

Modified deoxyoligonucleotides stable to exonuclease degradation in serum

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ABSTRACT

Unmodified deoxyoligonucleotides are rapidly degraded in serum-containing medium. Utilizing internally labelled deoxyoligonucleotides the deoxyribonuclease profile for fetal calf serum and human serum was determined. It was found that the predominate nuclease activity in both systems was 3' exonuclease. Deoxyoligonucleotides are protected from exonuclease degradation in sera and cell media by simple terminal modifications that maintain high binding affinity for the complementary DNA sequence.

INTRODUCTION

Sequence-selective inhibition of gene expression in mammalian cell culture has been demonstrated using antisense DNA oligonucleotides^{1,2}. The selectivity and flexibility of the antisense approach makes it an important research tool and an attractive class of therapeutic compounds for serious diseases with known genetic etiologies but poor therapeutic alternatives. Phosphorothioates have been shown to inhibit HIV in vitro by a sequence specific and sequence non-specific mechanism^{3,4}. Phosphoroamidate, phosphorothioate, and methane phosphonate oligonucleotide analogues contain substitutions of one of the nonbridging oxygen atoms in the oligonucleotide backbone and are known to significantly enhance nuclease stability in vitro^{5,6,7}. However, these types of uniform substitutions are known to give depressed hybridization affinities with the complementary DNA sequence^{5,8}. It has been reported that terminal phosphorothioate and methane phosphonate modifications stabilize an oligonucleotide to 3' and 5' exonucleases such as snake venom phosphodiesterase and spleen phosphodiesterase^{9,10}. A recent report has shown that terminal methane phosphonate modifications were useful in protecting deoxyoligonucleotides from exonuclease digestion in fetal calf serum¹⁹. Reported herein are two classes of conservative chemical modifications which confer protection from enzymatic exonucleolytic degradation. Phosphoroamidate substitutions at the terminal linkages and/or an inverted linkage have been used to stabilize oligonucleotides to exonuclease digestion without compromising binding to the complementary DNA sequence.

Oligonucleotides were modified at the terminal phosphate (3' and/or 5') with phosphoroamidate linkages (3 and 4) to block

exonucleolytic degradation. We have termed these terminal modifications as 5' or 3' 'end-caps'. The phosphoroamidate used for these analyses was derived from methoxyethylamine (MEA), as shown in Figure 1. Using internally labelled oligonucleotide analogues the nuclease profiles of human serum, fetal calf serum, and conditioned cell media were characterized.

EXPERIMENTAL

Synthesis of Deoxyoligonucleotides

Deoxyoligonucleotides were prepared on a Biosearch 8750 DNA synthesizer using standard H-phosphonate chemistry¹¹ on controlled pore glass (CPG). Phosphoroamidate and phosphorothioate analogues were prepared by the published procedures^{8,12}. The deoxyoligonucleotides were purified by reverse phase HPLC via DMT-on purification^{13,14}. The deoxyoligonucleotides with a terminal 3'-3' linkage (5a and 5b) require CPG with the first nucleoside attached via the 5' hydroxyl¹⁵ followed by chain elongation in standard 3'→5' fashion.

³⁵S Labeled Phosphorothioates

CPG containing the oligonucleoside H-phosphonate was washed with CH₂Cl₂, dried from Et₂O and treated with a mixture of ³⁵S₈ (150 μL of 3 mM, 15.2 μCi/μL) in toluene and ³²S₈ in pyridine/CS₂ (1/1, 150 μL of 20 mM). After 2 hr. incubation at room temperature 20 μL of anhydrous triethylamine was added and the mixture incubated for 16 hr after which time S₈ in pyr/CS₂ (400 μL of 200 mM) was added. After 2 hr the CPG was washed with Pyridine/CS₂ (3×500 μL), CH₃CN (2×500 μL) and Et₂O (2×500 μL). The product was deprotected and removed from the solid support with conc. NH₄OH (45°C/24hr.), evaporated, taken-up in H₂O and desalted with a C₈ SPE column (JT Baker), evaporated and used without further purification (specific activity = 1.6×10⁹ cpm/μmole).

Preparation of Internally-labelled Oligonucleotide

An 11-mer oligonucleotide (5'-TTTTTCTCCAT) was 5'-end-labelled using γ-³²P-ATP (ICN) and T4 polynucleotide kinase (USB). The labelled oligonucleotide was ligated to a 10-mer

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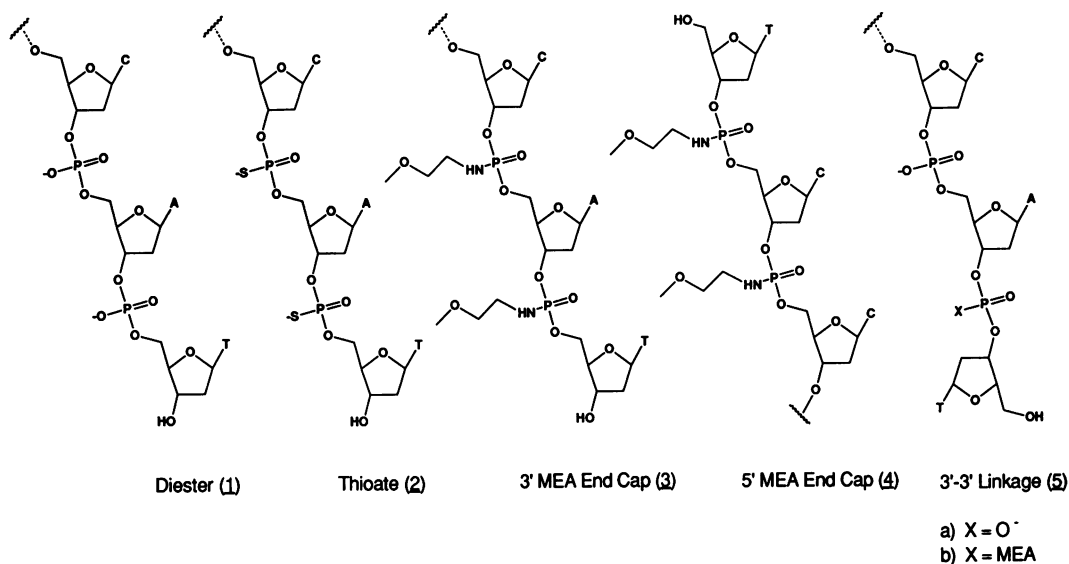


Figure 1: Oligonucleotide backbone structure; 5'-TCCAGTGATT³²pTTTTTCTCCAT-3'

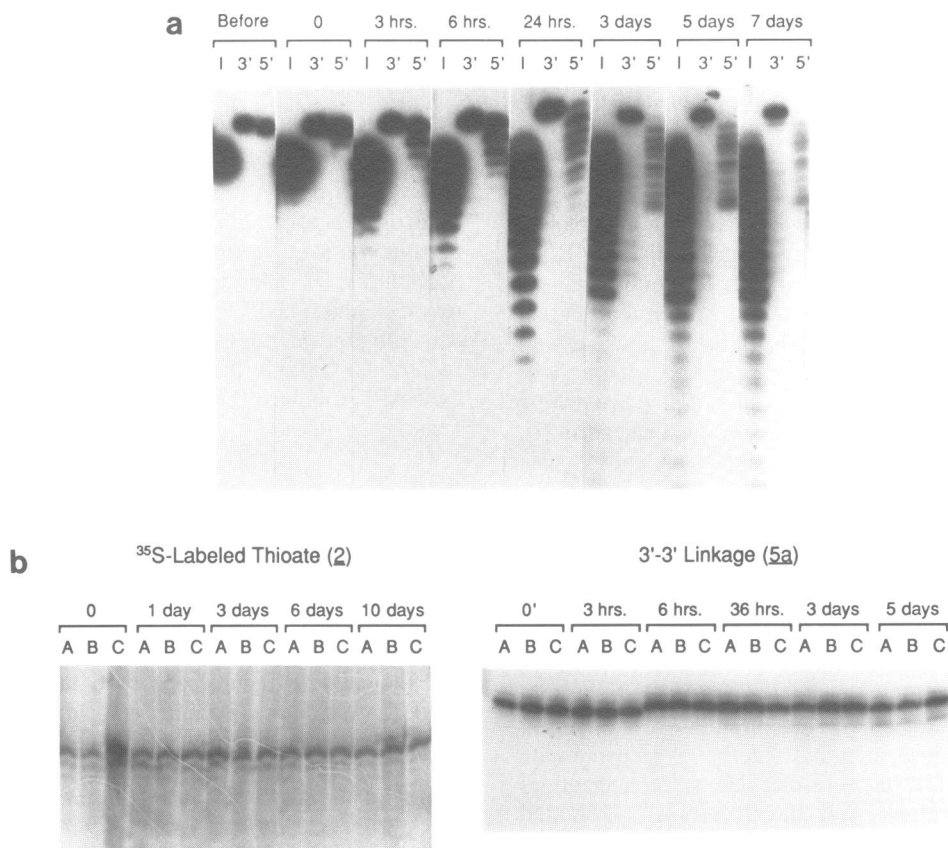


Figure 2: Oligonucleotide stability study; internally labeled oligonucleotide was added to H938 cell culture at a final concentration of 20 μ M. The oligonucleotides were recovered from the cell media at variable time-points and analyzed on 20% PAGE/7 M urea. 2a) I = internally labelled phosphodiester (1); 3' = 3'-end cap (3); 5' = 5'-end cap (4). 2b) ³⁵S labelled phosphorothioate (2); 3'-3' inverted linkage (5a); A, B, and C represent triplicate sets of experiments.

(5'-TCCAGTGATT) by T4 DNA ligase (USB) in the presence of the 33-mer template (5'-TCGCTGATGGAGAAAAAATCACTGGAGACCTC). The internally labelled 21-mer (5'-TCCAGTGATT³²pTTTTTCTCCAT) was purified by PAGE (20% Polyacrylamide/7 M urea) and desalted with a C₈ SPE column.

Thermal Denaturation Measurements

Thermal denaturation was carried out using a Gilford Response II temperature controlled spectrophotometer. The extinction coefficients for the deoxyoligonucleotides were calculated¹⁶ and

the final concentration of both strands was $\sim 2 \mu\text{M}$ in 150mM NaCl/10 mM Na_2HPO_4 (pH = 7.1). The heating rate was $0.5^\circ\text{C}/\text{min}$. from 20°C to 80°C and T_m values were determined by a first derivative plot of absorbance vs temperature.

Cell Culture

H938 (human T lymphoma cell lines) were grown in RPMI 1640 (Gibco) plus 10% heat inactivated fetal calf serum (Gibco, $56^\circ\text{C}/4$ hrs). The cells were plated at 2×10^5 cells/ml into 96 well plates and harvested at different time points.

Human Serum Preparation

Human serum was prepared as follows: freshly drawn human blood was coagulated at room temp for 30 min. Fibrin clots and cells were spun down, the serum (supernatant) was filter-sterilized and added to RPMI 1640 up to 10% for analysis.

RESULTS

Oligonucleotides labelled at the 5'-end using $\gamma\text{-}^{32}\text{P}\text{-ATP}$ and T4 polynucleotide kinase were found to rapidly dephosphorylate when added to cell culture media containing 10% heat-inactivated fetal calf serum, presumably due to high phosphatase activity present in serum (data not shown). In order to stably label oligonucleotides for degradation analyses, oligonucleotides twenty-one bases in length were radiolabelled with ^{32}P at an internal bridging phosphate by ligation of two shorter oligonucleotides in the presence of a complementary template oligonucleotide (see Materials and Methods). For oligonucleotide phosphorothioates the ligation results in a phosphodiester linkage in the center of the oligonucleotide. Alternatively, 21-mer phosphorothioate oligonucleotides were labelled with ^{35}S in a postsynthesis oxidation step (see Materials and Methods).

Stability analyses were performed by adding internally-labelled oligonucleotides (1–5) to serum-containing media in H938 human T lymphoma¹⁷ cell culture at a concentration of 10 nM either with or without 20 μM unlabelled oligonucleotide. The oligonucleotides were recovered from the cell media after incubation for variable times and the degree of degradation was analyzed by PAGE. Figure 2a shows a comparison of stability in cellular supernatant for unmodified oligonucleotides (1), 5' and 3' MEA phosphoroamidate end-capped oligonucleotides (3 and 4), and (in Figure 2b) phosphorothioate oligonucleotides (2). Oligonucleotides containing 5' or 3' terminal phosphoroamidate modifications migrate more slowly on PAGE (higher m/e ratio) than the unmodified diesters¹¹.

Under these experimental conditions the 3' end-capped oligonucleotide remains substantially undegraded for up to 7 days. Two 3'-terminal modifications (3) increased the half-life of intact oligonucleotide in 10% heat-inactivated serum from approximately 4 hours for the diester (1) (at 20 μM concentration) to greater than seven days. Phosphoroamidate end-caps at the 5' terminus (4) do not appear to significantly protect oligonucleotides from degradation in the presence of fetal calf serum and, while total amounts of labelled material differ between the two samples, in these analyses, 5' end-capped (4) and unmodified oligonucleotides (1) appeared to have similar patterns of degradation over time (Figure 2a). When the terminal 3' linkage of the oligonucleotide is inverted, giving rise to a 3'-3' linkage and an oligonucleotide with two 5' ends (5a), it was found to similarly stabilize the deoxyoligonucleotide to degradation in cell culture serum. An inverted diester (5a) was comparable to

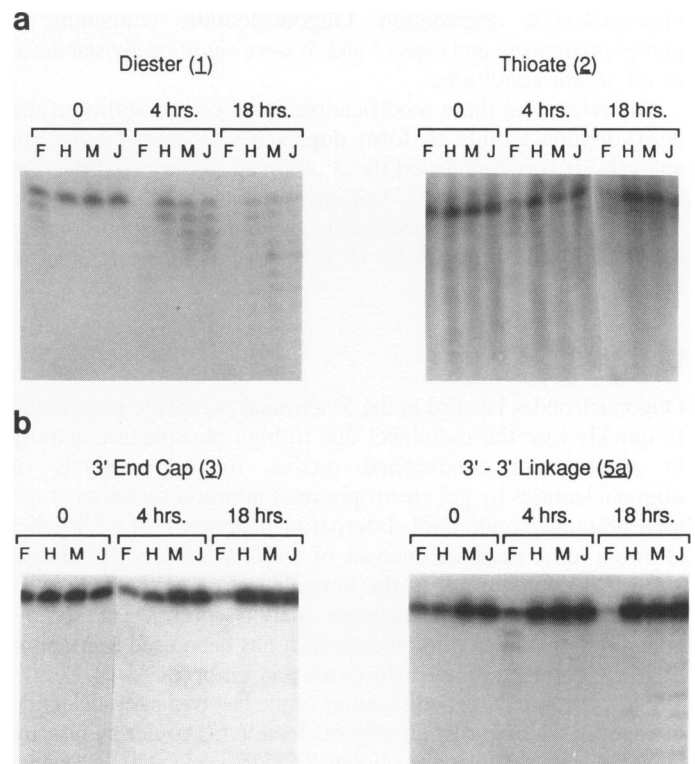


Figure 3: Oligonucleotide stability study in serum; internally labelled oligonucleotides were added to media containing 10% serum at a concentration of 10 nM and incubated at 37°C for 0, 4 or 18 hrs. Oligonucleotides were recovered and analyzed on 20% PAGE/7 M urea. F = fetal calf serum; H = heat inactivated fetal calf serum; M = sample 1 adult human serum; J = sample 2 adult human serum. 3a) Internally labeled phosphodiester (1) and ^{35}S labelled phosphorothioate (2). 3b) 3'-end cap (3) and 3'-3' inverted linkage (5a).

Table 1.

Oligonucleotide Analogue	FCS	Serum Half-Life Δ FCS	Cell Sup	T_m ($^\circ\text{C}$)
Diester (1)	< 5 min	~ 4 hr	~ 3 hr	61.5
Thioate (2)	~ 4 hr	$\gg 18$ hr	> 7 days	50.5
3'-EC (1 amidate) (3)	nd	nd	> 5 days	nd
3'-EC (2 amidate) (3)	~ 4 hr	$\gg 18$ hr	> 7 days	61.0
5'-EC (2 amidate) (4)	< 5 min	~ 4 hr	~ 3 hr	nd
3'-3' (diester) (5a)	~ 4 hr	$\gg 18$ hr	> 7 days	61.0
3'-3' (amidate) (5b)	~ 4 hr	$\gg 18$ hr	> 7 days	nd

FCS: fetal calf serum; Δ FCS: heat inactivated fetal calf serum; Cell Sup: cellular supernatant; concentration of the oligonucleotide was 10 nM, nd – not determined.

a phosphoroamidate inverted linkage (5b) (see Table 1). Uniformly substituted phosphorothioate oligonucleotides (2) showed little degradation after up to 10 days in cellular supernatant (Figure 2b).

A comparison of oligonucleotide degradation in fetal calf serum and adult human serum was performed (Figures 3a and 3b). Four serum conditions were examined, 10% fetal calf serum, 10% heat-inactivated fetal calf serum and two adult human serum samples (one male, one female; see Materials and Methods). In the presence of serum that had not been heat-inactivated, all oligonucleotides tested were degraded more rapidly than in heat-inactivated serum (Table 1). Heat-inactivated fetal calf serum and both adult human serum samples showed similar patterns of

oligonucleotide degradation. Oligonucleotides containing 3' phosphoroamidate end-caps (3 and 5) were significantly stabilized in all serum conditions.

The effect that these modifications have on the ability of the deoxyoligonucleotide to form duplexes was assessed by T_m analysis. It was found that the 3' end-cap (3) lowered the T_m by 0.5°C and that one 3'-3' linkage (5a) had a similar effect (see Table 1). In contrast, the uniformly substituted phosphorothioate (2) destabilized the duplex by 11°C relative to the diester control (1).

DISCUSSION

Oligonucleotides labelled at the 5'-terminal phosphate were found to quickly lose the radiolabel due to high phosphatase activity in serum and conditioned media, making analysis of oligonucleotides by gel electrophoresis nonrepresentative of the total oligonucleotide pool. Internal incorporation of a ³²P label allows a more accurate analysis of oligonucleotide degradation since it does not result in the immediate loss of the radiolabel by phosphatase or exonuclease activity present in serum-containing media. A similar approach has been used to monitor oligonucleotide stability in oocytes and embryos¹⁴.

Phosphoroamidate modifications of the last two internucleotide linkages at the 3' end of an oligonucleotide (3) confer significant nuclease stability when the oligonucleotide is added to cell media containing serum. The same modifications at the 5'-terminus (4) did not significantly alter oligonucleotide stability, suggesting that the predominant nuclease activity present in the serum samples tested is a 3' exonuclease. Oligonucleotides containing two methoxyethylamine (MEA) phosphoroamidate substitutions at the 3' end (3) retain excellent binding affinity for complementary DNA with a 0.5°C depression in T_m (Table 1). Previous studies have shown a large decrease in T_m with uniformly substituted phosphoroamidates and methane phosphonate deoxyoligonucleotides⁸ and these results suggest that only one or two such modifications are required for stability to exonuclease digestion^{9,10}. Oligonucleotides containing an inverted linkage at the 3'-end (5a and 5b) also stabilize the oligonucleotide to serum mediated degradation and retain excellent binding affinity to the complementary deoxyoligonucleotide (Table 1). Parallel duplexes have been demonstrated and have been shown to be thermodynamically less stable than antiparallel duplexes^{15,18}. Although not confirmed, we assume the terminal nucleoside in 5a and 5b is forming a base pair with the terminal nucleoside of the complementary deoxyoligonucleotide but with lower binding affinity. Uniformly substituted phosphorothioates stabilized the oligonucleotide to nuclease digestion but lead to a 11°C depression in T_m relative to phosphodiester oligonucleotide.

Non-heat inactivated fetal calf serum has greater nuclease activity than heat-inactivated fetal calf serum and adult human sera (which are comparable). All serum samples tested have predominately 3'-exonuclease activity. When oligonucleotide stability was assessed using only 10 nM oligonucleotide (radiolabelled material only) degradation was accelerated, suggesting that micromolar concentrations of oligonucleotide may have saturated available nuclease activity. However, the type of degradation seen and the relative stability of the modified oligonucleotides remained the same at all concentrations studied (data not shown). These results suggest that heat-inactivated fetal calf serum represents an accurate model for the nuclease content of human serum.

Simple 3' terminal modifications of deoxyoligonucleotides were used to protect the oligonucleotides from serum mediated degradation by exonucleases. Using an internally labelled oligonucleotide, the nuclease profile in fetal calf serum and adult human serum was determined to be predominately 3'-exonuclease. Both phosphoroamidate end-capped oligonucleotides and oligonucleotides with a 3'-3' linkage at the 3'-terminus were found to be very useful as terminal modifications to stabilize the oligonucleotides to exonuclease degradation.

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