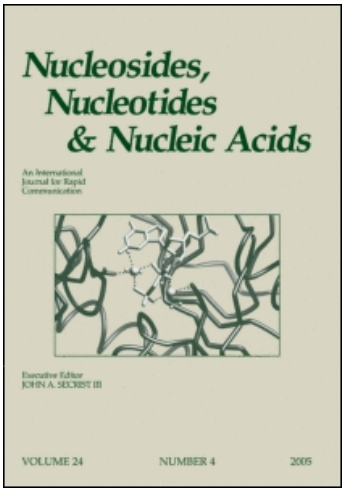


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# Natural and Phosphorothioate-Modified Oligodeoxyribonucleotides Exhibit a Non-Random Cellular Distribution

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# NATURAL AND PHOSPHOROTHIOATE-MODIFIED OLIGODEOXYRIBONUCLEOTIDES EXHIBIT A NON-RANDOM CELLULAR DISTRIBUTION

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ABSTRACT: Addition of specific antisense oligomers to L TK- cells infected with HSV-1 has been shown to decrease viral production (1,8). We have investigated the cellular components that contain these oligomers and a phosphorothioate derivative by Normarski light microscopy and gel analysis of sucrose gradient cell fractions. Fractionation analysis suggests that these oligomers are distributed throughout the cell in a non-random manner and gel analysis suggest that intact oligomers are not equally distributed in the cytosol, nuclear or membrane component. Information about the cellular location of antisense oligomers should aid in the understanding of their antiviral effect and in the design of more effective oligonucleotide derivatives as potential antiviral agents.



Several lines of evidence have shown that when specific oligodeoxyribonucleotides are added to tissue culture cells they can inhibit the synthesis of a specific gene product (1-4,13,14,22,25). In addition, antisense oligomers were also shown to inhibit viral replication, including HSV-1 and HIV (5-8). We have observed inhibition of both the expression of the alpha-TIF gene of HSV-1 and replication of HSV-1 in L TK- cells (1). These above data suggest that exogenous addition of oligomers or phosphorothioate-modified oligomers (5,6,9,10,14) that are complementary to specific regions of mRNA encoding

intracellular or viral proteins may be an important technique for inhibition of specific genes and for studying the role that particular gene products have in viral or in cell protein production.

Recently, Loke, et al. (11) described the cellular internalization of acridine conjugated oligomer (dN) by HL60 cells as determined by flow cytometric analysis and fluorescence microscopy. However, as the unconjugated acridine is also taken up by the cells (albeit by a passive mechanism rather than by the active mechanism of the acridine linked oligomer) the location of the conjugate oligomer may be dictated by the acridine moiety. Additional studies (23) have shown that when natural oligodeoxyribonucleotides are added to CEF and HeLa cells they become associated with the membrane/cytosol fraction. The work of Harel-Bellan et al. (25) showed that some of the binding of radio-labeled oligonucleotide to the membrane portion of T-lymphocytes was specific.



In this paper we use oligomers that were previously shown to have antiviral activity (1). We will describe the subcellular location of these end-labeled oligodeoxyribonucleotides and expand the study by including phosphorothioate-modified $^{32}$P-oligomers in L TK- cells and show that indeed what we describe associating within the cellular components are the input 18-mer oligomers and not free $^{32}$P. Further, we demonstrate that the ineffectiveness of an oligomer as a potential antiviral is not a function of its inability to reach a specific

cellular component or of its stability; rather, it is most likely sequence dependent.

## MATERIALS AND METHODS

### Cell growth and oligomer addition

L TK- cells were grown in EMEM media supplemented with 10% fetal calf serum, glutamine and penicillin-streptomycin. Approximately 5 x $10^6$ cells were plated in each of five T-75 flasks and allowed to grow overnight. The next day the media was removed and the monolayers were washed 2X with Hanks solution before the addition of radio-labeled oligomer 293 and 294 (1.5 x $10^5$ cpm/mcg - 4 x $10^5$ cpm/mcg, 50 mcg/2ml media per T-75 flask) and 293-S and 294-S (1.7 x $10^5$ cpm/mcg to 5 x $10^5$ cpm/mcg, 10 mcg/2 ml media per T-75 flask). After a 24 hour incubation, the oligomer solution was removed and the monolayers washed 2X with Hanks solution and trypsinized. Trypsinized cells were washed again in Hanks solution and spun at 2,000 rpm for 5 min. Cells were resuspended in two mls of RSB buffer (10 mM Tris-HCl, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$) for cell fractionation.

### End labelling of oligomers

Oligonucleotide sequences 5'-GTCCGCGTCCATGTCGGC-3' (293), 5'-CAAGAGGTCCATTGGGTG-3' (294) and their complete phosphorothioate derivatives (293-S and 294-S respectively), which are complementary to the mRNA sequence centered at the putative translation initiation codons for the open reading frame of the HSV-1 alpha-TIF protein were synthesized by Research Genetics, Huntsville, Alabama.

Oligomers were kinased with approximately 100 uCi of gamma-$^{32}$P ATP according to standard procedures (12). Unincorporated label was removed by Bio-Spin 6 column chromatography (Bio-Rad).

Nomarski light microscopy

Approximately 4 x $10^4$ L TK- cells were added to each well of an 8 chamber slide (LabTek) and allowed to grow overnight. Media was removed and monolayers washed 2X with Hanks solution. Labeled oligomer was added to each of the wells and uptake was allowed to occur for either a 6 or 24 hour period. At the end of the uptake process, monolayers were washed 2X in Hanks solution. The chamber portion of the slide was removed and the slides washed extensively (3x15 min with gentle shaking in PBS). Cells were fixed in 3.7% formaldehyde for 15 min., washed 2X in $H_2O$ and air dried. The slides were then dipped in Kodak NTB2 emulsion in the dark, dried and placed in a light tight box containing desiccant. The box was incubated at 4 $^{o}$C. Periodically, slides were developed (D-19 Kodak), fixed and examined with light microscopy using a Zeiss photomicroscope III at 40X magnification. Slides were photographed using Ektochrome 400 film.

Cell fractionation

Cells in 2 mls. of RSB buffer were dounced 20X with a tight fitting homogenizer on ice (15-17). Crude cytoplasm and nuclei were separated by spinning at 2500 rpm for 10 min. Microscopic analysis indicated that > 80% of the cells were disrupted by this technique. Aliquots were

saved for gel analysis and scintillation counting. The cytosol portion was kept on ice. The nuclei were washed again in RSB buffer and resuspended in 1.2 mls of TM buffer (50 mM Tris-HCl, pH 7.5, 2.5 mM $MgCl_2$) with 65% sucrose. The nuclear suspension (0.6 ml of suspension per tube) was transferred to Beckman ultraclear tubes. A decreasing sucrose-TM gradient was carefully layered on top of the sample: 0.6 ml 60%, 0.6 mls 55%, 1.2 mls 50%, and finally 25% sucrose to the top of the tube (15,17). Gradients were spun in an SW50.1 rotor for 1 hour at 20,000 rpm at 10 $^{o}C$. At the end of the spin a cloudy white band was observed at the 55%-60% sucrose boundary. This band was carefully removed, diluted to 50% sucrose-TM and rerun in a similar gradient for an additional hour at 20,000 rpm. The cloudy band at the 50%-55% boundary was again removed, diluted to 10% sucrose with TM and the nuclei pelleted by centrifugation in an Eppendorf microfuge for 15 min at 4 $^{o}C$ (19). Aliquots from the remaining first and second gradient runs were saved for gel analysis and scintillation counting.

The cytosol fraction was carefully layered in a Beckman ultraclear tube containing 1.5 mls 80% sucrose-TM and 2.5 mls 20% sucrose-TM. Gradients were spun at 35,000 rpm for 90 min in a Beckman SW50.1 rotor. The cytosol fractionated to the top 2/3 of the gradient and membranes appeared as a light grey band at the 20%-80% junction. Aliquots were again saved from the membrane, cytosol, and 80% sucrose component for counting and gel analysis.

Gel analysis

Aliquots of each fraction (10 ul) were denatured by heating in 80% formamide, 0.125% bromophenol blue for 3 min. at 90 $^{o}$C before being loaded on a 15% polyacrylamide, 8 M urea gel. Gels were electrophoresed at about 100-150 Volts until the dye had migrated about 2/3 to 3/4 the length of the gel. Gels were exposed to Kodak XAR-5 film with or without a Dupont intensifying screen from 1 to 4 days at -80 $^{o}$C.

RESULTS

Nomarski microscopic examination of L TK- cells incubated with $^{32}$P-end labeled antiviral oligomers 293 and 293-S revealed that the oligomers were distributed over the cell surface. (Fig. 1). The 293-S 24 hr. incubation was grossly overexposed to emphasize the observation that more oligomer was associating with the cells over the general background. The amount of oligomer associated with the cells did not appear to visually increase over a 24 hour time period. This was surprising as our previous results (20) had shown that the amount of oligomer associating with cells increases over a 24 hour period. Although these experiments suggested that the oligomers were able to associate with cells, they did not show whether any of the oligomers were actually internalized. Therefore we did a cell fractionation study that included oligomers which did not demonstrate antiviral activity by plaque reduction analysis (294 and 294-S, Table 1). We did not observe any significant cellular toxicity (<10%) with

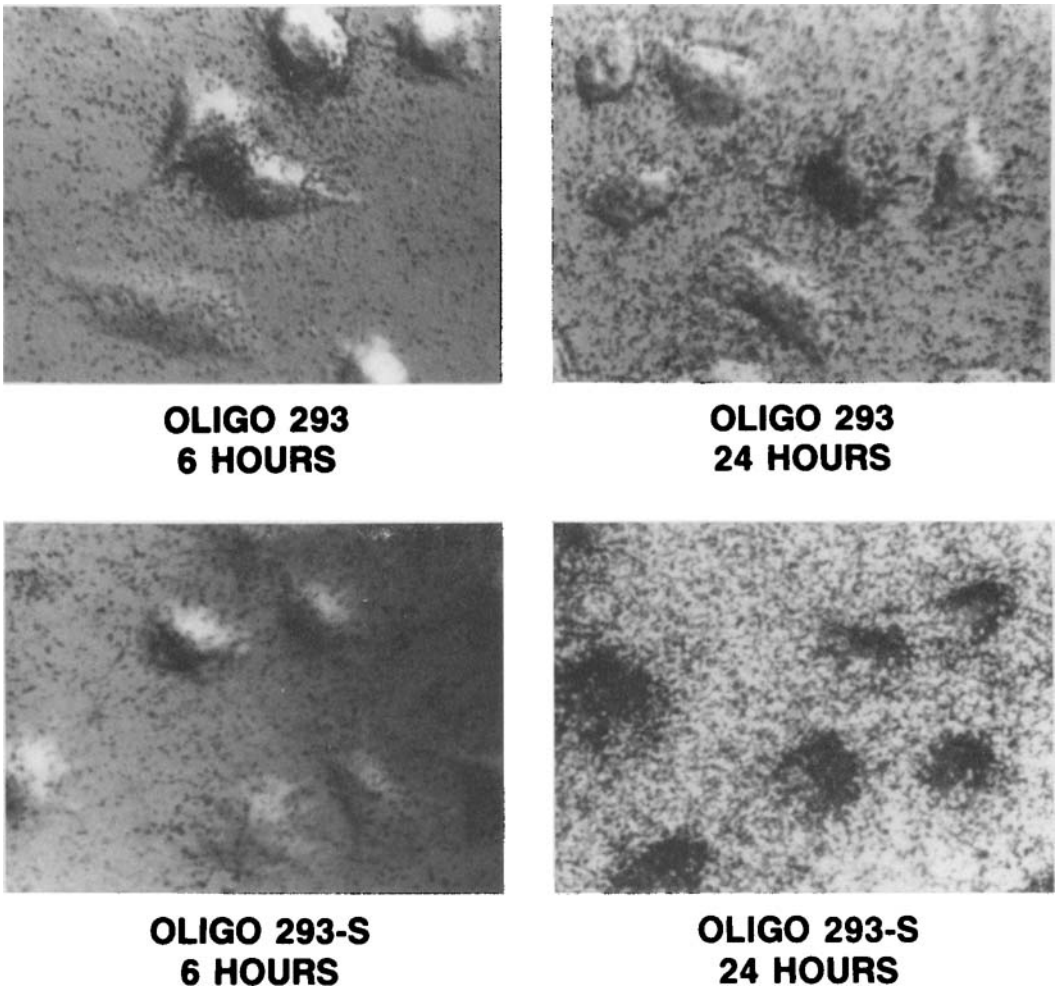


FIGURE 1
NOMARSKI MICROSCOPY OF CELL ASSOCIATED
OLIGONUCLEOTIDES



the natural oligomers while the amount of cellular toxicity for the phosphorothioate analogues ranged from 10% to 20%, as measured by $^{3}$H-leucine incorporation (data not shown).

Sucrose gradient fractionation

The cells were incubated with oligomers for a 24 hour period as our previous results suggested that oligomer

Table 1

Effect of Oligonucleotides
On Yield of Infectious HSV-1

| Oligomer | Representative virus yield | % Inhibition |
|---|---|---|
| 293 | -oligomer $5.7 \times 10^7$ | |
| | +oligomer $3.4 \times 10^7$ | 44 ± 11 |
| 294 | -oligomer $3.1 \times 10^7$ | |
| | +oligomer $3.0 \times 10^7$ | 1.5 ± 1.5 |
| 293-S | -oligomer $3.3 \times 10^7$ | |
| | +oligomer $0.8 \times 10^7$ | 72 ± 4.0 |
| 294-S | -oligomer $2.6 \times 10^7$ | |
| | +oligomer $2.7 \times 10^7$ | 0 ± 0 |

A typical plaque reduction experiment is shown. The method of this analysis is described elsewhere (1). Virus titrations were performed in duplicate. Data points represent the average of at least two replicate cultures. The % inhibition is represented by the reduction in viral titer of at least two separate experiments.
Error shown is standard error.



uptake was maximal at 24 hours (20). Cells were washed and trypsinized as explained in Materials and Methods. After douncing, aliquots of both crude nuclear and crude cytoplasm fractions of each oligomer experiment were counted and adjusted for sample volume. The percent total cell associated oligomer counts is the combination of volume adjusted counts from crude nuclear plus cytoplasmic fractions (Table 2). For each experiment the percent of a given oligomer associating with either the cytosol, nucleus or membrane component after sucrose gradient purification is also shown. Additional counts were found in the sucrose gradient itself after nuclei purification, presumably from

TABLE 2

L TK- Cell Fractionation

| Oligomer | N | % Cell associated | % Cytosol | % Nucleus | % Membranes |
|---|---|---|---|---|---|
| 293 | 2 | 6.9 ± 2.2 | 4.7 ± 1.6 | 0.02 ± 0.01 | 0.32 ± 0.11 |
| 294 | 2 | 21.0 ± 1.7 | 11.0 ± 1.0 | 0.01 ± 0.00 | 0.27 ± 0.23 |
| 293-S | 2 | 17.0 ± 1.3 | 12.8 ± 1.9 | 0.3 ± 0.2 | 0.65 ± 0.15 |
| 294-S | 2 | 10.0 ± 1.0 | 4.0 ± 0.0 | 0.6 ± 0.0 | 0.40 ± 0.00 |

Results are percent of total input counts from five T-75 monolayer flasks per oligomer experiment (refer to Materials and Methods for specific activity).
N= total number of times experiment was done.
% cell associated is the combination of crude cytoplasm plus crude nuclei.
Error shown is standard error.



cytosolic contaminants. This amount varied somewhat between the different oligomers: 1%, 3%, 3% and 3% of total input count for 293, 293-S, 294 and 294-S respectively. After the douncing procedure, aliquots were examined under the light microscope. This examination suggested that >80% of the nuclei remained intact (data not shown). The amount of oligomers associating with the small pellet after the first sucrose spin was about 1%-293, 3%-294 3%-293-S and 2% 294-S. Examination of this pellet suggested that it was comprised of cells which did not lyse during the douncing procedure. We did not attempt to increase the douncing procedure as the nuclei may have been damaged by further douncing. Quenching by the various fractions was 10% or less and thus did not have a significant effect on the results (data not shown).

Gel Analysis

To ascertain whether the radioactivity associated with each fraction was due to free label or intact oligomers, and to compare the relative integrity of each oligomer in the same fraction, we analyzed the fractions by gel electrophoresis. These results are not quantitative as equal cpm were not added to each lane. However, the results clearly indicate the relative integrities of oligomers in each fraction.

A portion of intact 293 oligomer was observed in all fractions but the nuclear component (Fig. 2a). The presence of intact oligomer in the crude nuclear fraction may be ascribable to cytosolic contamination. It appears that the cytosol and membrane components contain the greatest proportion of intact oligomer 293.



Neither crude nor purified fractions contained any intact oligomer 294 (compare control oligomer lane to cytosol, membrane and nuclei lanes Fig. 2b). The largest oligomer in any of the fractions was approximately 9 nucleotides in length. It is interesting to note that this oligomer does not demonstrate any antiviral activity (Table 1, ref. 1).

Every fraction was found to contain a portion of intact oligomer 293-S. This may be due in part to the higher resistance to nuclease that this molecule possesses (Fig. 2c). Again, the distribution of the amount of intact oligomer varied. It appears that most of the oligomer in the nuclear and membrane fractions was intact, however, a

significant portion of the oligomer in the cytosol had degraded. Like 293, 293-S also has demonstrated antiviral activity (Table 1).

We attempted to determine if the lack of antiviral activity by oligo 294 was due to the absence of full length 294 by using a more stable phosphorothioate-modified 294. Results from this analysis showed that the percent associating with each fraction was not significantly different than 293-S (Table 2). Full length 294-S was found in every fraction, although the distribution did vary between the various cellular components. Like 293-S, a significant portion of 294-S in the cytosol component was degraded (Fig. 2d). Phosphorothioate-modified 294 was also found not to inhibit HSV-1 replication (Table 1).



## CONCLUSIONS

In this study we have shown that alpha-TIF antisense oligomers 18 nucleotides in length, some (293 and 293-S) possessing antiviral activity, associate with L TK- cells. Our Nomarski analysis shows that there are no detectable differences in cell association between the 6 or 24 hour incubation with oligomer 293 or 293-S. This is surprising as our previous results showed that the amount of oligomer associating with cells increases over a 24 hour time period. It may be that the increase in the radioactive signal was below the sensitivity of this technique or that we did not use a synchronous culture of cells. We attempted a similar Nomarski study with MRC5 and WI38 primary lung cells. However, these experiments were

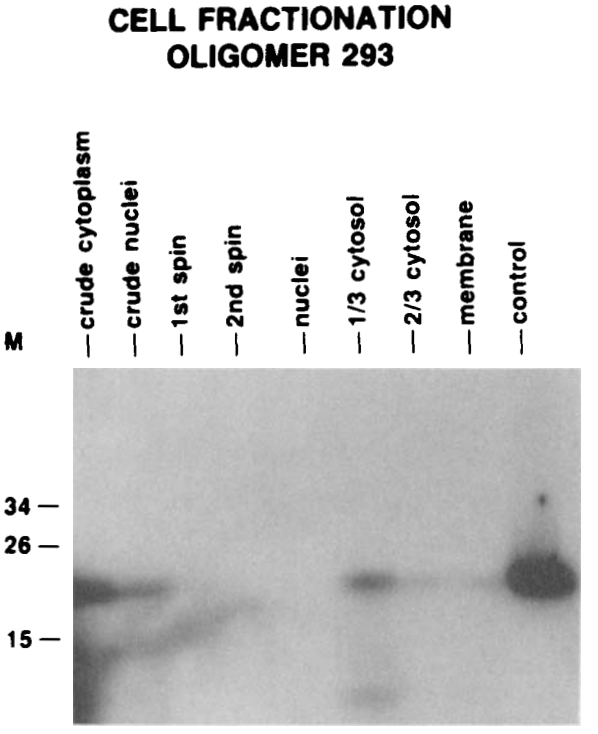


Fig. 2a.

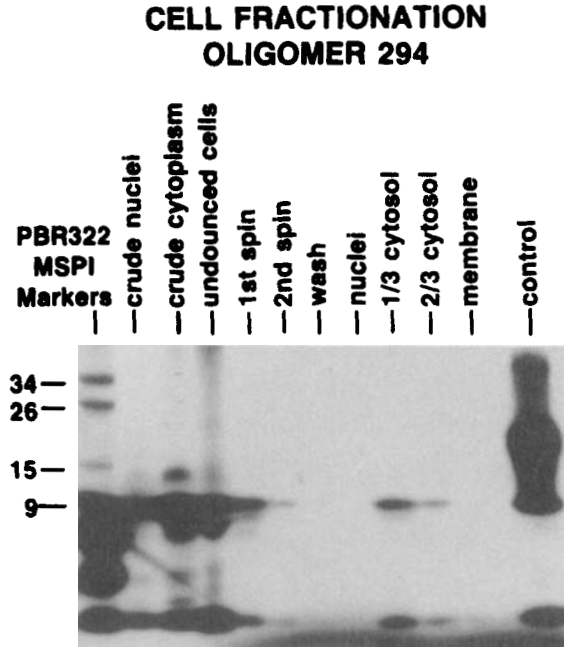


Fig. 2b.

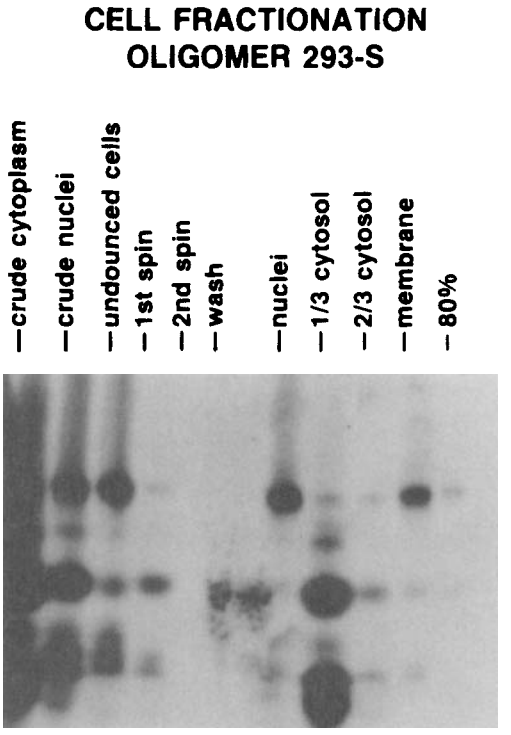


Fig. 2c.

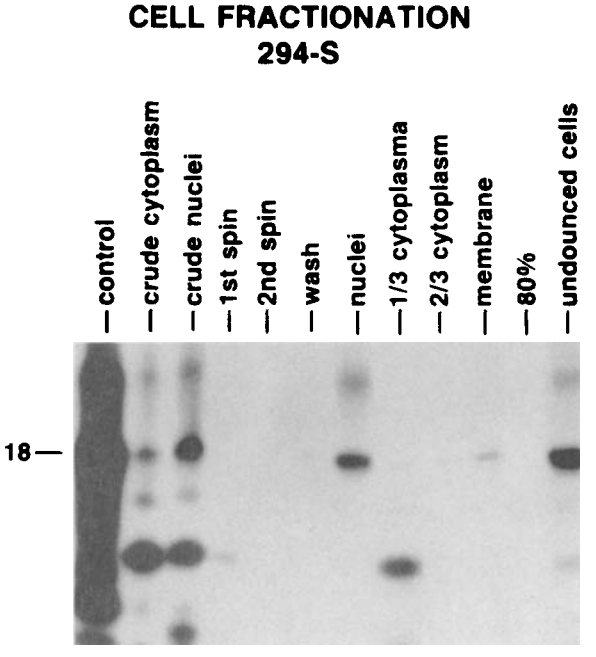


Fig. 2d.

difficult to interpret as during the many washes over 70% of the cells sloughed off the slides. Moreover, the Nomarski examination revealed nothing about the integrity of the oligomers. The L TK- cell fractionation studies addressed that issue as well as yielding more information about cellular localization. As shown by these fractionation studies, the majority of each oligomer is located in the cytoplasm with the membrane fraction possessing the next most abundant and the nuclear the least. There were, however, differences in integrity among the oligomers in each of the fractions. Intact oligomer 293 is located in the cytoplasm and membrane fractions but not in the nuclear fraction. Our in vitro S1 analysis (1) showed that oligomer 293 can hybridize with target mRNA molecules (1,14), supporting the theory that oligonucleotides such as 293 have the potential to interfere with translation, a cytoplasmic process. Unfortunately, a similar S1 analysis was impossible with the phosphorothioate analogues due to their inherent resistance to S1 nuclease.

No intact oligomer 294 was found in any of the fractions, which may explain its lack of antiviral activity. Why oligomer 294 is more degraded in all fractions than oligomer 293 is unclear. Oligomers 293 and 294 are unmodified oligomers and have a 72% and 55% GC content respectively and should be equally susceptible to extra- and intra- cellular nucleases.

Oligomer 293-S, which also affects HSV-1 production (Table 1, 23), was found, unlike 293, to be more intact in

the membrane and nuclei component than in the cytoplasmic fraction. The observation that an oligomer with this modification is present in these components suggests the possibility that it may affect cellular/viral processes other than translation.

Our fractionation studies show that the distribution of 294-S in the various cellular components is similar to the that of oligomer 293-S. Although oligo 294's lack of any antiviral effect may be due to the absence of full length oligomer in any of the fractions examined, the observation that full length 294-S was present yet had no antiviral effect suggests that the presence of intact oligomer is not sufficient for antiviral effect. This also suggests that the S-moiety per se does not affect viral growth. Our preliminary results with a random 18 nucleotide phosphorothioate oligomer supports this argument as this oligomer also shows no antiviral effects.

In summary the observation that oligomers 294-S, 293 and 293-S are found in the membrane and/or nuclear as well as the cytosol components suggests the possibility that they may be able to affect processes other than translation, such as RNA transport (nuclear) or receptor function (membrane). Even if these additional processes are affected, it is most likely sequence specific as antiviral effects were observed only with oligomers 293 and 293-S, not 294-S. These additional mechanisms were not explored in this study, but, as mentioned above, we had previously demonstrated that the natural oligomers have the

potential to hybridize to its target mRNA in vitro (1). To further explore the potential of oligomers as chemotherapeutic agents, additional studies are necessary to ascertain the mechanism of oligomer action, cellular uptake and resistance to nucleases.

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