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Cellular Proteins Prevent Antisense Phosphorothioate Oligonucleotide (SdT18) to Target Sense RNA (rA18): Development of a New in Vitro Assay

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ABSTRACT: There are numerous indications that the "antisense" mechanism alone cannot account for the observed effects in living cells. Despite that, interactions between antisense oligonucleotides (ASO) and cellular proteins are usually not considered. In this work, we have tested the ability of antisense phosphorothioate (SdT) oligonucleotides and natural deoxyoligonucleotides (dT) for their ability to interact with target RNA in the presence of cellular proteins. We show that the affinity for cellular proteins is an essential factor that determines the success of RNA targeting. We have used a simple nuclease digestion assay to detect RNA/ASO hybrid formation in the presence of proteins. The results show the inability of a phosphorothioate oligonucleotide (SdT18) to reach the target RNA (rA18) in vitro in the presence of proteins. However, if proteins are absent, the RNA targeting was successful, as is usual in in vitro assays. Note that the target RNA concentration exceeded physiological values by several orders of magnitude while the crude protein extract was 20-fold diluted in the reaction tube. This finding is compatible with the notion that therapeutic properties of phosphorothioates could largely derive from a so-called "aptamer" effect.

Modified oligonucleotides and their analogues represent an exciting class of new agents with potential therapeutic and diagnostic applications (*1*−*8*). Selective hybridization to target nucleic acids, efficient cellular uptake, nuclease stability in biological fluids, and the ability to elicit RNase H activity are features that make modified oligonucleotides better candidates for antisense oligonucleotides (ASO)[1] technology (*7*, *8*). It is usually hard to get the right combination of good features in one therapeutic agent (*2*). In the past, the most extensively investigated analogues were phosphorothioates (*6*). Due to the very promising combination of the aforementioned features and to the therapeutic effects detected in some cell culture experiments, phosphorothioates are rapidly entering the clinical trial stage of drug development. However, in some cases they exhibit significant toxicity that might be related to their high affinity for serum and cellular proteins (*9*−*14*).

In this work, we emphasize the importance of the intrinsic propensity of ASO for nonspecific protein binding as a critical factor in the early stage evaluation of ASO as drug candidates. Our results show that the crude protein binding features of our ASO are closely linked to their ability to reach the targeted RNA. The relative ability of different ASO to bind cellular proteins were first detected by a simple gel shift assay. The sequestration of ASO by cellular proteins might result in a limited ASO availability for targeting nucleic acids in a cellular context. To determine how much ASO is reaching target RNA, we carried out additional nuclease digestion experiments (*15*). They are designed to detect hybrid formation in the presence of crude protein extracts. The outcome of these experiments shows that in vitro RNA targeting by our ASO is impaired by the presence of mouse liver homogenate protein extracts. We compared phospohorothioate oligonucleotides (SdT) and ASO containing natural linkages (dT).

MATERIAL AND METHODS

Synthesis and Labeling of ASO. Oligomers were prepared as described previously (*14*) and characterized by PAGE and MALDI-TOF mass spectrometry or provided by GIBGO BRL. The 5′-end labeling of oligonucleotides was done as described (*16*). Briefly, 100 pmol of oligonucleotides were 5′-end-labeled with 30 μCi of [γ^{32}P] ATP using 20 units of T4 polynucleotide kinase. Reaction conditions were 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 5 mM DTT in 100 μL volume. The mixture was incubated for 30 min at 37 °C. The DNA was recovered by ethanol precipitation and purified on 16% polyacrylamide gels.

Nuclease Stability of ASO in Crude Protein Extract Made from Mouse Liver Homogenate. The mouse liver was homogenized in an equal volume of buffer B [20 mM Tris Cl (pH 7.9), 60 mM KCl, 1 mM DTT, and 12% glycerol] (*16*). The 50% homogenate thus obtained was centrifuged 10 min at 3000 rpm (Eppendorf 5145 C) to remove membrane particles and cell debris. The reaction was mixed with equal volumes (1 μL) of homogenate and single-stranded oligonucleotide (ASO, 1 pmol, 10 000 cpm) in a

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[1] Abbreviations: ASO, antisense oligonucleotide(s); SdT, antisense phosphorothioate(s); dT, deoxyoligonucleotide(s).

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total volume of 2 μL. The 5′-end labeled ASO was incubated for 0, 20, 30, 40, 50, and 60 min at room temperature. The reaction was quenched by adding the loading buffer (98% deionized formamide, 10 mM EDTA, 1 mg/mL bromophenol blue, and 1 mg/mL of xylene cyanol) and boiled for 5 min. The reaction products were resolved on a 16% sequencing gel containing 7 M urea and visualized by autoradiography (Kodak X-OMAT film).

Gel shift assay was used to estimate the differences in the relative binding affinities between different types of ASO (18-mers) and crude protein extract from mouse liver homogenate (*16*). The ASO were 5′-end-labeled by [γ^{32}P]-ATP. Antisense oligonucleotides and crude protein extracts from mouse liver homogenate were left on ice for 5 min. An additional excess of cold ASO was then added to the mixture (see Figure 2). After 10 min of incubation, the mixture was loaded onto a 20% nondenaturing polyacrylamide gel. The EDTA was absent, while 5 mM $MgCl_2$ was present both in the gel and in the gel running buffer. The gel was run for 24 h at 4 °C at 8 V/cm.

The RNase H assay was carried out at 22 °C in 10-μL aliquots that included 1 pmol of ^{32}P 5′-end-labeled template RNA and 8 pmol of ASO. The crude protein extract was 5% when present. The reaction was started by addition of *Escherichia coli* RNase H (0.4 U) (MCB, Canada) in the presence of buffer A [60 mM Tris-HCl (pH 7.8), 2 mM dithiothreitol, 60 mM KCl, and 2.5 mM $MgCl_2$ as metal activators]. The reaction was quenched after a 20-min incubation by adding loading buffer (98% deionized formamide, 10 mM EDTA, 1 mg/mL bromophenol blue, and 1 mg/mL of xylene cyanol) and boiling for 5 min. The reaction products were resolved on a 16% sequencing gel containing 7 M urea and visualized by autoradiography (Kodak X-OMAT film). The particular lines were scanned by UN-SCAN-IT, Automated Digitizing System (Silk Scientific Inc., USA).

The propensity of different ASO to form hybrids in the presence of a 5% crude protein extract was detected by adding *E. coli* RNase H into the mixture. The differences between background degradation, where only extract was added, and degradation in the presence of externally added RNase H, reflect the propensity of ASO for hybrid formation. If a hybrid is formed, the RNA will be cleaved at a rate that exceeds the control (crude protein extract). The order of addition of RNA, ASO, and extract is also presented under each illustration.

RESULTS

We used a gel shift assay to document the propensity of ASO to bind to crude proteins from a mouse liver extract. This assay detects free ASO versus ASO in a complex with proteins. Surprisingly, the propensity of phosphorothioates (SdT) and deoxyphosphates (dT) for binding to crude cellular proteins was different (Figure 1). The titration of premade radiolabeled ASO-crude protein complexes with the excess of unlabeled ASO revealed an unexpected differential behavior. In the time scale of the experiment (about 10−15 min), the unlabeled SdT (phosphorothioate) ASO, even in 50 times molar excess, was unable to release the labeled ASO from the protein complexes. On the other hand, the titration with cold dT ASO liberated a part of the ASO from ASO−protein complexes. A phosphorothioate oligonucleotide with a different sequence showed the same apparent protein "stickiness" (data not shown). Also, the electrophoretic mobility of free phosphorothioate oligonucleotides in nondenaturing gels was consistently slower than for the rest of ASO. This could be the result of charge/flexibility differences of phosphorothioates as compared to other ASO.

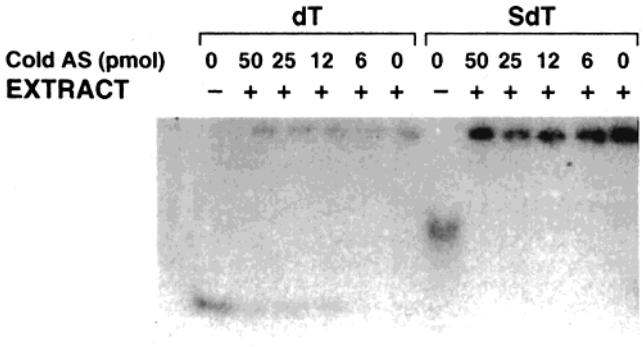

FIGURE 1: Gel mobility assay showing differential relative affinities of various ASO for crude protein extract (5% w/v): phosphorothioate oligonucleotides (SdT) and antisense oligonucleotide containing natural linkages (dT). The presence of cold ASO (pmol) is depicted at the top of each gel lane. The higher mobility bend in lane 1 for each particular ASO corresponds to the free ASO (not in the complex with protein extract).

The gel mobility shift experiments described above suggest that the ability of different ASO to target RNA could be differentially affected by the presence of crude cellular proteins. To test this hypothesis, we have designed an experimental setup intended to detect ASO/RNA hybrid formation using RNase H (hybrid degradation).

The ability of ASO (dT18 and SdT18) to form hybrids and elicit cleavage of 18-mer long RNA in RNA/ASO hybrid was measured using an RNase H digestion study (RNA/ASO hybrid degradation assay) (Figure 2). Radioactive rA18 is first shown not to be degraded by RNase H in the absence of ASO (either dT18 or SdT18). When a "classical" RNase H assay was done, both ASOs elicited enzymatic cleavage of RNA and produced very similar digestion profiles. These data suggest that in the absence of mouse protein extracts the impact of two different ASOs is similar (Figure 2, lanes 1 and 5 for each ASO). However, when we compared rA18 degradation without and with RNase H in the presence of a mouse protein extract (Figure 2, lanes 2 and 4 for each ASO), the results are surprisingly different. When the ASO is dT18, the rA18 degradation is nearly complete (Figure 2, lane 4). In fact, the degradation is more complete than with RNase H and ASO alone (Figure 2, compare lanes 4 and 5). However, for the SdT18 ASO, the degradation by RNase H is hard to detect, when the mouse proteins are present (Figure 2, compare lanes 2 and 4). This effect is most readily observed by comparing densitometric scans of lanes 2 (protein extract) and 4 (protein extract + RNase H) for different ASO (Figure 3). Differential behavior of ASO also arises from the fact that dT18 stimulates RNase H-like activity in the mouse protein extract (without exogenous RNase H) more than SdT18 (Figure 3, compare lanes 2 for two ASOs). Since SdT18 binds to crude protein extracts more tightly than does dT18, the amount of available ASO that can enhance RNase H-like activity is higher for dT18 than for SdT.

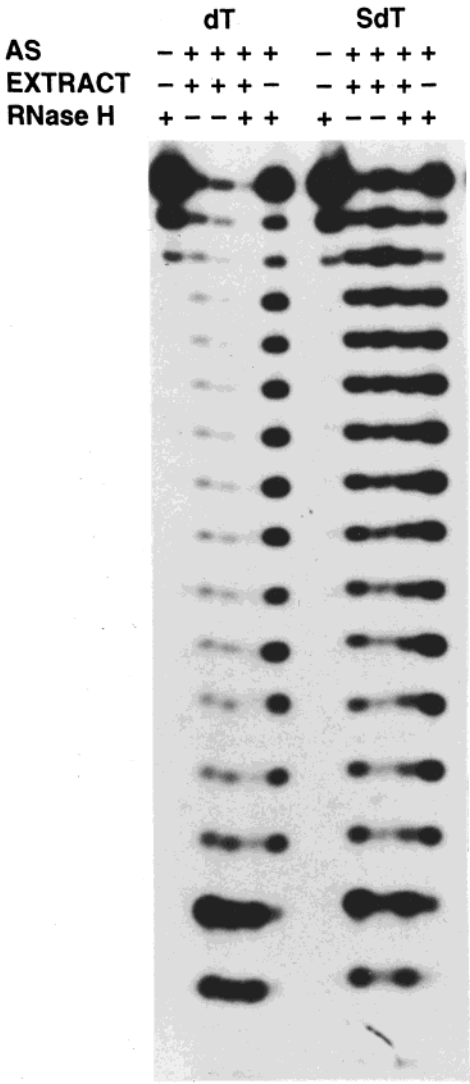

FIGURE 2: RNA/ASO hybrid degradation assay. The same experimental procedure was applied to each ASO (dT and SdT). Lane 1 in each set is a negative control, i.e., standard RNase H assay done on rA18, without ASO and without crude protein homogenate. Lanes 2 and 3 are digestions of RNA + ASO in the presence of 5% crude protein extract. The order of compounds addition in lane 2 was ASO, extract, RNA, while in the lane 3 ASO was preannealed with RNA, and then protein extract was added. Note that preannealing of ASO with rA18 (lane 3) produces mild nuclease protective effect toward rA18. Lane 4: the ASO was mixed with 5% crude extract, and 5 min later the target RNA was added together with *E. coli* RNase H. Lane 5: the standard RNase H assay was done on RNA/ASO hybrids (without 5% crude homogenate).

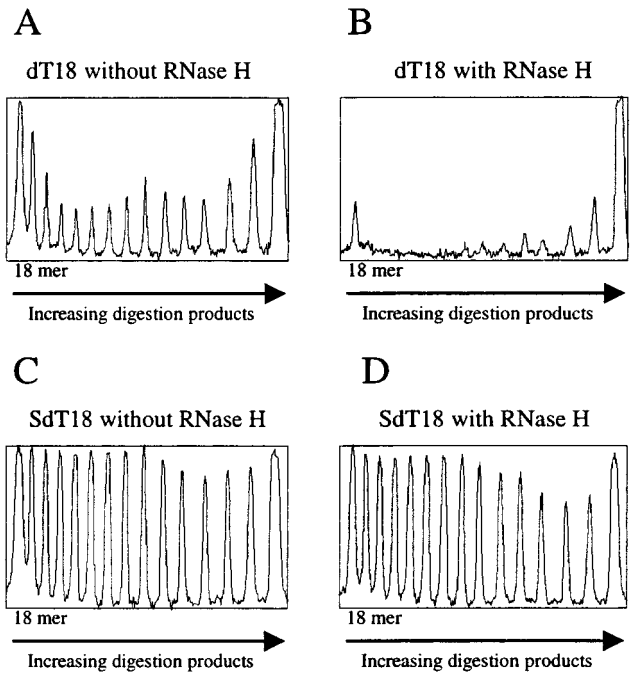

FIGURE 3: RNA/ASO hybrid degradation assays in the presence of protein extracts without (A and C) or with RNase H (B and D). Relative digestability of hybrids is illustrated by density profiles of the lanes 2 and 4 of Figure 2. Oligodeoxynucleotide (dT18) degradation is shown on A and B while phosphorothioate oligonucleotide (SdT18) degradation is shown on C and D. Longer fragments are on the left while smaller digestion products are on the right of the densitometric scans.

DISCUSSION

Direct information about interactions between an antisense molecule and proteins is often missing (*2*−*10*). Therefore, we developed a new nuclease digestion assay that estimates the success of RNA targeting in the presence of proteins. The results show that the overall propensity of ASO to bind proteins directly influences the efficiency of RNA targeting. In our opinion, phosphorothioate SdT oligonucleotides do not reach their target RNA in the presence of added proteins. It is important to note that in our experiments, the concentration of target RNA exceeded physiologically expected values by several orders of magnitude. At the same time, the concentration of protein effectively represented a 20-fold dilution from that found in living tissues. In the case of SdT ASO, proteins must be considered as "antisense" targets. In addition to protein affinity, the relatively low thermal stability of rA/SdT duplex could also explain, at least theoretically, the inability of SdT to form stable hybrids with RNA (*14*). However, this is clearly not the case since RNase H did cleave rA/SdT hybrids in the buffer without proteins. Also, note that our nuclease digestion experiments are done at 22 °C and not at 37 °C, where concerns about thermal stability might be relevant.

In light of the presented data, it seems very possible that the therapeutic effects of phosphorothioates reported so far are "aptamer" effects (*2*, *7*−*10*). Overall, protein affinity seems to be a crucial factor in the ability of ASO to reach their target RNA, which is compatible with previous reports (*7*−*10*). Therefore, future ASO assays should be complemented with estimation of ASO−crude protein affinity (*18*, *19*).

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