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Morpholino and Phosphorothioate Antisense Oligomers Compared in Cell-Free and In-Cell Systems

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ABSTRACT

Morpholino and phosphorothioate (S-DNA) antisense oligos were compared in both cell-free and in-cell translation systems. In the most stringent test of specificity in the cell-free system, a globin-targeted S-DNA oligo was found to inhibit its target sequence at concentrations of 10 nM and above, but the sequence-specific component of this inhibition dropped below 50% at concentrations of 100 nM and above. A corresponding Morpholino oligo achieved even higher inhibition at 10 nM, but in contrast to the S-DNA, with the Morpholino, the sequence-specific component of this inhibition remained above 93% at a concentration of 3000 nM. In this same cell-free test system, several S-DNA oligos exhibited substantial undesired nonantisense effects at concentrations of 300 nM and above, whereas corresponding Morpholino oligos exhibited little or no nonantisense activity through a concentration of 3000 nM. In scrape-loaded HeLa cells, both globin-targeted and HBV-targeted S-DNAs (both antisense and control oligos) generally failed to achieve significant translational inhibition at extracellular concentrations up to 3000 nM. In contrast, the Morpholino oligos achieved effective and specific translational inhibition at extracellular concentrations ranging from 30 nM to 3000 nM.

INTRODUCTION

SEQUENCE-SPECFIC BINDING OLIGOMERS designed to inactivate selected messenger RNAs (the sense strands) are commonly called "antisense" oligos. Such antisense oligos hold promise as tools for studying the function of genes, as well as the prospect of safe and effective therapeutics for a wide variety of diseases. First-generation antisense oligos, comprising natural genetic material (Zamecnik and Stephenson, 1978), often with added crosslinking moieties for irreversibly binding their targeted genetic sequences (Belikova et al., 1967; Surnmerton and Bartlett, 1978a,b; Summerton, 1979), were found to be rapidly degraded in biologic systems. Methylphosphonate-linked DNA oligos (MP-DNAs) (Miller, 1989) were the first to provide resistance to enzymatic degradation but were found to afford limited efficacy and poor aqueous solubility. In an effort to overcome such limitations, a number of other structural types have been developed over the past decade (Crooke and Lebleu, 1993). Of these, phosphorothioate-linked DNA oligos (SDNAs, Fig. 1) have come to dominate the antisense field, being easily prepared and moderately resistant to enzymatic degradation and providing higher efficacies and much better aqueous solubility than MP-DNAs (Stein and Cohen, 1989). However, with their widespread use, it is becoming apparent that S-DNAs provide reasonable sequence specificity only within a narrow concentration range (Stein and Cheng, 1993), and they generate a plethora of nonantisense effects, due at least in part to interactions with extracellular and cellular proteins (Jansen et al., 1995; Krieg et al., 1995; Yaswen et al., 1993; Perez et al., 1994-1 Krieg and Stein, 1995; Stein, 1995). With the goal of overcoming these remaining limitations, we have developed Morpholino oligos (Fig. 1), a novel structural type that contains six-membered morpholine backbone moieties joined by non-ionic phosphorodiamidate intersubunit linkages (Summerton and Weller, 1993; Partridge et al., 1996; Hudziak et al., 1996).

MATERIALS AND METHODS

mRNAs, plasmids, and oligos

In these studies, two messenger RNAs (mRNAs) were used, one comprising the 5' leader sequence of rabbit α-globin mRNA joined to the amino acid-coding sequence of luciferase

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and the other comprising a highly conserved portion of the 5' leader sequence of the hepatitis B virus (HBV) 3.5 kb mRNA joined to the coding sequence of luciferase. For the cell-free studies, the globin and HBV mRNA constructs were transcribed from plasmids pAVI-l and pAVI-2, respectively, containing the T7 promoter. The plasmids were linearized with a restriction nuclease and transcribed with T7 polymerase following the protocol in Ambion mMessage rnMachine Instruction Manual (catalog 1344, Ambion Inc., Austin, TX). For in-cell studies, the globin and HBV-containing mRNA constructs were transcribed from plasmids pAVI-3 and pAVI-4, respectively, stably transfected into HeLa cells, with both plasmids containing the mouse mammary tumor virus promoter inducible by dexamethasone.

Figure 2 shows the 5' regions of these two mRNA constructs and corresponding antisense and control oligos used in these experiments.

S-DNA oligos having the structure shown in Figure I and the sequences shown in Figure 2 were purchased from Biosource International, Keystone Division (Camarillo, CA). Corresponding Morpholino oligos were synthesized at ANTIVIRALS Inc. by methods similar to those detailed elsewhere (Summerton and Weller, 1993). (Morpholino oligos and the plasmids used in these studies are commercially available from ANTIVIRALS Inc., 4575 S.W. Research Way, Corvallis, OR 97333.)

Cell-free assays

Cell-free translation reactions, run in triplicate, were modeled after those in Novagen protocol TB012. For each translation reaction, 2 µl of oligo in H_20 was added to 6 µl of mRNA solution containing 3 µl rabbit reticulocyte lysate without methionine (Novagen catalog 69360), 0.25 µl RNasin (Promega catalog N2512), 0.5 µl translation mix without methionine (Novagen catalog 69360), 0.275 µl 2.5 M KCl, 0.125 µl 25 mM magnesium acetate, 0.85 µl of H20 (for reactions without RNase H) or RNase H solution (0.235 *U/*µl from US Biochemical (catalog 70054), and 1.0 µl mRNA construct in H_20. This mixture was incubated for 60 minutes in a 37°C air incubator (to preclude evaporation and recondensation on the walls of the tube). To start the translation process, 16 µl of lysate solution was added, comprising 11.25 µl Promega nuclease-treated reticulocyte lysate (catalog L4970), 3.75 µl H_20, and 1.0 µl 1 mM amino acid mix (Promega catalog L4461). Oligo concentrations indicated in Figures 3, 4, 5, and 6a refer to concentrations in these final translation reaction mixes. Each translation reaction was incubated for 90 minutes at 37°C and then chilled on ice. Quantitation of the luciferase activity generated in the translation reaction entailed adding 10 µl of the chilled preparation to 50 µl of ambient-temperature Promega luciferase assay reagent (catalog 1483), mixing 30 seconds, and measuring the light emission for 15 seconds in a Turner Model TD-20e luminometer (Turner Designs, Inc., Mountain View, CA). Separate experiments showed that in this translation mixture and in the luciferase assay solution, neither S-DNA nor Morpholino oligos at concentrations ranging from 10 nM to 10,000 nM have a significant effect on the activity of preformed luciferase.

Percent inhibition of luciferase activity values was calculated as

% Inhibition = 100(l - (+oligo/-oligo))

where +oligo is the light emitted from a preparation treated with oligo and -oligo is the average of the light emitted from three control preparations not treated with oligo.

In-cell assays

In-cell inhibition assays in cells scraped in the presence of Morpholino oligos were carried out in triplicate as described in Partridge et al. (1996). Corresponding assays with S-DNAs were modified such that at the end of the posttreatment 16-hour incubation period, both free-floating and readhered cells were collected, washed three times by gentle centrifugation, and assessed for luciferase and total protein, as described in Partridge et al. (1996). The modified assay for cells scrape-loaded with SDNA oligos was necessary because a substantial fraction of cells treated with 300 nM or greater concentration of S-DNA oligo (either antisense or control) during or following scraping fails to readhere to the culture plate in the subsequent 16-hour posttreatment incubation. In this regard, we have found that when the S-DNA oligo is added a few minutes after scraping [so as to preclude oligo entry into the cytosol/nuclear compartment, which occurs predominantly within the first minute after scraping (Partridge et al., 1996)], the nonadherent cells undergo normal levels of induction, transcription, and translation of the plasmid-coded mRNAs. Further, if the S-DNA oligo is removed from the medium after the scrape-load procedure, the SDNA-treated cells readhere normally. Based on these findings, we postulate that lack of readherence in the presence of S-DNA may simply be because of the S-DNA blocking sites on the cell surface or culture plate or both required for cell readherence.

In-cell inhibition assays using S-DNA complexed with LIPOFECTIN™ were modeled after procedures provided by Life Technologies Inc., from which the LIPOFECTIN™ was purchased. This entailed seeding 2 X 10^5 cells per 60-mm well and incubating in growth medium (containing 10% serum and antibiotics) for 24 hours. LEPOFECTIN™ reagent (20 µl*)* (1 mg/ml) (Life Technologies, Inc., Catalog 18292-011) was diluted into 80 µl serum-free medium (without antibiotics) and allowed to stand 40 minutes at ambient temperature, after which it was mixed with 100 µl serum-free medium (without antibiotics) containing the S-DNA antisense oligo. After 15 minutes at ambient temperature, another 1.8 ml of serum-free medium was added to the S-DNA/LIPOFECTIN™ solution. The growth medium was removed from the cells, and the cells were washed with serum-free medium (without antibiotics). Then the S-DNA/LIPOFECTIN™ solution was added, and the cells were incubated for 6 hours at 37°C. The S-DNA/LIPOFECTIN™ solution was removed and replaced with growth medium containing 10% serum, antibiotics, and dexamethasone (1 µl*),* and incubation continued for 16 hours. Cells were then assessed for luciferase activity.

As described in Partridge et al. (1996), quantitation of Inciferase activity in samples from the in-cell experiments entailed both measurement of light emission from a sample and measurement of total protein in that sample. The light emission value was then divided by the total protein value to give a normalized light emission value. These normalized light emission values correct for sample to sample variations in the cell scrap-

TABLE 1. THERMAL MELT VALUES FOR OLIGO/RNA DUPLEXES

	S-DNA/RNA	*Morpholino/RNA*
0-mispair oligo	70°C	86°C
4-mismatch oligo	46°C	59°C

ing and transfer steps. Percent inhibition of luciferase activity was calculated as in the cell-free experiments using these normalized light emission values.

RESULTS

Cell-free studies

To illustrate the relative RNA binding affinities of these two structural types, Table 1 gives the thermal melt (T_m) values for the α-globin S-DNA and Morpholino antisense oligos of Figure 2 paired to complementary target RNA, as well as the corresponding four-mismatched control oligos paired with the same target RNA.. These T_m values were obtained with oligo and target RNA each at 5 µM in salinelphosphate buffer (0.15 M NaCl, 0.05 M NaH^2PO_4 NaOH to pH 7.2).

Initial studies were carried out in a simple reticulocyte lysate translation system to provide an assessment of translational inhibition independent of the complexities associated with cell permeability barriers and subcellular compartmentation. Relative efficacies of these two structural types were assessed in both the absence and presence of added RNase H using perfectly paired antisense oligos at concentrations ranging from 10 nM to 3000 nM, with their respective target mRNAs at a concentration of 1 nM. Figure 3a shows efficacies against a globin target in the absence of added RNase H, Figure 3b shows efficacies against this same globin target in the presence of added RNase H, and Figure 3c shows efficacies against an HBV target in the presence of added RNase H.

Antisense therapeutics that lack adequate sequence specificity are expected to inhibit translation of inherent cellular mRNAs, which could be manifested as toxicity for patients. Based on an estimated cellular RNA pool complexity of approximately 200 million bases of unique-sequence RNA, we estimate that for a typical 25-base viral genetic sequence (not recently plagiarized from the host) one expects to find at least four mismatches relative to any given sequence in the inherent pool of cellular RNAs in humans. Thus, the globin four-mismatched control oligo of Figure 2 is expected to provide a reasonable emulation of the closest match a viral-targeted oligo is likely to have with any cellular RNAs of a patient. To provide a stringent assessment of relative sequence specificities of the two structural types, we used the globin zero-mismatch oligo to provide a measure of the total inhibition afforded by that oligo type and the corresponding globin four-mismatch oligo (containing a 10-contiguous-base match to the target sequence) to provide an estimate of the low-specificity component of the inhibition that could cause toxicity in patients. The difference between these two values provides a measure of the high-specificity component, which we denote as "sequence-specific inhibition" (Fig. 4a). Results from a similar but somewhat less stringent specificity study using the HBV four-mismatch oligo (containing a 7-contiguous-base match to the target sequence) are shown in Figure 4b.

Because S-DNAs have been reported to bind a variety of proteins (Krieg and Stein, 1995) and may affect translation via such binding, it is also desirable to estimate the fraction of translational inhibition that is largely unrelated to pairing between the oligo and its target RNA. To this end, oligos having negligible complementarity to the target mRNAs were assessed for their inhibitory activity. This inhibition, denoted as "nonantisense inhibition," is shown in Figure 5.

In-cell studies

A growing body of evidence, including our fluorescence microscopy studies, suggests that in the absence of special delivery reagents, most or all antisense oligos enter unperturbed cultured animal cells predominantly or solely via endocytosis and are subsequently exocytosed or sequestered (or both) in lysosomes, with little or no intact oligo entering the cytosol/nuclear compartment (Wagner et al., 1993). However, our fluorescence microscopy studies with fluorescein-tagged oligos also indicate that both S-DNA and Morpholino oligos can be delivered rapidly and efficiently into the cytosol of adherent cells in culture by a simple scrape-load procedure (Partridge et al., 1996; unpublished observations). These studies further show that in a typical scrape-load procedure, both S-DNA and Morpholino oligos appear to enter the cytosol/nuclear compartment to about the same extent, with oligos entering about 85% of the scraped cells. In this context, it is noteworthy that Farrell et al. have reported that normal smooth muscle cells of artery walls do not take up significant S-DNA, but when those cells are perturbed in the course of balloon angioplasty, a substantial amount of S-DNA is seen to enter the cytosol/nuclear compartment of the perturbed cells (Farrell et al., 1995).

In agreement with our fluorescence microscopy observations, results from functional assays (Fig. 6) suggest that both S-DNA and Morpholino oligos fail to enter the cytosol of unperturbed HeLa cells to a significant extent during a 16-hour incubation but can readily enter the cytosol via scrape-loading. Specifically, Figure 6a shows that both S-DNA and Morpholino oligos at low nanomolar concentrations are quite effective against their mRNA target sequence in a cell-free translation system. Figure 6b shows that these same oligos at a

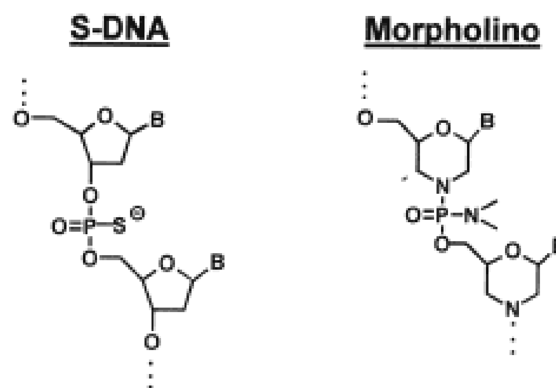

FIG. 1. Oligo structural types.

Translational start site

| ←------ α-globin leader ------→ ⇓ ←--- luciferase --→

5'-GGGUCGACACUUCUGGUCCAGUCCGACUGAGAAGG A ACCACC **AUG**GAAGACGC.....

Globin antisense oligo GGUCAGGCUGACUCUUCCUUGGUGG-5' 0-Mismatch

Globin control oligo GGACAGCCUGACUCUUCGUUGCUGG-5' 4-Mismatch

Translational start site

| ←------ HBV leader ------→ ⇓ ←--- luciferase --→

5'-GCA ACUUUUUCACCUCUGCCUAAUCAUCUCUUGUACAUGUC...........GGC**AUG**GACCUCGAGGAC.........

CGUUGAAAAAGUGGAGACGGAUUAG-5' 0-Mismatch HBV antisense oligo

CGUUCAAAAUGUGGAGAGGGAAUAG-5' 4-Mismatch HBV control oligo

FIG. 2. Messenger RNA constructs and oligos.

FIG. 3. Efficacies in cell-free system. (a) Translation reactions, without added RNase H, contained globin antisense oligo and globin mRNA. (b) Translation reactions as in (a) but with added RNase H (25 U/ml). (c) Translation reactions as in (b) contained HBV antisense oligos and HBV mRNA. Open circles, S-DNA; closed circles, Morpholino.

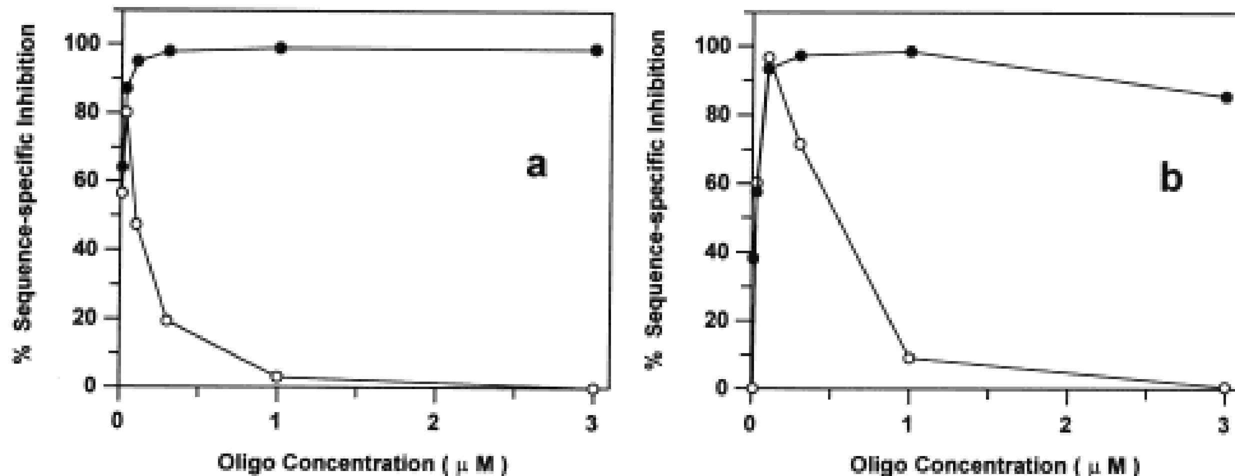

FIG. 4. Sequence-specific inhibition in cell-free system. Translation reactions, with added RNase H, were carried out with the four-mismatch control oligos, and the resulting inhibition values at each concentration were subtracted from the corresponding inhibition values for the 0-mismatch oligos to give % sequence-specific inhibition values. Any negative inhibition values (presumably due to stimulation of luciferase synthesis) were set to zero in calculating these % sequence-specific inhibition values. (a) Globin-targeted oligos/globin mRNA. (b) HBV-targeted oligos/HBV mRNA. Open circles, S-DNA; closed circles, Morpholino.

thousandfold higher concentration in the extracellular medium fail to inhibit the same mRNA target within unperturbed HeLa cells. Finally, Figure 6c demonstrates that when the Morpholino oligo is introduced into cells via scrape-loading, it is quite effective against its mRNA target therein. We used this scrape-load oligo delivery procedure to assess in HeLa cells the activities of S-DNA and Morpholino antisense oligos, with oligo concentrations ranging from 30 nM to 3000 nM in the extracellular medium.

LIPOFECTIN™ has been used to deliver S-DNAs into cultured cells (Wagner et al., 1993; Dean et al., 1994). Therefore, to provide a comparison with delivery via scrape-loading, we also -treated unperturbed HeLa cells with varying concentrations of S-DNA antisense oligo complexed with LIPO-

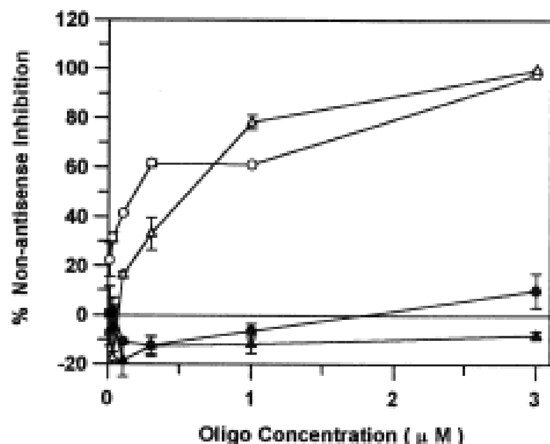

FIG. 5. Nonantisense activities in cell-free system. Translation reactions as per Figure 3, with added RNase H. Open circles, S-DNA, and closed circles, Morpholino IIBV antisense oligo and globin mRNA; open triangles, S-DNA, and closed triangles, Morpholino globin antisense oligo and HBV mRNA.

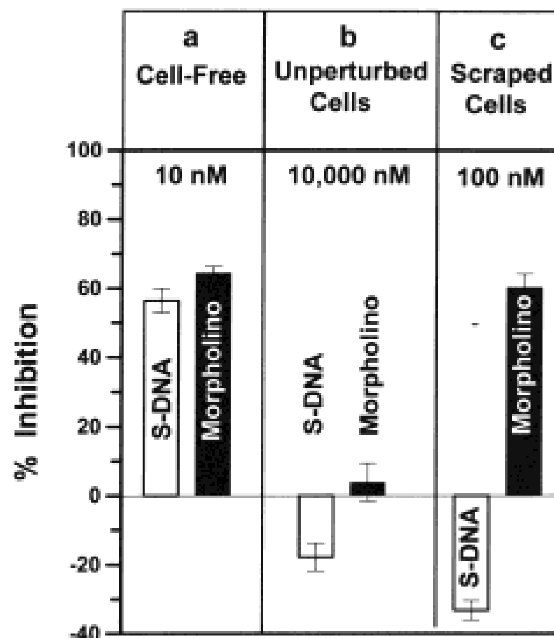

FIG. 6. Cell-free and in-cell activities. (a) Cell-free translations as per Figure 3b with 10 nM oligos. (b) Inhibition of in-cell expression of globin/luciferase mRNA in unperturbed HeLa cells treated for 16 hours with oligos at a concentration of 10,000 nM in the extracellular medium. (c) Inhibition of in-cell expression of globin/luciferase mRNA in HeLa cells scrape-loaded with oligos at a concentration of 100 nM in the extracellular medium

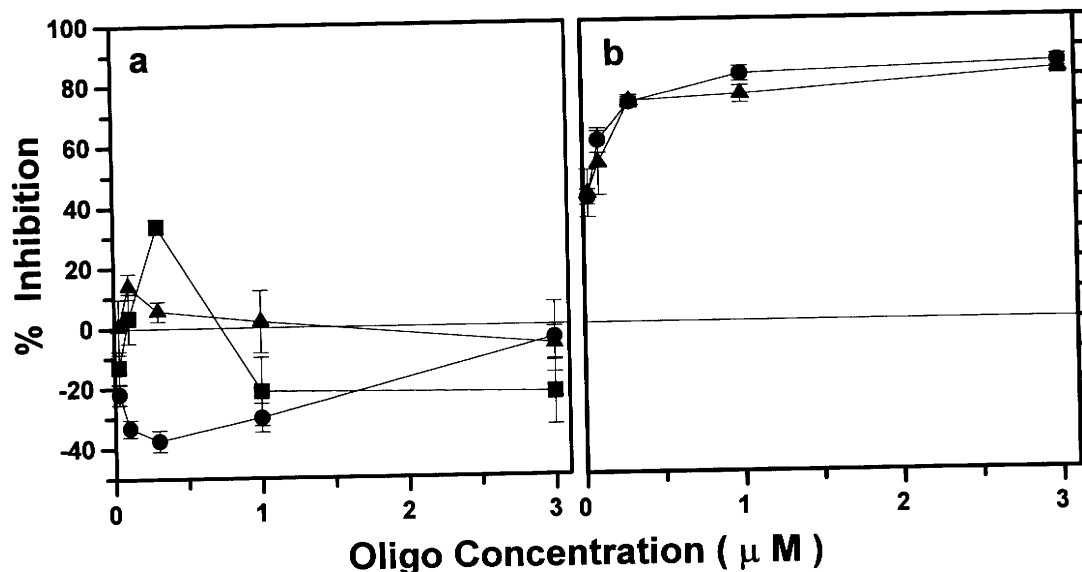

FIG. 7. Efficacies in cells. (a) S-DNA oligos. (b) Morpholino oligos. Closed circles, globin antisense oligo with globin mRNA in scraped cells; closed triangles, HBV antisense oligo with HBV mRNA in scraped cells; closed squares, globin antisense oligo-LIPOFF)CTE'Tm complex with globin mRNA in unperturbed cells.

FECTIN™ Figure 7a shows the activities of S-DNA antisense oligos in scraped cells containing the target sequence for the respective oligos. It aso shows the activity of an S-DNA antisense oligo complexed with LIPOFECTIN™ in unperturbed cells containing the target sequence for that oligo. Figure 7b shows the activities of corresponding Morpholino antisense oligos in scraped cells.

We also used this scrape-load oligo delivery procedure to test for nonantisense activities of S-DNA and Morpholino oligos in cells. To this end, oligos were delivered into cells containing a luciferase-coding plasmid that lacked the target sequence for said oligos. Specifically, globin-targeted antisense oligos were delivered into cells containing the HBV plasmid, and HBV-targeted antisense oligos were delivered into cells containing the globin plasmid. Figure 8a shows the non-antisense activities of S-DNA oligos in scraped cells, and Figure 8b shows the nonantisense activities of corresponding Morpholino oligos.

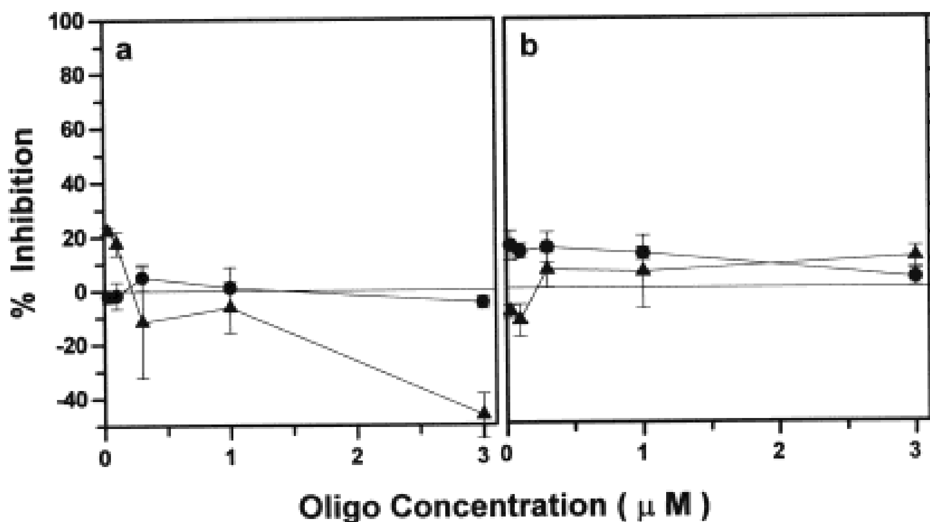

FIG. 8. Non-antisense activities in cells. (a) S-DNA oligos. (b) Morpholino oligos. Closed circles, HBV antisense oligo with globin mRNA in scraped cells; closed triangles, globin antisense oligo with HBV mRNA in scraped cells.

DISCUSSION

Cell-free results

In the cell-free studies in the presence of RNase H, both SDNA and Morpholino antisense oligos achieved reasonable efficacies at low oligo concentrations, averaging about 25% for S-DNAs and 50% for Morpholinos at just 10 nM, with both structural types achieving nearly quantitative inhibition of their targeted mRNAs at 100 nM.

With regard to specificity of S-DNAs, in the most stringent test of specificity (Fig. 4a), the sequence-specific component of the inhibition by the globin-targeted S-DNA dropped below 50% at an oligo concentration of 100 nM and approached 0% at an oligo concentration of 1000 nM. Further, the S-DNAs exhibited significant nonantisense effects at an oligo concentration of 100 nM, and these effects dominated at oligo concentrations above about 500 nM. It is noteworthy that Cazenave et al. (1989) have also reported poor sequence specificity by S-DNAs in a cell-free translation system.

In comparison with the S-DNAs, the Morpholino oligos afforded greater sequence specificity over a far broader concentration range, evidenced by the sequence-specific component of the inhibition by the globin-targeted Morpholino oligos averaging about 90% at an oligo concentration of 3000 nM. Further, the Morpholino oligos exhibited little nonantisense activity over the full 10 nM-3000 nM concentration range.

In-cell results

Although the S-DNA antisense oligos at a concentration of 100 nM afforded near quantitative inhibition of their targets in the cell-free system, in studies with scrape-loaded cells containing the same targets, these S-DNA oligos appear not to inhibit translation of their targeted mRNA at S-DNA concentrations up to 3000 nM in the extracellular medium. At the higher concentration of 10,000 nM (data not shown), however, the globin-targeted S-DNA antisense oligo did afford a modest 34% inhibition. In the presence of LIPOFECTINTM, the S-DNA antisense oligo was moderately inhibitory (33%) at 300 nM but appeared inactive at lower and higher concentrations.

In contrast to the S-DNAs, the Morpholino antisense oligos gave results similar to what they showed in the cell-free system, affording significant inhibition of their targets at all concentrations tested, ranging from 41% at 30 nM to 85% at 3000 nM. It should be noted that in this scrape-load procedure, our fluorescence microscopy observations indicate that oligos enter only about 85% of the cells (Partridge et al., 1996). Thus, at extracellular concentrations of 300 nM and above, target inhibition appears to be nearly complete in those cells that received oligo.

In the specificity study wherein oligos were delivered into cells lacking their targeted sequences, the Morpholino oligos also behaved as one would predict from the cell-free results, exhibiting little inhibition of nontargeted mRNAs over the full 30 nM-3000 nM concentration range. In regard to specificity of Morpholinos in cells, we have reported previously that the globin-targeted four-mismatch control Morpholino oligo (Fig. 2), at concentrations ranging from 30 nM to 3,000 nM in the extracellular medium, does not inhibit its target in scrape-loaded cells (see Figure 7 in Partridge et al., 1996).

It is puzzling that in the cell-free system, both Morpholino and S-DNA antisense oligos achieved near quantitative target inhibition at oligo concentrations of 100 nM, but in the in-cell system, only the Morpholino oligos achieved high efficacy (near quantitative inhibition at 300 nM), with the S-DNAs affording only very low efficacy (less than 50% inhibition at 10,000 nM). Our first guess as to basis for this discrepancy was that our scrape-load procedure might be effective for delivering non-ionic Morpholinos into the cytosol/nuclear compartment of cells but much less effective for delivering polyaniortic SDNAs. However, subsequent fluorescence microscopy studies with fluorescein-tagged Morpholino and S-DNA oligos indicated that in our scrape-load procedure, the S-DNAs entered the cytosollnuclear compartment even better than did the Morpholinos.

Another possible explanation for the great discrepancy between in-cell efficacies of these two antisense structural types comes from recent evidence reported by scientists at Gilead (Moulds et al., 1995) that suggests the presence of a strand-separating activity in mammalian cells. Specifically, they have shown that when unmodified S-DNAs are preannealed with their target mRNAs and then microinjected into either the nucleus or the cytoplasm of cultured cells, the blocked mRNAs are effectively reactivated, evidenced by translation of their protein products, In this context, although the duplex of the polyanionic S-DNA and its target RNA appears to be readily recognized and acted on by this strand-separating activity, we postulate that the corresponding duplex of the Morpholino oligo and its target RNA may be immune to this strand-separating activity because the structure of the non-ionic Morpholino component of the duplex differs so radically from that of natural nucleic: acids.

Other properties of Morpholino oligos

In addition to their cell-free and in-cell efficacy and specificity properties detailed here, it is noteworthy that these nonionic Morpholino oligos are surprisingly water soluble (1 g of representative 22-mer dissolves in 4 ml water), are immune to a wide variety of degradative enzymes and serum (Hudziak et al., 1997), and in large-scale production are expected to be substantially less expensive to produce than S-DNA and other DNAlike antisense structural types (Summerton, 1989, 1992). Because of this composite of desirable properties, Morpholino oligos may be particularly suited for antisense research and therapeutic applications.

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