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Oligonucleotide circularization by template-directed chemical ligation

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ABSTRACT

An efficient method for producing the covalent closure of oligonucleotides on complementary templates by the action of BrCN was developed. A rational design of linear precursor oligonucleotides was studied, and the effect of factors such as oligonucleotide concentration and oligomer-template length ratio was evaluated. The efficiency of circularization was shown to correlate well with the secondary structure of the precursor oligomer (as predicted by a simple computer analysis), hairpin-like structures bearing free termini clearly favouring the circularization reaction. A novel idea, consisting of the incorporation of non-nucleotide insertions in the precursor oligomer (namely, 1,2-dideoxy-D-ribofuranose residues), may render this method universal and highly effective. An original set of assays was developed to confirm the circular structure of the covalently closed oligonucleotides.

INTRODUCTION

Extensive studies of chemical reactions in DNA duplexes have made it possible to develop a procedure (modelling the DNA ligase reaction) for the effective condensation of oligonucleotides on complementary templates using chemical reagents (chemical ligation) (1). Chemical ligation gives the possibility to incorporate various modifications of the sugar–phosphate backbone at a particular site when DNA duplexes are being assembled (2), and its efficiency has been shown to depend strongly on the nature of the reacting nucleotide residues (3). Obviously, such approach is more convenient than enzymatic ligation for the design of topologically unusual DNA.

We are interested in studying the potentialities of chemical ligation in producing the circularization of natural and modified oligonucleotides. One of the major interest of circular oligonucleotides concerns their use as antisense compounds, due to their increased resistance to degradation by cellular exonucleases. By using enzymatic ligation with T4 DNA ligase, we were able to circularize 42-mer oligonucleotides carrying a self-complementary sequence bearing the ligation site (Fig.1A; M.B., unpublished results and ref.4). *In vitro* studies showed that the resulting covalently closed oligonucleotides have a $t_{1/2}$ in serum that is at least 100 times higher than the $t_{1/2}$ of a linear oligomer (4). Most importantly, circular oligonucleotides can properly hybridize to complementary RNAs all along the complementary region, as shown by S1 mapping, forming DNA/RNA hybrids that are efficiently digested by RNase H (4). The enzymatic approach, while being useful in providing material for analytical studies, cannot be envisaged for producing large quantities of circular oligonucleotides, as required for *ex vivo* or *in vivo* antisense experiments.

Recently, the successful chemical cyclization of oligonucleotides having two runs of pyrimidines has been described (5). A homopurine template was used to form a triple-helical complex and, thus, to align the reactive ends. However, this technique can only be used to circularize homopyrimidine-containing precursors, thus considerably limiting the number and type of targets that might be addressed by this approach.

Here we describe the obtention by chemical ligation of circular oligomers containing mixed sequences, not restricted to polypyrimidine ones. Our study provides the basis for a rational design of oligonucleotide precursors in order to obtain an efficient circularization.

MATERIALS AND METHODS

Buffer solutions

Buffers used in this work were: A (for BrCN-induced ligation): 0.25 M MES adjusted with $(C_2H_5)_3N$ to pH 7.5, 0.02 M $MgCl_2$; B (for EDC-induced ligation): 0.05 M MES adjusted with $(C_2H_5)_3N$ to pH 6.0, 0.02 M $MgCl_2$; C (for snake venom phosphodiesterase and bacterial alkaline phosphatase): 0.2 M Tris–HCl, pH 8.5, 0.04 M $MgCl_2$; D (for exonuclease VII): 0.07 M Tris–HCl, pH 8.0, 0.008 M EDTA, 0.01 M 2-mercaptoethanol; E (for T4 polynucleotide kinase): 0.05 M Tris–HCl, 0.01 M $MgCl_2$, 0.005 M 2-mercaptoethanol, 0.002 M spermidine, pH 9.0.

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Oligonucleotides

Oligonucleotides used in this study are depicted in Table I. Oligodeoxyribonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer, using standard phosphoramidite chemistry. Phosphoramidite 1,2-didesoxy-D-ribose was prepared according to ref. (6). Incorporation of the 1,2-didesoxyribose monomers in oligonucleotides was performed according to the standard protocol for the Applied Biosystems automated synthesizer, using condensation steps of 2 min. The coupling efficiency of the modified monomer was essentially the same as that of the non-modified ones. Subsequent deblocking and isolation procedures were carried out according to standard phosphoramidite protocols.

Oligonucleotides with a 3′-terminal phosphate group were obtained as described in ref. (7,8). 5′-^{32}P phosphorylation of oligonucleotides carrying a 3′-phosphate group was performed by standard procedures (9).

Circularization of 3′-phosphorylated oligonucleotides (phosphodiester linkage formation)

Linear precursors and template oligomers were combined in a 1:2 ratio and dissolved in buffer A. Oligonucleotide concentration (per monomer) was 10^{-3}, 10^{-4} or 5×10^{-5} M. Reaction mixtures were incubated at 95°C for 1−2 min and slowly cooled to 0−4°C during 2−3 hours. Then, 5 M BrCN in dry acetonitrile was added (1/10 of the total volume). After 1−3 min at 0−4°C, oligonucleotides were precipitated with ethanol, dried and analyzed by denaturing polyacrylamide gel (20%) electrophoresis (PAGE). EtBr staining was used to visualize the DNA fragments.

Circularization of 5′, 3′-diphosphorylated oligonucleotides (pyrophosphate linkage formation)

Circular oligomers were obtained using EDC, imidazole or *N*-hydroxybenzotriazole as described in ref. (10−12), respectively. Reaction mixtures were analyzed by PAGE as described above.

Analysis of ligation products

Phosphodiesterase and phosphatase treatment. Gel-purified oligonucleotides were incubated with a mixture of snake venom phosphodiesterase (PDEI) and bacterial alkaline phosphatase (BAP) in buffer C for 1 hour at 37°C. After ethanol-precipitation, oligonucleotides were analyzed by PAGE on 20% denaturing gels. EtBr staining was used to visualize the DNA fragments.

Exonuclease VII treatment. Digestion of gel-purified ligation products with Exo VII (Gibco-BRL) was carried out for 1 hour at 37°C in buffer D. After incubation, reaction mixtures were analyzed as described above.

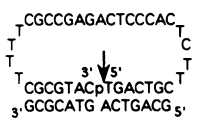
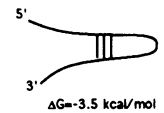
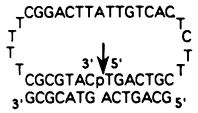
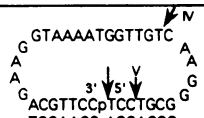
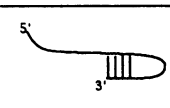
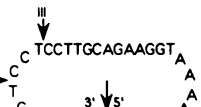
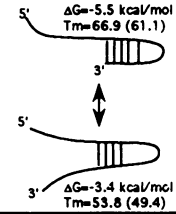
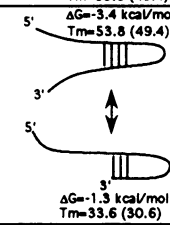
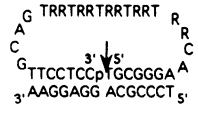
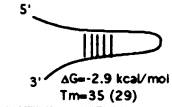
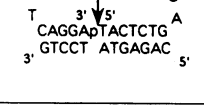
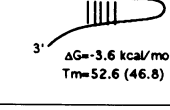
Radioactive phosphorylation with T4 polynucleotide kinase. Aliquots of the reaction mixtures after cyclization of 3′-phosphorylated precursors were precipitated with ethanol and further incubated in buffer E with T4 polynucleotide kinase and γ-^{32}P-ATP, using standard procedures (9). Reaction products were electrophoretically separated on a 20% denaturing gel and then subjected to autoradiography at −80°C with X-Omat X-ray film and intensifying screen.

Maxam−Gilbert sequencing. Gel-purified ^{32}P-labeled ligation products, obtained by circularization of 5′-^{32}P, 3′-diphosphorylated precursors, were cleaved at dG residues by the standard Maxam−Gilbert sequencing procedure (9), and further analyzed by PAGE and autoradiography as described above.

Computer analysis

Secondary structure of oligonucleotides was analyzed by the OLIGO 4.O Primer Analysis Software (National Biosciences, Inc.), which bases the calculations on the measurement of nearest-neighbour ΔG values (13, 14).

Table I. Correlation between the predicted conformations of the oligonucleotide precursors used in this work and the observed yield of circularization

NUMBER	DUPLEX COMPOSITION #	PREDICTED SECONDARY STRUCTURE OF PRECURSOR *	YIELD OF CIRCULAR PRODUCT (%) §
I			80-90
II		–	20-30
III			2-5
IV			20-30
V			20-30
VI			95
VII			70-80

3′/5′ indicates the ligation site in each precursor. Precursors III, IV and V contain the same sequence but differ in the position of the ligation sites. The alternative sites are shown by additional arrows marked with the precursor number. R indicates 1,2-dideoxy-D-ribofuranose.

*Precursor structural analysis was performed as described in Materials and Methods. Vertical bars represent Watson−Crick base-pairings. ΔG values were calculated for 4°C and 1 M NaCl. Tm values are calculated for 0.25 M MES and 0.05 M MES (in brackets), and are expressed in C.

§BrCN-induced ligation reactions were performed under optimal conditions (see text). S.D. ± 5%

RESULTS AND DISCUSSION

Direct solid-phase synthesis of cyclic oligonucleotides has been recently reported to produce short-size oligomers (15–17). While this technique might be used for the synthesis of short circular oligonucleotides, useful as tools for DNA conformational studies, it is however inadequate to obtain longer sequences, as required for an antisense application. An alternative for producing circular oligonucleotides is to use the ligation of a linear precursor forming a double-helix with a template oligomer (Fig.1B). The best strategy to perform efficient ligation of such complexes is to use chemical ligation methods (1), since enzymatic ligation might be greatly influenced by some structural features of the resulting precursor–template duplexes.

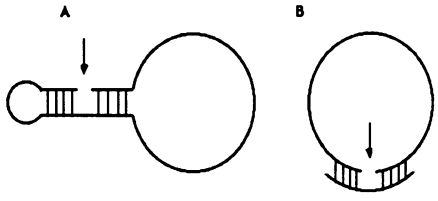


Figure 1. Strategies for obtaining circular oligonucleotides. **A** represents a 'dumbbell-like' precursor oligonucleotide that can be closed without need of a template. The ligation site, represented by an arrow, is contained in the double-stranded region. **B** shows the scheme for obtaining circular oligonucleotides by means of template-directed ligation. The linear precursor oligonucleotide has to hybridize on both ends to a short complementary template; ligation site is represented by an arrow.

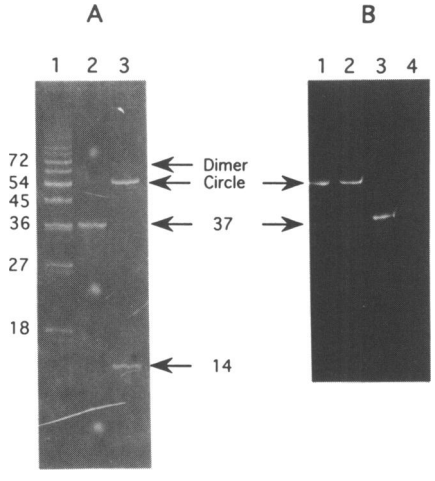


Figure 2. BrCN-induced circularization of a 37-mer oligonucleotide using a 14-mer template. **A.** 3′-phosphorylated precursor and template in duplex I (Table I) were subjected to chemical ligation with BrCN as described in Materials and Methods. Total nucleotide concentration was 10^{-4} M and precursor to template ratio was 1:2. The reaction mixture (lane 3) was analyzed by PAGE on a 20% denaturing gel; EtBr staining was used to visualize the DNA fragments. Lane 2 shows a control reaction in which the template was omitted. Lane 1 displays a ladder of 9-mer concatemers as size markers. The arrows indicate the positions of the putative linear dimer (74-mer), the putative circular monomer, the 37-mer precursor and the 14-mer template, respectively. **B.** Gel-purified oligomers corresponding to the linear precursor (lanes 3 and 4) and the presumed circular oligonucleotide (lanes 1 and 2) were digested with BAP and PDEI (lanes 2 and 4), as indicated in Materials and Methods. Lanes 1 and 3 show the undigested controls. After reaction, the mixtures were analyzed by PAGE on a 20% denaturing gel. Detection was performed by EtBr staining of the gel.

Previous data concerning chemical ligation (1–3, 10–12) allowed us to predict some properties concerning the design of the linear precursors. Firstly, the length of the oligonucleotide precursor should be sufficient to provide a stable duplex formation with the template, bringing the reactive ends adjacent to one another. For that, it seems reasonable that the precursor has at least 6+6 nucleotides (nt) complementary to the template. In addition, the loop size has to exceed the number of nucleotides involved in base-pairing with the template (>12 nt), in order to be sure that the ligation site is completely hybridized to the template. Secondly precursors should preferentially bear a 3′ phosphate group, since the internucleotide linkages form more readily with the reactive phosphate at this end of the oligomer (1, 2). Thirdly, since the chemical ligation efficiency depends strongly on the nature of the nucleotide residues to be ligated (3), precursors should be designed carrying the most 'productive' contacts in the ligation site, namely −C 3′ p + 5′ T−, −T 3′ p + 5′ T− or −A 3′ p + 5′ T−.

In addition to the above mentioned features, the following factors have to be considered for obtaining optimal circularization conditions: the relationship between oligomer circularization and competing concatemerization reactions, and the influence of nucleotide concentration on both processes; the optimal molar ratio of linear precursor to template and their respective lengths; the influence of the secondary structure of the linear precursors on the efficiency of circularization; and finally, the search of optimal condensing reagents. Also, several criteria have to be established to clearly confirm the circular structure of the ligation products.

Since one of the putative applications of circular oligonucleotides is to use them as antisense compounds, we

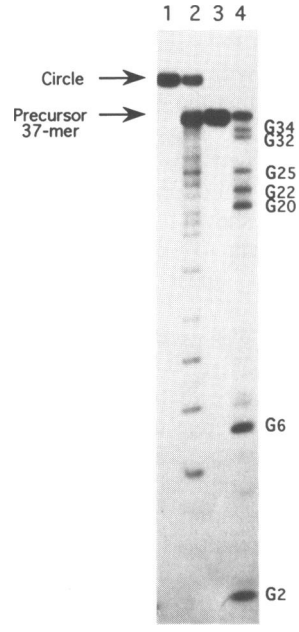


Figure 3. Maxam–Gilbert sequencing analysis of the circular ligation product. Gel-purified 5′ ^{32}P-labeled precursor I 5′ p*TGACTGCTTCTCACCCTCA-GAGCCGCTTTTCGCGTAC 3′ (lanes 3 and 4) and its circular product (lanes 1 and 2), obtained as described in the text, were subjected to the Maxam–Gilbert dG-specific cleavage (even lanes) and further analyzed by PAGE and autoradiography, as detailed in Materials and Methods. Odd lanes show the unreacted controls.

synthesized 30−40 nt-long precursors, so that the final circles had no size constraints to allow hybridization with mRNAs over 15−18 nucleotides. Such oligomers, as well as the corresponding 3′ or 5′ phosphorylated derivatives, can nowadays be easily obtained on DNA synthesizers by the standard phosphoramidite procedure.

Our first model system consisted of a 37-mer linear precursor and a 14-mer template (Table I, duplex number I). Computer analysis predicted that the 37-mer precursor I might fold into only one probable structure having a negative DG at the reaction temperature (4°C). As shown in Table I, the predicted structure consisted in a stable hairpin leaving both 3′ and 5′ ends free to interact with a complementary sequence, and having a Tm= 65.7°C under the BrCN reaction conditions (250 mM MES). We first subjected duplex I to chemical ligation in the presence of BrCN at a total nucleotide concentration (Co) equal to 10^{-4} M and a precursor:template ratio of 1:2, and we analyzed the reaction products by electrophoresis on 20% denaturing gels. As shown in Figure 2, under these conditions the 37-mer precursor I almost quantitatively (85−90%) gave rise to a slower migrating ligation product, comigrating with a 54-mer size marker (Fig.2A, lane 3). In addition, a very faint band (less than 5% of the original precursor) was observed migrating at about the position of a 72 bases-long marker (Fig.2A, lane 3). This band probably corresponded to the linear precursor dimer (74 nt) that results from the binding of one mole of template to two moles of precursor. The dimeric nature of this band was confirmed by sequencing analysis (see below).

As previously described (1), chemical ligation was dependent on the presence of a template, since no ligation products were observed when the 14-mer template oligonucleotide was omitted (Fig.2A, lane 2).

The same pattern was observed when the precursor-template ratio was 1:3 (data not shown); on the contrary, increasing Co to 10^{-3} M resulted in the preferential obtention of the linear dimer (results not shown).

In order to confirm that the major reaction product (Fig.2A, lane 3) corresponded to the expected circular 37-mer, we incubated the gel-purified ligation product with a mixture of bacterial alkaline phosphatase and snake venom phosphodiesterase. As shown in Figure 2B, the ligation product was completely resistant to exonucleolytic degradation under conditions in which the linear 37-mer precursor I was totally degraded (compare lanes 2−4). Similar results were obtained when exonuclease VII was used (data not shown). The absence of free termini in the putative 37-mer circle was further confirmed by its complete resistance to be labeled with γ-^{32}P-ATP and polynucleotide kinase, whereas the 37-mer linear precursor I and the 74-mer linear dimer were efficiently labeled (data not shown).

A 5′ and/or 3′ end modification of the linear precursor, not involving any circularization, would equally account for the exonuclease or kinase results as described above. A more direct confirmation of the circular nature of the chemical ligation product was provided by a sequencing analysis. For doing so, we replaced the 3′P- linear precursor by 5′-^{32}P 37-mer carrying a 3′ OH, so as to obtain radioactive products after BrCN condensation. As previously reported, ligation of oligonucleotides carrying 5′ phosphate groups is less effective than that of 3′ phosphorylated oligomers (1). In agreement with these results, after ligation of the radioactive 37-mer precursor I we observed 40% of unreacted product, 40% of putative circle comigrating with a 54-mer marker, and about 20% migrating at the 74-mer dimer position (results not shown). After gel purification, the different radioactive bands were subjected to a Maxam−Gilbert cleavage reaction specific for dG, and further analyzed by PAGE on 20% denaturing gels and autoradiography. This original approach unambiguously allows to the identification of a circular monomeric oligonucleotide from a linear precursor and a linear dimer. Thus, the linear precursor I gives a set of bands corresponding to the expected dG positions in the sequence (Fig.3, lane 4), while the cyclic product gives a main band comigrating with the 37-mer precursor oligonucleotide, with no intermediate bands appearing between the unreacted circle and the linear precursor (Fig.3, lane 2). This pattern corresponds exactly to that expected for a radioactive circular molecule, since each unique cleavage at a dG position will regenerate the linear 37-mer. Moreover, as the Maxam−Gilbert reaction is performed so as to cleave only once per molecule, it is normal to obtain the linear 37-mer as the major band. In addition, double-cleavage reactions at different dG positions give a more complicated pattern of bands ranging from 5 to 35 nt, but still recognizable and specific for each oligonucleotide (Fig.3, lane 4). An even more complicated band pattern is obtained after cleavage of a linear dimer (data not shown), since such a compound contains ^{32}P at two positions. However, the main characteristic of a dimer is that a continuous ladder of radioactive bands appears between the dimer and the monomer upon cleavage, contrasting with the sharp transition between the circle and the monomer, already discussed. Therefore, the sequencing approach provides a unique way to confirm the circular or multimeric condition of a ligation product.

Concerning the condensing reagent, BrCN-induced ligation of 3′P 37-mer precursor I to produce a phosphodiester linkage, was more efficient than EDC-induced condensation and preactivation by imidazole or *N*-hydroxybenzotriazole of 5′, 3′-diphosphorylated 37-mer precursor I to form a pyrophosphate linkage (data not shown). Since this is not the case when considering linear template-dependent chemical ligation (2), it is possible that the EDC-ligation conditions (50 mM MES) are too stringent and thus, the hairpin conformation required for circle synthesis is not formed.

To test the effect of varying the precursor to template length ratio, we studied the circularization of precursor I under optimal ligation conditions, using templates of increasing length. These experiments showed that the precursor oligonucleotide must be at least twice as long as the template in order to obtain circular and not concatemeric oligomers (data not shown).

Next, we investigated the influence of precursor secondary structure on the efficiency of circularization in the BrCN-induced ligation. As already demonstrated, the 37-mer precursor I was circularized to almost 90%, when subjected to optimal experimental conditions (see above). To check the influence of the precursor conformation (Table I) on ligation efficiency, we designed a 37-mer precursor II, containing 8 base-changes respect to the former oligonucleotide, which destroyed the putative hairpin conformation without changing the base-pairing with the 14-mer template (Table I, duplex II). Under optimal ligation conditions, the yield of circular product obtained from precursor II was 20−30%, the dimer as well as higher molecular weight forms being equally obtained (results not shown). This data strongly indicates that a particular conformation, rather than a linear structure, is probably needed in order to allow the condensation reaction to take place intra- and not intermolecularly.

Essentially similar results were obtained with another oligonucleotide, precursor VII, differing from precursor I both

in length and sequence. As shown in Table I (duplex VII), when the 27-mer precursor VII was subjected to BrCN-induced ligation using a 12-mer template, it gave 70–80% of circular monomer. In addition, computer analysis predicted for precursor VII a similar hairpin structure as for precursor I (Table1). Therefore, efficient oligonucleotide circularization correlates well with the prediction of a hairpin conformation.

To better understand the structural requirements of the template-directed chemical ligation, we designed a 36-mer oligonucleotide consisting of 18 bases complementary to the acceptor splice junction of herpes simplex virus type 1 (HSV-1) pre-mRNA IE4 and IE5 (18), plus 18 bases of random sequence. We chose the three possible -C 3′/5′ T- sites present in the circularly represented sequence, and we synthesized the corresponding 3′P precursors and 14-mer templates, as depicted in Table I (duplexes III, IV and V). Table I shows the most probable predicted conformation of each precursor, as well as the yield of the resulting circular products, obtained after ligation under optimal conditions.

Several important characteristics can be inferred from these results. First, as in the case of precursor III, the presence of a stable base-pairing involving the 3′ or the 5′ ends completely inhibits chemical ligation, not only for the formation of circular oligomers, but also for the synthesis of linear multimers. Confirming results were obtained with two other oligonucleotides containing 4 or 5 mutations respect to precursor III, and which present a similar predicted structure (data not shown). Second, when other alternative conformations, such as hairpins having free termini, were also predicted (Table I, precursors IV and V), the total ligation increased and the yield of circular products rose to 20–25%. It is interesting to note that in the case of precursor I, which circularize to 80–90%, and of precursor VII, which yielded 70–80% of circular product, the hairpin structure was the only predicted stable conformation. Therefore, hairpins with free 3′ and 5′ ends seem to be favourable structures for oligonucleotide circularization, and the yield of the circularization reaction most probably reflects the equilibrium concentration of the hairpins in the starting reaction conditions.

The previous examples strongly suggest that secondary structure is the major factor that influences the efficiency of template-directed oligonucleotide circularization. It is also clear, that inadequate conformations cannot systematically be avoided when designing 30–40 bases-long oligonucleotides, as theoretically required for antisense circular molecules. One way to restrict the conformational problems, is to replace the irrelevant non-antisense sequences in the linear precursors by non-nucleotidic insertions. This would substantially decrease the number of intramolecular base-pairings, thus allowing a more rational design of the precursors. In addition, the resulting circular oligonucleotides should also present a reduced potential for non-specific interactions with RNA within cells, which constitutes a major problem for the therapeutic application of antisense oligonucleotides (19).

As a first approach, we studied the effect of including repeats of alternating residues of 1,2-dideoxy-D-ribofuranose and thymidine. Table I depicts the structure of precursor VI, which represents a substituted form of precursor V. Computer analysis of precursor VI, in which 16 bases of oligomer V were replaced by five (TTR) repeats, predicts the presence of only one stable conformation having a negative DG, corresponding to a stable hairpin having both 3′ and 5′ free termini (Table I, compare precursor V to VI). When 3′P precursor VI was subjected to BrCN-induced ligation using the corresponding 14-mer template (Table I, duplex VI), the linear precursor was almost quantitatively transformed into a circular oligomer, the cyclic structure being confirmed by all the analytical methods already mentioned (data not shown). Again, as previously discussed, it seems clear that hairpins with free 3′ and 5′ ends favour template-dependent circularization of oligonucleotides (Table I, compare yield of cyclic product for precursor V and VI, respectively). Thus, non-nucleotidic insertions may be very useful for eliminating 'unproductive' oligonucleotide conformations, and must clearly be considered as a powerful tool for rational oligonucleotide design.

CONCLUSIONS

Single-stranded circular oligonucleotides can be obtained by using template-directed chemical ligation. Under optimal ligation conditions, the efficiency of the circularization reaction seems to depend on the oligonucleotidic precursor secondary structure and the precursor-template length ratio. The principal criteria for the design of precursors are to avoid the formation of 3′ and/or 5′ base-paired ends, and to choose sequences that will preferentially adopt a hairpin-like structure bearing free terminal ends. Any rational design that would increase the proportion of oligonucleotide molecules in such favorable conformation, should consequently result in an increased production of circular molecules. One way of doing so is to replace irrelevant sequences in the oligonucleotide precursors by non-nucleotidic insertions, like 1,2-dideoxyribose repeats.

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