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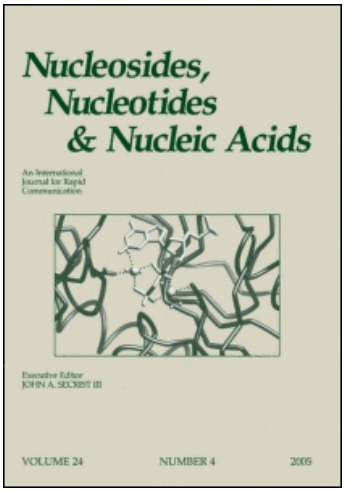

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NUCLEOSIDES & NUCLEOTIDES, 16(7-9), 1677-1682 (1997)

STABILITY OF STEREOREGULAR OLIGO(NUCLEOSIDE PHOSPHOROTHIOATE)S IN HUMAN CELLS. DIASTEREOSELECTIVITY OF CELLULAR 3'-EXONUCLEASE

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ABSTRACT: Stability of oligo(nucleoside phosphorothioate)s (PS-oligos) in HUVEC (human umbilical vein endothelial cells) has been studied. Cytosolic fraction of HUVEC possesses 3'-exonucleolytic activity which is responsible for degradation of natural oligomers and PS-oligos. The enzyme is R_P-specific, i.e. it cleaves internucleotide phosphorothioate function of R_P- and not S_P-configuration at phosphorus atom.

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INTRODUCTION

It is well known that phosphorothioate analogues of oligonucleotides (PS-oligos) are much more stable than unmodified phosphodiester oligomers. However, some nucleolytic enzymes are able to hydrolyze internucleotide phosphorothioate linkages and the rate of degradation strongly depends upon the absolute configuration at the phosphorus atom of internucleotide function [1-3]. Since the diastereoselectivity of several nucleolytic enzymes (nuclease P1, snake venom phosphodiesterase, endonuclease from *Serratia marcescens*) towards PS-oligos is well documented [4], we decided to investigate the influence of absolute configuration of phosphorus atoms involved in internucleotide phosphorothioate linkages on the stability of PS-oligos in HUVEC lysates. Such studies became feasible due to availability of stereoregular PS-oligos, synthesized by oxathiaphospholane method recently developed in our laboratory [3].

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MATERIALS AND METHODS

Cell culture

Human endothelial cells from umbilical vein were cultured in RPMI 1640 medium supplemented with 20% fetal calf serum, heparin (90 μg/ml), ECGS (150 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a 90-95% humidified atmosphere of 5% CO_2 at 37°C. The cells were cultured in bottles (area 25 cm^2) covered with 1% gelatin. Nuclear extract (NE) and cytosolic fraction (CF) were prepared according to Johnson [5] and stored at -80°C. The protein concentration was 1.4 mg/ml for NE and 3.0 mg/ml for CF.

Synthesis of oligonucleotides

An unmodified oligonucleotide **1** was prepared by the phosphoramidite method on an ABI 391 synthesizer. PS-oligos **1-3** were synthesized using the oxathiaphospholane method as described elsewhere [3]. Purification of all oligonucleotides was carried out by two-step RP-HPLC (DMT-on and DMT-off).

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Oligonucleotides labeling

Oligonucleotides used for the studies were 5'-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. A mixture (20 μl) containing 10 mM $MgCl_2$, 7 mM ß-mercapto-ethanol, 25-50 μM oligonucleotide (0.5-1.0 nmole, 0.1 A_{260} unit), 1 μl (10 μCi) of [γ-^{32}P]ATP and T4 polynucleotide kinase (5 units) was incubated for 3 h at 37°C, then heat-denatured and stored at -20°C.

Assay for nucleolytic activity of NE and CF

The samples of oligonucleotides (0.25-0.5 nmole) were incubated in 50% NE or CF (a total volume of 50 μl) at 37°C. At various time points (usually 0, 1, 2, 4 and 8 h) 10 μl aliquots were withdrawn and the enzymatic reaction was quenched by heating for 2 min at 95°C. Then water (50 μl) was added to each denaturated sample. After vigorous shaking, the protein precipitates were spun down and the aqueous solutions were dried in a Speed Vac rotary evaporator. The resultant samples were then dissolved in formamide containing 0.03% bromophenol blue and 0.03% xylene cyanol (5-8 μl) and analyzed by 20% polyacrylamide/7M urea gel electrophoresis. The autoradiograms were scanned using an LKB Ultroscan XL densitometer.

RESULTS

Oligonucleotides listed in the TABLE have been used for the studies of activity and diastereoselectivity of intracellular 3'-exonuclease from human cells. Although there is

TABLE: The oligonucleotides used for the studies of activity of 3'-exonuclease from HUVEC.

Compounds No.	Sequence 5'→3'
1	$d[T_{12}]$
1a	[Mix-PS]-$d[T_{12}]$
1b	[R_P-PS]-$d[T_{12}]$
1c	[S_P-PS]-$d[T_{12}]$
1d	[R_P-but-last one-PS]-$d[T_{12}]$
2a	[Mix-PS]-d[GGGAATTCCC]
2b	[R_P-PS]-d[GGGAATTCCC]
2c	[S_P-PS]-d[GGGAATTCCC]
3a	[Mix-PS]-d[GAGGGCTGGAGACATC]
3b	[R_P-PS]-d[GAGGGCTGGAGACATC]
3c	[S_P-PS]-d[GAGGGCTGGAGACATC]

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no evidence that the 3'-exonucleolytic activity of HUVEC results from the presence of a single enzyme, we use the term "3'-exonuclease" for this activity. The substrates labeled at the 5'-end with [^{32}P]phosphate were incubated in 50% NE or CF, then separated from precipitated protein and analyzed on 20% polyacrylamide gel containing 7M urea.

In our preliminary studies we examined the stability of unmodified oligomer **1** in nuclear extract and in cytosolic fraction. The substrate appeared to be much more stable in NE than in CF (FIG.1). Its $t_{1/2}$ in NE was longer than 4 h, while $t_{1/2}$ observed for the same substrate incubated in CF was 1 h.

In the following set of experiments [R_P-PS]-, [S_P-PS]- and [mix-PS]-forms of $d[T_{12}]$ and d[GGGAATTCCC] were used as substrates for 3'-exonuclease. After 8 h incubation the [S_P-PS] constructs remained intact, while [R_P-PS] counterparts were partially degraded (see FIG.2). These results indicate that the 3'-exonuclease from human cells is an R_P-specific enzyme. Moreover, the presence of a single phosphorothioate internucleotide bond of S_P configuration at the 3'-end completely protects the oligomer **1d** against enzymatic degradation from the 3'-end (FIG.2). It means that the degradation of PS-oligos in CF is caused by 3'-exonuclease only; no traces of endonucleolytic activity have been observed. The observations obtained for homooligonucleotide $d[T_{12}]$ and its phosphorothioate analogues have been confirmed using oligomers **2** and **3** (data not shown).

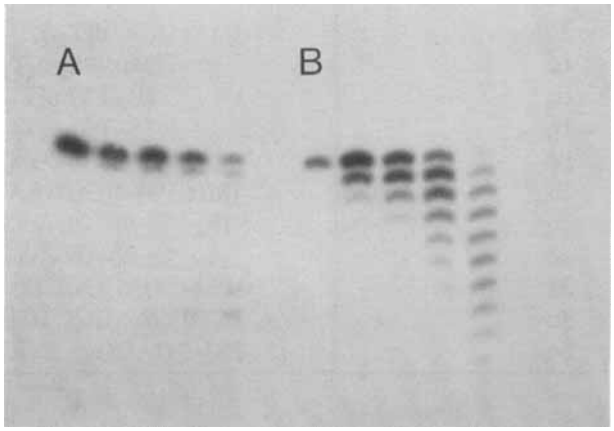

FIG. 1. Stability of phosphodiester dodecanucleotide d[T_{12}] in nuclear extract (NE) and cytosolic fraction (CF) of HUVEC.

A: d[T_{12}] in 50% NE after 0, 15, 30, 60 and 120 min of incubation at 37°C;

B: d[T_{12}] in 50% CF incubated as above.

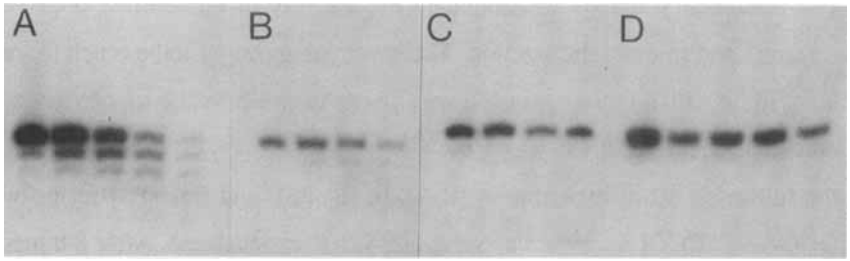

FIG. 2. Degradation of the phosphorothioate oligonucleotide d[$(T_{PS})_{11}$T] by 3'-exonuclease from cytosolic fraction of HUVEC.

A: [all-R_P]-isomer after 0, 1, 2, 4 and 8 h incubation in 50% CF at 37°C;

B: [mix]-form incubated for 0, 1, 2 and 4 h;

C: [all-S_P]-isomer incubated for 0, 1, 2 and 4 h.

D: [all-R_P-but-last-one] d[T_{12}] after 0, 1, 2, 4 and 8 h incubation in 50% CF.

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DISCUSSION

The results described above clearly indicate that internucleotide phosphorothioate groups of R_P configuration are hydrolyzed by 3'-exonuclease present in cytosolic fraction. The presence of the R_P-specific enzyme(s) in cytoplasm may be especially detrimental for the PS-oligo molecules which are enriched in R_P linkages [6]. This observation is of great importance since our previous studies show that [all-R_P]-PS-oligos seem to have better antisense activity than [mix]-forms or [all-S_P] isomers [7].

In independent studies we have observed that PS-oligos [labeled at their 5'-end with fluorescein isothiocyanate (FITC)] were taken up by endothelial cells and localized almost exclusively within the cytoplasm. Even after 24 hours incubation of the cells with the solution containing PS-oligos, the FITC-labeled oligonucleotides were observed mostly in endothelial vesicles [8]. Therefore, it is possible that oligonucleotides localized in cytoplasm are strongly exposed to 3'-exonucleolytic degradation.

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The aforementioned observations prompted us to propose another strategy for protection of antisense oligonucleotides against 3'-exonucleolytic degradation. Because internucleotide phosphorothioate function of S_P configuration is resistant to this enzyme, we propose to protect PS-oligos against its action introducing at the 3'-end at least one internucleotide phosphorothioate linkage of S_P configuration.

Further studies on intracellular 3'-exonuclease are in progress.

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