Inhibitory properties of double helix forming circular oligonucleotides

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Received August 27, 1997; Revised and Accepted October 30, 1997

ABSTRACT
Several circular oligonucleotides were synthesized and characterized by electrospray ionization mass spectrometry. Experiments on termination of primer extension catalysed by DNA polymerases, Klenow fragment and Tth have demonstrated that a double helix forming circular 2′-deoxyribooligomer containing a 25mer sequence complementary to the target single-stranded DNA along with a 34mer random mismatching stretch appears to be a potent inhibitor of replication in vitro. Studies on inhibition of luciferase gene expression in a cell-free transcription–translation system have shown that a duplex forming circular 2′-deoxyribooligonucleotide containing a 25mer sequence complementary to the target mRNA and a 14mer random mismatching stretch can serve as an effective antisense compound as a standard linear complementary oligomer. Features of double helix forming circular oligonucleotides composed of 2′-deoxyribonucleosides seem to be useful for the design of new antigene and antisense agents.

INTRODUCTION
Antigene (1) and antisense (2–4) oligonucleotides have been found to display properties useful for drug design. These compounds are often chemically modified to improve their pharmacokinetics and stability. Extensive research has been focused on modification of the sugar–phosphate backbone in an attempt to prepare potential therapeutics based on phosphorothioates, methylphosphonates and 2′-O-alkylribooligomers (5–10). Some reports show that excessive use of some of these modifications changes the affinity of an oligonucleotide for a target nucleic acid and, more interestingly, leads to formation of complexes of a conformation that appears to alter interaction with enzymes involved in the biochemical process of interest (10–12). It is well known, for example, that although introduction of certain chemical modifications leads to an enhanced affinity of antisense compounds for RNA, their complexes with nucleic acids appear to be poor RNase H substrates. Our recent investigations on binding of triplex forming circular oligonucleotides and a hybrid complex forming looped oligonucleotides with single-stranded DNA and their influence on replication in vitro (13) led us to the conclusion that inhibitory effects result from the spatial structure of oligonucleotide–target complexes rather than from mere differences in their thermodynamic stability. This conclusion led to the idea of utilization of duplex forming circular oligonucleotides as mediators of certain biochemical reactions.

We report here results on the preparation and characterization of several disulphide cross-linked circular oligonucleotides. We also include some experiments employing these compounds and describing termination of primer extension catalysed by two different DNA polymerases and inhibition of luciferase reporter gene expression in a cell-free system.

MATERIALS AND METHODS
Solid supports and monomers for oligonucleotide synthesis

The oligomers were prepared on solid support 1 bearing an ester linkage (14), on support 2 bearing a disulphide bond (15) or on commercial nucleoside-bound solid supports (Glen Research). Monomer 3, bearing an ester function, was prepared as described earlier (16) and monomer 4, having a disulphide bond, was a commercial product (Glen Research). 2′-Deoxyribonucleoside 3′-phosphoramidites and 2′-O-methylribonucleoside 3′-phosphoramidites were from Glen Research.

Oligonucleotides

Oligomers reported here were assembled on a PE Applied Biosystems 392 DNA synthesizer on a 1 µM scale. Introduction of the cystamine residue into assembled oligonucleotides bearing ester functions was achieved with 1 M cystamine (free base) in water overnight. Free thiol groups were generated from the disulphide functions by treating with dithiothreitol in the presence of triethylamine (17). After isolation of oligomers bearing thiol functions on both the 5′- and the 3′-termini cross-linking was achieved as reported earlier (13,17).

Characterization
Gel electrophoresis and HPLC. PAGE was carried out as described (10,14). Anion exchange HPLC to purify and characterize

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RESULTS AND DISCUSSION

Synthesis

To synthesize the circular disulphide cross-linked oligonucleotides solid supports 1 and 2 and monomeric blocks 3 and 4 were employed (Fig. 1).

Circular oligonucleotides. The applicability of disulphide bonds for cyclization of oligomers composed of 2′-deoxy- and 2′-O-methylribo nucleosides has previously been demonstrated (13,17). Basically, the same approach was used to prepare 5–14 (Fig. 2) employing various disulphide-containing tethers and their combinations for subsequent cross-linking. The procedures used to introduce a disulphide function at both the 5′- and 3′-termini of a linear precursor of the circular oligomer differed slightly, depending on the 5′-monomer and solid support employed. Thus linear 2′-O-methylribo oligonucleotide 5 was assembled on solid support 1 and completed with monomer 3. 1 and 3 both containing an ester function. After assembly the solid support was first treated with aqueous cystamine to cleave the oligomer from the solid support and to introduce a disulphide function at the 5′-terminus and the 3′-terminal non-nucleosidic unit derived from 3. Deprotection of 5 was then completed with ammonia. The disulphide bonds were subsequently reductively cleaved and the oligomer containing two mercapto functions was cross-linked to give circular 2′-O-methylribo oligonucleotide 6 (Fig. 2). 2′-Deoxyribo oligomers 7, 9, 11 and 13 were assembled utilizing cDNA upstream of the T7 site. Reactions were performed at 30 °C for 90 min, according to the kit protocol. After completion of transcription–translation the amount of luciferase expressed was verified by measuring light emission on a luminometer (Bio-Orbit) using standard luciferase assay reagent [20 mM tricine, 1.07 mM Mg(OH)2·5H2O, 2.67 mM MgSO4, 0.1 mM EDTA, 33.3 mM DTT, pH 7.8, 2 μM coenzyme A, 470 μM luciferin and 530 μM ATP].

Repeition termination experiments

Replication termination experiments were conducted on synthetic 75mer single-stranded DNA template M1 or M2. These experiments employed Escherichia coli Klenow fragment (Phar macia) or Tth polymerase (Hytest) and were performed as reported earlier (13). A mixture of 1 pmol template and 10–100 pmol inhibitory oligomer was used in this study.

Luciferase expression assay

Expression of luciferase was studied using a coupled transcription–translation system (TNT T7/SP6 Coupled Wheat Germ Extract System; Promega). RSV-Luc luciferase expression plasmid (0.2 μg) and different concentrations of oligonucleotides were employed. The plasmid was used in its circular form utilizing cDNA upstream of the T7 site. Reactions were performed at 30 °C for 90 min, according to the kit protocol. After completion of transcription–translation the amount of luciferase expressed was verified by measuring light emission on a luminometer (Bio-Orbit) using standard luciferase assay reagent [20 mM tricine, 1.07 mM Mg(OH)2·5H2O, 2.67 mM MgSO4, 0.1 mM EDTA, 33.3 mM DTT, pH 7.8, 2 μM coenzyme A, 470 μM luciferin and 530 μM ATP].
on disulphide-containing solid support 2, employing the same 5'-terminal non-nucleosidic monomer 3 (Fig. 2). Here cystamine was used to cleave the oligomers from the solid support and to derivatize the 5'-terminal non-nucleosidic unit. Additionally, disulphide exchange of the 3'-hydroxypropylthio group to a 2-aminoethylthio function took place. Subsequent reduction of the 5'- and 3'-terminal disulphide bonds and cross-linking then gave circular 8, 10, 12 and 14 (Fig. 2). Yields of oligonucleotides 5–14 are given in Table 1. Commercial disulfide monomer 4 was also tried in the preparation of circular oligomers. We employed support 2 and assembly in this case was completed with 4. Alternatively, a commercial thymidine-containing solid support was used to introduce monomer 4 as the second and last unit. After deprotection with ammonia the linear oligonucleotides were isolated in good yield (50–75%). Treatment with dithiothreitol and separation from the excess of reagent did not, however, result in a reasonable yield of circular oligonucleotide. Presumably formation of a disulphide linkage is somehow unfavourable with the thiol group resulting from the non-nucleoside unit derived from 4.

**Characterization**

*Gel electrophoresis and HPLC.* The 15% denaturing PAGE showed that circular oligomers migrated 10–15% slower than their linear precursors or linear oligonucleotides having two terminal mercapto groups. These were obtained by treating either precursor 5, 7, 9, 11 or 13 or circular oligomer 6, 8, 10, 12 or 14 with dithiothreitol in the presence of triethylamine prior to application to the gel. Anion exchange HPLC also appeared to be characteristic of circular oligonucleotides, which displayed retention times ~2 min greater than their linear precursors (Table 1). RP HPLC showed the purity of circular oligomers to be >95% (Table 1).
Figure 3. Negative ion ESI mass spectrum of circular oligonucleotide 8. The major multiply charged ions represent negative charge states from 10 to 15. Sample, 3 µl 8 at 12 pmol/µl concentration in 25 mM triethylamine in 80% acetonitrile. Flow rate, 3 µl/min.

Table 1. Overall yields, ion exchange (IE) and reversed phase (RP) HPLC retention times (rt) and purity of the oligonucleotides synthesized

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>No. bases</th>
<th>No. phosphodiester bonds (%)</th>
<th>Overall yield</th>
<th>IE HPLC a rt (min)</th>
<th>RP HPLC b rt (min)</th>
<th>Purity c (%)</th>
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<tbody>
<tr>
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<td>59</td>
<td>60</td>
<td>51</td>
<td>24.0</td>
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<td>–</td>
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<td>6</td>
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<td>7</td>
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<td>67</td>
<td>24.3</td>
<td>21.9</td>
<td>99.6</td>
</tr>
</tbody>
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aColumn, PolyWax LP; buffer A, 0.05 M KH2PO4 in 50% aqueous formamide, pH 5.60; buffer B, A + 0.6 M (NH4)2SO4; flow rate 1 ml/min; linear gradient 5–60% B over 30 min.
bColumn, Nucleosil C18; buffer A, 0.05 M ammonium acetate; buffer B, 0.05 M ammonium acetate in 50% CH3CN; flow rate 0.76 ml/min; linear gradient 5–35% B over 30 min.
cAs assessed from RP HPLC by peak integration at 260 nm.

Electrospray ionization mass spectrometry. Electrospray ionization mass spectrometry (ESI-MS) has proven to be a gentle and very sensitive method for analysis of natural and modified oligonucleotides (18–21). Figure 3 shows a typical ESI mass spectrum obtained after injection of 3 µl circular 60mer 6 at a concentration of 12 pmol/µl. A mass deconvolution program was then used for calculation of the molecular weight of the oligomers using m/z values of the multiply deprotonated molecules (Fig. 4). The measured and theoretically calculated average molecular weights of the compounds are shown in Table 2. The error between calculated and measured molecular weights was <2 mass units (<0.01%).

Inhibitory properties of double helix forming circular oligonucleotides

We have investigated inhibitory effects of double helix forming circular oligonucleotides on two processes. Firstly, we studied the
influence of these oligomers on primer extension on a single-stranded DNA template catalysed by two different DNA polymerases, *E. coli* Klenow fragment and thermostable Tth polymerase. Secondly, circular oligonucleotides were employed to inhibit luciferase reporter gene expression *in vitro*.

### Table 2. Measured and theoretically calculated average molecular weights of the oligonucleotides synthesized

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Molecular weight (mass units)</th>
<th>Calculated</th>
<th>Measured</th>
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<tr>
<td>14</td>
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**Influence of circular oligonucleotides on replication *in vitro*.** It is known that most DNA polymerases easily overcome normal duplex structures encountered upon primer extension on a single-stranded DNA template. Usually no inhibition of replication is observed *in vitro* when linear double helix forming oligonucleotides are used as potential terminators (22). On the other hand, triplex formation often leads to substantial inhibition of replication (22, 23). Our recent results (13) with different polymerases showed that while triplex forming circle T1 and hybrid (triplex/duplex) complex forming loop L1, both composed of 2′-O-methylribooligonucleosides, exhibit very strong termination on replication *in vitro*, their 2′-deoxyribo analogues T2 and L2 display a very weak, if any, inhibitory effect (Fig. 5). The dramatic differences observed in the inhibitory effects of these oligonucleotides upon replication *in vitro*, catalysed by different DNA polymerases, cannot be explained by mere differences in the stability of their complexes with the target. Circular oligonucleotide T1, for example, formed a weaker complex (−ΔG°_{37°C} = 23.0 kcal/mol) with the complementary sequence than complex L2 (−ΔG°_{37°C} = 27.0 kcal/mol) but inhibited DNA polymerase-catalysed replication *in vitro* much more strongly. Oligonucleotide L1, in turn, formed a much more stable complex with the target (−ΔG°_{37°C} = 29.9 kcal/mol) than T1 (−ΔG°_{37°C} = 23.0 kcal/mol) but inhibited replication to a comparable extent. Although we can give no clear explanation for this phenomenon, it appears reasonable to think that such a profound difference in relation to the enzyme may be attributed to distinct spatial structures of the complexes formed by the target with the above-mentioned oligonucleotides. At the beginning of the present work we assumed that the same inhibition of replication *in vitro* may possibly be achieved with a circular oligonucleotide that incorporates a sequence complementary to the target and, additionally, possesses a random mismatching stretch. This circular oligomer will form a relatively stable duplex and simultaneously bring a random mismatching sequence into proximity to the template. When encountered, this unusual structure might prevent the DNA polymerase from carrying out further primer extension. Circular oligomers 6 and 8 studied in this work were able to form duplexes with templates M1 and M2 near their 5′-termini (Fig. 5). The primer employed was a 15mer that hybridized with the 3′-terminal part of both templates. Figure 6 shows PAGE of primer extension reactions on templates M1 and M2 catalysed by Klenow fragment in the presence and absence of various circular and linear oligonucleotides. As seen, only circular 2′-deoxyribooligonucleotide 8 exhibits a termination effect upon the reaction performed on template M2. The
Inhibition of luciferase gene expression in an in vitro transcription–translation system. The circular oligonucleotides used contained sequences (15–25 bases) complementary to the initiation codon region of firefly luciferase mRNA (Fig. 8). Linear antisense oligonucleotides binding to this region of analogous result was obtained with similar circular 2′-deoxyribo-oligomer 6a, complementary to template M1 (data not shown). Neither the linear control oligonucleotides C3 and C4 nor, more interestingly, the circular 2′-O-methylribooligomer 6 on M1 showed any clear termination under the same experimental conditions. On the other hand, termination by circular 2′-deoxyribo-oligomer 8 was clear, primer extension being stopped about three bases before the duplex region. In summary, compound 8 appears to work as an inhibitor of E.coli Klenow fragment. The results obtained with thermostable Tth polymerase at elevated temperature, 55°C (Fig. 7), basically resemble those for Klenow fragment. With linear C3 and C4 or circular 2′-O-methylribo-oligomer 6 no termination took place. In contrast, circular 2′-deoxyribo-oligomer 8 displayed distinct termination close to the region of M2/8 duplex. In this case a higher concentration of 8 was required to show the clear effect, the M2/8 ratio being 1:10 with Klenow fragment and 1:50 with the thermostable polymerase. This fact may be attributed to the lower stability of the M2/8 double helix at 55°C. Thus the present work demonstrates that circular 2′-deoxyribo-oligomeranodes are capable of displaying an inhibitory effect upon replication in vitro. As in the previous paper, we can give no clear explanation of the considerable difference observed in termination properties of 2′-deoxyribio- and 2′-O-methylribo-oligomers. One can only speculate again that the spatial structure of the duplex formed by circular oligomer and template is of extreme importance with respect to the inhibitory properties of oligonucleotide analogues.

Figure 5. Structures of circular oligonucleotides 6, 8, 6a, T1 and T2, looped oligonucleotides L1 and L2, synthetic templates M1 and M2 and control oligonucleotides C1, C2, C3 and C4 employed in replication termination experiments. 2′-O-Methylribo-oligomeric units are given in lower case and 2′-O-methylribo-oligomeric units in upper case. Matching sequences are given not underlined not italic and random mismatching sequences are underlined italic. Z, 3′-deoxyribo- and 2′-deoxyribo-oligonucleotides are shown. Neither the linear control oligonucleotides nor, more interestingly, the circular 2′-ribo-oligomer showed any clear termination under the same experimental conditions. On the other hand, termination by circular 2′-deoxyribo-oligomer 8 was clear, primer extension being stopped about three bases before the duplex region. In summary, compound 8 appears to work as an inhibitor of E.coli Klenow fragment. The results obtained with thermostable Tth polymerase at elevated temperature, 55°C (Fig. 7), basically resemble those for Klenow fragment. With linear C3 and C4 or circular 2′-O-methylribo-oligomer 6 no termination took place. In contrast, circular 2′-deoxyribo-oligomer 8 displayed distinct termination close to the region of M2/8 duplex. In this case a higher concentration of 8 was required to show the clear effect, the M2/8 ratio being 1:10 with Klenow fragment and 1:50 with the thermostable polymerase. This fact may be attributed to the lower stability of the M2/8 double helix at 55°C. Thus the present work demonstrates that circular 2′-deoxyribo-oligomeranodes are capable of displaying an inhibitory effect upon replication in vitro. As in the previous paper, we can give no clear explanation of the considerable difference observed in termination properties of 2′-deoxyribio- and 2′-O-methylribo-oligomers. One can only speculate again that the spatial structure of the duplex formed by circular oligomer and template is of extreme importance with respect to the inhibitory properties of oligonucleotide analogues.

Inhibition of luciferase gene expression in an in vitro transcription–translation system. The circular oligonucleotides used contained sequences (15–25 bases) complementary to the initiation codon region of firefly luciferase mRNA (Fig. 8). Linear antisense oligonucleotides binding to this region of

Figure 7. Influence of circular 6 and 8 on Th-catalysed polymerization on templates M1 and M2. Lane 1, [32P]M2; lane 2, control oligonucleotide [32P]C2; lanes 3 and 18, control oligonucleotide [32P]C1; lane 4, influence of 6 on polymerization on M1 (6:M1 10:1); lane 5, influence of 6 on polymerization on M1 (6:M1 50:1); lane 6, influence of 6 on polymerization on M1 (6:M1 100:1); lane 7, influence of 8 on polymerization on M2 (8:M2 10:1); lane 8, influence of 8 on polymerization on M2 (8:M2 50:1); lane 9, influence of 8 on polymerization on M2 (8:M2 100:1); lane 10, polymerization on M1 in the absence of inhibitor; lane 11, polymerization on M2 in the absence of inhibitor; lane 12, influence of C3 on polymerization on M1 (C3:M1 10:1); lane 13, influence of C3 on polymerization on M1 (C3:M1 50:1); lane 14, influence of C3 on polymerization on M1 (C3:M1 100:1); lane 15, influence of C4 on polymerization on M2 (C4:M2 10:1); lane 16, influence of C4 on polymerization on M2 (C4:M2 50:1); lane 17, influence of C4 on polymerization on M2 (C4:M2 100:1); lane 19, [32P]M1.

Figure 8. Structures of linear 9, 11 and 13, circular 10, 12 and 14, control C5, C6 and C7 oligonucleotides and the fragment of firefly luciferase mRNA employed in inhibition of luciferase gene expression in an in vitro transcription–translation system. 2′-Deoxyribonucleoside and ribonucleoside units are given in upper case and 2′-O-methylribonucleoside units are in lower case letters. Matching sequences are given in bold and random mismatching sequences are underlined italic. ∼SS∼, disulphide linker.

Figure 9. Influence of linear 9, 11 and 13 and circular 10, 12 and 14, control C5, C6 and C7 oligonucleotides on luminescence caused by firefly luciferase activity.
oligonucleotides should contain a relatively short random mismatching sequence. Circularization itself does not seriously influence these antisense properties and the advantage of using circular oligomers lies in their higher stability against nuclease degradation in comparison with their linear counterparts (25–27). It is also interesting to note that linear 2′-O-methylribooligonucleotide C7, complementary to the same –9 to +16 region of the mRNA, had no effect upon luminescence at concentrations of 0.05–3 µM. Since 2′-deoxyl oligonucleotide C5 and 2′-O-methylribooligonucleotide C7 demonstrated completely different results in our experiments we can reasonably assume that RNAs H mediates cleavage of mRNA in the transcription–translation assay employed in this work. Therefore, it is most probably this process that is reflected in the loss of luminescence in the presence of both linear and circular antisense oligonucleotides. The ability of the circular oligomers to cause cleavage of RNA by RNase H has been demonstrated previously (28).

CONCLUSION

The present work demonstrates that double helix forming circular 2′-deoxyribooligonucleotides containing sequences complementary to the target nucleic acids and possessing random mismatching sequences may be employed to influence certain key biochemical processes. By tuning the structure of these compounds one can form nucleic acids complexes which would bring about either inhibition or preservation of the enzymatic reactions of interest. Additionally, these compounds may display new properties (e.g. increased stability). Although knowledge of the spatial structures of complexes of modified circular oligonucleotides with nucleic acids has yet to be gained and although certain efforts concerning proper tuning of these compounds lie ahead, we believe that interesting features of the cross-linked oligonucleotides reported here may be useful for the design of new antigen and antisense compounds.

ACKNOWLEDGEMENT

Financial support from the Ministry of Education, Academy and Technology Development Centre of Finland, is gratefully acknowledged.

REFERENCES