

## A Simple Method for Delivering Morpholino Antisense Oligos into the Cytoplasm of Cells

MICHAEL PARTRIDGE, ALEXANDRA VINCENT, PAULA MATTHEWS, JOHN PUMA, DAVID STEIN,  
and JAMES SUMMERTON

### ABSTRACT

We report a simple and effective means for delivering Morpholino antisense oligos into the cytosol of cultured anchorage-dependent animal cells. This method, referred to as scrape-loading, is carried out in a matter of seconds, uses a common inexpensive laboratory implement, and has minimal detrimental impact on the cell. Using this delivery method, a Morpholino oligo present at 0.1  $\mu\text{M}$  and 1  $\mu\text{M}$  in the extracellular medium inhibited its targeted genetic sequence within cultured Hela cells at levels of 56% and 85%, respectively. Lack of inhibition by two control Morpholino oligos at concentrations up to 3  $\mu\text{M}$  indicates good sequence specificity by this structural type. Also described is a test system for simple, rapid, and sensitive quantitation of antisense activity in cultured cells.

### INTRODUCTION

DELIVERY OF ANTISENSE OLIGOS into the cytosolic/nuclear compartment of mammalian cells has proven to be one of the greatest challenges in the antisense field. Typically, in cultured cells, antisense oligos are endocytosed, but then they appear to remain largely in the endosomal/lysosomal compartment until degraded or exocytosed (Shoji et al., 1991; Oberhauser and Wagner, 1992; Wagner et al., 1993; Bennett, 1993; Stein and Cheng, 1993; Tonkinson and Stein, 1994; Bonham et al., 1995). In either case, most or all of the endocytosed oligos fail to gain access to their targeted genetic sequences within the cell.

Various strategies have been devised to achieve cytosolic delivery of antisense oligos. Some of the more effective include direct microinjection (Wagner et al., 1993) and complexing with cationic liposomes (Colige et al., 1993; Bennett et al., 1994). Although these methods appear to work, microinjection is tedious and cationic liposomes are somewhat toxic to cultured animal cells.

In 1984, McNeil et al. reported a method for delivering macromolecules into adherent cells, which they refer to as scrape-loading. This method entails gently scraping adherent cells from the surface of a culture dish with a rubber policeman in the presence of the macromolecules to be loaded into the cells. These workers suggest that scraping cells from the surface to which they are adhered generates transient holes in the plasma membrane at those sites that were tightly adhered to the surface. They postulate that macromolecules in the medium enter the cytosol by passage through such holes (McNeil et al., 1984). Scrape-loading has also been used

for delivering plasmid DNA into cultured cells (Fechheimer et al., 1987).

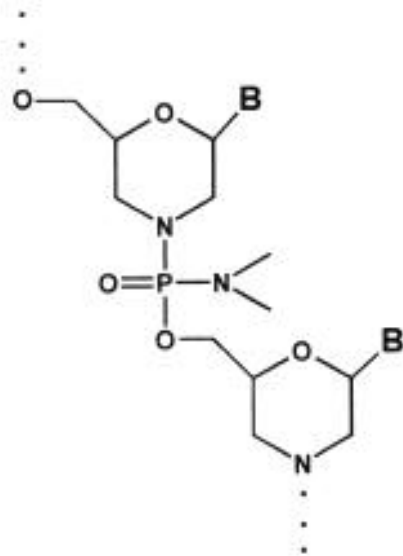
Although this scrape-loading procedure appears primarily suited for research applications with anchorage-dependent cultured cells, a recent report from research workers at Amgen (Farrell et al., 1995) suggests that an analogous scrape-loading of antisense oligos may occur in smooth muscle cells permeabilized in the course of balloon angioplasty, a procedure commonly used to open partially occluded arteries.

Herein we describe the use of scrape-loading for delivering into cultured animal cells a novel antisense structural type, which we refer to as Morpholino (Summerton, 1992; ANTIVIRALS Inc. Technical Reports 2 and 3, 1993; Summerton and Weller, 1993). As illustrated in Figure 1, Morpholino oligos have a backbone consisting of 6-membered morpholine rings joined by nonionic phosphorodiamidate intersubunit linkages. Entry of these oligos was assessed using cells stably transfected with plasmids designed for simple, rapid, and sensitive quantitation of antisense activity within cells.

### MATERIALS AND METHODS

#### *Test system*

The two plasmids used in these studies contain the mouse mammary tumor virus (MMTV) promoter controlling an



**B = adenine, cytosine, guanine, uracil**

FIG. 1. Morpholino oligo

mRNA transcription sequence comprising the leader sequence of rabbit  $\alpha$ -globin mRNA or the leader sequence of hepatitis B virus (HBV) mRNA, followed by the amino acid coding sequence for firefly luciferase. Both plasmids were transfected into HeLa cells following the procedures described in Promega Technical Bulletin No. 116 (Promega Corp., Madison, Wisconsin). The plasmid containing the  $\alpha$ -globin leader was also transfected into rat FT02B liver cells from Scripps Institute. Transfected cell lines were selected on the basis of high inducibility of the MMTV promoter with dexamethasone and high production of luciferase in the induced state. Figure 2 is a schematic of such a test plasmid and its use in assessing antisense activity within cells.

*Oligos*

Figure 3A shows the 5' region of the mRNA transcribed from the  $\alpha$ -globin leader/luciferase plasmid used in these studies. The globin antisense oligo is shown in position on its target sequence in that mRNA. Also shown are two control oligos, control I being the globin antisense sequence, but with four mispairs to its target sequence, and control 2 being the globin sense sequence. Figure 3B shows the 5' region of the mRNA transcribed from the hepatitis B leader/luciferase plasmid used in these studies. The HBV antisense oligo is shown in position on its target sequence in that

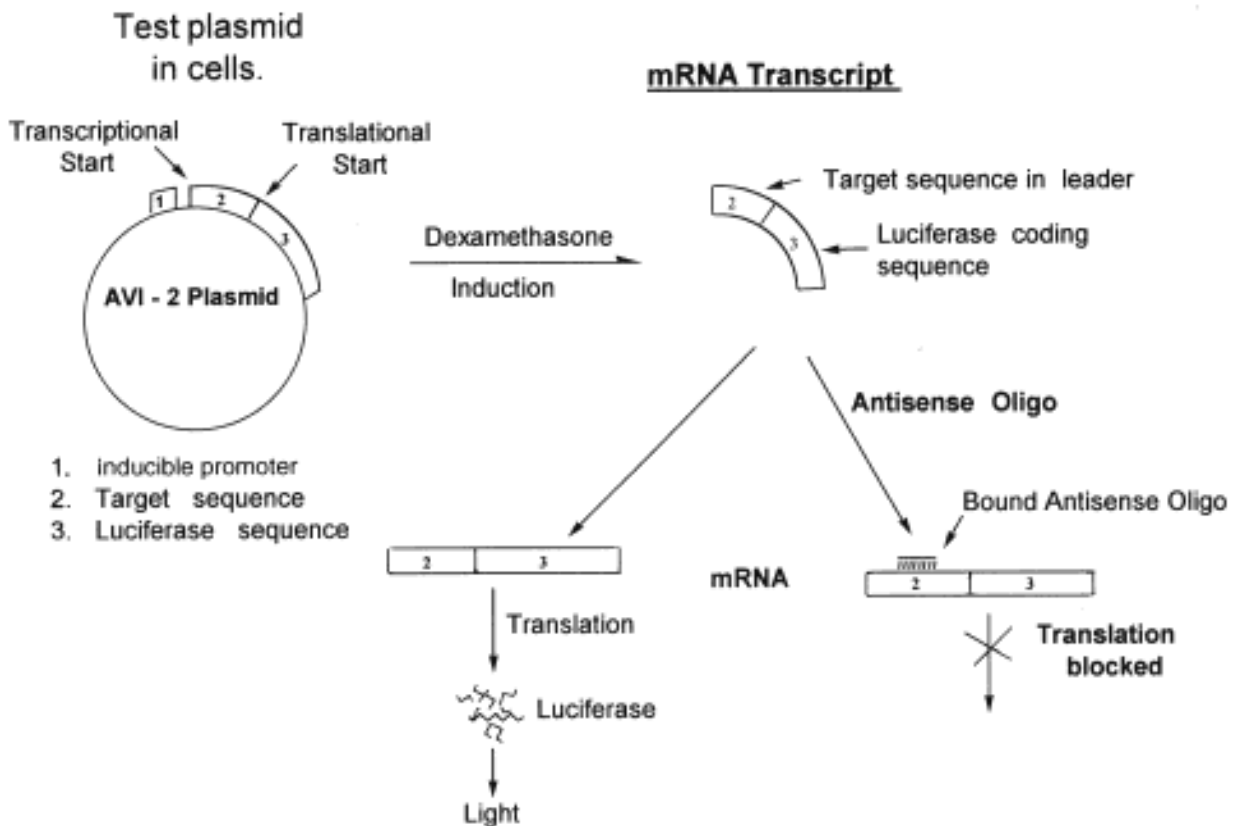


FIG. 2. Cell test system.

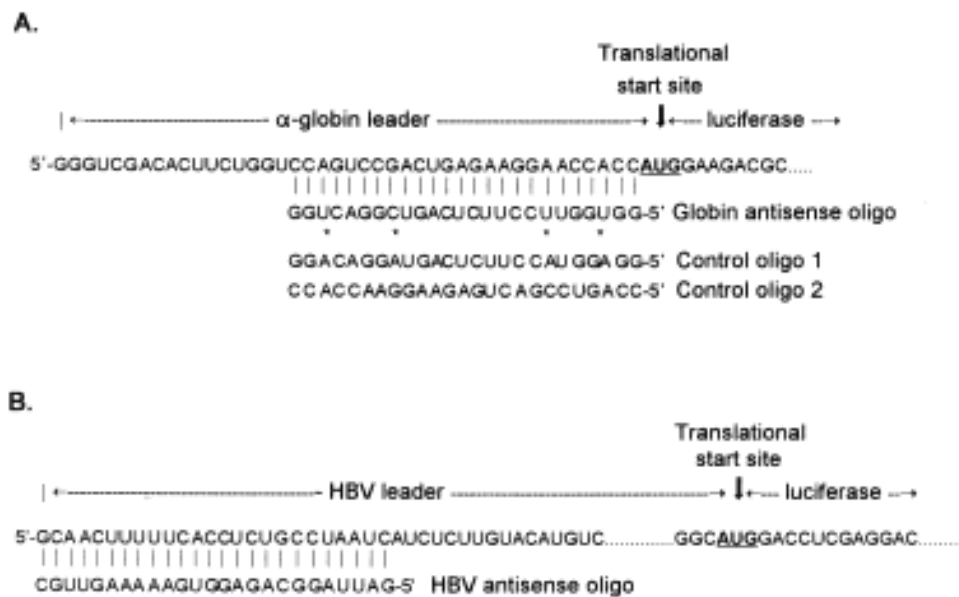


FIG. 3. Messenger RNA and oligos.

mRNA. Morpholino oligos having a backbone structure of the type shown in Figure 1 and the sequences shown in Figure 3 were synthesized (Summerton and Weller, 1993) and purified at ANTIVIRALS Inc. Analyses by laser desorption time-of-flight mass spectrometry suggest purities greater than 90%, with most of the remaining material having a mass corresponding to a 24mer.

### Test procedure

The typical scrape-loading procedure entails seeding 106 trypsinized cells in 2 ml of DME/F12 growth medium (Hyclone Laboratories, Catalog B-1002 AX) supplemented with 100 U/ml penicillin, 75 U/ml streptomycin, and 10% fetal bovine serum (FBS) (Life Technologies, Catalog 26300-053) into a well of a Falcon 3046 six-well tissue culture plate.

After incubating at 37°C for 24 hours, antisense or control oligo is added to the medium to give the desired concentration and swirled briefly, and the cells are scraped with a rubber policeman (Catalog No. 53801-008 and 59060-047, VWR Scientific, Seattle, WA). Best results are achieved by keeping the plate flat on the work surface and using a low-force sweeping motion to scrape cells off in sheets. Rubber policemen that have been autoclaved more than about eight times lose their resiliency, after which they yield poor results. Using a 2 ml disposable pipet, the cells are gently transferred with the medium to the well of another culture plate. Best results are obtained when the cells are not disaggregated by repeated pipetting. Thirty minutes later, dexamethasone is added to a concentration of 1 μM to induce transcription of the target mRNA. After another 16 hours incubation, the medium is removed, and the adherent cells are lysed, and the lysate is assessed for luciferase activity. Thereafter, the same lysate sample is assessed for protein content to provide a measure of the number of cells contributing to that lysate. Light units from the luciferase assay are divided by this protein value to give normalized luciferase activity. Each value in the Results section represents the mean of three or more experimental samples.

Cell lysis and quantitation of luciferase activity entail placing

the culture plate on ice, aspirating off the culture medium, adding 1 ml of phosphate-buffered saline (PBS), aspirating the PBS, and adding another 1 ml of PBS. The washed cells are then scraped from the surface of the plate. The PBS containing scraped cells is centrifuged for 45 seconds at 16,000g, and the supernatant is aspirated off. The cell pellet is resuspended in 25 μl of 1X Cell Culture Lysis Reagent (Catalog E153A, Promega Corp.) and let stand at ambient temperature for 20 minutes. The nuclei are pelleted by spinning at 16,000g for 15 seconds. Ten microliters of the lysate supernatant is added to 50 μl of Promega Luciferase Assay Reagent (Catalog E1483) at ambient temperature and mixed for 30 seconds. Then light emission is measured for 15 seconds in a Model TD-20e luminometer (Turner Designs, Inc., Mountain View, CA). Use of this luciferase assay reagent, which uses coenzyme A (CoA) as the energy source, affords higher and more extended light emissions relative to assay reagents that use ATP as the energy source.

Following assessment of the light emission, protein in the lysate/Luciferase Assay Reagent sample is quantitated by adding 2.5 ml of 1X filtered Protein Dye Reagent (Catalog No. 500-0006, BioRad Laboratories, Hercules, CA), vortexing 5 minutes and then reading the optical absorbance in a spectrophotometer at 595 nm. Light units from the luciferase assay are divided by this protein value to give normalized luciferase activity.

Values of percent inhibition of luciferase are calculated as

$$\% \text{ Inhibition} = 100 (1 - (+\text{oligo}/-\text{oligo}))$$

where +oligo refers to the normalized luciferase activity from preparations treated with oligo and -oligo refers to the normalized luciferase activity from preparations not treated with oligo.

### Visualization of oligo in cells

5-Carboxyfluorescein was linked to the N-terminus of the Morpholino globin antisense oligo. This oligo was suspended

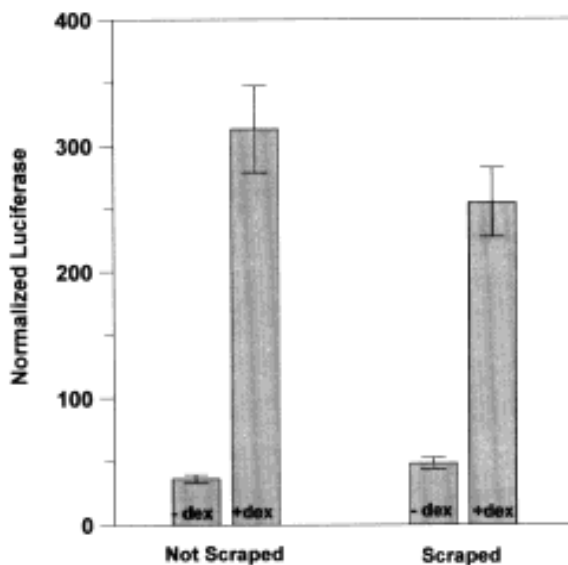


FIG. 4. Effect of scraping on luciferase expression.

in culture medium at a concentration of 10  $\mu\text{M}$  and added to two wells of HeLa cells that had been plated 24 hours earlier. Cells in one well were released from the surface by trypsinizing, and cells in the other well were released by scraping. After 15 minutes, the suspended cells were transferred to centrifuge tubes containing 3 ml of culture medium and centrifuged, the supernatants were removed, the cells were resuspended in 5 ml of culture medium and again centrifuged, the supernatants were removed, the cells were resuspended in 10 ml of culture medium, and 1 ml was plated per well in 2-well glass chambered slides. After 24 hours, the cells were visualized by fluorescence microscopy using a water-immersion 40X objective (Nikon CF Achromat WI 40X, Cat. 85063).

Each field of cells was photographed four times: (1) exposure for 1 second in fluorescence mode, (2) exposure for 4 seconds in fluorescence mode, (3) exposure for 15 seconds in fluorescence mode, and (4) a double exposure comprising exposure for 4 seconds in fluorescence mode, followed by exposure for 0.004 second in phase contrast mode. The four photographs of the field were then assessed for number of highly labeled cells (visible in the 1 second fluorescence exposure), number of moderately labeled plus highly labeled cells (visible in the 4 second fluorescence exposure), total number of labeled cells (visible in the 15 second fluorescence exposure), and total number of cells in the field (visible in the fluorescence/phase contrast double exposure).

## RESULTS

### *Effect of scraping on luciferase induction and production*

Figure 4 shows normalized luciferase activity from transfected HeLa cells that were not scraped or scraped and then either not induced or induced with dexamethasone. The plasmid in these cells contained the  $\alpha$ -globin leader sequence. These results suggest that in scraped cells luciferase activity is reduced only moderately relative to that in unperturbed cells.

### *Distribution of oligo in trypsinized and scraped cells*

Table 1 gives the cellular distribution of oligo in cells that were trypsinized and in cells that were scraped in the presence of fluorescein-labeled Morpholino oligo. These results indicate that in trypsinized cells there was little entry of oligo into the cell in the 15 minutes that cells were exposed to labeled oligo. However, if the trypsinized cells are exposed to labeled oligo for many hours, significant labeled oligo enters cells, wherein it is distributed in a perinuclear punctate pattern characteristic of localization in the endosomal/lysosomal compartment.

In scraped cells, oligo entry is clearly heterogeneous. In cells with a relatively low concentration of oligo, one generally sees diffuse labeling throughout the cell, with more dense labeling in the nucleus. In cells with a high concentration of oligo, the labeling is generally homogeneous throughout the cell.

### *Antisense activity in unperturbed, trypsinized, and scraped cells*

Figure 5 shows the percent inhibition of luciferase in HeLa cells containing the  $\alpha$ -globin plasmid, which were unperturbed, trypsinized, or scraped in the presence of 10  $\mu\text{M}$  globin antisense oligo. Clearly, this Morpholino antisense oligo exhibits little or no inhibitory effect in both unperturbed and trypsinized cells but exhibits significant inhibitory activity in scraped cells.

### *Time course of cell entry*

Morpholino oligo entry into HeLa cells containing the globin plasmid was assessed as a function of time after scraping. Figure 6 shows the percent inhibition afforded by 10  $\mu\text{M}$  globin antisense oligo present during scraping ( $t = 0$ ) or added at 1, 10, or 100 minutes after scraping. These results suggest that the majority of Morpholino oligo entry into cells occurs within the first minute after scraping.

### *Cell types*

Table 2 gives calculated percent inhibition by 10  $\mu\text{M}$  globin antisense oligo in two cell lines transfected with the globin plasmid. The cell lines are HeLa (human) and FF02B liver (rat). Also shown are the corresponding calculated percent inhibition values for control oligo 1 and control oligo 2. These re-

TABLE 1. FRACTION OF CELLS LABELED AND SUBCELLULAR DISTRIBUTION

	<i>Treatment</i>	
	<i>Trypsinized</i>	<i>Scraped</i>
Highly labeled	0%	62%
Moderate + high	0%	81%
Total labeled	0%	83%
Subcellular distribution	Perinuclear Punctate	Diffuse throughout cell Denser in nucleus

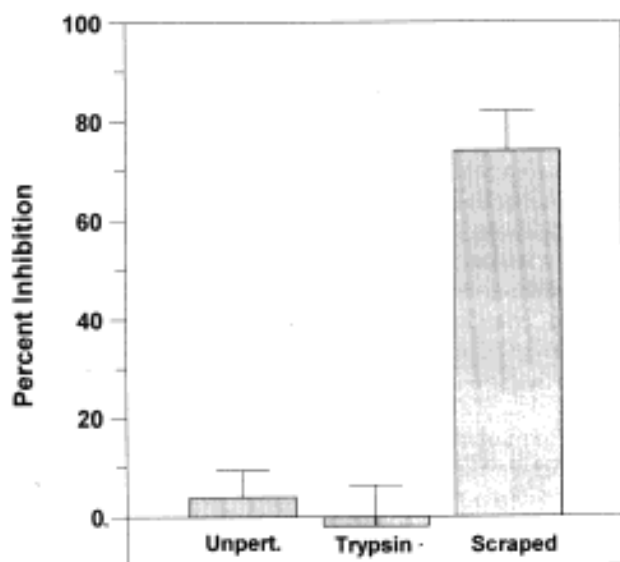


FIG. 5. Cell entry with different treatments. Unpert., unperturbed.

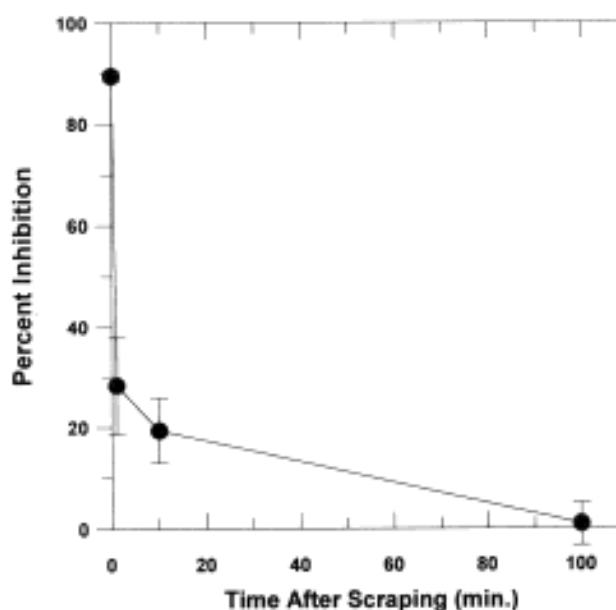


FIG. 6. Time course of cell entry.

sults suggest that scrape loading of antisense oligos may be effective in a variety of cell types.

From Table 2 it is apparent that the calculated percent inhibition values for the antisense oligo are quite different in the two clones tested. However, much of this difference may be artifactual, caused by differences in luciferase inducibility between the two clones. Specifically, luciferase expression is induced 8.5-fold by dexamethasone in HeLa clone 47, whereas it is induced only 3.4-fold in FT02B clone 8. As a consequence, relative to postinduction levels, preinduction levels of luciferase in FT02B clone 8 are proportionately higher (29%) than in HeLa clone 47 (12%). Thus, this lower inducibility of FT02B clone 8 results in a larger relative baseline level of luciferase, which, in turn, leads to a lower calculated percent inhibition value compared to that of HeLa clone 47. In principle, corrections to the calculated percent inhibition value can be made. However, it is generally simpler to select clones that afford a high multiplicity of induction by dexamethasone (preferably 6-fold or greater), thereby minimizing the error due to luciferase present before translational inhibition. Alternatively, the treated cells can be cultured for a longer period after the scrape-loading procedure to more completely dilute out the pretreatment luciferase.

#### Dose-response and specificity

To provide a measure of efficacy and specificity of Morpholino oligos within cells, both antisense and control oli

gos were assessed for activity when present in concentrations ranging from 30 nM to 3  $\mu$ M in the extracellular medium. Figure 7 shows activities of the globin antisense oligo and the two globin control oligos, as a function of oligo concentration added to HeLa cells transfected with the globin plasmid. In this scrape-load test system, the Morpholino antisense oligo exhibited significant antisense activity (56%) at an extracellular concentration of 100 nM and very good activity (85%) at 1  $\mu$ M.

At concentrations up to 3  $\mu$ M, the control oligos were not inhibitory. At 10  $\mu$ M, the sense oligo (control 2) did not inhibit significantly, but the 4-mispaired oligo (control 1) afforded a modest 21% inhibition.

#### Cross-targeting

If the inhibitory activity exhibited by Morpholino oligos in these scrape-load studies is indeed due to a true antisense mechanism, the globin-targeted oligo should and the HBV-targeted oligo should not inhibit expression of the globin plasmid. Likewise, the HBV-targeted oligo should and the globin-targeted oligo should not inhibit expression of the HBV plasmid. Figure 8 shows dose-response results from four such cross-targeting experiments using the two plasmids and the two antisense oligos of Figure 3. These results constitute good evidence that the Morpholino antisense oligos are acting in a true antisense mode.

TABLE 2. INHIBITION OF EXPRESSION IN TWO CELL LINES

Cell line	% Inhibition		
	Antisense oligo	Control oligo 1	Control oligo 2
HeLa clone 47	92	21	-27
FT02B clone 8	57	3	-26

## DISCUSSION

Our results show that in unperturbed or trypsinized HeLa cells, a Morpholino antisense oligo at a concentration of 10  $\mu\text{M}$  in the extracellular medium affords little or no inhibition of its targeted mRNA. In contrast, in scraped HeLa cells, the same antisense oligo at one-hundredth that concentration achieves substantial (56%) inhibition of its genetic target. Lack of significant inhibition by two morpholino control oligos suggests that this inhibition is both sequence dependent and quite specific. Sequence specificity is further supported by the results from the cross-targeting experiments, wherein a globin oligo is only effective against a globin target and an HBV oligo is only effective against an HBV target. Our results further indicate that most of the oligo entry into the cytosolic compartment of cells occurs within the first minute after scraping. This scrape-loading of oligos is shown to be effective in both a human and a rat cell line, suggesting that it may be effective in a variety of cell lines.

We suggest that this simple and effective method for delivering Morpholino oligos into the cytosol of adherent cells may allow investigators using antisense agents to achieve more effective and reproducible results in studies on anchorage-dependent cultured mammalian cells.

In recent years, there has been considerable interest in using antisense oligos for preventing restenosis of arteries following balloon angioplasty (Morishita et al., 1994; Bennett and Schwartz, 1995; Villa et al., 1995). In this regard, it is

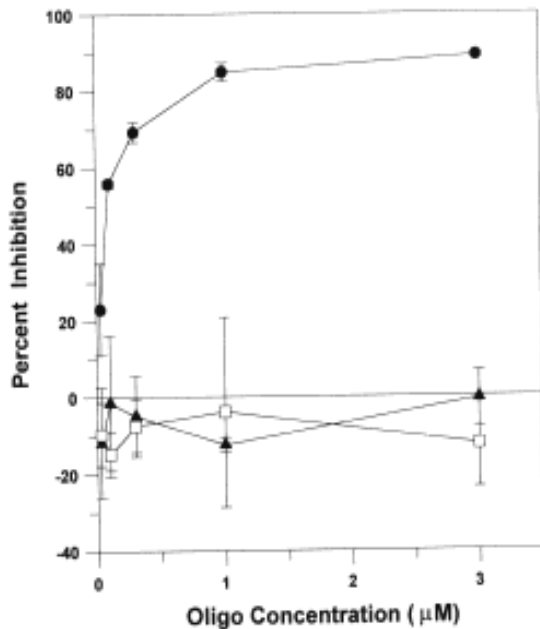


FIG. 7. Dose-response and specificity. Antisense oligo (●); control oligo I (▲); control oligo II (□).

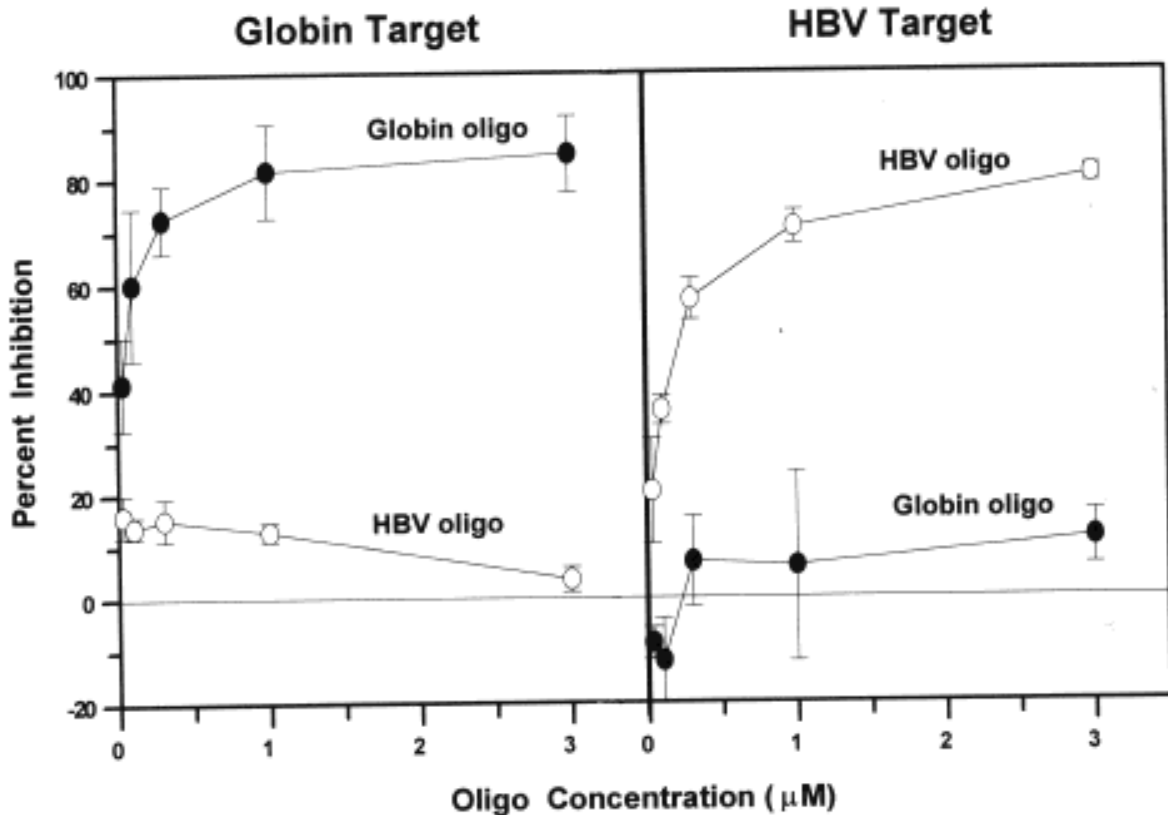


FIG. 8. Cross-targeting dose-response.

## DELIVERING MORPHOLINO OLIGOS INTO CELLS

noteworthy that Farrell et al. (1995) have demonstrated that normal smooth muscle cells of artery walls do not take up significant antisense oligo. However, when those same cells were scraped in the course of balloon angioplasty, a significant amount of antisense oligo (phosphorothioate-linked DNA) was seen to enter the cytosolic/nuclear compartment of the scraped cells. If the permeability generated in scraping adherent cells from a culture dish is similar to the permeability generated in smooth muscle cells during balloon angioplasty, we suggest, because of the very transient nature of the cytosolic entry process, that delivery into the cytosol of the scraped smooth muscle cells can best be achieved by presaturating the artery tissue with antisense oligo. This should ensure that the oligo is in close proximity to the cells at the moment they are scraped, thereby maximizing entry of the therapeutic oligo into the target cells.

### ACKNOWLEDGMENTS

We thank Dwight Weller for helpful discussions during this project, Elisabeth Barofsky of the Mass Spectrometry Lab, Department of Agricultural Chemistry and Environmental Health Science Center, Oregon State University, for assessing the purity of the Morpholino oligo preparations, and the ANTIVIRALS oligomer synthesis group for the preparation and purification of the Morpholino oligos.

### REFERENCES

- ANTIVIRALS Inc. Technical Report 2. (1993). Comparative studies of translation inhibition in reticulocyte lysates. *Antisense Res. Dev.* 3(1), Technical Reports 2, following page 153.
- ANTIVIRALS Inc. Technical Report 3. (1993). Comparative studies of efficacy and specificity of translation inhibition in reticulocytelysates. *Antisense Res. Dev.* 3(3), Technical Reports 3, following page 306.
- BENNETT, M., and SCHWARTZ, S. (1995). Antisense therapy for angioplasty restenosis: Some critical considerations. *Circulation* 92,1981-1993.
- BENNETT, R. (1993). As nature intended? The uptake of DNA and oligonucleotides by eucaryotic cells. *Antisense Res. Dev.* 3,235-241.
- BENNETT, R., CONDON, T.P., GRIMM, S., CHAN, H., and CHIANG, M.-Y. (1994). Inhibition of endothelial cell adhesion molecule expression with antisense oligonucleotides. *J. Immunol.* 152,3530-3540.
- BONHAM, M., BROWN, S., BOYD, A., BROWN, P., BRUCKENSTEIN, D., HANVEY, J., THOMSON, S., PIPE, A., HASSMAN, F., ISI, J., FROEHLER, B., MATTEUCCI, M., WAGNER, R., NOBLE, S., and BABISS, L. (1995). An assessment of the antisense properties of Rnase H-competent and steric-blocking oligomers. *Nucleic Acids Res.* 23, 1197-1203.
- COLIGE, A., SOKOLOV, B.P., NUGENT, P., BASERGA, R.,; PROCKOP, D.J. (1993). Use of an antisense oligonucleotide to inhibit expression of a mutated human procollagen gene (COL1A1) in transfected mouse 3T3 cells. *Biochemistry* 32, 7-11.
- FARRELL, C., BREADY, J., KAUFMAN, S., QUIAN, Y., and BURGESS, T. (1995). The uptake and distribution of phosphorothioate oligonucleotides into vascular smooth muscle cells in vitro and in rabbit arteries. *Antisense Res. Dev.* 5, 175-183.
- FECHHEIMER, M., BOYLAN, J., PARKER, S., SISKEN, J., PATEL, G., and ZIMMER, S. (1987). Transfection of mammalian cells with plasmid DNA by scrape loading and sonication loading. *Proc. Natl. Acad. Sci. USA* 84, 8463-8467.
- MCNEIL, P., MURPHY, R., LANNI, F., and TAYLOR, D. (1984). A method for incorporating macromolecules into adherent cells. *J. Cell Biol.* 98,1556-1564.
- MORISHITA, R., GIBBONS, G., KANEDA, Y., OGIHARA, T., and DZAU, V. (1994). Pharmacokinetics of antisense oligodeoxyribonucleotides (cyclin B, and CDC 2 kinase) in the vessel wall in vivo: Enhanced therapeutic utility for restenosis by HVJ-liposome delivery. *Gene* 149, 13-19.
- OBERHAUSER, B., and WAGNER, E. (1992). Effective incorporation of 2'-O-methyl-oligoribonucleotides into liposomes and enhanced cell association through modification with thiocholesterol. *Nucleic Acids Res.* 20,533-538.
- SHOJI, Y., AKHTAR, S., PERIASAMY, A., HERMAN, B., and JULIANO, R. (1991). Mechanism of cellular uptake of modified oligodeoxynucleotides containing methylphosphonate linkages. *Nucleic Acids Res.* 19, 5543-5550.
- STEIN, C., and CHENG, Y. (1993). Antisense oligonucleotides as therapeutic agents-Is the bullet really magical? *Science* 261, 1004-1012.
- SUMMERTON, J. (1992). Cost-effective antisense structures, In: *Biotechnology International* (Century Press, London), pp. 73-77.
- SUMMERTON, J., and WELLER, D. (1993). Uncharged morpholino-based polymers having phosphorus containing chiral intersubunit linkages. US Patent 5,185,444.
- TONKINSON, J., and STEIN, C. (1994). Patterns of intracellular compartmentalization, trafficking and acidification of 5'-fluorescein-labeled phosphodiester and phosphorothioate oligodeoxynucleotides in HL60 cells. *Nucleic Acid Res.* 22,4268-4275.
- VILLA, A., GUZMAN, L., POPTIC, E., LABHASETWAR, V., D'SOUZA, S., FARRELL, C., PLOW, E., LEVY, R., DICORLETO, P., and TOPOL, E. (1995). Effects of antisense c-myc oligonucleotides on vascular smooth muscle cell proliferation and response to vessel wall injury. *Circ. Res.* 76, 505-513.
- WAGNER, R., MATTEUCCI, M., LEWIS, J., GUTIERREZ, A., MOULDS, C., and FROEHLER, B. (1993). Antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines. *Science* 260, 1510-1513.

Address reprint requests to:

James Summerton, Ph.D.

ANTIVIRALS Inc.

4575 S.W. Research Way, Suite 200

Corvallis, OR 97333

Received March 4, 1996; accepted in revised form May 13,1996