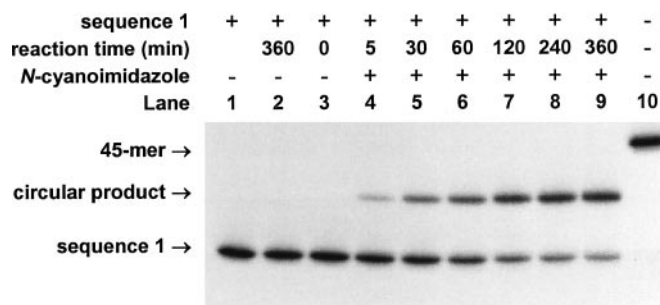**Figure 2.** Structural representation of fluorescence-labeled circular G-quadruplexes.

Table 1. Nucleotide sequences of oligodeoxyribonucleotides used in the current investigation

Oligomer name	Nucleotide sequences
Sequence 1	5′-GGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGG-3′
Mis-1	5′-TGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGG-3′
Mis-2	5′-TGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGT-3′
Mis-3	5′-TTTTTTGGGGTTTTGGGGTTTTGGGGTTTTGT-3′
Mis-4	5′-TTTTTTGGGGTTTTGGGGTTTTGGGGTTTTTT-3′
Rec-1	5′-GTTTTGGGGTTTTGGGGTTTTGGGGTTTTGG-3′
Rec-2	5′-TTTTGGGGTTTTGGGGTTTTGGGGTTTTGG-3′
Rec-3	5′-TTTTGGGGTTTTGGGGTTTTGGGGTTTTG-3′
Rec-4	5′-TTTTGGGGTTTTGGGGTTTTGGGGTTTT-3′
Lp-1	5′-GGTGGGGTGGGGTGGGGTGG-3′
Lp-2	5′-GGTTGGGGTTGGGGTTGGGGTTGG-3′
Lp-3	5′-GGTTTGGGGTTTGGGGTTTGGGGTTTGG-3′
Lp-5	5′-GGTTTTTGGGGTTTTTGGGGTTTTTGGGGTTTTTGG-3′
Sequence 2	5′-GGTTTGGGTTTGGGTTTGGGTTTG-3′
Sequence 3	5′-GGTTAGGGGTTAGGGGTTAGGGGTTAGG-3′
G-0	5′-GGTTTTGGGGTTTTTTTTTTTTGGGGTTTTGG-3′
G-1	5′-GGTTTTGGGGTTTTGTTTTTTTGGGGTTTTGG-3′
G-2	5′GGTTTTGGGGTTTTGTTTTTTGGGGTTTTGG-3′
G-3	5′-GGTTTTGGGGTTTTGGGTTTTTGGGGTTTTGG-3′
45mer	5′-T$_3$CGT$_2$CGTGCT$_2$GCT$_3$GCTCT$_2$G$_3$T$_2$AG$_5$T$_2$AG$_2$T$_4$

Figure 3. Construction of unimolecularly circular oligodeoxyribonucleotides on the template basis of G-quadruplex and time course of the ligation reaction. Chemical ligation reactions were performed as described in Materials and Methods. The reaction times for these ligation reactions were 0 (lane 3), 5 (lane 4), 30 (lane 5), 60 (lane 6), 120 (lane 7), 240 (lane 8) and 360 min (lane 9), respectively. Lane 1, sequence 1 alone; lane 2, same reaction as the one loaded in lane 9, except for the absence of *N*-cyanoimidazole; and lane 10, the 45mer of 5′-T$_3$CGT$_2$CGTGCT$_2$GCT$_3$GCTCT$_2$G$_3$T$_2$AG$_5$T$_2$AG$_2$T$_4$-3′ as a molecular weight marker.

corresponding 5′ hydroxyl-ended oligodeoxyribonuleotides catalyzed by T4 polynucleotide kinase. *N*-Cyanoimidazole was synthesized from imidazole and cyanogens bromide (41), purified by sublimation and stored under argon at −20°C prior to use. Chemical ligation products were analyzed via 20% denaturing PAGE and visualized by autoradiography. Concentration of oligodeoxyribonucleotides was measured by using a ultraviolet (UV) spectrophotometer (UV-2101PC, Shimadzu, Kyoto, Japan) at $\lambda = 260$ nm. Radioactive bands were quantified by PhosphoImager analysis (SI system, Molecular Dynamics, Mountain View, CA) and yield of the circularization reaction was calculated based on the ratio of the radioactivity of product to those of both product and reactant.

Chemical ligation reactions

A stock solution of sequence 1 (2 μM) in 100 mM MES (pH 6.0) and 100 mM KCl were kept at 100°C for 3 min followed by cooling the mixture to 25°C over a period of 120 min to allow the formation of unimolecular G-quadruplexes. The reaction mixtures consisting of 1 μM of sequence 1 taken from the stock solution, 100 mM MES (pH 6.0), 100 mM KCl, 10 mM MnCl$_2$ and 10 mM

N-cyanoimidazole in a total volume of 20 μl were then prepared and incubated at 25°C for 6 h. The reaction was next terminated by the addition of loading buffer and the resultant ligation product was analyzed by PAGE.

Structural verification of the identified circular product

Exonuclease VII hydrolysis. The identified circular product with a slower rate of mobility shift (Figure 3, lane 9) was purified through PAGE. A reaction mixture containing 1′ exonuclease VII buffer (100 mM Tris–HCl, 100 mM potassium phosphate, 16.6 mM EDTA and 20 mM 2-mercaptoethanol, pH 7.9), 20 U of exonuclease VII (42) and the identified circular product or sequence 1 in a total volume of 20 μl was subsequently prepared and incubated at 37°C for 2 h. This reaction product was next analyzed through PAGE.

Partial hydrolysis of the identified circular product by DNAse I. A reaction mixture containing 1× DNAse I buffer (100 mM sodium acetate and 5 mM magnesium sulfate, pH 5.0), 10 U DNAse I and the identified circular product in a total volume of 20 μl was incubated at room temperature for 30 min. This hydrolysis reaction was terminated by freezing the samples in liquid nitrogen and the reaction product was analyzed through PAGE.

RESULTS AND DISCUSSION

Synthesis of circular oligodeoxyribonucleotides on the template basis of unimolecular G-quadruplex and time course of the ligation reaction

Figure 1 illustrates our strategy for manipulating the unimolecular entity of G-quadruplex to facilitate the construction of guanine-rich circular oligodeoxyribonucleotides. A linear sequence, 5′-GG(TTTTGGGG)₃TTTTGG-3′ (sequence 1), was selected as the precursor of our circularization reaction. It is anticipated that under certain conditions, this linear oligodeoxyribonucleotide would self-assemble into a desired unimolecular complex, within which its two termini are forced to align in a proximal position guided through Hoogsteen interaction (6). Once this self-assembling entity ('g' in Figure 1) is generated, subsequent chemical ligation reaction between the two open termini would lead to a desired circular G-quadruplex constituted of a single strand of oligodeoxyribonucleotide.

Sequence 1 was hence incubated in a pH 6.0 buffer to allow the desired structure of G-quadruplex to generate (Figure 1). The 5′ terminal phosphate within the unimolecular complex was next activated by the addition of *N*-cyanoimidazole to facilitate the formation of a phosphodiester bond with its adjacent 3′ hydroxyl group (Figure 1, 'g' and 'h'). As shown in Figure 3, a new product (lane 9) was generated from this ligation reaction with its rate of mobility shift slower than that of sequence 1 (lane 1). It was demonstrated earlier by Wang and Kool (43) that a 34mer circular oligonucleotide moved slower than that of its corresponding linear precursor. Based on this information, the newly formed product (Figure 3, the higher band in lane 9) from our ligation reaction was identified tentatively as the circular oligodeoxyribonucleotide generated from its corresponding linear precursor, sequence 1. The yields of these ligation reactions were found to be dependent on reaction time: circularization of the linear precursor proceeded in 13, 29, 42, 59, 67 and 71% yield when the corresponding reactions continued for 5 (lane 4), 30 (lane 5), 60 (lane 6), 120 (lane 7), 240 (lane 8) and 360 min (lane 9), respectively. In addition, the overall yield of the circularization process was 39% after a period of 6 h reaction, which was calculated based on the ratio of OD volume of sequence 1 initially used in the ligation reaction to that of circular product purified from polyacrylamide gel.

It should be noticed that the circular oligodeoxyribonucleotide possessing three guanines in a row in its sequence, <TTAGGGTTAGGGTTAGGGTTAGGG>, have not yet been obtained using the same methodology as discussed above starting from a linear precursor of 5′-GGTTAGGGT-TAGGGTTAGGGTTAG-3′ or 5′-GTTAGGGTTAGGGT-TAGGGTTAGG-3′. Since these two linear oligodeoxyribonucleotides are closely correlated with the sequences of telomeres of higher organisms, effort in finding out a proper condition for circularizing the above sequences will be continuing in our laboratory.

Confirmation of circular nature of our ligation product

Exonuclease VII is an exonuclease that hydrolyzes nucleotides from both 3′ and 5′ ends of single-stranded linear sequence of


Figure 4. Hydrolysis of the identified circular products by exonuclease. Lane 1, sequence 1 alone; lane 2, sequence 1 after treatment with 20 U of exonuclease VII at 37°C for 2 h; lane 3, the identified circular products alone; and lane 4, the identified circular product after treatment with 20 U of exonuclease VII at 37°C for 2 h.
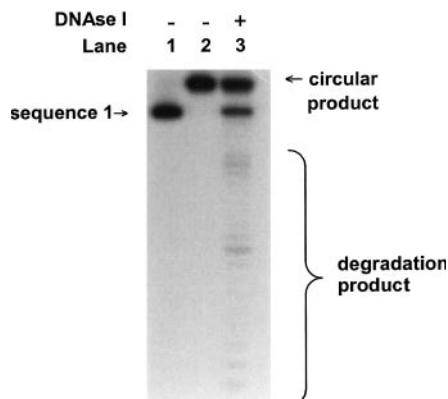
Figure 5. Partial hydrolysis of the identified circular products by DNAse I. Lane 1, sequence 1 alone; lane 2, the identified circular products alone; and lane 3, the identified circular product after treatment with 10 U of DNAse I at 25°C for 30 min.

deoxyribonucleic acids. Circular oligodeoxyribonucleotides are known to resist the degradation by this enzyme (31,42) due to the absence of open termini within their structures. In order to confirm the circular nature of its phosphate–sugar backbones, the newly formed product from our ligation reaction (Figure 3, lane 9) was purified via denaturing PAGE and then hydrolyzed by this exonuclease. As shown in Figure 4, there was absence of degradation product observable from the reaction of our identified circular product with exonuclease VII (lane 4). As a positive control, the hydrolysis reaction of sequence 1 with exonuclease VII was also carried out, which consequently led to lower molecular-weight products in quantitative yield (lane 2). The complete resistance of our ligation product to the hydrolysis by this exonuclease should be considered as the indication that there is absence of open terminus within its macromolecular structure.

Earlier gel electrophoresis studies (42,43) revealed that partially randomized hydrolysis of circular sequences of oligonucleotides resulted in a new major band with its rate of mobility shift correspondent to that of its linear precursor. The reason leading to this observation is that cleavage of
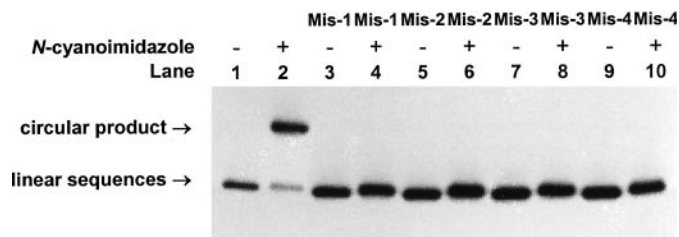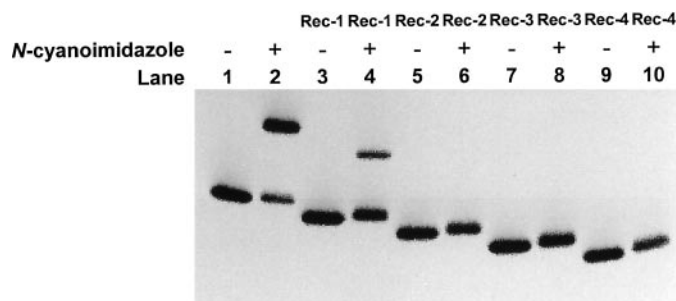
Figure 6. Effect of mismatched sequences on the circularization reaction. Ligation reactions were carried out in the same way as the one loaded in lane 9 in Figure 3, except for replacing sequence 1 with the corresponding mismatched sequences. Lane 1, sequence 1 alone; lane 2, reaction mixture of sequence 1; lane 3, Mis-1 (5′-**T**GT$_4$G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_2$-3′) alone; lane 4, reaction mixture of Mis-1; lane 5, Mis-2 (5′-**T**GT$_4$G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G**T**-3′) alone; lane 6, reaction mixture of Mis-2; lane 7, Mis-3 (5′-**TT**T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G**T**-3′) alone; lane 8, reaction mixture of Mis-3; lane 9, Mis-4 (5′-**TT**T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$**TT**-3′) alone; and lane 10, reaction mixture of Mis-4.

any of the phosphodiester bonds within a circular structure will give rise to the corresponding linear products possessing identical molecular weight (42). With the aim of further confirming the circular nature of our ligation product, partial hydrolysis tests were carried out during our investigation using DNAse I, an enzyme that hydrolyzes both single and duplex forms of DNA in a randomized pattern. As shown in Figure 5, a major band was observed upon partial hydrolysis of our ligation product that exhibited the same rate of mobility shift as that of its linear precursor (lane 3). In addition, there was absence of intermediate bands between our ligation product (higher band in lane 3) and the major band produced from it, which was the characteristic pattern of randomized partial hydrolysis of circular oligonucleotides by enzyme or chemicals (42,43).

Conformation dependency of the circularization reactions

If the designed self-assembly of G-quadruplex ('g' in Figure 1) is indeed an intermediate structure of our ligation reactions, any alteration from this conformation should have an effect on our circularization courses. In order to examine the effect of this conformation dependency, four new sequences were designed during our investigations, in which one, two, three or four non-guanine nucleotides appeared at their 5′ or 3′ end. As shown in Figure 6, none of the four mismatched sequences exhibited any observable amount of circularization product (lanes 3–10) under the same reaction condition as the one designed for sequence 1 (lane 2). These experimental results indicated that a single-base variation led to a distorted conformation of G-quadruplex that was consequently incapable of sustaining correct proximity between the 5′ and 3′ termini of the same sequence as required for further chemical ligation reaction.

In order to further probe the effect of sequence variation on our circularization reaction, four additional linear oligodeoxyribonucleotides were selected that were shorter than sequence 1 by one (rec-1), two (rec-2), three (rec-3) or four (rec-4 in Figure 7) nucleotide units. Our expectation from the design of these new sequences was that G-quadruplex, once it was formed from rec-1, rec-2, rec-3 or rec-4, would possess recessive ends in the middle of its columnar structure. As shown in Figure 7, a small amount of circularization

Figure 7. Effect of recessive sequences on the circularization reaction. Reactions were carried out in the same way as the one loaded in lane 9 in Figure 3, except for replacing sequence 1 with the corresponding recessive sequences. Lane 1, sequence 1 alone; lane 2, reaction mixture of sequence 1; lane 3, Rec-1 (5′-GT$_4$G$_4$T$_4$G$_4$T$_4$G$_2$-3′, 31mer) alone; lane 4, reaction mixture of Rec-1; lane 5, Rec-2 (5′-T$_4$G$_4$T$_4$G$_4$T$_4$G$_2$-3′, 30mer) alone; lane 6, reaction mixture of Rec-2; lane 7, Rec-3 (5′-T$_4$G$_4$T$_4$G$_4$T$_4$G-3′, 29mer) alone; lane 8, reaction mixture of Rec-3; lane 9, Rec-4 (5′-T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$-3′, 28mer) alone; and lane 10, reaction mixture of Rec-4.
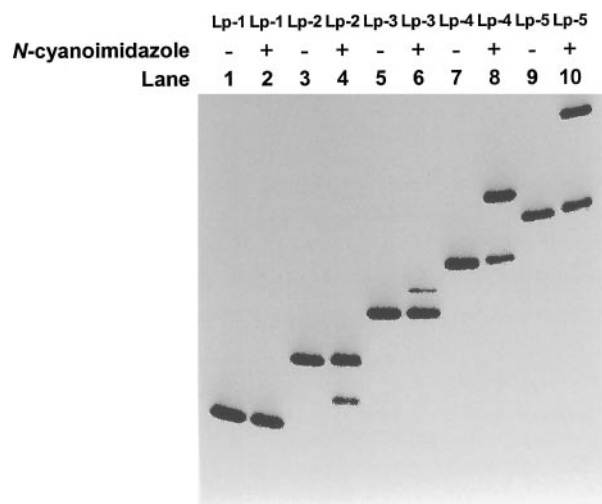
Figure 8. Effect of loop size on the circularization reaction. Reactions were carried out in the same way as the one loaded in lane 9 in Figure 3, except for replacing sequence 1 with Lp-1 (5′-G$_2$TG$_4$TG$_4$TG$_4$TG$_2$-3′), Lp-2 (5′-G$_2$**T**$_2$G$_4$**T**$_2$G$_4$**T**$_2$G$_4$**T**$_2$G$_2$-3′), Lp-3 (5′-G$_2$**T**$_3$G$_4$**T**$_3$G$_4$**T**$_3$G$_4$**T**$_3$G$_2$-3′) and Lp-5 (5′-G$_2$**T**$_5$G$_4$**T**$_5$G$_4$**T**$_5$G$_4$**T**$_5$G$_2$-3′), respectively. Lane 1, Lp-1 alone; lane 2, reaction mixture of Lp-1; lane 3, Lp-2 alone; lane 4, reaction mixture of Lp-2; lane 5, Lp-3 alone; lane 6, reaction mixture of Lp-3; lane 7, sequence 1 (=Lp-4) alone; lane 8, reaction mixture of sequence 1; lane 9, Lp-5 alone; and lane 10, reaction mixture of Lp-5.

product was generated when the ligation reaction began with rec-1 (lane 4), while there was absence of circular product observable in the reactions where the sequences of rec-2 (Figure 7, lane 6), rec-3 (lane 8) and rec-4 (lane 10) were used as reactants. These observations could be the indication that a proper structural feature of G-quadruplex can still be generated from a linear oligodeoxyribonucleotide shorter than sequence 1 by 1 nt unit, but not by the others that are two or more nucleotide units shorter.

Loop-size dependency of our circularization reactions

One of the distinctive features of G-quadruplex in structure is its possession of connecting loops that link the guanine tracts

within the unimolecular and bimolecular complexes of G-quadruplexes (44–52). For example, the guanine-rich sequences of DNA are capable of forming looped quartet structures whose stabilities increase with the increase in the number of intervening thymine residues within their loops (53–55). Five new sequences were accordingly designed in our studies, in which the numbers of thymine nucleotide between guanine tracts are one (Lp-1 in Figure 8), two (Lp-2), three (Lp-3), four (Lp-4) and five (Lp-5), respectively. As shown in Figure 8, our circularization reaction starting with Lp-2 (lane 4), Lp-3 (lane 6), Lp-4 (lane 8) and Lp-5 (lane 10) proceeded in 31, 20, 72 and 60% yield, respectively. Conversely, there was absence of circularization product observable when the ligation reaction began with Lp-1 (lane 2). These results could be the indication that the guanine-rich sequence possessing a single thymine in its intervening loop might not be able to sustain the intermediate structure required for our designed circularization reaction.

Alkali-ion dependency of our circularization course

Selective interaction with cations that fit well in the cavities formed by the stacking of guanine tetrads is a distinguishable characteristic of G-quadruplex from any other structural features of nucleic acids. In the alkali series, the order of ions preferred by G-quartet is $K^+ \gg Na^+ > Rb^+ > Cs^+ > Li^+$ (1). With the intention of knowing whether alkali ion affects our circularization course, the efficiency of our circularization course in the presence of these ions was examined during our investigation. As shown in Figure 9, there was absence of circular product observable when lithium (lane 2), sodium (lane 3), rubidium (lane 4) and cesium ions (lane 5) were used to facilitate the formation of G-quadruplex structures. This took place most likely because these alkali ions might not be able to sustain a stable structure of G-quadruplex as potassium ion does under our standard condition for chemical ligation reactions.

pH dependency of the designed ligation reactions

The effect of pH values on our circularization course was examined during our investigation in the range from 5.5 to 7.5. As shown in Figure 10, the efficiency of our ligation reactions decreased with the increase in pH values of the corresponding buffer solutions. These ligation reactions proceeded in 75, 78, 71, 49, 9 and 1% yield at pH 5.5 (lane 2), 6.0 (lane 3), 6.5 (lane 4), 7.0 (lane 5) and 7.5 (lane 6), respectively. Earlier CD spectroscopic analysis (54) revealed that the variation of pH values of the corresponding buffer solutions
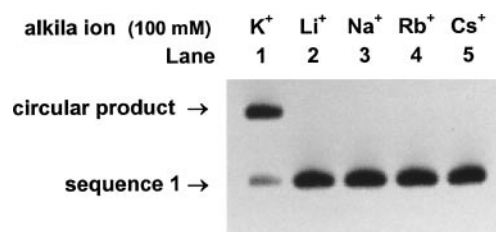
ranged from 5 to 8 alone exhibited little effect on the stability of G-quadruplex. The relatively low efficiency of our ligation reaction at higher pH values (lanes 5 and 6) should be attributed to the fast decomposition of *N*-cyanoimidazole, the chemical agent required for the ligation reaction, under basic conditions.

Potassium ion concentration dependency of our ligation reaction

Possession of cations by the structural feature of G-quartet as a part of its structure determines that the formation of G-quadruplex from its precursor of unstructured sequences is an ion-concentration dependent process. Earlier capillary electrophoresis studies (56) revealed that G-quadruplex was not observable in the absence of cations, while the amount of this tetraplex structure increased with the increase in the concentration of potassium ion. When the concentration of potassium chloride reached up to 100 mM (56), single-stranded form of DNA was converted to G-quadruplex structures completely. With the aim of finding out whether our designed unimolecular structure of G-quadruplex ('g' in Figure 1) is affected by the variation of ion concentration, our ligation reaction in the presence of different amount of potassium ion was accordingly examined during our investigation. As shown in Figure 11, the efficiency of the circularization reactions increased with the increase in potassium ion concentration (lanes 2–8) and the ligation product in over 50% yield was obtained when potassium ion concentration was kept at 100 mM (lane 2). These results were consistent with the early suggestion that there is equilibrium between the unstructured linear sequence and G-quadruplex structures,

Figure 10. pH dependency of the circularization reaction. Reactions were carried out in the same way as the one loaded in lane 9 in Figure 3, except for that pH of the buffer solutions were 5.5 (lane 2), 6.0 (lane 3), 6.5 (lane 4), 7.0 (lane 5), and 7.5 (lane 6), respectively; lane 1, sequence 1 alone.

Figure 11. Effect of potassium ion concentration on the circularization reaction. Ligation reactions were carried out in the same way as the one loaded in lane 9 in Figure 3, except for that the concentration of potassium chloride was kept at 200 mM (lane 1), 100 mM (lane 2), 40 mM (lane 3), 20 mM (lane 4), 10 mM (lane 5), 4 mM (lane 6), 2 mM (lane 7), 1 mM (lane 8), 0.2 mM (lane 9), 0 mM (lane 10) instead.
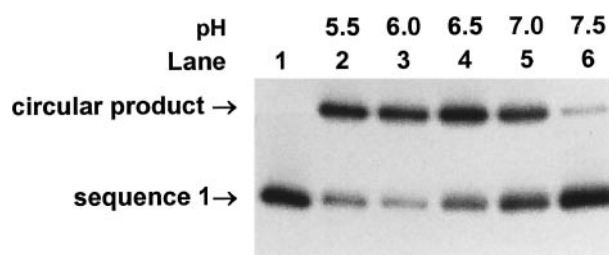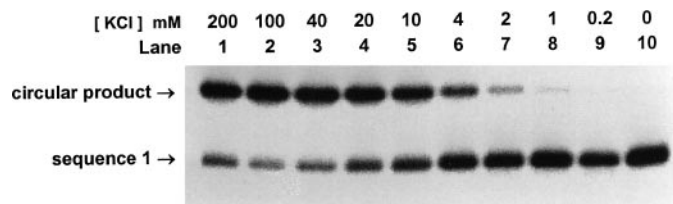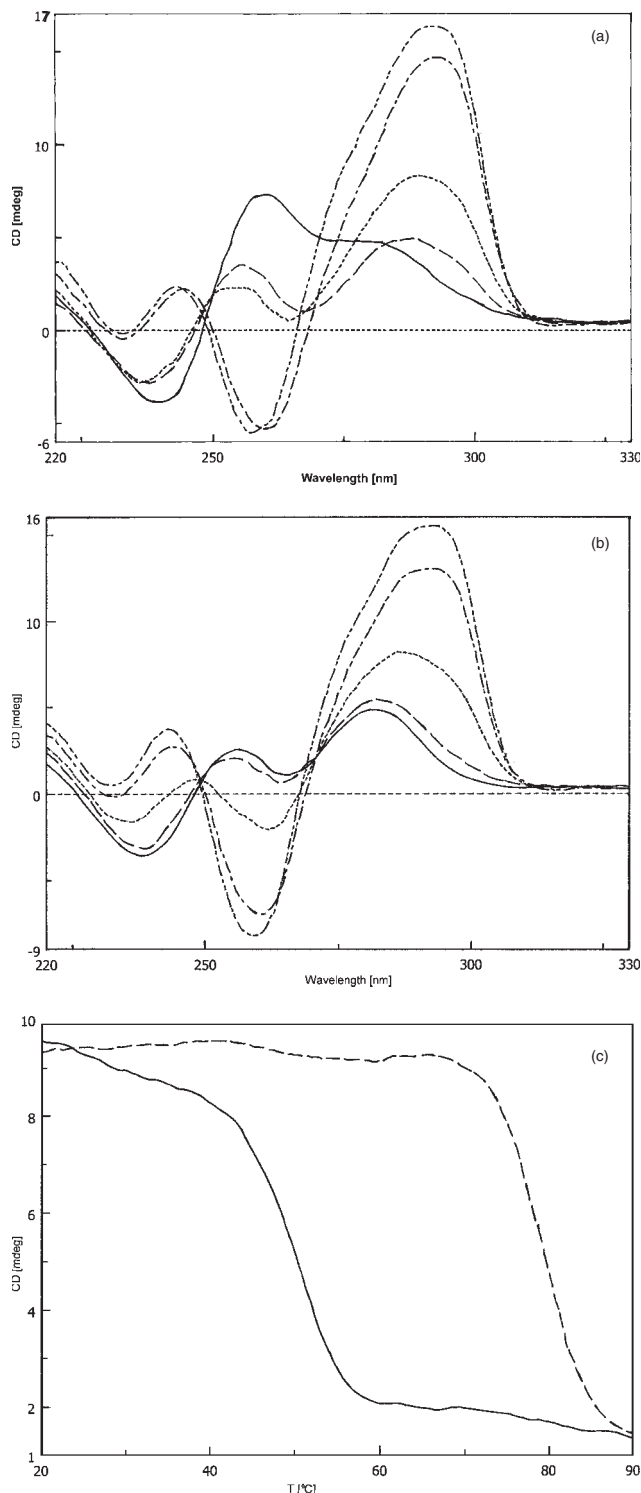

Figure 9. Effect of alkali ions on the circularization reaction. Lane 1, same as lane 9 in Figure 3; lanes 2–5, reactions were carried out in the same way as the one loaded in lane 9 in Figure 4, except for replacing KCl with 100 mM of LiCl (lane 2), NaCl (lane 3), RbCl (lane 4) and CsCl (lane 5), respectively.

Figure 12. CD spectroscopic measurement of linear and circular oligodeoxyribonucleotides. (**a**) Spectrum of circular <GGTT-TTGGGGTTTTGGGGTTTTGGGGTTTTGG> (20 µM) in 10 mM Tris–HCl buffer (pH 7.0) containing 0 mM (———), 0.1 mM (— — —), 0.2 mM (– – – – –), 2 mM (– – – –) and 20 mM (– – ———–) KCl; (**b**) spectrum of linear GGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGG in 10 mM Tris–HCl buffer (pH 7.0) containing 0 mM (———), 0.5 mM (— — —), 1.5 mM (– – – – –) 2 mM (– – – –) and 20 mM (– – ———–) KCl; and (**c**) melting point measurement of linear (20 µM) (———) and circular (20 µM) (– – – – –) oligodeoxyribonucleotides in 10 mM Tris–HCl buffer (pH 7.0) containing 20 mM KCl.

in which a higher ion concentration favors the formation of structured DNA.

Verification of the formation of G-quadruplex by our newly synthesized circular oligodeoxyribonucleotides

With the aim of verifying that the structural feature of G-quadruplex could indeed be generated by the newly synthesized circular oligodeoxyribonucleotides, certain CD spectroscopic examinations were carried out on the corresponding ligation products. A parallel G-quadruplex usually exhibits a maximum near 265 nm and a minimum near 240 nm, while an anti-parallel G-quadruplex is characterized by a maximum near 290 nm and a minimum near 260 nm, respectively (57,58). As shown in Figure 12a, our circular oligodeoxyribonucleotide displayed spectra characterized by positive maxima at 293 nm and a negative minimum at 265 nm with the increase in potassium chloride concentration, which are the typical features for the formation of anti-parallel G-quadruplex from random conformations of oligodeoxyribonucleotides. The result shown in Figure 12a could be the indication that a guanine-rich circular oligodeoxyribonucleotide, like its linear counterpart (Figure 12b), is capable of forming anti-parallel G-quadruplex in the presence of potassium chloride.

In addition, the stabilities of our circular oligodeoxyribonucleotide and its linear precursor were investigated by monitoring the positive band at 295 nm on a Spectropolarimeter with variation in temperature. The observed melting points of our circular product and its linear precursor were 79 and 52°C, respectively (Figure 12c). The higher melting point of the circular product could be attributed to the absence of free end movement within the newly formed circular structure.

CONCLUSIONS

Our current studies demonstrate that certain unimolecularly circular G-quadruplexes can indeed be attained on the template basis of G-quadruplex through chemical ligation reactions. These circularization courses are highly effective and sequence-specific under our standard reaction condition. The circular structure of G-quadruplex possessing two, three, four or five thymine nucleotides in its intervening loops is readily achievable, while the linear sequences possessing a single thymine nucleotide between its guanine tracts is not a proper precursor for our circularization reaction. Potassium ion is the only one in the alkali series that promotes the formation of circularly unimolecular G-quadruplex, which indicates that this circularization process is susceptible to variation of the nature of metal ion in the cavity of G-quartet. In addition, this circularization course can be affected by other factors such as pH values of the corresponding buffer solutions as well as the concentration of potassium ions. We accordingly anticipate that the unimolecularly circular G-quadruplexes achieved in our studies could be useful molecular probes for investigating the winding and resolving mechanisms of G-quadruplex promoted by G-quadruplex-promoting and disintegrating proteins. More importantly, the current studies do lay a firm experimental foundation for further constructing fluorescence-tagged unimolecularly circular G-quadruplexes ('j' in Figure 2) in the near future.

ACKNOWLEDGEMENTS

REFERENCES

1. Simonsson,T. (2001) G-quadruplex DNA structures-variations on a theme. *Biol. Chem.*, **382**, 621–628.
2. Mills,M., Lacroix,L., Arimondo,P.B., Leroy,J.-L., Francois,J.-C.e, Klump,H. and Mergny,J.-L. (2002) Unusual DNA conformations: implications for telomeres. *Curr. Med. Chem.*, **2**, 627–644.
3. Belmont,P., Constant,J.F. and Demeunynck,M. (2001) Nucleic acid conformation diversity: from structure to function and regulation. *Chem. Soc. Rev.*, **30**, 70–81.
4. Rhodes,D. and Giraldo,R. (1995) Telomere structure and function. *Curr. Opin. Struct. Biol.*, **5**, 311–322.
5. Lane,A.N. and Jenkins,T.C. (2001) Structures and properties of multi-stranded nucleic acids. *Curr. Org. Chem.*, **5**, 845–869.
6. Patel,D.J., Bouaziz,S., Kettani,A. and Wang,Y. (1999) Structures of guanine-rich and cytosine-rich quadruplexes formed *in vitro* by telomeric, centromeric, and triplet repeat disease DNA sequences. In Neidle,S. (ed.), *Oxford Handbook of Nucleic Acid Structure*, Oxford University Press, Oxford, UK, pp. 389–453.
7. Mergny,J.-L.and Helene,C. (1998) G-quadruplex DNA: a target for drug design. *Nature Med.*, **4**, 1366–1367.
8. Pilch,D.S., Plum,G.E. and Breslauer,K.J. (1995) The thermodynamics of DNA structures that contain lesions or guanine tetrads. *Curr. Opin. Struct. Biol.*, **5**, 334–342.
9. Rippe,K., Kuryavyi,V.V.,Westhof,E. and Jovin,T.M. (1992) Polymorphism and possible biological functions of parallel-stranded DNA. In Lilley,D.M.J., Heumann,H. and Suck,D. (ed.), *Structural Tools for the Analysis of Protein–Nucleic Acid Complexes*, Birkhauser, Basel, Switzerland, pp. 81–107.
10. Jin,R., Gaffney,B.L., Wang,C., Jones,R.A. and Breslauer K.J. (1992) Thermodynamics and structure of a DNA tetraplex: a spectroscopic and calorimetric study of the tetramolecular complexes of d(TG3T) and d(TG3T2G3T). *Proc. Natl Acad. Sci. USA*, **89**, 8832–8836.
11. Blume,S.W., Guarcello,V., Zacharias,W. and Miller,D.M. (1997) Divalent transition metal cations counteract potassium-induced quadruplex assembly of oligo(dG) sequences. *Nucleic Acids Res.*, **25**, 617–625.
12. Wang,Y. and Patel,D.J. (1993) Solution structure of the human telomeric repeat d(AG3(T2AG3)3] G-tetraplex. *Structure*, **1**, 263–282.
13. Lu,M., Guo,Q. and Kallenbach,N.R. (1993) Thermodynamics of G-tetraplex formation by telomeric DNAs. *Biochemistry*, **32**, 598–601.
14. Deng,H. and Braunlin,W.H. (1996) Kinetics of sodium ion binding to DNA quadruplexes. *J. Mol. Biol.*, **255**, 476–483.
15. Kankia,B.I. and Marky,L.A. (2001) Folding of the thrombin aptamer into a g-quadruplex with Sr(2+): stability, heat, and hydration. *J. Am. Chem. Soc.*, **123**, 10799–10804.
16. Parkinson,G.N., Lee,M.P.H. and Neidle,S. (2002) Crystal structure of parallel quadruplexes from human telomeric DNA. *Nature*, **417**, 876–880.
17. Phan,A.T., Modi,Y.S. and Patel,D.J. (2004) Propeller-type parallel-stranded G-quadruplexes in the human c-myc promoter. *J. Am. Chem. Soc.*, **126**, 8710–8716.
18. Wang,K.Y., Swaminathan,S. and Bolton,P.H. (1994) Tertiary structure motif of Oxytricha telomere DNA. *Biochemistry*, **33**, 7517–7527.
19. Padmanabhan,K., Padmanabhan,K.P., Ferrara,J.D., Sadler,J.E. and Tulinsky,A. (1993) The structure of alpha-thrombin inhibited by a 15-mer single-stranded DNA aptamer. *J. Biol. Chem.*, **268**, 17651–17654.
20. Kang,C., Zhang,X., Ratliff,R., Moyzis,R. and Rich,A. (1992) Crystal structure of four-stranded Oxytricha telomeric DNA. *Nature*, **356**, 126–131.
21. Laughlan,G., Murchie,A.I.H., Norman,D.G., Moore,M.H., Moody,P.C.E.; Lilley,D.M. and Luisi,B. (1994) The high-resolution crystal structure of a parallel-stranded guanine tetraplex. *Science*, **265**, 520–524.
22. Aboul-ela,F., Murchie,A.I. and Lilley,D.M. (1992) NMR study of parallel-stranded tetraplex formation by the hexadeoxynucleotide d(TG4T). *Nature*, **360**, 280–282.
23. Chen,J., Liu,D., Lee,A.H.F., Qi,J., Chan,A.S.C.and Li,T. (2002) G-quadruplex as a new class of structural entities for directing the formation of circular oligodeoxyribonucleotides. *Chem. Comm.*, **22**, 2686–2687.
24. Williamson,J.R., Raghuraman,M.K. and Cech,T.R. (1989) Monovalent cation-induced structure of telomeric DNA: the G-quartet model. *Cell*, **59**, 871–880.
25. Simonsson,T., Pecinka,P. and Kubista,M. (1998) DNA tetraplex formation in the control region of c-myc. *Nucleic Acids Res.*, **26**, 1167–1172.
26. Hammond-Kosack,M.C., Kilpatrick,M.W. and Docherty,K. (1992) Analysis of DNA structure in the human insulin gene-linked polymorphic region *in vivo*. *J. Mol. Endocrinol.*, **9**, 221–225.
27. Jing,N., Marchand,C.Liu,J., Mitra,R., Hogan,M.E. and Pommier,Y. (2000) Mechanism of inhibition of HIV-1 integrase by G-tetrad-forming oligonucleotides *in vitro*. *J. Biol. Chem.*, **275**, 21460–21467.
28. Macaya,R.F., Schultze,P., Smith,F.W., Roe,J.A. and Feigon,J. (1993) Thrombin-binding DNA aptamer forms a unimolecular quadruplex structure in solution. *Proc. Natl Acad. Sci. USA*, **90**, 3745–3749.
29. Erlitzki,R. and Fry,M. (1997) Sequence-specific binding protein of single-stranded and unimolecular quadruplex telomeric DNA from rat hepatocytes. *J. Biol. Chem.*, **272**, 15881–15890.
30. Kool,E.T. (1998) Recognition of DNA, RNA, and proteins by circular oligonucleotides. *Acc. Chem. Res.*, **31**, 502–510.
31. Kool,E.T. (1996) Circular oligonucleotides: new concepts in oligonucleotide design. *Annu. Rev. Biophys. Biomol. Struct.*, **25**, 1–28.
32. Fang,G. and Cech,T.R. (1993) Characterization of a G-quartet formation reaction promoted by the β-subunit of the Oxytricha telomere-binding protein. *Biochemistry*, **32**, 11646–11657.
33. Fang,G. and Cech,T.R. (1993) The subunit of Oxytricha telomere-binding protein promotes G-quartet formation by telomeric DNA. *Cell*, **74**, 875–885.
34. Giraldo,R. and Rhodes,D. (1994) The yeast telomere-binding protein RAP1 binds to and promotes the formation of DNA quadruplexes in telomeric DNA. *EMBO J.*, **13**, 2411–2420.
35. Harrington,C., Lan,Y. and Akman,S.A. (1997) The identification and characterization of a G4-DNA resolvase activity. *J. Biol. Chem.*, **272**, 24631–24636.
36. Baran,N., Pucshansky,L., Marco,Y., Benjamin,S. and Manor,H. (1997) The SV40 large T-antigen helicase can unwind four stranded DNA structures linked by G-quartets. *Nucleic Acids Res.*, **25**, 297–303.
37. Sun,H., Karow,J.K., Hickson,I.D. and Maizels,N. (1998) The Bloom′s syndrome helicase unwinds G4 DNA. *J. Biol. Chem.*, **273**, 27587–27592.
38. Fry,M. and Loeb,L.A. (1999) Human werner syndrome DNA helicase unwinds tetrahelical structures of the fragile X syndrome repeat sequence d(CGG)n. *J. Biol. Chem.*, **274**, 12797–12802.
39. Browning,D.D., Mc,S.M., Marty,C. and Ye,R.D. (2001) Functional analysis of type 1alpha cGMP-dependent protein kinase using green fluorescent fusion proteins. *J. Biol. Chem.*, **276**, 13039–13048.
40. Wehrl,W., Niederweis,M. and Schumann,W. (2000) The FtsH protein accumulates at the septum of *Bacillus subtilis* during cell division and sporulation. *J. Bacteriol.*, **182**, 3870–3873.
41. Luebke,K.J. and Dervan,P.B. (1992) Nonenzymic ligation of double-helical DNA by alternate-strand triple helix formation. *Nucleic Acids Res.*, **20**, 3005–3009.
42. Dolinnaya,N.G., Blumenfeld,M., Merenkova,I.N., Oretskaya,T.S., Krynetskaya,N.F., Ivanovskaya,M.G., Vasseur,M. and Shabarova,Z.A. (1993) Oligonucleotide circularization by template-directed chemical ligation. *Nucleic Acids Res.*, **21**, 5403–5407.
43. Wang,S. and Kool,E.T. (1994) Circular RNA oligonucleotides. Synthesis, nucleic acid binding properties, and a comparison with circular DNAs. *Nucleic Acids Res.*, **22**, 2326–2333.
44. Clark,G.R., Pytel,P.D., Squire,C.J. and Neidle,S. (2003) Structure of the first parallel DNA quadruplex-drug complex. *J. Am. Chem. Soc.*, **125**, 4066–4067.
45. Haider,S.M., Parkinson,G.N. and Neidle,S. (2003) Structure of a G-quadruplex-ligand complex. *J. Mol. Biol.*, **326**, 117–125.

46. Kang, Sang-G. and Henderson,E. (2002) Identification of non-telomeric G4-DNA binding proteins in human, *E.coli*, yeast, and *Arabidopsis*. *Mol. Cell*, **14**, 404–410.

47. Lyonnais,S, Gorelick,R.J, Mergny, Jean-L., Le,C.E. and Mirambeau,G. (2003) G-quartets direct assembly of HIV-1 nucleocapsid protein along single-stranded DNA. *Nucleic Acids Res.*, **31**, 5754–5763.

48. Hurley,L.H. (2001) Secondary DNA structures as molecular targets for cancer therapeutics. *Biochem. Soc. Trans.*, **29**, 692–696.

49. Wen,J.D. and Gray,D.M. (2002) The Ff gene 5 single-stranded DNA-binding protein binds to the transiently folded form of an intramolecular G-quadruplex. *Biochemistry*, **41**, 11438–11448.

50. Ying,L., Green,J.J., Li,H., Klenerman,D. and Balasubramanian,S. (2003) Studies on the structure and dynamics of the human telomeric G quadruplex by single-molecule fluorescence resonance energy transfer. *Proc. Natl Acad. Sci. USA*, **100**, 14629–14634.

51. Kim,M-Y., Gleason-Guzman,M., Izbicka,E., Nishioka,D. and Hurley,L.H. (2003) The different biological effects of telomestatin and TMPyP4 can be attributed to their selectivity for interaction with intramolecular or intermolecular G-quadruplex structures. *Cancer Res.*, **63**, 3247–3256.

52. Siddiqui-Jain,A., Grand,C.L., Bearss,D.J. and Hurley,L.H. (2002) Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress c-MYC transcription. *Proc. Natl Acad. Sci. USA*, **99**, 11593–11598.

53. Balagurumoorthy,P., Brahmachari,S.K., Mohanty,D., Bansal,M. and Sasisekharan,V. (1992) Hairpin and parallel quartet structures for telomeric sequences. *Nucleic Acids Res.* **20**, 4061–4067.

54. Guo,Q., Lu,M. and Kallenbach,N.R. (1993) Effect of thymine tract length on the structure and stability of model telomeric sequences. *Biochemistry*, **32**, 3596–3603.

55. Smirnov,I. and Shafer,R.H. (2000) Effect of loop sequence and size on DNA aptamer stability. *Biochemistry*, **39**, 1462–1468.

56. Scaria,P.C., Shire,S.J. and Shafer,R.H. (1992) Quadruplex structure of d(G3T4G3) stabilized by potassium or sodium is an asymmetric hairpin dimer. *Proc. Natl. Acad. Sci. USA*, **89**, 10336–10340.

57. Giraldo,R., Suzuki,M., Chapman,L. and Rhodes,D. (1994) Promotion of parallel DNA quadruplexes by a yeast telomere binding protein: a circular dichroism study. *Proc. Natl. Acad. Sci. USA*, **91**, 7658–7662.

58. Guo,Q., Lu,M. and Kallenbach,N.R. (1993) Thermodynamics of G-tetraplex formation by telomeric DNAs. *Biochemistry*, **32**, 598–601.