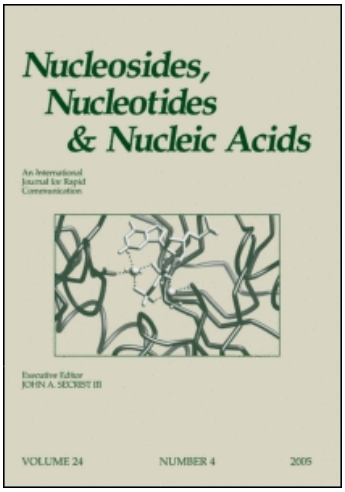

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Oligonucleotide Analogues with Terminal 3'-3'- and 5'-5'-Internucleotidic Linkages as Antisense Inhibitors of Viral Gene Expression

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OLIGONUCLEOTIDE ANALOGUES WITH TERMINAL 3'-3'- AND 5'-5'-INTERNUCLEOTIDIC LINKAGES AS ANTISENSE INHIBITORS OF VIRAL GENE EXPRESSION

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Abstract: Oligodeoxynucleotides with terminal 3'-3'- and 5'-5'-internucleotidic linkages were found to be stabilized towards intracellular degradation and to inhibit gene expression in vitro and in vivo at concentrations of 10-30 μM.

INTRODUCTION

The concept of antisense oligonucleotides as potential therapeutics has produced a wide variety of structural modifications designed to meet the requirements of a) cellular uptake, b) stable and discriminating hybridization and c) stability towards intracellular degradation.[1] In view of problems arising e.g. from chirality or hydrophobicity, as well as from the risk of toxicity and antigenicity in vivo, we have taken interest in looking at analogous structures, which minimally deviate from biological oligonucleotides, although still showing an inhibitory effect on gene expression. As candidates, we studied oligonucleotides, which have terminally inverted polarity, i.e. 3'-3'- and 5'-5'-internucleotidic linkages ("INV"-linkages) at the ends of the sequence. This concept is based on the assumption that intracellular degradation if mainly due to exonucleases and thus, modification at the ends will suffice to stabilize the sequence.[2] "INV"-linkages have often been mentioned as products of undesired phosphorylation, and the properties of "INV"-dimers have been studied.[3] Oligomers containing an internal[4] or terminal[5] "INV"-linkage have been made for protection and

TABLE 1

No.	Sequence
(1)	HO(3')T(5'-5')TTTTTTTTTTTTTTTTTT(3'-3')T(5')OH
(2)	5' TTTTTTTTTTTTTTTTTTT 3'
(3)	HO(3')T(5'-5')CCATCTTTGCAAAGC(3'-3')T(5')OH
(4)	5' TCCATCTTTGCAAAGCT 3'
(5)	5' AGCTTTGCAAAGATGGA 3'
(6)	HO(3')T(5'-5')CTCTGTTTAAAACTTTATCCATCTTTGCAAAGC(3'-3')T(5')OH
(7)	5' TCTCTGTTTAAAACTTTATCCATCTTTGCAAAGCT 3'
(8)	5' AGCTTTGCAAAGATGGATAAAGTTTTAAACAGAGA 5'

labelling or for structural studies, however, oligonucleotides with two "INV"-ends to our knowledge have not been taken into account as potential "antisense" agents.

RESULTS

Synthesis and Sequence Analysis: Oligonucleotides with "INV" termini were made by routine automated phosphoramidite or H-phosphonate preparations[6], except that 3'-DMTr-nucleosides[7] - 5'-linked to CPG support[6] - were used at the start and - as 5'-phosphoramidites or 5'-H-phosphonates - at the end of the synthesis. Sequences thus prepared, together with unmodified references, are shown in Table 1. Alternatively, we used "INV"-dimer blocks, the preparation of which will be discussed elsewhere.

Structural proof for the "INV"-linkages was obtained from ^{1}H-NMR spectroscopy of the respective dithymidylates[8]. Sequence analysis was done by the method of Maxam and Gilbert after phosphorylation of the 5'-OH group protruding from the 3'-3'-end with γ-^{32}P-ATP/polynucleotide kinase. The sequence is, thus, read from the "reverse", i.e. 3'-side of the 3'-5'-linked "core" sequence.

Behaviour towards snake venom phosphodiesterase: Snake venom phosphodiesterase is known to cleave biological oligonucleotides from their 3'-end within 5-15 min. at 37°C. When (1) was incubated with this enzyme under standard conditions[9], we observed immediate loss of one pdT-unit from the 5'-5'-

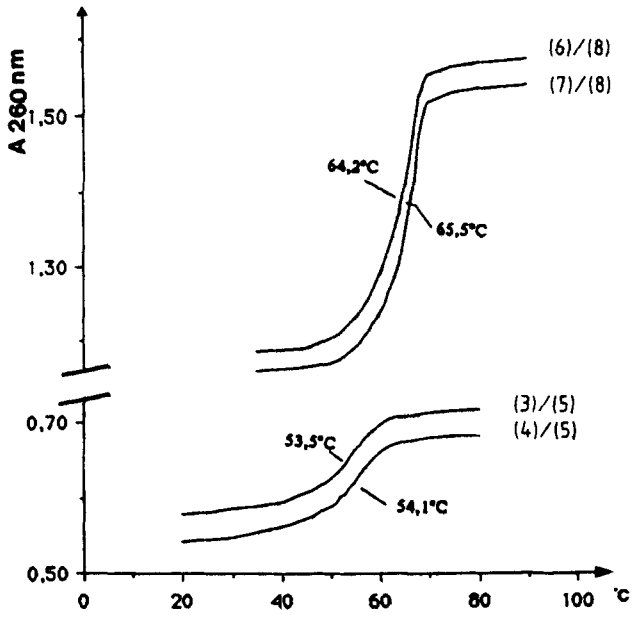

FIG. 1
Hybridization behaviour of INV- and unmodified antisense sequences (explanations see text)

end. The residual molecule, exhibiting a 5'-OH and 3'-3'-phosphate end as expected[9], was only slowly degraded within ca. 90 min., the appearance of a ladder of truncated chains suggesting an assistance by contaminating endonuclease activity[10].

Hybridization Studies: As an example, the melting points of the hybrids formed between the SV40-specific antisense sequences (3) and (6), together with unmodified (4) and (7), and the corresponding sense strand fragments (5) and (8) were determined in 10 mM sodium cacodylate/100 mM NaCl buffer at pH7. As shown in Fig. 1, the difference of melting points between duplexes with modified and unmodified ends was found to be around 1°. Thus, there is little change of melting behaviour due to end inversion.

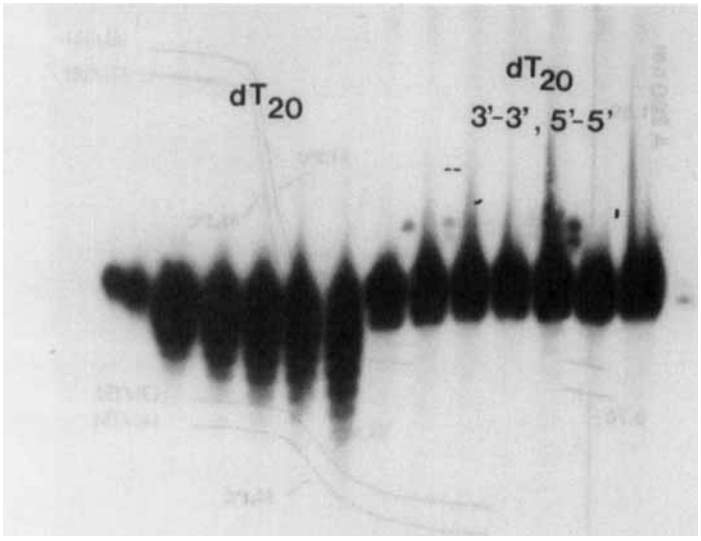

FIG. 2

Test for degradation in human blood serum: lanes 1 to 6: (2) after treatment for 0, 5, 8, 11, 15 and 30 min.; lanes 7 to 13: (1) after treatment for 0, 5, 8, 11, 25, 30 and 90 min.

Stability in Human Serum: Serum stability was tested by incubating 0.01 $O.D._{260}$ (1 µl) ^{32}P-labelled (1) in fresh human serum, as described by Wickstrom[11] at 37°C. As a reference, (2) was similarly incubated. As shown in Fig. 2, the eicosathymidylate with inverted ends (1) was completely undegraded after 90 min., whereas significant breakdown was observed in case of the biological oligomer (2) after 30 min..

Inhibition of Gene Expression in Cell Culture System: A model system, consisting of SV40-infected COS 1 cells, was selected to test for inhibition of gene expression. Antisense oligonucleotides (3) and (6) used in this study were directed towards the translation start of T-antigen, which is constitutively expressed in these cells. The respective sense strand fragments (5) and (8) were used as a reference. Ca. 10^6 cells were grown as a monolayer, washed and incubat-

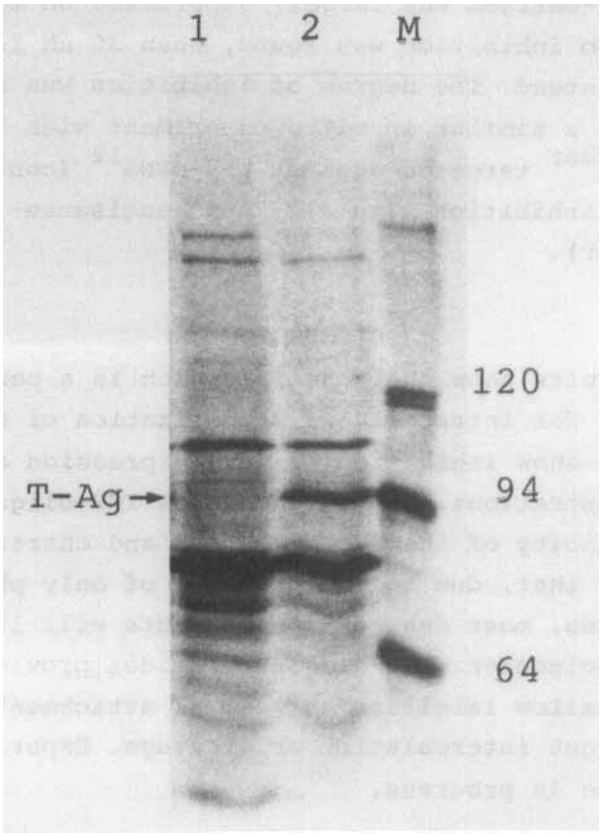

FIG. 3

Autoradiograph of protein gel from lysate of SV40-T-antigen producing COS 1 cells after incubation (lane 1) with 30 µM (3) resp. (lane 2) with 30 µM (5). Lane 3: length standards

ed with one of the modified oligonucleotides and with ^{35}S-methionine for 30 min. at 37°C. After cell lysis, T-antigen was immunoprecipitated[12]. The immunoprecipitates were washed as described[12], lyophilized, redissolved by boiling in Lämmli buffer and applied on 1% SDS-PAGE. The gel bands were visualized on X-ray film. As shown in Fig. 3, the band corres-

ponding to T-antigen was largely suppressed on addition of 30 μM (3). No inhibition was found, when 30 μM (4) or (5) were used instead. The degree of inhibition was determined to be 70% in a similar in vitro experiment with 10 μM INV-antisense 25^{mer} targeted against p53-mRNA^{12} (control: insignificant inhibition with all 3'-5'-antisense- or sense-penteicosamer).

DISCUSSION

Our results show that end inversion is a new and efficient method for intracellular stabilization of oligonucleotides, which show inhibition of gene expression at relatively low concentrations. The advantage of INV-oligonucleotides is the simplicity of their preparation and characterization and the fact that, due to the presence of only phosphodiester linkages, most degradation products will likely be biological molecules. INV-oligonucleotides provide for end groups that allow labelling as well as attachment of residues for target intercalation or cleavage. Experiments on this line are in progress.

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