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Effects of phosphorothioate capping on antisense oligonucleotide stability, hybridization and antiviral efficacy versus herpes simplex virus infection

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ABSTRACT

Efforts have been made to improve the biological stability of phosphodiester (PO) oligonucleotides by the addition of various modifications to either the 3′, 5′ or both the 3′ and 5′ ends of an oligonucleotide. ISIS 1080, a phosphorothioate (PS) 21-mer oligonucleotide complementary to the internal AUG codon of UL13 mRNA in HSV-1, reduces the infectious yield of HSV-1 in HeLa cells to 9.0% ± 11%. PO analogs of ISIS 1080 containing three PS linkages placed on the 3′ (ISIS 1365), 5′ (ISIS 1370), both the 3′ and 5′ (ISIS 1364) ends or with four linkages in the middle (ISIS 1400) demonstrated reduced antiviral efficacy compared to fully PS ISIS 1080. Thermal denaturation profiles demonstrated that these oligonucleotides hybridized to complementary DNA or RNA with equivalent binding affinities. All were able to support *E. coli* RNase H cleavage of the HSV mRNA to which they were targeted. The stability of the congeners in cell culture medium containing 10% fetal calf serum (FCS), HeLa cytosolic extract, HeLa nuclear extract and in intact HeLa cells revealed that ISIS 1080 was most resistant to nucleolytic digestion through 48 hours. Partial PS oligonucleotides exhibited increased degradation compared to the fully thioated oligonucleotide by exonuclease activity in FCS and endonuclease activity in cell extracts or intact cells. Thus, the reduced efficacy of partial compared to fully PS oligonucleotides against HSV-1 in HeLa cells may result from increased degradation of the mixed PO/PS oligonucleotides.

INTRODUCTION

Antisense oligonucleotides have been shown to act as specific inhibitors of gene expression in a variety of *in vitro* systems (1, 2). One of the major problems encountered using the naturally occurring phosphodiester oligonucleotides (PO) is their rapid degradation by various nucleolytic activities in cells or culture medium (3, 4). Modifications of the phosphodiester backbone have been used to generate oligonucleotides with enhanced nuclease stability. Since the phosphorous center is the site of nucleolytic attack, many modifications have been made in the internucleotide linkage. Of these modifications, methylphosphonate (MeP; 5, 6) and phosphorothioate (PS; 7, 8) oligonucleotides have been extensively characterized. MeP oligonucleotides are resistant to purified exonucleases (phosphodioesterases) and endonucoleases (S1 nuclease). PS oligonucleotides also demonstrate increased resistance to both exo- and endonucleases. With a fully modified PS backbone, nuclease susceptibility to endonuclease (S1 nuclease) and the endo- and exonuclease (P1 nuclease) ranged from 2-45 times slower than that observed for PO congeners (8). The PO oligonucleotide containing PS linkage 'caps' on the 3′ and 5′ termini demonstrated nuclease stability comparable to the full PS (8). PS oligonucleotides also have been reported to exhibit nuclease resistance in intact cells. In *Xenopus* oocytes or embryos, internally labeled PO oligonucleotides were rapidly degraded with a half-life of 30 min when microinjected into cells (9). With full PS oligonucleotides the half-life was greater than 3 hours, in oocytes. In embryos the half-life of the full PS oligonucleotide depended upon the concentration injected. At low concentration (<5ng) the half-life was less than 30 min while at higher concentrations the half-life increased, suggesting that the process may become saturated.

Although PS oligonucleotides provide increased nuclease resistance, thermal denaturation studies suggest that the introduction of PS linkages reduce their ability to form stable hybrids with RNA (8). With PS oligonucleotides there was a reduction in Tm compared to PO congeners. Based upon nuclease stability and Tm values, it was concluded that PS capped oligonucleotides appear to be ideal modifications for antisense oligonucleotides (8). These data have been supported by the nuclease resistance of a single 3′-PS linkage to degradation imposed by exonuclease III, a 3′-5′ exonuclease (10). Also, PS linkages near the 5′ end of PO oligonucleotides provide protection from the 5′-3′ exonuclease activity of *E. coli* DNA polymerase I (11).

Use of 3′ and/or 5′ end caps of phosphoroamidates (PA) or phosphorothioates on PO oligonucleotides, significantly increased stability of a PO oligonucleotide (12). Positioning of two PA or two PS linkages at the 3′ end of the oligonucleotide was sufficient to confer stability from nuclease digestion in both cell culture medium containing fetal calf serum or in human serum. Modifications at the 5′ end of the oligonucleotide did not confer nuclease stability. This data demonstrated that 3′-3′ diester and 3′-3′ amidate linkages at the 3′ end of an oligonucleotide also conferred significant nuclease stability under the conditions tested (12).

We have investigated the potential for PS capping of PO oligonucleotides to provide better efficacy in reducing the infective yield of HSV-1 in infected HeLa cells. The rational for this approach was based upon the conclusions (8) that: 1) capping should provide nuclease stability in both the medium and within the cellular milieu and 2) PS capped oligonucleotides may provide an increased heteroduplex stability when hybridized to the target mRNA. For this study, we chose to examine the antiviral activity, hybridization stability, nuclease resistance, and RNase H (*E. coli*) activity against the mRNA/oligonucleotide heteroduplex. Comparisons are made between PO, PS and partial PS oligonucleotides that were capped on either the 3′, 5′ or 3′and 5′ ends or a PO oligonucleotide containing 4 PS linkages in the middle.

EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis

DNA oligonucleotides were synthesized using the Applied Biosystem 380B DNA synthesizer (Applied Biosystems, Incorporated, Foster City, CA). PO oligonucleotides were synthesized using standard phosphoramidate chemistry. For the PS oligonucleotides, sulfuration was performed after each coupling using 0.2 M 3H-1,2-benzodithol-3-one-1,1-dioxide dissolved in acetonitrile as described by Iyer et al.(13). To insure complete thioation, the growing oligonucleotides were capped after each sulfuration step. Mixed PS/PO oligonucleotides were oxidized or sulfurized after each coupling as appropriate. Following cleavage from the support matrix, deprotection, and detritylation, all oligonucleotides were lyophilized and purified by two ethanol precipitations.

Phenoxyacetyl protected phosphoramidates (ABN, Hayward, CA) were used for synthesis of RNA oligonucleotides on the ABI 380B synthesizer. For RNA synthesis the standard DNA synthesis cycle was modified by increasing to 15 min the wait step after pulse delivery of tetrazole and base. Removal of base protection groups was accomplished by overnight incubation in methanolic ammonia at 25°C. RNA oligonucleotides were dried, resuspended in tetrahydrofuran containing 1 M tetrabutylammoniumfluoride for removal of the 2′ protecting group, t-butyldimethylsilyl. Final RNA oligonucleotide product was desalted using SEP-PAK Cartridges (Waters, Milford, MA). Oligonucleotides were suspended in 6×SSC and loaded onto cartridges that were previously washed and equilibrated in the loading buffer. Cartridges were washed with 6×SSC, deionized water, and 10% methanol. Oligonucleotides were eluted from the cartridge with 90% methanol and dried in a SpeedVac Concentrator (Savant, Farmingdale, NY).

DNA and RNA oligonucleotides were assayed for purity by analytical polyacrylamide gel electrophoresis and all were greater than 90% full length oligomers. Concentrations of final oligonucleotide stock solutions were calculated using molar extinction coefficients calculated by Puglisi and Tinoco (14) and optical density at 260 nm.

[^{35}S]-Labeling of Oligonucleotides

The 5′ end of ISIS 1080 or ISIS 1364 was labeled using T4 polynucleotide kinase and gamma-[^{35}S]ATP (SA 1174 Ci/mmole). Fortyfive (45) pmoles of each oligonucleotide was heated to 70°C in 150 mM Tris-HCl, pH 8.0, 25 mM $MgCl_2$, 10 mM DTT and then slow cooled to 25°C. Labeling was carried out using 70 μCi gamma-[^{35}S]ATP and 150 units T4 polynucleotide kinase for 1 hr at 37°C. End labeled oligonucleotides were purified form unincorporated label using SEP-PAK Cartridges (as described above). Following purification oligonucleotides were resuspended in distilled water. and added to unlabeled oligonucleotides to yield a final concentration of 0.4 mM for addition to cells and quantitated via spectroscopy and liquid scintillation counting. The final specific activity of ISIS 1080 and ISIS 1364 were 9 and 18 Ci/mmole, respectively.

Cells and Virus

HeLa cells (ATCC #CCL2) were grown in Dulbecco's Modified Essential Medium (D-MEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 U/ml), and L-glutamine (2 mM). FCS was heat inactivated at 65°C for 0.5 hr prior to use. Stock cultures of HSV-1, strain KOS, were grown in HeLa cells using D-MEM with 10% FCS and supplemented as above using low multiplicity infections (multiplicity of infection [MOI] = 0.02 plaque forming units [PFU] per cell). Cultures were incubated at 37°C in the presence of 5% CO_2.

HSV Infectious Yield Assay

To assess ability of oligonucleotides to inhibit HSV replication, an infectious yield assay was employed. HeLa cells were seeded at a density of 7×10^5 cells per well in 6 well tissue culture plates. Three wells were set for each experimental condition to be tested. Cells were overlaid with 2 ml D-MEM + 10% FCS and incubated for 48 hr at 37°C. After incubation, unattached cells were removed by rinsing with phosphate buffered saline (PBS, pH 7.4) and the monolayer was covered with 1 ml D-MEM + 10% FCS containing the appropriate oligonucleotides. Control treatment involved no added oligonucleotide. Following an 18 hr incubation at 37°C, all wells were rinsed with PBS, resuspended in 0.5 ml D-MEM + 10% FCS and infected with HSV-1 at an input MOI = 0.5. Virus and cells were incubated for 90 min at 37°C with rocking. Following viral adsorption, unabsorbed virus was removed by rinsing with PBS and cells were incubated an additional 48 hr. Post-infection incubation was followed by scraping of cells into overlaying media. All replicates were harvested and frozen at −80°C. Prior to viral quantitation by plaque assay, the viral preparations were freeze-thawed three times (37 and −80°C). Additionally, HSV-1 samples were drawn through a 20 gauge needle three times immediately prior to plaque assay. The yield of infectious virus in each pooled sample was determined by plaque assay on Vero cells. All oligonucleotides were assigned a relative antiviral activity expressed as the percentage of control viral yield (% control yield = [titer of virus in oligonucleotide treated sample]/[titer of virus in concurrently run untreated sample]×100).

Intracellular Fate of Labeled Oligonucleotides

For determining the intracellular fate of the end labeled oligonucleotides, 5′-[^{35}S]-labeled oligonucleotides were added to HeLa cells at a final concentration of 4 uM (labeled and unlabeled oligonucleotide) under conditions outlined above. Following the 18 hr exposure to oligonucleotide, cells were infected with HSV-1 as above. After an additional 24 hr incubation in the presence of oligonucleotide, the cells were harvested by trypsinization. Supernatant and harvested cells were each extracted using an equal volume of phenol (saturated with buffer). Phenol extracted samples were precipitated with ethanol and resuspended in 9 M urea/2×TBE (0.2 M Tris-HCl, pH 8.3, 0.2 M sodium borate,

4 mM EDTA). Samples were analyzed by gel electrophoresis on 20% polyacrylamide gels containing 7 M urea. Equal amounts of cpms were analyzed for the oligonucleotides extracted from cells for comparison. Gels were dried and autoradiography was performed to visualize oligonucleotide length and degradation products.

Cell Fractionation

HeLa cells were washed three times and then scraped into 1 ml ice cold PBS. Cells were pelleted at 500 g for 10 min at 4°C. Cells were resuspended in 20 mM Tris-HCl (pH 7.3), 1 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5% NP-40 and kept at 4°C for 15 min. Lysed cells were clarified of nuclei and cellular debris by centrifugation at 800 g. The supernatant was used as the cytoplasmic preparation and the pellet was resuspended in the above Tris/ $MgCl_2$ /$CaCl_2$/NP-40 buffer and homogenized with a tight fitting pestle in a Dounce homogenizer. Microscopic visualization revealed disruption of nuclei and this fraction was used as a crude nuclear preparation. Protein concentration on each fraction was quantitated with BSA as a standard (15).

Nuclease Digestion

Determination of the rate of nuclease degradation of the oligonucleotides in medium containing 10% fetal calf serum (FCS) was carried out in DMEM containing 10% heat inactivated FCS. Heat inactivation of the FCS was carried out at 65°C for 0.5 hour prior to addition into media. Oligonucleotide was added to a final concentration of 66 ug/ml (approximately 10 uM). Following incubation at 37°C, 15 ul aliquots (containing 1 ug oligonucleotide) were removed and added to 15 ul of a solution of 9 M urea in 2× TBE. Aliquots were mixed and frozen at −20°C until electrophoresis on 20% polyacrylamide/7M urea slab gels. Following electrophoresis, gels were stained using 'Stains All' (Sigma Chem. Co., St. Louis, MO). Following destaining, gels were analyzed via laser densitometry using the UltraScan XL (Pharmacia LKB Biotechnology, Uppsala, Sweden). Integrations were performed and the data presented as the percentage decrease from (prior to 37°C incubation) full length, n, to n-1 length oligonucleotides. Nuclease susceptibility using the cytoplasmic and crude nuclear preparations was carried out as described for the 10% FCS experiments except cell extract was added at 0.33 mg total protein/ml in the cell fractionation buffer minus NP-40.

Thermal Denaturation

Oligonucleotides were mixed with either the DNA or RNA complementary oligonucleotide at 4 uM each strand in 100 mM Na^+, 10 mM phosphate, 0.1 mM EDTA, pH 7.0. Absorbance vs temperature profiles were measured at 260 nm using a Gilford Response II spectrophotometer (Ciba-Corning Diagnostics Corp., Oberlin, OH). Free energy of duplex formation (ΔG°_{37}) and melting temperature (Tm) were obtained from non-linear least square fit of the absorbance vs temperature curves to a modified two state model with sloping baselines (16).

RNase H Analysis

Oligonucleotides (2-fold molar excess to RNA) and 5 ug (3.1 kb) of complementary *in vitro* synthesized HSV-1 UL13 mRNA (17) were incubated in 5 µl RNAse H hybridization buffer (150 mM KCl, 100 mM HEPES, pH 7.0, and 5 mM EDTA) for 30 min at 60°C. Samples were slow cooled to room temperature, then adjusted to 3.7 mg/ml BSA, 3 mM DTT, 100 mM KCL, 3 mM $MgCl_2$, 60 mM Tris-HCl, pH 7.5. Twenty units *E. coli* RNAse H (Promega, Madison, WI) were added and the mixture incubated for 30 min at 37°C. Samples were phenol extracted, ethanol precipitated, and analyzed by electrophoresis on 1.2% agarose gels. Bands were visualized by ethidium bromide and fluorography. Markers were run to determine approximate length of RNA samples.

RESULTS

Antiviral Efficacy of Oligonucleotides

Table 1 shows the sequence and positioning of the PS linkages in oligonucleotides used for this study. ISIS 1047 is a phosphodiester oligonucleotide targeted to an internal AUG start

Table 1. ISIS Number, sequence and position of phosphorothioate linkages in oligonucleotides.

ISIS Number	Sequence (5'-3')*
1047	ACCGAGGTCCATGTGCGTACGC
1080	A*C*C*G*A*G*G*T*C*C*A*T*G*T*C*G*T*A*C*G*C
1364	A*C*C*GAGGTCCATGTCGTA*C*G*C
1365	ACCGAGGTCCATGTCGTA*C*G*C
1370	A*C*C*GAGGTCCATGTCGTACGC
1400	ACCGAGGT*C*C*A*TGTCGTACGC

* Positions of PS modifications are shown with an *.

Table 2. HSV-1 antiviral activity of phosphorothioate containing oligonucleotides.

Oligonucleotide	HSV-1 Viral Yield *% Control Level**
1080	9.0 ± 11.0
1364	77.1 ± 22.9
1365	75.1 ± 14.9
1370	55.4 ± 10.8
1400	71.5 ± 0.5
1047	81.6 ± 23.4

* Values reported represent the mean ± SD, n=3.

Table 3. Comparison of Duplex Stability for PO or PS Modified Oligonucleotides with their DNA or RNA Complements.

OLIGO (ISIS #)	Tm*	ΔG°_{37}**
	vs DNA	
1080	61.1	−17.7
1364	67.1	−22.7
1365	67.6	−22.5
1370	68.1	−22.7
1400	67.1	−21.4
1047	68.9	−22.9
	vs RNA (PO)	
1080	59.3	−15.8
1364	65.2	−19.5
1365	66.0	−20.5
1370	66.6	−19.7
1400	66.7	−20.1
1047	67.6	−16.0

* Tms were determined using absorbance vs temperature profiles at 260 nm as described in Experimental Procedures. Values are reported in degrees C.
** Values for ΔG°_{37} are reported in kcal/mole.

codon of HSV-1 UL13 mRNA. ISIS 1080 is the fully PS oligonucleotide, ISIS 1370 and ISIS 1365 are the 5′ and 3′ PS-capped oligomers, respectively . ISIS 1364 contains both the 3′ and 5′ PS-caps while ISIS 1400 has a block of 4 PS linkages located at positions 8−11.

Oligonucleotides (at 4 μM) were tested for their antiviral efficacy by assaying the yield of HSV-1 from infected HeLa cells. Table 2 lists the ISIS oligonucleotide and its antiviral activity. The fully thioated compound (ISIS 1080) decreased viral yields of HSV-1 by an average of 90% in our assays. The partially thioated oligonucleotides showed much less antiviral activity (55 to 77% of untreated controls). The PO oligonucleotide, ISIS 1047, reduced viral yield to 82% of control.

Thermal Stability

The Tm (melting transition temperature for 50% strand separation) and the ΔG°_{37} (free enengy of duplex formation) were determined against complementary DNA or RNA oligonucleotide (Table 3). Against the DNA complement, the ΔG°_{37} values for ISIS 1047 and ISIS 1080 were −22.9 and −17.7 kcal/mol, respectively. The loss in hybridization stability due to the PS linkages was also reflected in the decrease in Tm from 68.9 for ISIS 1047 (PO oligomer) to 61.1°C for the PS oligonucleotide, ISIS 1080. The Tm depression observed for the PS oligonucleotide, ISIS 1080, is consistent with data that has been reported in the literature (8). The values for Tm and ΔG°_{37} for the capped and internal blocked PS oligonucleotides were closer to that of the PO oligonucleotide, ISIS 1047. Thus, the presence of the end caps of PS linkages or the internal block of 4 PS linkages does not significantly alter the stability of the DNA-DNA duplex.

Against RNA complement, Tm and ΔG°_{37} values for all of the oligonucleotides were slightly lower than the corresponding values obtained with DNA complement. The DNA-RNA hybrids exhibited the same trend with the PS capped or internal blocked oligonucleotides as was observed with the DNA-DNA duplexes. The values for ΔG°_{37} or Tm are more similar to the values for the PO oligonucleotide than they are to the values of the all PS oligonucleotide.

E.coli RNase H Activity

The ability of *E. coli* RNase H to cleave the RNA portion of a heteroduplex between each oligonucleotides and *in vitro* synthesized HSV-1 target mRNA is shown in Figure 1. All combinations exhibited cleavage of the RNA. The mRNA is approximately 3.1 kb and the oligonucleotides hybridizes at a site approximately 0.7 kb from the 5′ end of the RNA. Thus, cleavage at the site of oligonucleotide hybridization results in two fragments of RNA, one 0.7 kb and the other 2.4 kb. As is seen in Figure 2, two bands of approximately the correct length are present in all lanes that contained hybrid complexes and RNase H. Therefore, each of the oligonucleotides binds to the mRNA in the correct region and supports RNase H cleavage of the mRNA. When considered in context with hybridization data,

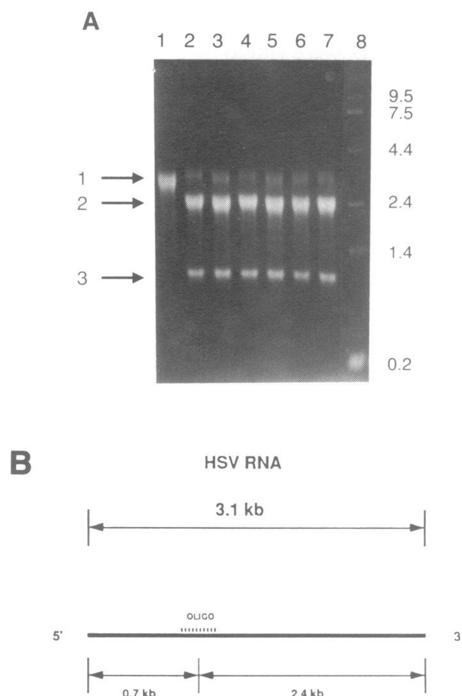

Figure 1. Ability of *E. coli* RNase H to cleave HSV target mRNA when heteroduplexed with PO, PS, and partial PS oligonucleotides. A) Gel analysis of cleavage reactions. Lanes: 1) RNA; 2) RNA + ISIS 1080; 3) RNA + ISIS 1047; 4) RNA + ISIS 1400; 5) RNA + ISIS 1370; 6) RNA + ISIS 1365, 7) RNA + ISIS 1364; 8) RNA sizing ladder. Base length is shown next to lane 8. Arrows denote: 1) full length RNA; 2) 2.4 base RNase H fragment; 3) 0.7 base RNase H fragment. B) Schematic of HSV target RNA, position of oligonucleotide binding site and expected fragment size following cleavage of the RNA by RNase H.

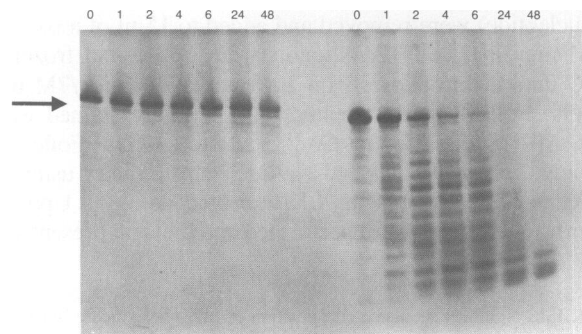

Figure 2. Denaturing PAGE analysis of HeLa cytoplasmic extract degradation of ISIS 1080 (left) and ISIS 1364 (right). Arrow denotes position of full length 21-mer oligonucleotide. Numbers at top of gel represent time in hours.

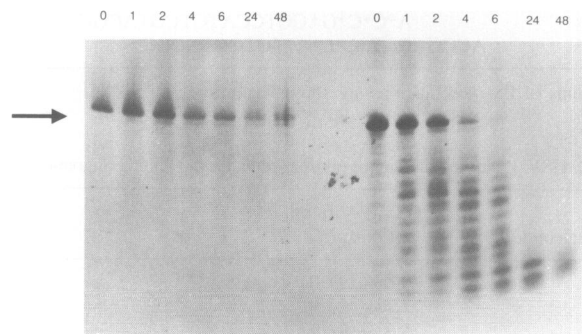

Figure 3. Denaturing PAGE analysis of HeLa nuclear extract degradation of ISIS 1080 (left) and ISIS 1364 (right). Arrow denotes position of full length 21-mer oligonucleotide. Numbers at top of gel represent time in hours.

these results suggest reduction in antiviral efficacy with partial PS oligonucleotides is not due to inability of the oligonucleotides to form stable hybrids with target RNA.

Stability of Oligonucleotides to Nucleolytic Degradation

The stability of the various oligonucleotides in cell culture medium containing heat-inactivated fetal calf serum (FCS) demonstrated that both the uniform PS oligonucleotide (ISIS 1080) and oligonucleotides containing a 3′ PS cap (ISIS 1364 and ISIS 1365) were nuclease resistant (data not shown). Oligonucleotides containing the 5′ PS cap (ISIS 1370), the internal block of PS (ISIS 1400) or without PS linkages (ISIS 1047) were rapidly degraded (data not shown). This is in agreement with previous literature reports that the predominant nucleases in FCS are 3′-exonucleases (12, 18). Of the oligonucleotides incubated in the presence of HeLa cytosolic preparation or the HeLa nuclear preparation, only the PS oligonucleotide (ISIS 1080) exhibited any degree of stablity to nuclease degradation (Figures 2 and 3). Only the slight appearance of bands representing degradation products could be observed. Oligonucleotide containing PS caps on both the 3′ and 5′ ends (ISIS 1364) was rapidly degraded in both extracts (Figures 2 and 3). ISIS 1364 was analyzed by ^{31}P NMR for quantitation of the extent of thioation. Integration of the chemical shifted PS peaks revealed that 27.4%, of the expected 30%, of the P atoms were acutally PS (data not shown). Thus, the PS capped oligonucleotide used in these experiments was 91% PS capped oligonucleotide.Interestingly, the majority of shorter bands were approximately 15−13 bases in length (Figure 4) and single base laddering, expected with processive exonuclease activity, was not evident. This suggests endonucleases may have been the predominant nuclease activity acting in this system. The full length fully PS oligonucleotide was totally degraded in the cytoplasmic extract within 24 hrs and in the nuclear extract by 48 hrs. All of the single capped, internal blocked PS, and the full PO oligonucleotides were rapidly degraded in both extracts (data not shown).

Intracellular Fate of Oligonucleotides

ISIS 1080 or ISIS 1364 oligonucleotides were 5′ end labelled using gamma[^{35}S]-ATP. Oligonucleotides containing the 5′-end label were isolated and applied to HeLa cells using the same protocol as in the infectious yield assay. The [^{35}S]-label on the 5′-end of the oligonucleotides was not removed by incubation in FCS for 48 hr (data not shown). This time interval corresponds to the time of oligonucleotide exposure to FCS in the infectious yield assay. Figure 5 shows the result of gel analysis and autoradiography of oligonucleotides recovered from both culture medium and cells as well as untreated controls. As is seen, full length ISIS 1080 was present in both the medium and cellular extract. ISIS 1364, however, was present as the full length 21-mer only in medium and exhibited a reduction in length in the cell extract. These results match those observed for cell extracts and suggest stability of ISIS 1080 and ISIS 1364 in cellular extracts may be an accurate reflection of their intracellular stability.

DISCUSSION

There have been reports of capping PO oligonucleotides with moieties that are resistant to nucleolytic degradation for the purpose of increasing oligonucleotide stability (8, 12, 19). The majority of these studies have utilized isolated nucleases, serum nucleases or cell supernatants. The increased resistance to FCS nucleases of oligonucleotides containing either two phosphoramidates or two phosphorothioates at the 3′ end of a PO oligonucleotide (12) are in agreement with our data. However, in our study capped oligonucleotides appear to be rapidly degraded by intracellular endonucleases. Capping of oligonucleotides with nuclease resistant modifications may not be sufficient for sustaining pharmacological activities of oligonucleotides in cells.

HSV UL13 mRNA was chosen as a target based upon the following: 1) conservation of nucleotide sequence between serotypes; 2) UL13 encodes a virion associated protein kinase and, thus, may be necessary for viral function Treatment with ISIS 1080 at a concentration of 4 uM shows significant reductions in HSV-1 viral infection in HeLa cells (reduction to 9 ± 11 of the untreated control). ISIS 1047, the full PO oligonucleotide reduced the viral titers to 88.6 ± 23.4% of untreated controls. Using oligonucleotides which contain PS linkages internally or at the 3′, 5′+ 3′ and 5′ termini, results in decreased efficacy and increased yields of the virus (Table 2). Because the Tm and ΔG°_{37} for binding of the partial PS oligonucleotides to complementary DNA or RNA was comparable to the PO or fully PS oligonucleotides (Table 3) duplex stability can not account for reduction in antiviral efficacy. All of the oligonucleotides were

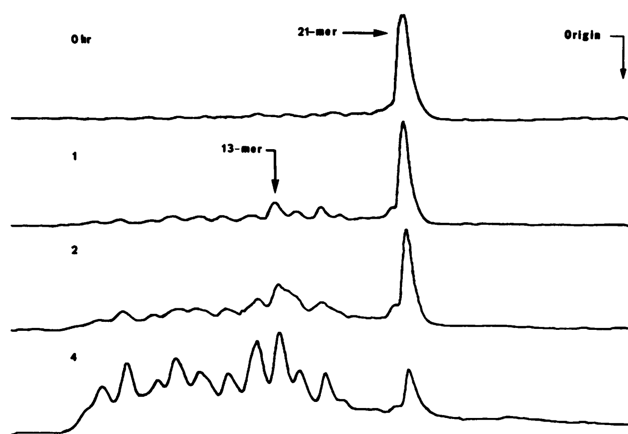

Figure 4. Densitometric tracing of PAGE analysis for degradation of ISIS 1364 in HeLa cytoplasmic extract. Full length 21-mer and degradation product, 13-mer, are shown. Laser densitometry of the gel depicted in Figure 2 for 0 through 4 hours is represented in the tracings.

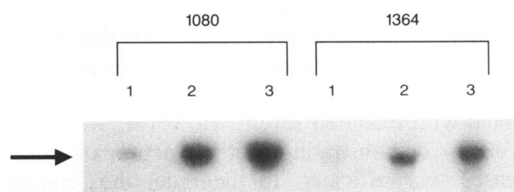

Figure 5. Autoradiogram of PAGE analysis of the radioactivity recovered from cell culture medium or cells following incubation of HSV-1 infected HeLa cells with [^{35}S]-labeled ISIS 1080 or ISIS 1364. Numbers represent: 1) Cell; 2) Culture Medium; 3) Untreated oligonucleotide markers.

capable of binding to *in vitro* synthesized RNA and were capable of acting as a substrate for *E. coli* RNase H suggesting that binding to the mRNA or reduced activity with RNase H also may not account for reduction in antiviral activity (Figure 1). It is a possible, however, that end-capped oligonucleotides can not serve as substrates for mammalian RNase H. Differences in bacterial and mammalian RNAase H do exist (19). Presently, we are investigating the potential for hybrids of these modified oligonucleotides to serve as templates for nuclear RNase H from HeLa cells.

Here we report antiviral efficacy of the PS oligonucleotide, ISIS 1080, is reduced when only the 3′, 5′, or 3′ and 5′ ends of the oligonucleotide are capped with three-PS linkages. In extracts of HeLa cells, there is significant degradation of the partial PS oligonucleotides compared to the PS oligonucleotide (Figures 2 and 3). Intracellular degradation of partial PS oligonucleotides by endogenous endonucleases in the cell could account for the observed reduction in antiviral efficacy.

Using 5′-[^{32}P]-end labeled oligonucleotides, degradation of a PO oligonucleotide in HeLa postmitochondrial cytoplasmic extract occurred within two hours (3). In these experiments, loss of label oligonucleotide could have been due to either the presence of nucleases released from subcellular compartments during extract preparation or the activity of phosphatases on the 5′-terminal phosphate. This latter case would not reflect actual oligonucleotide degradation. To determine if endonucleolytic degradation occurs *in vivo*, 5′-[^{35}S]-end labeled ISIS 1080 or ISIS 1364 was incubated with HeLa cells. Extraction of the oligonucleotides and size analysis by denaturing PAGE revealed that full length 5′-[^{35}S]-end labeled ISIS 1080 could be recovered (Figure 5), whereas full length ISIS 1364 was not observed. Thus, reduction in antiviral efficacy demonstrated with end capped PS oligonucleotides may be explained by the activity of cellular endonucleases.

Nonspecific inhibition of HSV-2 and, to a less extent, HSV-1 by phosphorothioate homopolymers has been previously observed (20, 21). The inhibition of viral replication by these PS homopolymers has been attributed to their acting as anti-template inhibitors of viral DNA polymerase (20). PS-dC_{28} and PS-dT_{28} did not reduce the number of HSV-1 (KOS strain) viral plaques compared to untreated control but did reduce the size of the plaques (21). In comparisons of ISIS 1080 and a scrambled sequence PS control oligonucleotide for efficacy against HSV-1 viral plaque formation we have routinely seen a 3- to 10-fold reduction in potency for ISIS 1080 (data not shown). Thus, there appears to be a significant degree of sequence specificity in ISIS 1080 inhibition of HSV-1. The reduced potency of PS-capped oligonucleotides compared to fully thioated ISIS 1080 would, therefore, appear to reside in the cellular instability of the capped and partially thioated oligonucleotides.

In conclusion, we confirm previous reports that the presence of simple 3′ or 3′+ 5′ modifications may provide protection from degradation by exonucleases present in cell culture medium supplemented with fetal calf serum. However, we extend these observations by demonstrating that the action of intracellular endonucleases is sufficient to degrade the end-modified oligonucleotides and may potentially reduce the ability of these capped oligonucleotides to act as antisense inhibitors of gene expression. These results suggest that uniform modification throughout an oligonucleotide may be required for sufficient stability from nucleases in cells.

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REFERENCES

1. Stein,C.A. and Cohen,J.S. (1988) *Cancer Res.*, **48**, 2659–2668.
2. Uhlmann,E. and Peyman,A. (1990) *Chem. Rev.*, **90**, 543–584.
3. Wickstrom,E. (1986) *J. Biochem. Biophys. Meth.*, **13**, 97–102.
4. Cazenave,C., Chevrier,M., Thuong,N.T. and Helene,C. (1987) *Nucleic Acid Res.*, **15**, 10507–10520.
5. Miller,P.S., Dreon,N., Pulford,S.M. and McParland,K.B. (1980) *J. Biol. Chem.*, **255**, 9659–9665.
6. Tidd,D.M. and Warenius,H.M. (1989) *Br. J. Cancer*, **60**, 343- 350.
7. Froehler,B., Ng,P., and Matteucci,M. (1988) *Nucleic Acid Res.*, **16**, 4831–4839.
8. Stein,C.A., Subasinghe,C., Shinozuka,K., and Cohen,J.A. (1988) *Nucleic Acid Res.*, **16**, 3209–3221.
9. Woolf,T.M., Jennings,C.G.B., Rebagliati,M. and Melton,D.A. (1990) *Nucleic Acid Res.*, **18**: 1763–1769.
10. Putney,S.D., Benkovic,S.J. and Schimmel,P.R. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7350–7354.
11. Ott,J. and Eckstein,F. (1987) *Biochem.*, **26**, 8237–8241.
12. Shaw,J.-P., Kent,K.,Bird,J., Fishback,J., and Froehler,B. (1991) *Nucleic Acid Res.*, **19**, 747–750.
13. Iyer,R.P., Phillips,L.R., Egan,W., Regan,J., and Beaucage,S.L. (1990) *J. Org. Chem.*, **55**, 4693–4699, 1990.
14. Puglisi,J.D. and Tinoco, Jr,I. (1989) *Methods Enzymol.*, **180**, 304–324.
15. Petersheim,M. and Turner,D.H. (1982) *Biochem.*, **22**, 256–263.
16. Draper, K. and V.B. Driver (Submitted
17. Cerrizzi,M. and Draper,K. (1989) *Nucleoside and Nucleotide*, **8**, 815–818.
18. Couch,R.J. (1990) *New Biologist*, **2**, 771–777.
19. Agrawal,S. and Goodchild,J. (1987) *Tetrahedron Lett.*, **28**, 3539- 3542.
20. Gao,W., Stein,C.A., Cohen,J.S., Dutschman,G.E. and Cheng,Y-c. (1989) *J. Biol. Chem.*, **264**, 11521–11526.
21. Gao,W-Y., Hanes,R.N., Vazquez-Padua,M.A., Stein,C.A., Cohen,J.S., and Cheng,Y-c. (1990) *Antimicrob. Agents Chemother.*, **34**, 808–812.