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Resistance to Degradation by Nucleases of (2′S)-2′-Deoxy-2′-C-methyloligonucleotides, Novel Potential Antisense Probes

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ABSTRACT

An oligonucleotide must fulfill two main requirements to become a potential antisense probe: effective hybridization properties with the complementary sequence and stability toward nucleases. In this article the degradation pattern of a new class of potential antisense fragment, (2′S)-2′-deoxy-2′-C-methyloligonucleotides, is analyzed. The results described here show that the modification introduced in these oligonucleotides confers an enhanced stability toward purified nucleases, human sera, and HeLa cell extracts.

INTRODUCTION

The development of new antisense oligonucleotides faces the difficulty of addressing two different requirements (Cohen, 1989). On the one hand, the modified fragment must recognize the complementary target sequence and on the other it must be stable to the degradative activity of nucleases present in the biological systems in which it is expected to exert its action. The latter requirement is particularly important for the development of therapeutic agents.

Several modifications have been proposed in order to obtain suitable fragments for antisense strategies (English and Gauss, 1991). Among these, the introduction of different substituents in the 2′-position has provided oligonucleotides with interesting antisense characteristics (Cook, 1991). Representative example are 2′-*O*-alkyloligonucleotides, which are now being widely used (Sproat et al., 1989).

(2′*S*)-2′-Deoxy-2′-C-methyloligonucleotides (Fig. 1) (Cicero et al., 1993a) is the first member of a new family of 2′-modified oligonucleotides, carrying substituents in an *arabino* orientation at all the nucleotide positions except the 3′ end. The synthesis of the pyrimidine derivatives has already been achieved and the polymers have shown hybridization properties comparable to those of the corresponding natural sequences (Cicero et al., 1993a).

In this article the stability of these new antisense fragments toward nucleases is evaluated.

MATERIALS AND METHODS

Nucleases and T4 polynucleotide kinase were purchased from Boehringer Mannheim (Indianapolis, IN). [γ-^{32}P]ATP (10 mCi/ml, 6000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Human serum and HeLa cell extracts were kindly supplied by A. Nicosia and S. Colloca, respectively (IRBM, Rome).

Oligonucleotide synthesis

Oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) synthesizer model 380B using β-cyanoethyl phosphoramidite chemistry. (2′*S*)-2′-Deoxy-2′-C-methyloligonucleotides were synthesized from protected building blocks as described elsewhere (Cicero et al., 1993a). The solid-phase support used was the commercially available derivatized controlled pore glass (CPG).

The sequence studied in this article is the following: (UCCUCCCUCUUCCUCUCC)$_{me\ up}$dC, where $C_{me\ up}$ = (2′*S*)-2′-deoxy-2′-C-methylcytidine and $U_{me\ up}$ = (2′*S*)-2′-deoxy-2′-C-methyluridine.

Oligonucleotide labeling

Oligonucleotides (10 pmol) were ^{32}P-labeled using T4 polynucleotide kinase (1 unit), [γ-^{32}P]ATP (5 μl), and 10× ki-

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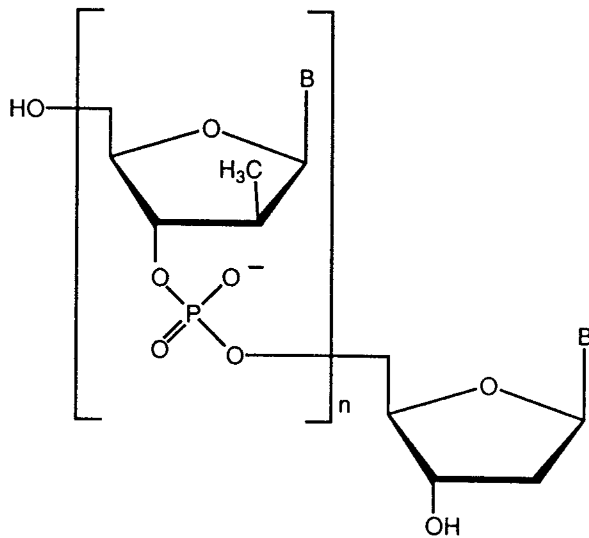

FIG. 1. General structure of oligonucleotides carrying (2′*S*)-2′-deoxy-2′-C-methylnucleotides at all positions except for the 3′ end.

nase buffer (2 μl) in a total volume of 15 μl. Solutions were incubated at 37°C for 45 min and the reactions were stopped by heating at 70°C for 10 min. The reaction mixture was first purified by spin-column chromatography with Sephadex G-50 and then by electrophoresis in 20% polyacrylamide gels run in 1× Tris–borate–ethylenediaminetetraacetic acid (EDTA) (TBE) buffer at 25 mA.

Nuclease digestion

Digestion with nucleases was carried out by incubating 1 pmol of kinased oligonucleotide and different enzyme concentrations for 1 hr at 30°C. Buffer conditions were those recommended by the supplier (Boehringer Mannheim, Indianapolis, IN) of each enzyme and the final volume was 10 μl.

Kinased oligonucleotides (1 pmol) were incubated in human serum (40 μl) at 37°C. To the incubation mixture were added sodium azide (0.02%) and mineral oil (40 μl). Aliquots were taken at different times and extracted first with phenol–chloroform and then with chloroform. Samples were analyzed by electrophoresis in 20% polyacrylamide–7 *M* urea denaturing gels run in 1× TBE buffer at 25 mA.

In the case of cellular extracts, the kinased oligonucleotides (1 pmol) were incubated in HeLa extract at 0.33 mg of total protein/ml in the cell fractionation buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES, pH 7.9], 10 mM potassium chloride, 1.5 mM magnesium chloride, and 0.5 mM dithiothreitol [DTT]). Sodium azide and mineral oil were added as previously mentioned. Preparation and analysis of the samples were carried out as described for the human serum experiments.

RESULTS

The stability of this new modified oligonucleotide toward three representative nucleases was assessed. *Bal* 31, mung bean, and P1 nucleases have dual single-stranded (ss) RNA/DNA specificity, although *Bal* 31 does not degrade ssRNA efficiently (Sambrook et al., 1989). The results obtained showed that in all three tests (2′*S*)-2′-deoxy-2′-C-methyloligonucleotides were resistant because there was a concentration of enzyme at which the normal deoxy sequence is cleaved while the modified one is stable.

As seen in Fig. 2, mung bean nuclease hydrolyzes unmodified oligodeoxyribonucleotides approximately 100 times more efficiently.

In the case of *Bal* 31 (Fig. 3), the (2′*S*)-2′-deoxy-2′-C-methyloligonucleotide is possibly 100-fold more stable than the corresponding unmodified sequence. Finally, P1 nuclease (Fig. 4) is not able to cleave the modified fragment at a concen-

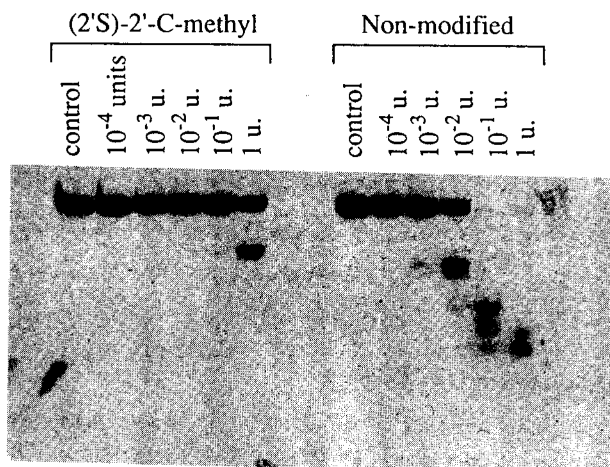

FIG. 2. Sensitivity of oligonucleotides to mung bean nuclease degradation. (2′*S*)-2′-C-Methyl, (2′*S*)-2′-deoxy-2′-C-methyloligonucleotides, $(UCCUCCCUCUUCCUCUCC)_{me}$ $_{up}$dC; non-modified, oligodeoxy ribonucleotides, d(UCCUCCCUCUUCCUCUCCC).

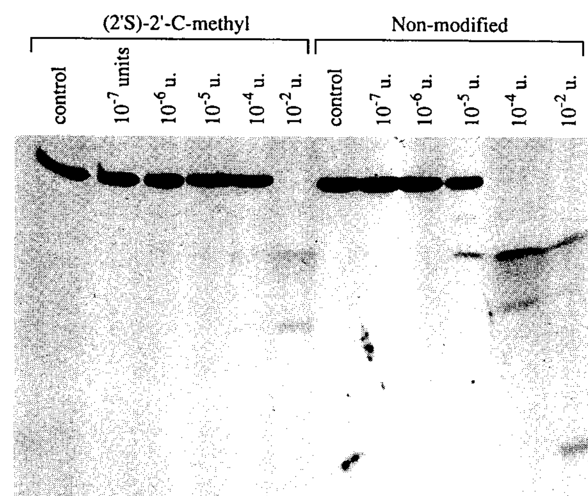

FIG. 3. Sensitivity of oligonucleotides to *Bal* 31 nuclease degradation. For the analyzed sequences see caption to Fig. 2.

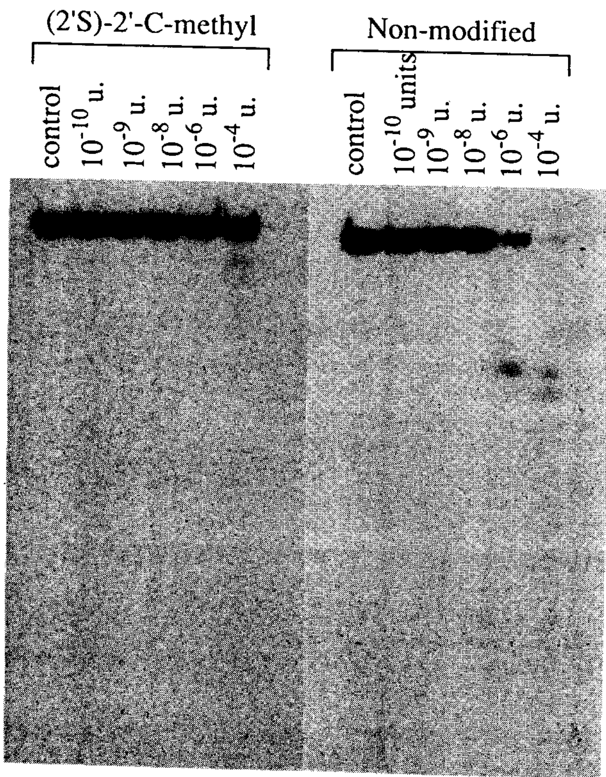

FIG. 4. Sensitivity of oligonucleotides to P1 nuclease degradation. The analyzed sequences are shown in the caption to Fig. 2.

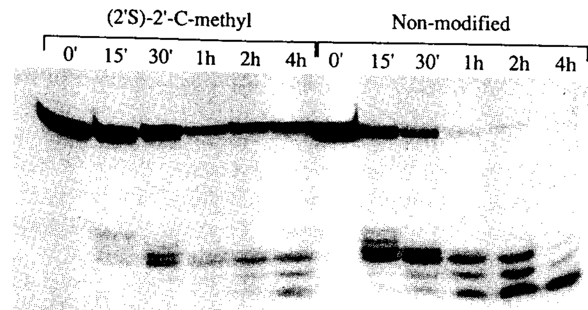

FIG. 6. Degradation of oligonucleotides by HeLa cell extract. Incubation times: 0, 15 min, 30 min, 1 hr, 2 hr, and 4 hr. The sequences of the oligonucleotides are shown in the caption to Fig. 2.

tration at which the normal oligodeoxyribonucleotide is completely degraded.

The results obtained by incubating the modified and non-modified oligonucleotides in human serum provided additional evidence of their relative stability. Whereas the natural sequence was completely degraded after 1 hr, the (2′*S*)-2′-deoxy-2-C-methyloligonucleotide was stable after several hours of incubation (Fig. 5). After 5 days only partial degradation by 3′-exonucleases was observable, being the main source of degradation of phosphatase activity present in the serum.

Incubation in HeLa cell extracts showed that natural oligodeoxyribonucleotides are almost completely hydrolyzed under these conditions after a 1-hr incubation whereas the full-length sugar-modified sequence was still visible after 4 hr (Fig. 6).

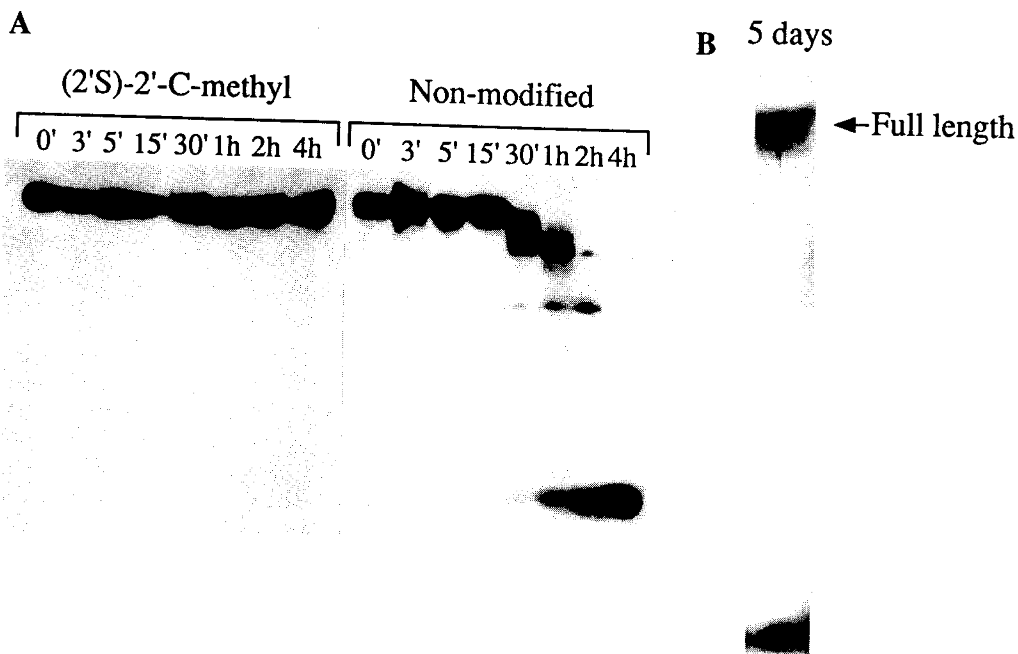

FIG. 5. Human serum degradation of oligonucleotides. (A) Incubation times: 0, 3 min, 5 min, 15 min, 30 min, 1 hr, 2 hr, and 4 hr. (B) After 5 days of incubation. The sequences of the oligonucleotides are given in the caption to Fig. 2.

DISCUSSION

The dual RNA/DNA nucleolytic activity displayed by the tested enzymes (mung bean, P1, and *Bal* 31) appears to be appropriate for these modified oligonucleotides, because on the one hand they are 2′-deoxy derivatives but on the other the C-3′ endo conformation preferred by the sugar of the monomers (Cicero et al., 1993b) suggests that the structure of the oligo may be more related to RNA. In addition, these kinds of nu cleolytic enzymes have been shown to be the most active in degrading other 2′-modified oligonucleotides (Iribarren et al., 1990).

They are also representative because they have different functions: *Bal* 31 and P1 are single-stranded endonucleases and mung bean is predominantly a 3′-single-stranded exonuclease.

The analysis of the polyacrylamide gels (Figs. 2–4) showed that in all cases the modified oligonucleotide was stable at concentrations at which the natural deoxy sequence was completely degraded. The stabilization due to the introduction of the 2′-C-methyl group was evidenced by the higher concentration of nucleases required for its degradation.

The study of the stability toward human serum nucleases is relevant in view of the potential use of antisense fragments in human therapy. The results obtained show the particular stability of the (2′*S*)-2′-deoxy-2′-C-methyloligonucleotide in this medium. This compound was completely stable after several hours of incubation and only partial degradation was observed after 5 days (Fig. 5). The pattern of degradation observed on the autoradiograph, although not clearly visible in Fig. 5B, corresponded to the expected 3′-exonuclease activity because this is the main degradative process in sera (Shaw et al., 1991).

To explore further the stability of this kind of compounds the resistance in HeLa cell extracts was investigated. As can be observed in Fig. 6, the natural sequence is almost completely degraded after 1 hr of incubation at 37°C whereas the respective (2′*S*)-2′-deoxy-2′-C-2′-methyloligonucleotide is still observable after 4 hr of incubation.

All of these results, together with the hybridization properties observed for this new class of compounds (Cicero et al., 1993a), suggest that (2′*S*)-2′-deoxy-2′-C-2′-methyloligonucleotides are potentially useful antisense probes.

Further studies regarding the synthesis of the purine building blocks and physical and biological properties of these new modified oligonucleotides are being carried out.

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