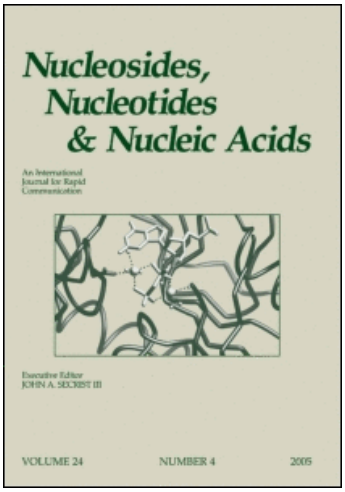

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The Intracellular and Extracellular Fate of Oligodeoxyribonucleotides in Tissle Culture Systems

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THE INTRACELLULAR AND EXTRACELLULAR FATE OF OLIGODEOXYRIBONUCLEOTIDES IN TISSUE CULTURE SYSTEMS

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Abstract. In this study, we analyzed the intracellular and extracellular stability of an oligodeoxyribonucleotide (oligo 293) which exhibits antiviral activity against Herpes Simplex Virus Type 1 (HSV-1) in tissue culture. The uptake and stability of oligo 293 varied when cell type or internucleoside linkage was changed.

In bacteria, the levels of expression from select mRNAs is controlled by the presence of complementary (antisense) RNA molecules[1]. Complementary nucleic acid sequences have been used to inhibit gene expression in Drosophila embryos and replication of virus in tissue culture[2,3]. We have found that an 18 base oligodeoxyribonucleotide (oligo) with sequence complementarity to the region encompassing the translation initiation codon of the HSV-1 Vmw65 mRNA inhibited replication of HSV-1 in tissue culture. At room temperature, this oligo (oligo 293) stably bound to Vmw65 RNA and inhibited in vitro translation of that RNA. Exposure of a Vmw65 expressing L cell line (MTX5)[4] to oligo 293 resulted in reduced transcriptional activation of the HSV ICP4 promoter, which requires functional Vmw65 protein for expression in these cells. The level of genetic inhibition observed with oligo 293 in tissue culture was surprisingly high and led to the following analysis of the molecular fate of oligo 293.

Preparations of oligo 293 (5'-dGTCCGCGTCCATGTCGGC) used in this study were custom synthesized and purified by Research Genetics, 2130 Memorial Parkway S.W., Huntsville, AL. The phosphorothioate analog of oligo 293 (oligo 293-S) was prepared by synthesis and hydrogen-phosphonate sulfurization of oligo 293 (D. Deyo, Chemical Research, Schering Corporation). Subsequent purification of oligo 293-S

was accomplished using an oligonucleotide purification cartridge (Applied Biosystems, 850 Lincoln Center Drive, Foster City, CA) as directed by the manufacturer. Aliquots of oligo 293 and oligo 293-S were end labeled with γ-32P-ATP using T4 polynucleotide kinase (New England Biolabs, 32 Tozer Road, Beverly, MA) according to published procedures[5].

To assess the capacity of oligo 293 to penetrate the cell membrane, ^{32}P-end labeled preparations of oligo 293 (specific activity = 1.6×10^5 - 2.4×10^5 cpm/μg, 50 μg / 2 ml of medium / T75 flask) were added to monolayer cultures of various cell types and incubated at 37°C for prescribed periods of time. Cells were rinsed twice with Hank's Balanced Salt Solution, trypsinized and harvested in a total volume of one ml. One-half milliliter aliquots of the cell preparations were centrifuged through 1.0 ml cushions of 20% sucrose (12000 xg, 2 min. in 1.5 ml Eppendorf tubes). Cell pellets were rinsed with phosphate buffered saline and uptake of oligo 293 was quantified from Cerenkov radiation present in each pellet. Cell associated radiolabel increased throughout the observed 24 hr incubation period in each of the 5 cell types tested. The apparent level of oligo 293 uptake varied somewhat depending upon cell type and experiment, but generally cells were capable of internalizing between 0.27% and 2.36% of the input ^{32}P label (see Table 1). The conversion of oligo 293 to the phosphorothioate analog (oligo 293-S) did not change 24 hr. uptake levels of the 18 mer in L cells and resulted in lower levels of uptake in the lung lines (Table 1). The examination of internalized oligo using high resolution acrylamide gels revealed the presence of a shortened oligo fragment (16 nucleotides in length), whose abundancy increased in direct proportion to the abundancy of intact oligo 293. The presence of the shorter oligo species was independent of cell type or nucleoside linkage.

Although increased levels of intracellular ^{32}P labeled oligo 293 were observed when cold oligo was added to the extracellular medium, the uptake of oligo by cells in culture was not linearly dependent upon extracellular concentrations of the molecule. To further understand the basis of this observation, the stability of oligo 293 in cell free medium containing 10% serum was examined. Samples of ^{32}P end-labeled oligo 293 (50 ng) were incubated at 37°C for various lengths of time,

TABLE 1. Cellular internalization of oligo 293 and oligo 293-S.

Cell Type	% Input ^{32}P label which is cell associated*				
Hours after oligo addition	1	3	5	7	24
293					
L cell (Tk-)	0.004	0.012	0.037	0.090	0.27
HeLa	0.036	0.167	0.239	0.453	1.25
HFF (Foreskin)	0.120	0.450	0.628	0.937	1.88
WI38 (Lung)	0.128	0.314	0.388	0.814	2.14
MRC5 (Lung)	0.130	0.334	0.890	0.952	2.36
293-S					
L cell (Tk-)	0.028	0.049	0.069	0.088	0.25
WI38 (Lung)	0.191	0.518	0.737	0.775	1.14
MRC5 (Lung)	0.200	0.380	0.331	0.633	1.19

*Due to differences in cell size between the various cell types, the number of cells in a T75 flask varies. Therefore, values were normalized to levels expected for 5 x 10^6 cells.

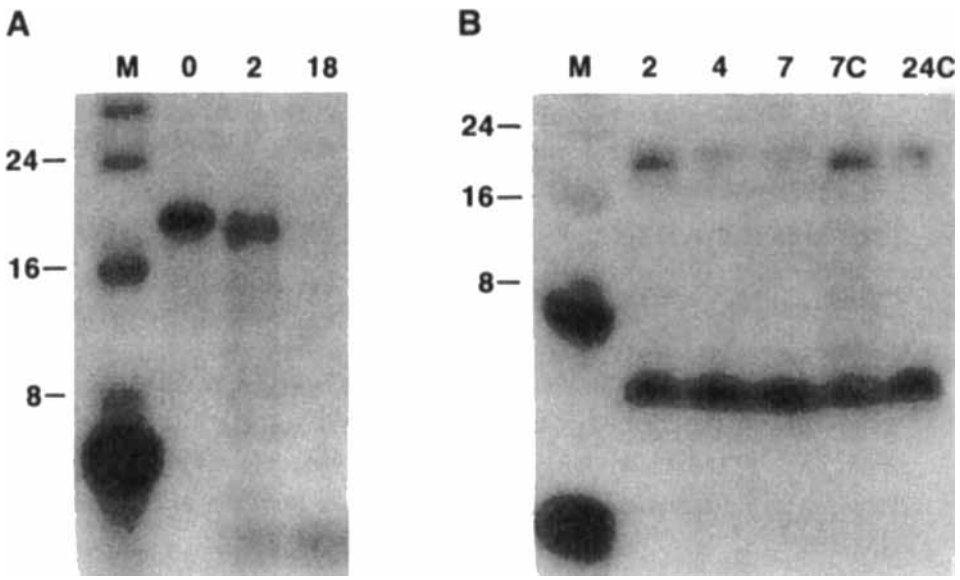

Figure 1. Oligo 293 stability in EMEM-10% serum. Hours of incubation are above lanes. 7C, 24C; 7, 24 hr. incubation in presence of 25 μg/ml cold oligo 293. Sizes of marker oligo (M) are shown in left margin.

deproteinized by phenol/chloroform extraction and analyzed by electrophoresis in denaturing acrylamide gels. As shown in Figure 1A, oligo 293 was completely hydrolyzed during 18 hr of incubation with 10% serum. Examination of oligo integrity at earlier times revealed a sharp drop in levels of intact oligo 293 after two hours of incubation (Figure 1B, Lanes 2-4). The addition of cold oligo 293 (25 μg/ml) to the incubation medium protected radiolabeled oligo from nucleoytic attack (Lane 5), even after 24 hr of incubation (Lane 6). Oligos containing lower GC/AT ratios showed similar digestion patterns in these assays. Judging from gel patterns (Figure 1A), a 3'-5' exonuclease activity in serum was responsible for most of the extracellular oligo degradation. In contrast to data using other phosphorothioate substituted oligos[6], oligo 293-S was very resistant to digestion from the 3'-5' exonuclease activity of our serum preparations.

In summary, we have shown that oligo stability in tissue culture involves many interdependent variables. Conversion of a normal oligomer (oligo 293) to its phosphorothioate analog (oligo 293-S) increased resistance to serum nuclease but did not increase the 24 hr. levels of cellular internalization. Studies of oligo 293-S subcellular localization are in progress.

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