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Transformed and Immortalized Cellular Uptake of Oligodeoxynucleoside Phosphorothioates, 3′-Alkylamino Oligodeoxynucleotides, 2′-O-Methyl Oligoribonucleotides, Oligodeoxynucleoside Methylphosphonates, and Peptide Nucleic Acids

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ABSTRACT. Direct quantitative comparisons of cellular uptake across a wide variety of analogs and cell types are necessary for the design of oligonucleotide diagnostic and therapeutic applications. This work reports quantitative cellular uptake and nuclear localization of [^{14}C]oligodeoxynucleoside phosphorothioates (PS), 3′-alkylamino oligodeoxynucleoside phosphodiesters (PO-NH_2), 2′-O-methyl oligoribonucleoside phosphodiesters (2OM), peptide nucleic acids (PNA), and oligodeoxynucleoside methylphosphonates (MP) in several transformed or immortalized cell lines. All analogs demonstrated active cellular uptake in that intracellular concentrations greatly exceeded the extracellular 1 μM concentration within 1–3 hr. However, by 9–24 hr, cellular accumulations of PS exceeded those of PO-NH_2 and 2OM by 3- to 5-fold, PNA by 6- to 7-fold, and MP by 8- to 10-fold. Similar results were observed in two transformed cell lines, HL-60 leukocytes and H-*ras* transformed fibroblasts, using three different heterogeneous sequences. H-*ras* and IGF-1R transformed fibroblasts had a 2- to 5-fold higher uptake of all analogs than non-transformed immortalized fibroblasts. Nuclear levels of the PO-NH_2, PS, and MP analogs were approximately 25% of total cellular uptake, while nuclear percentages of 2OM and PNA were less than 20%, suggesting some differences in nuclear localization among the analogs. These observations provide a direct quantitative comparison of cellular uptake as a function of oligonucleotide modification, and imply that transformation enhances cellular uptake. From the perspective of therapy and diagnosis, clear trade-offs were apparent between efficiency of uptake on the one hand, and nuclease resistance and hybridization strength on the other. BIOCHEM PHARMACOL 53;10:1465–1476, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. antisense; oligonucleotides; cell uptake; phosphorothioates; methylphosphonates; PNA, 2′-O-methyl oligoribonucleotides

Nuclease-resistant oligonucleotide analogs designed to hybridize to specific RNA or DNA sequences are being considered for diagnostic and therapeutic applications [1–3]. Quantitative comparisons of a few oligonucleotide analogs have been reported for several biological properties such as nuclease susceptibility, hybridization, and RNase H activation [4–9]. However, few direct quantitative comparisons of cellular uptake among a variety of available analogs and cell types exist, although there are a number of studies examining cellular uptake of specific analogs by individual cell lines [1, 8, 10].

The simplest oligodeoxynucleotide modification involves addition of a blocking group on the 3′ terminus, an addition that serves to impede the activity of 3′ exonucleases, the predominant extracellular degradative mechanism for oligodeoxynucleotides [6, 11, 12]. Numerous modifications focus on the internucleoside linkage, altering the PO‡ linkages to PS, MP, or other less commonly studied analogs [1, 4, 8, 13, 14]. Modifications of the ribose component include addition of 2′-O-alkyl groups [5, 15, 16]. The most radical modifications are found in PNA where both the phosphodiester linkages and sugars are replaced with a peptide-like backbone of (*N*-2-aminoethyl) glycine units,

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‡ *Abbreviations:* PO, oligodeoxynucleoside phosphodiesters; PS, oligodeoxynucleoside phosphorothioates; MP, oligodeoxynucleoside methylphosphonates; PNA, peptide nucleic acids; IGF-1R, insulin-like growth factor 1 receptor; DMEM, Dulbecco's modified Eagle's medium; PO-NH_2, 3′-alkylamino oligodeoxynucleoside phosphodiesters; 2OM, 2′-O-methyl oligoribonucleoside phosphodiesters; PS-Fl, 3′-fluoresceinyl oligodeoxynucleoside phosphorothioates; and MP-Fl, 3′-fluoresceinyl oligodeoxynucleoside methylphosphonates.

with the bases directly attached by methylene-carbonyl linkers [17, 18]. Each of these modifications alters the structural characteristics of oligonucleotides. Thus, for example, the PNA and MP analogs are uncharged molecules, while the PS and MP analogs contain chiral phosphates, with Rp or Sp diastereomers at each linkage. These structural changes may affect not only nuclease susceptibility but other biological properties of oligonucleotides critical for the efficacy of hybridization activity such as nuclease susceptibility, cellular trafficking, and RNase H activation [1, 8].

Two different mechanisms have been described for internalization of oligonucleotides, and these mechanisms are clearly affected by structural characteristics of the analogs [1, 8, 10]. Negatively charged oligonucleotides (PS, PO) are internalized both by receptor-mediated endocytosis and fluid-phase endocytosis [10, 19–21], whereas uncharged MP are internalized only by the latter mechanism [22]. Even among negatively charged oligonucleotides, differences in uptake can be substantial. When the amounts of cellular uptake of three negatively charged (PO, PS, and mixed PO-MP), fluorescently labeled analogs were compared directly [23], PS oligonucleotides evidenced the highest uptake in cultured spleen cells, exceeding PO by a factor of almost 10, while MP-PO had the lowest uptake. However, the comparison did not include uncharged oligonucleotides or oligoribonucleotide analogs.

The present study was designed to compare cellular uptake for a range of different analogs in the absence of any specific vector system, and to examine the effect of several variables such as cell type, cellular transformation, and sequence on uptake of the analogs. The five analogs selected are relatively stable biologically and represent several different structural classes. Subcellular fractionation was chosen over microscopy as a means to isolate and analyze compartmental distribution due to advantages in quantitation. Quantitative, side-by-side comparisons were obtained of cellular uptake by several oligonucleotide analogs in both transformed and immortalized cells, revealing contrasts between efficiency of cellular uptake and nuclear localization on the one hand, and nuclease resistance and hybridization strength on the other.

MATERIALS AND METHODS

Cells

Six cell lines were used for oligonucleotide uptake experiments. The 504 and T24 cells are transformed NIH-3T3 mouse fibroblasts containing multiple copies of an activated Val12 H-*ras* oncogene derived from a human bladder carcinoma [24, 25]. The p6 cells are transformed Balb/c-3T3 mouse fibroblasts containing multiple copies of the human gene for IGF-1R under the control of an SV40 promoter [26]. These transformed cell lines and their non-transformed but immortalized parental cell lines were maintained at 37° under 5% CO_2 DMEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and 2 mM glutamine. HL-60 cells are human promyelocytic leukemia cells containing multiple copies of the c-*myc* gene [27, 28]. They were maintained at 37° under 5% CO_2 in RPMI-1640 medium supplemented as described above.

Oligonucleotides

Three sequences were employed in the study: (a) H-*ras*15 (15-mer): 5′-dCAC ACC GAC GGC GCC; (b) K-*ras*15 (15-mer): 5′-dTAC GCC AAC AGC TCC or rUAC GCC AAC AGC UCC in the case of 2OM; and (c) PN14 (14-mer): 5′-dACC CTC CAC CCA TC. PO-NH_2 oligonucleotides were synthesized by standard automated β-cyanoethyl phosphoramidite chemistry [29] using 3′-C7 amino CPG columns from Glen Research (Sterling, VA). 2OM oligonucleotides were similarly prepared using 2′-O-methyl phosphoramidites and 2′-O-methyl nucleoside CPG columns (Glen Research). PS oligonucleotides were synthesized using standard β-cyanoethyl phosphoramidite chemistry but with stepwise sulfurization of the phosphite linkages using sulfurizing reagent (No. 40-4036, Glen Research). MP oligonucleotides were prepared by a modified phosphoramidite procedure [30], using methylphosphonamidites from Prime Synthesis (Acton, PA). PS and MP oligonucleotides containing a 3′ fluorescein group (PS-Fl, MP-Fl) were also synthesized as described above except for the use of fluorescein CPG solid support (Glen Research). PNA were synthesized by HBTU coupling of Boc-PNA monomers [31]. They contained an N-acetylated glutamic acid on the N-terminus (comparable to the 5′ end of an oligonucleotide), with the C terminus converted to an amide. The 2OM, MP, and PNA were purified (> 90%) by reverse-phase HPLC [30–32], and MP-Fl was purified (> 90%) by TLC on silica gel plates (No. JT7002-5, J. T. Baker, Phillipsburg, NJ) developed with isopropanol: NH_4OH:H_2O (70:10:20). PO-NH_2 and PS were purified by repeated butanol precipitations [33], with purity (> 90%) confirmed by denaturing gel electrophoresis of 1-nmol aliquots on 20% polyacrylamide gels visualized with Stains-all (No. 19171, Bio-Rad, Richmond, CA). Purified oligonucleotides were sterilized by filtration through sterile 0.22 μm pore Durapore filters (No. SLGVL040S, Millipore, Bedford, MA).

Radiolabeling of Oligonucleotides

Oligonucleotides were radioactively labeled using [^{14}C]formaldehyde in a reductive formylation reaction to methylate specifically the exocyclic amino groups of nucleotide bases [34, 35], a procedure appropriate for all five oligonucleotide analogs. PO-NH_2, PS, 2OM, and PNA were dissolved in 0.2 M sodium phosphate buffer (pH 8.0) to a concentration of 1 mM, while MP was dissolved in the same buffer containing 20% ethanol to a concentration of 0.5 mM. The dissolved oligonucleotides (50 nmol) were mixed with 200 nmol [^{14}C]formaldehyde (No. NEC-039H,

40–60 Ci/mol, Dupont-NEN, Boston, MA) and incubated for 1 hr at room temperature with periodic mixing. Sodium cyanoborohydride (7 μL of a fresh 100 mM solution) was added to the mixture, and the incubation was continued for an additional 4 hr with periodic mixing. The PO-NH_2, PS, 2OM, and MP oligonucleotides were purified by *n*-butanol precipitation followed by solid phase extraction with C_{18} Sep-Pak cartridges (No. 51910, Waters, Milford, MA). PNA was purified by gel filtration on NAP10 columns (No. 17-0854-01, Pharmacia, Milwaukee, WI).

Purity of the labeled oligonucleotides was evaluated by analyzing a small aliquot using reverse-phase HPLC for PO-NH_2, PS, 2OM, and MP [30, 36] or TLC on cellulose plates (No. 1366061, Eastman Kodak, Rochester, NY) developed with *n*-butanol:glacial acetic acid:H_2O (4:1:5) for PNA. Specific activities of the labeled oligonucleotides were estimated by measuring concentrations from A_{260} in HPLC elution peaks, and UV-absorbing TLC bands extracted with water, and ^{14}C radioactivity using liquid scintillation counting at 75% counting efficiency. For PO-NH_2, the specific activities of several preparations ranged from 27 to 49 Ci/mol; for PS, the range was 13–45 Ci/mol; for 2OM, the activity was 45 Ci/mol; for MP, the range was 31–51 Ci/mol; and for PNA, the range was 5.3–11 Ci/mol.

The activity range indicated a methylation ratio of 0.12 to 0.79 mol methylated sites/mol oligonucleotide. Specific activities of the purified labeled oligonucleotides did not differ by more than 10% from those of the crude labeled preparations before purification, implying lack of degradation during labeling and purification.

Stability of the Radiolabeled Oligonucleotides

The biological stabilities of [^{14}C]oligonucleotides in the absence of cells were compared following incubation for 8 hr in cell-free DMEM containing 10% heat-inactivated fetal bovine serum at 37°. The incubation concentration was 25 μM, a value necessary to obtain an accurate evaluation of oligonucleotide degradation by the analytical methods described above. The specific activities of the [^{14}C]oligonucleotides were too low to permit measurements of labeled oligonucleotide stabilities in cells. Following incubation, oligonucleotides were extracted from medium using an equal volume of acetonitrile for MP and PNA, or phenol:chloroform:isoamyl alcohol (25:24:1) in the case of PO-NH_2, PS, and 2OM. The samples were then dried and stored at −20° for subsequent analysis of degradation and specific activity by HPLC (PO-NH_2, PS, 2OM, MP) or TLC (PNA) as described above.

Cellular Uptake of Fluoresceinyl Phosphorothioates

The 504 cells were plated in 12.5 cm^2 flasks and grown for 2 days to 50–80% confluence. The medium was then removed and replaced with fresh medium containing 1 μM K-*ras*15 PS-Fl or MP-Fl, pre-warmed to 37°; control cells received medium with no oligonucleotide. After incubation at 37° for various lengths of time, the cells were rinsed with PBS, incubated with trypsin–EDTA (0.25% trypsin, 1 mM EDTA) for 10 min, transferred to a microcentrifuge tube, and washed twice with room temperature PBS. After the last wash, the cells were fixed with 0.5% paraformaldehyde in PBS and stored in the dark at 5°. Flow cytometry was performed using a Coulter Profile II fluorescence activated cell sorter.

HL-60 cells were plated in 6-well microwell plates (10^6 cells/well) in medium containing 1 μM K-*ras*15 PS-Fl or MP-Fl; control cells received no oligonucleotide. The cells were transferred to a microcentrifuge tube after various lengths of time and washed at room temperature four times: once with fresh medium, once with PBS, once with a high salt/low pH buffer (1 M NaCl, 0.4 M acetate, pH 2.3), and once again with PBS. The cells were then fixed and stored for flow cytometry as described above.

Cellular Uptake of Radiolabeled Oligonucleotides

HL-60 cells were plated in 6-well microwell plates (10^6 cells/well) and incubated with a 1 μM concentration of the various [^{14}C]oligonucleotide analogs and washed as described above; control cells received no [^{14}C]oligonucleotide. After the final wash, the cells were lysed in 1 mL of 1% SDS in H_2O. Fifty microliters of the lysate was allocated for total protein measurement, and ^{14}C activity in the remaining lysate was measured by liquid scintillation counting, from which background counts were subtracted. Background samples from control lysates not treated with [^{14}C]oligonucleotides typically yielded 12–15 cpm, corresponding to less than 1 pmol even at 10 Ci/mol, or 0.2 pmol at a typical specific activity of 40 Ci/mol. Thus, an experimental sample with twice the background cpm would contain 0.2 to 1 pmol, depending on the specific activity, which may be considered the limit of detection. Cell counts, on the order of 10^6 per sample, were performed on untreated control samples using a hemocytometer just prior to lysis, and these values were combined with total protein measurements for these samples to generate a standard curve relating cell number and total protein. The curve was used to estimate cell counts from the total protein measurements of treated cells, and radioactivity counts were used to calculate picomoles of oligonucleotide using the specific activity of each labeled oligonucleotide. Values for cell number and picomoles of cell-associated oligonucleotide were therefore obtained for each treated sample. An estimate of cell volume was obtained for HL-60 and 504 cells in suspension by microscopic evaluation of cell diameters using a micrometer and subsequent calculation of an average cell volume, as described [37].

The 504, T24, NIH-3T3, p6, and Balb/c-3T3 cells were plated in 12.5 cm^2 flasks and grown for 2 days to 50–80% confluence, on the order of 10^6 cells/flask. The medium was then removed and replaced with fresh medium containing a 1 μM concentration of the various [^{14}C]oligonucleotides,

pre-warmed to 37°; control cells received medium with no [^{14}C]oligonucleotide. After incubation for various times, washing was performed in one of two ways. For one experiment, the cells were washed in serum-free medium, trypsinized, and washed twice more in PBS before lysis with 1% SDS. For the remaining experiments, attached cells were washed directly in the flasks using four washes: once with fresh medium, once with PBS, once with high salt/low pH buffer, and once with PBS. This procedure had the advantage of removing both non-internalized oligonucleotide and any dead cells, which can accumulate large amounts of oligonucleotide and thereby skew any uptake measurements [23]. Following the last wash, the cells were lysed, and the samples were processed for total protein measurement and liquid scintillation counting as described above. Control samples of varying confluence were trypsinized following the last wash, resuspended in PBS for cell counting, and then lysed to obtain total protein values. This allowed the generation of a standard curve corresponding to total protein versus cell number.

Distribution of Radiolabeled Oligonucleotides in Nuclear and Cytoplasmic Cellular Compartments

The 504 cells were incubated as above with [^{14}C]oligonucleotides at 1 μM for 8 hr. The cells were washed with serum-free medium, trypsinized, and washed twice with PBS. The cells were then resuspended in 500 μL of 0.5% NP-40/PBS and passed ten times through a 25-g needle or a 200-μL pipet tip during a 10-min incubation period. A 5-μL aliquot was mixed with an equal volume of 0.1% Trypan Blue/PBS and examined under a microscope (1000×) to verify lysis and removal of the cytoplasm from nuclei. The remaining sample was centrifuged, and the supernatant containing the cytoplasmic fraction was removed. A portion of the supernatant (50 μL) was employed for total protein measurement, with the remainder used for liquid scintillation counting. The pellet containing the nuclear fraction was washed once with PBS, centrifuged, lysed in 0.5 mL of 1% SDS, and counted by liquid scintillation counting. In one experiment, the nuclear pellet was washed once with PBS, centrifuged, and processed for electron microscopy [38].

RESULTS

Cellular Uptake of Fluoresceinyl Phosphorothioates

Cellular uptake of PS-Fl and MP-Fl oligonucleotides was evaluated in both 504 and HL-60 cells following incubation with oligonucleotides from 1 to 24 hr at 37°. Incubation of the cells with 1 μM MP-Fl did not yield measurable fluorescence values above controls, even after 24 hr of incubation. In contrast, incubation of both 504 and HL-60 cells with 1 μM PS-Fl produced substantial cell-associated fluorescence, a fluorescence that increased with longer incubation times (Figs. 1 and 2). Moreover, at each time point, the fluorescence distribution indicated a single cell

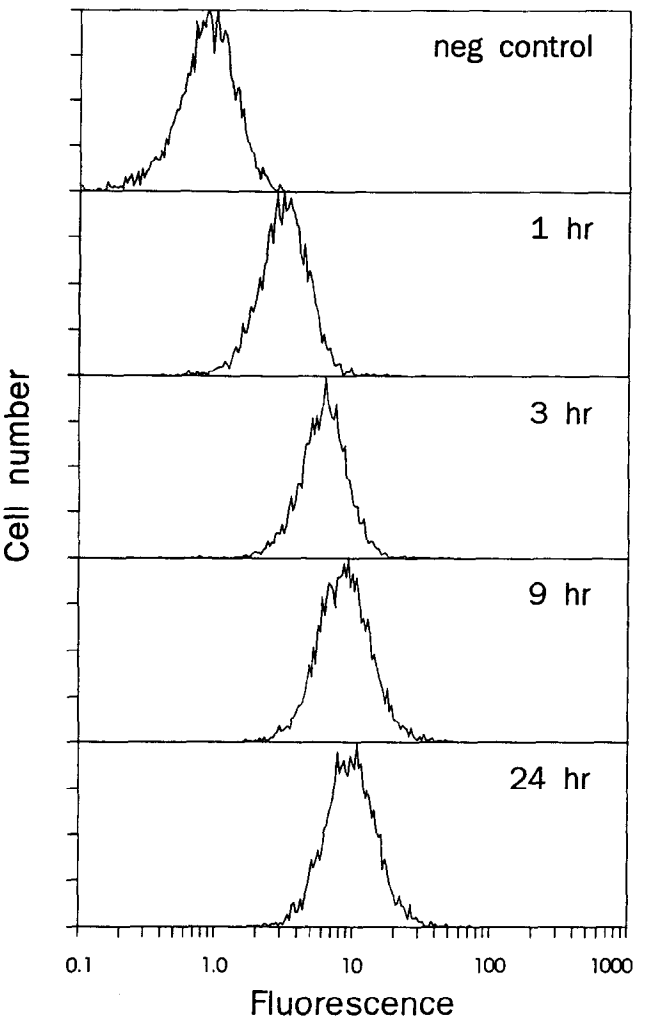

FIG. 1. Cellular uptake of fluorescent PS oligonucleotide by 504 cells with increasing time of incubation, measured by flow cytometry. Relative cell number is plotted on the ordinate as a function of fluorescence channel (log scale) on the abscissa for 10,000 504 cells incubated for various times in the presence of 1 μM fluoresceinated K-*ras*15 PS oligonucleotide. Negative control cells received no oligonucleotide.

population. Since the cells were proliferating in a non-synchronous manner with a cell cycle of approximately 12–18 hr, the absence of more than one population at any point during 24 hr of incubation suggests that oligonucleotide uptake for phosphorothioates is not strictly limited to any particular part of the cell cycle in these two transformed cell lines.

Stability of Radiolabeled Oligonucleotides

After an 8-hr incubation in medium containing 10% serum, in the absence of cells, all of the [^{14}C]oligonucleotide analogs remained largely intact, measured by HPLC (PO-NH_2, PS, 2OM, MP) or TLC (PNA). MP and PNA demonstrated no detectable degradation, while 2OM, PO-NH_2, and PS evidenced approximately 12–15% degraded products (data not shown). In each case, the intact oligo-

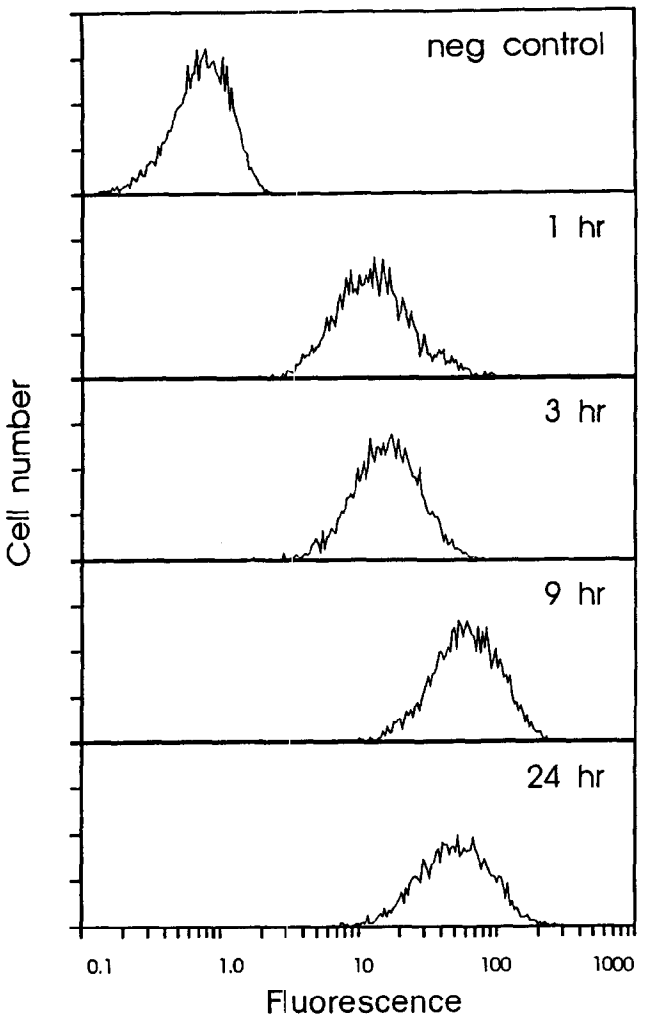

FIG. 2. Cellular uptake of fluorescent PS oligonucleotide in HL-60 cells with increasing time of incubation, measured by flow cytometry. Relative cell number is plotted on the ordinate as a function of fluorescence channel (log scale) on the abscissa for 10,000 HL-60 cells incubated for various times in the presence of 1 μM fluoresceinated K-*ras*15 PS oligonucleotide. Negative control cells received no oligonucleotide.

nucleotide fraction had specific activities within 10% of unincubated oligonucleotide.

Cellular Uptake of Radiolabeled Oligonucleotide Analogs—Time Course

Evaluation of oligonucleotide uptake requires the removal of free, non-internalized oligonucleotide from cells following a specified incubation time, and an initial experiment was conducted to evaluate washing protocols. The 504 and HL-60 cells were incubated with a charged (PS) or uncharged (MP) [^{14}C]oligonucleotide at 1 μM for 4 hr. They were then subjected to either one, three, five, or seven washings, with the latter three protocols including a high salt/low pH buffer wash. Following the last wash, the cells were lysed, and the lysate was analyzed for total protein and radioactivity.

The values for cell-associated picomoles of [^{14}C]oligonucleotide, relative to cell numbers estimated from total protein measurements, leveled off between three and five washes, an indication that four washes were sufficient to eliminate all removable oligonucleotide (data not shown). Since the remaining oligonucleotide survived a number of washes including the high salt/low pH wash, the working assumption is that this strongly cell-associated fraction represents internalized oligonucleotide. Equivalent results were obtained with HL-60 cells (data not shown).

The time course for cellular uptake of PO-NH_2, PS, and MP oligonucleotides was examined in 504 and HL-60 cells using the H-*ras*15 sequence (Figs. 3 and 4). The data are presented in terms of both picomoles of internalized oligonucleotide per 10^6 cells and estimated intracellular concentration. The average volume for fibroblast cells was 3 pL while the average volume for the HL-60 cells was 1.7 pL. These values were used to calculate an estimated cellular concentration for cell-associated oligonucleotide. All three analogs demonstrated some important similarities in uptake. Each evidenced a biphasic increase in cellular levels over time, with an initial rapid accumulation over a period of 3 hr followed by a more gradual increase over the next 21 hr. For all three analogs, uptake was an active process, based on the criterion that estimated intracellular concentrations greatly exceeded the extracellular 1 μM concentration within 3 hr. However, the three analogs differed substantially in the level of uptake. PS had the highest accumulation, PO-NH_2 evidenced an intermediate level, and MP showed the lowest accumulation. The differences were

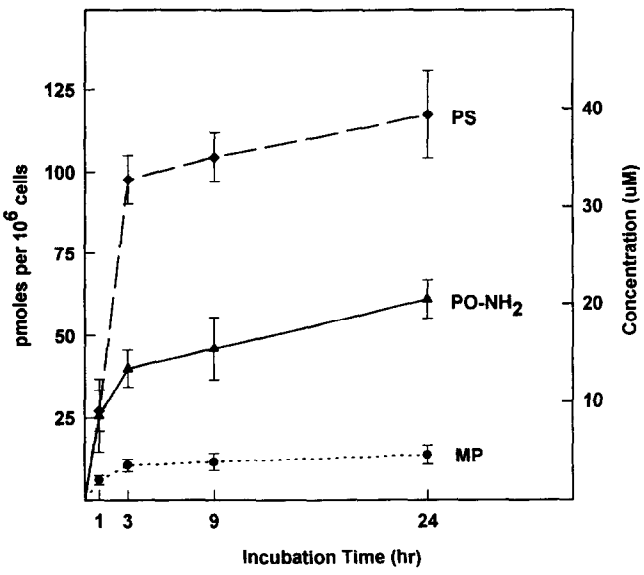

FIG. 3. Cellular uptake of PS, PO-NH_2, and MP oligonucleotide analogs as a function of incubation time in 504 cells. Cells were incubated in the presence of 1 μM H-*ras*15 [^{14}C]oligonucleotide for various time and processed for measurement of cell-associated radioactivity. Data are presented both in terms of picomoles oligonucleotide per 10^6 cells, and estimated intracellular concentration; each data point represents the mean ± SEM of four replicates.

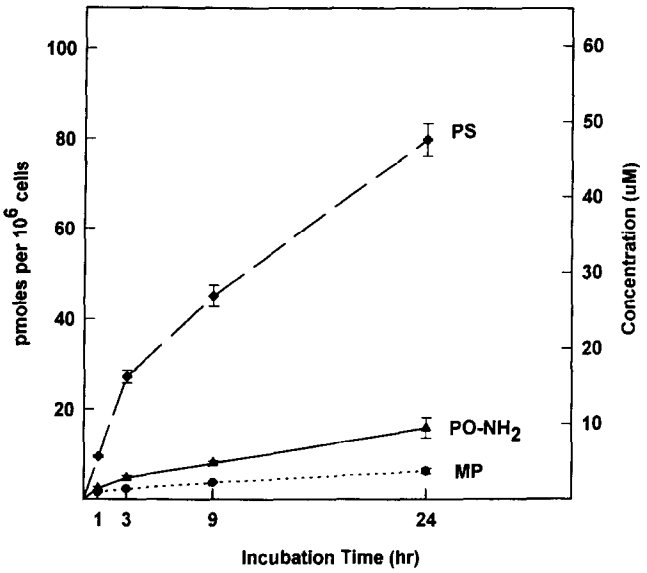

FIG. 4. Cellular uptake of PS, PO-NH_2, and MP oligonucleotide analogs as a function of incubation time in HL-60 cells. Cells were incubated in the presence of 1 μM H-*ras*15 [^{14}C]-oligonucleotide for various time and processed for measurement of cell-associated radioactivity. Data are presented both in terms of picomoles oligonucleotide per 10^6 cells, and estimated intracellular concentration; each data point represents the mean ± SEM of four replicates.

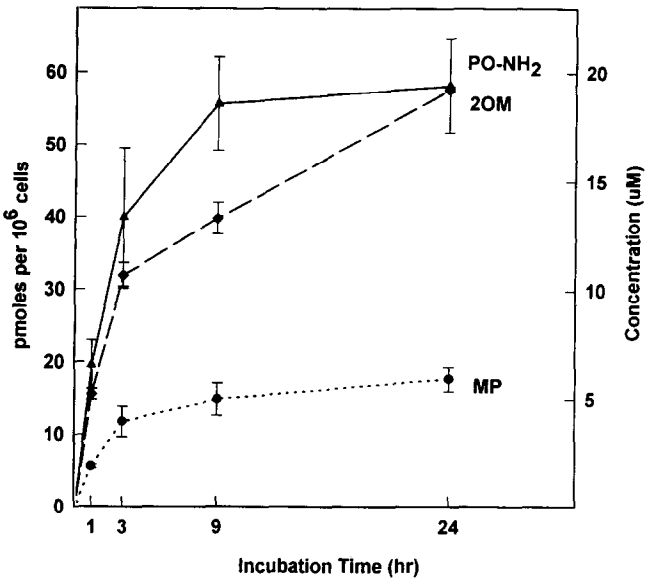

FIG. 5. Cellular uptake of PO-NH_2, MP, and 2OM oligonucleotide analogs as a function of incubation time in 504 cells. Cells were incubated in the presence of 1 μM K-*ras*15 [^{14}C]oligonucleotide for various time and processed for measurement of cell-associated radioactivity. Data are presented both in terms of picomoles oligonucleotide per 10^6 cells, and estimated intracellular concentration; each data point represents the mean ± SEM of four replicates.

evident within 1–3 hr, with PS exceeding PO-NH_2 by approximately 3-fold. The time course, overall levels of accumulation, and relative differences among the three analogs were similar in 504 and HL-60 cells.

In a second experiment, cellular accumulations of MP, PO-NH_2, and 2OM were compared, using the K-*ras*15 sequence (Figs. 5 and 6). In both 504 and HL-60 cells, 2OM and PO-NH_2 showed similar patterns and overall levels of uptake over the 24-hr period, demonstrating a slight difference only at the 9-hr time point. Both PO-NH_2 and 2OM exhibited substantially higher uptake than MP at all time points.

The time course for PNA uptake was compared with that of MP using the PN14 sequence in 504 and HL-60 cells (Figs. 7 and 8). PNA uptake was substantially higher than MP after 9–24 hr of incubation, apparently due to differences in the pattern of uptake over time. PNA demonstrated an almost linear increase over the 24-hr time period, rather than the biphasic pattern typical of the other analogs.

Cellular Uptake of Radiolabeled PO-NH_2, PS, and MP Oligonucleotides—Different Sequences

It should be noted that three different oligonucleotide sequences were employed in the time-course experiments described above, with similar results for each sequence. To confirm these findings, two different heterogeneous sequences (H-*ras*15 and K-*ras*15) were directly compared following 8 hr of incubation in 504 cells. The sequences were selected because they differ in two important ways. First, the sequences differ at five separate sites out of a total of fifteen bases, yielding a different structural sequence and overall base composition. Also, the H-*ras*15 sequence is complementary to the human H-*ras* oncogene. This onco-

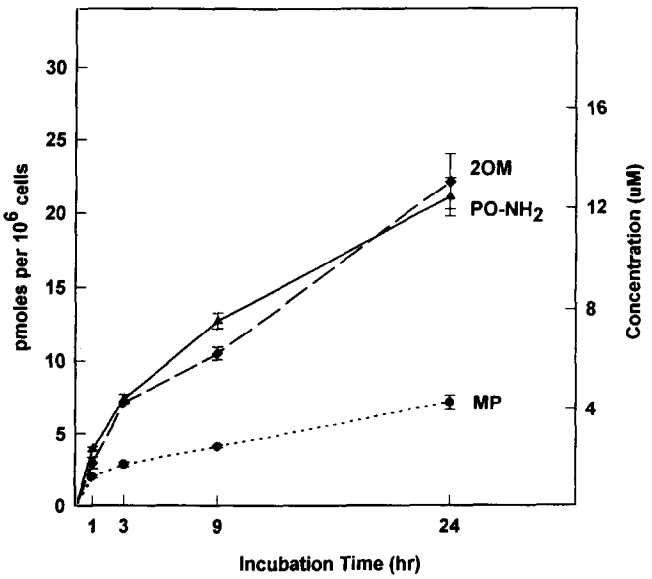

FIG. 6. Cellular uptake of PO-NH_2, MP, and 2OM oligonucleotide analogs as a function of incubation time in HL-60 cells. Cells were incubated in the presence of 1 μM K-*ras*15 [^{14}C]-oligonucleotide for various time and processed for measurement of cell-associated radioactivity. Data are presented both in terms of picomoles oligonucleotide per 10^6 cells, and estimated intracellular concentration; each data point represents the mean ± SEM of four replicates.

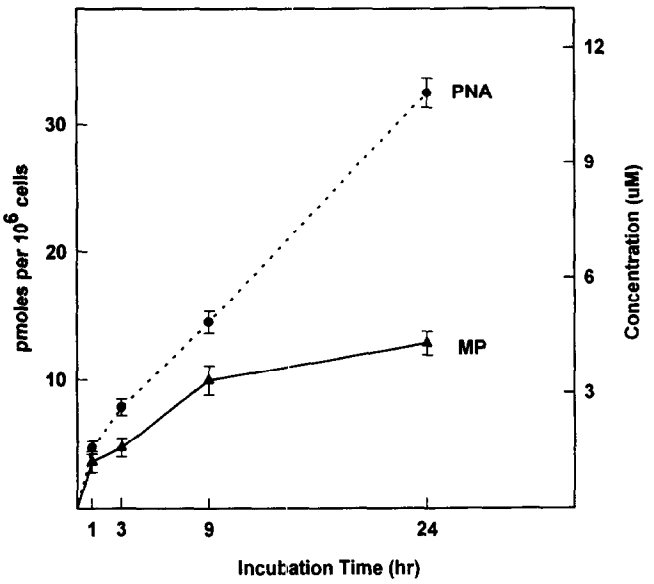

FIG. 7. Cellular uptake of MP and PNA oligonucleotide analogs as a function of incubation time in 504 cells. Cells were incubated in the presence of 1 μM PN14 [^{14}C]oligonucleotide for various time and processed for measurement of cell-associated radioactivity. Data are presented both in terms of picomoles oligonucleotide per 10^6 cells, and estimated intracellular concentration; each data point represents the mean ± SEM of four replicates.

gene is constitutively overexpressed in the 504 cells and, therefore, provides a target RNA transcript. In contrast, the K-*ras*15 sequence is complementary to a human K-*ras* oncogene and has no known complementary RNA transcript in the mouse. The results for the two sequences were essentially equivalent for all three analogs, suggesting that the differences between the two sequences had no obvious effect on cellular uptake for the PO-NH_2, PS, and MP modifications examined in this experiment.

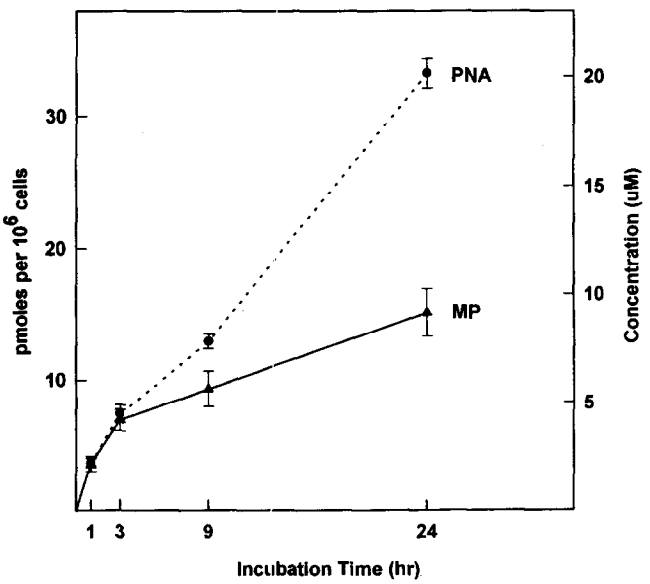

FIG. 8. Cellular uptake of MP and PNA oligonucleotide analogs as a function of incubation time in HL-60 cells. Cells were incubated in the presence of 1 μM PN14 [^{14}C]oligonucleotide for various time and processed for measurement of cell-associated radioactivity. Data are presented both in terms of picomoles oligonucleotide per 10^6 cells, and estimated intracellular concentration; each data point represents the mean ± SEM of four replicates.

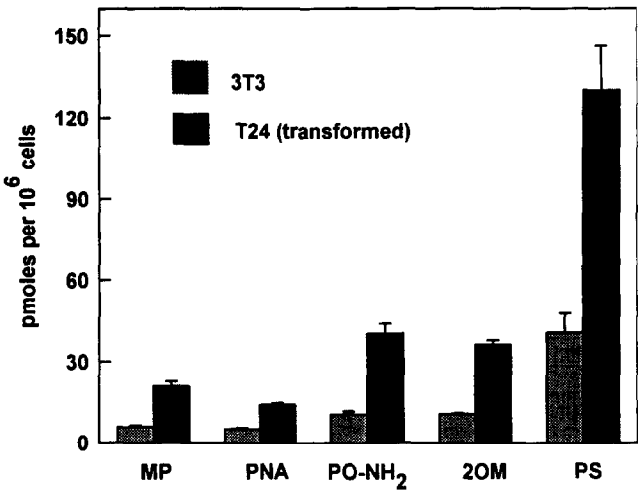

FIG. 9. Cellular uptake of various oligonucleotide analogs in H-*ras* transformed and non-transformed mouse fibroblasts. Cells were incubated in the presence of 1 μM H-*ras*15 [^{14}C]-oligonucleotide and processed for measurement of cell-associated radioactivity. Data represent the means ± SEM of 3–4 replicates for: T24 (transformed), NIH-3T3 cells permanently transfected with multiple copies of the T24 H-*ras* oncogene; and 3T3, cells of the original NIH-3T3 cell line.

Cellular Uptake of Radiolabeled Oligonucleotides—Effect of Transformation

Anticipating oligonucleotide therapy, it would be advantageous for transformed cells to take up oligonucleotides to a greater extent than normal cells. The effect of cellular transformation on the uptake of oligonucleotides was investigated by comparing cells transformed by oncogene transfection with the parental cell line. In one case, cells permanently transformed by multiple copies of the T24 H-*ras* oncogene were compared with the parental NIH-3T3 cell line. The transformed cells demonstrated substantially higher uptake of all analogs, exceeding accumulations in NIH-3T3 cells by factors of 2–4 (Fig. 9). It should be noted that these differences were not due to differences in cell confluence since the experiments were conducted at a confluence of 60–70% for all cells.

A second experiment examined the effects of transformation by a different gene and yielded similar results. P6 cells, which are permanently transformed by multiple copies of the human gene for IGF-1R, exhibited approximately 2-fold higher uptake than non-transformed cells of the parental Balb/c-3T3 cell line (Fig. 10).

Distribution of Radiolabeled Oligonucleotides in Nuclear and Cytoplasmic Cellular Compartments

The above experiments demonstrated significant, active cellular uptake for each of the five analogs examined,

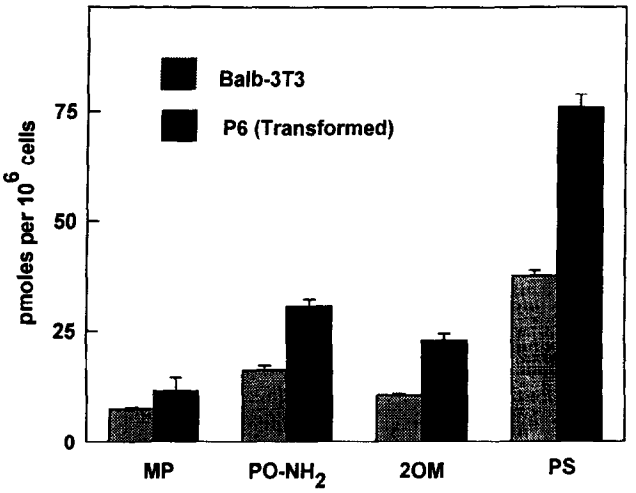

FIG. 10. Cellular uptake of various oligonucleotide analogs in IGF-1R transformed and non-transformed mouse fibroblasts. Cells were incubated in the presence of 1 μM H-*ras*15 [^{14}C]-oligonucleotide and processed for measurement of cell-associated radioactivity. Data represent the means ± SEM of 4 replicates for: P6 (Transformed), Balb/c-3T3 cells permanently transfected with multiple copies of the human gene for IGF-1R; and Balb-3T3, cells of the original Balb/c-3T3 cell line.

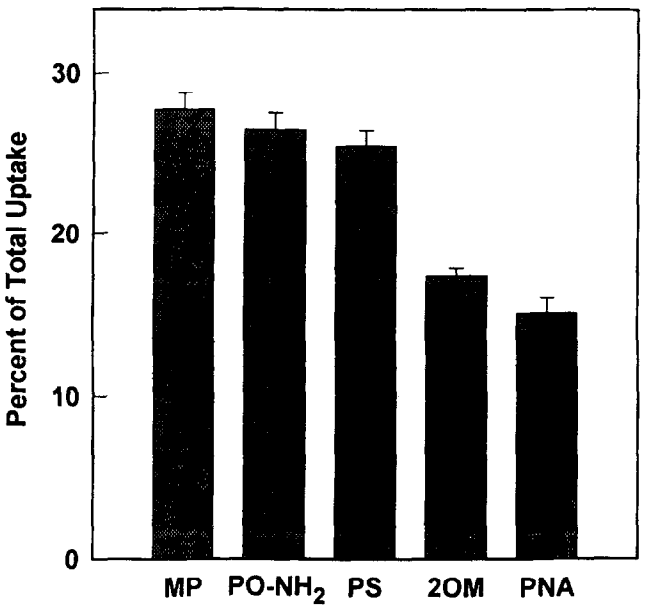

FIG. 11. Nuclear accumulation of various oligonucleotide analogs in 504 cells. Cells were incubated for 8 hr in the presence of 1 μM H-*ras*15 [^{14}C]oligonucleotide. Data represent the means ± SEM of four replicates and are presented as a percentage of nuclear-associated radioactivity relative to total cell-associated radioactivity, from the data of Table 1.

although the degree of accumulation differed substantially. A subsequent question concerns the distribution of accumulated oligonucleotides within the cells. Following oligonucleotide uptake, the nuclear and cytoplasmic compartments of cells were separated by using a low concentration of non-ionic detergent to lyse differentially the plasma membrane, leaving the nuclear envelope intact. The accumulated [^{14}C]oligonucleotides in each compartment were quantitated by liquid scintillation counting of compartmental lysates. The validity of the procedure was verified by microscopic analysis of the nuclear fraction in two ways. Each nuclear fraction was examined by light microscopy to ensure that the nuclei were intact and essentially free of attached cytoplasmic fragments. Also, in one experiment the nuclear fraction was examined by electron microscopy. It revealed the presence of intact nuclei and occasional mitochondria, but no intact endosomal organelles.

TABLE 1. Distribution of intracellular oligonucleotides between nuclear and cytoplasmic compartments

Oligonucleotide	Oligonucleotide distribution (pmol/10^6 cells)	
	Nuclear	Cytoplasmic
H-*Ras*15 Sequence		
MP	2.9 ± 0.4	7.5 ± 0.8
PO-NH_2	7.7 ± 1.0	21.6 ± 1.7
PS	24.1 ± 3.2	70.0 ± 6.9
K-*Ras*15 Sequence		
MP	2.9 ± 0.7	8.4 ± 0.4
PO-NH_2	8.0 ± 0.9	23.3 ± 0.8
2OM	5.0 ± 0.2	23.7 ± 0.4
PN14 Sequence		
MP	2.7 ± 0.1	8.5 ± 0.5
PNA	1.9 ± 0.1	13.2 ± 0.9

Data represent means ± SEM of 3–4 replicates. H-*ras* transformed 504 cells were incubated for 8 hr with 1 μM oligonucleotide.

Nuclear and cytoplasmic accumulations for the various analogs were obtained following incubations in 504 cells for 8 hr at 1 μM. Table 1 presents the results in terms of picomoles per 10^6 cells. The nuclear compartment evidenced measurable amounts of all analogs although the amounts were substantially less than cytoplasmic accumulations. As noted above, sequence-dependent differences were not observed. Comparisons of cytoplasmic values among the various analogs yielded the same relative pattern seen with measurements of total cellular uptake: PS > PO-NH_2, 2OM > PNA, MP. However, the pattern for nuclear values was somewhat different due to the behavior of 2OM and PNA. Their nuclear levels were lower relative to total cellular levels than those for the other three analogs. As illustrated in Fig. 11, nuclear levels for the three DNA derivatives were approximately 25% of total cellular uptake, whereas the levels for 2OM and PNA were less than 20%.

DISCUSSION

These experiments directly and quantitatively compared cellular uptake and nuclear localization in several transformed or immortalized cell types for a variety of oligonucleotide analogs: two charged DNA analogs (PO-NH_2, PS), a charged RNA analog (2OM), and two uncharged species (MP, PNA). All analogs demonstrated some degree of active cellular uptake, in that intracellular concentrations

exceeded the extracellular concentration within 1–3 hr and increased over the 24-hr incubation period. The incubation concentration of 1 μM was chosen because cellular uptake of some charged oligonucleotides (PO, PS) at this concentration is primarily through receptor-mediated endocytosis [19, 21]. This concentration should therefore maximize differences between oligonucleotides internalized by receptor-mediated endocytosis and those that rely on fluid phase endocytosis [22].

There were substantial differences in the level of uptake among the various analogs. The charged oligonucleotides had higher uptake than the uncharged analogs, but there were also significant differences within the two groups. PNA demonstrated higher uptake than MP, although the difference was evident only after 3–9 hr of incubation due to the more linear increase in uptake shown by PNA. PS evidenced substantially higher uptake than the other two charged analogs, PO-NH_2 and 2OM. Most of the differences among the analogs were evident within 1–3 hr, and by 9 hr, cellular accumulation of PS exceeded that of PO-NH_2 and 2OM by about 3- to 5-fold and exceeded MP by about 8- to 10-fold. Similar relative differences have been reported for some analogs [20, 23], but no previous study has directly compared cellular uptake for all of these analogs in a quantitative manner.

The differences between charged and uncharged analogs most likely reflect differences in the mechanism of uptake. Uncharged MP are internalized essentially by fluid-phase endocytosis [22], and a similar mechanism probably accounts for PNA uptake. Fluid-phase endocytosis is less efficient than receptor-mediated endocytosis, the process characteristic of charged PO and PS analogs, and this would account for the lower uptake values for the uncharged analogs. The RNA analog, 2OM, is charged, and its similarity in uptake with PO-NH_2 suggests that it is also internalized by a receptor-mediated process. PS exhibit higher uptake than the other charged analogs, an effect presumably due to higher cell-surface binding affinities for PS [23]. This factor would increase both receptor-mediated and fluid phase endocytosis for PS analogs.

Several other variables did not affect cellular uptake in the present study. Similar results were obtained in two types of cell lines (mouse fibroblasts and human promyelocytic leukemia cells) and three different heterogeneous sequences. Although limited in scope, these findings indicate that the uptake differences described above are not unique to a particular cell line or sequence and may therefore have some generality. Contrary to an earlier report [39], we found no evidence that the presence or absence of a complementary RNA target affected cellular accumulation of oligonucleotides.

Cellular transformation had a substantial effect on the cellular uptake of all analogs. This was evident in cells permanently transformed by overexpression of either an H-*ras* oncogene or IGF-1 receptor. A similar effect of transformation on PS and PO uptake has been reported in lymphocytes activated by an HTLV-1 *tax* gene or mitogens, an effect possibly related to increased membrane activity [23, 40–42]. The present results support this supposition, extending the effect to a variety of different analogs and to fibroblast-type cells. It is worth noting that the effect may have some role in the clinical application of oligonucleotides against cancer since tumor cells may also exhibit higher oligonucleotide uptake than normal cells *in vivo*.

Cellular uptake of the oligonucleotides was associated with significant accumulations in the nucleus, as measured by cell fractionation. The results for PO-NH_2 and PS oligonucleotides are comparable to previous results obtained using various physicochemical techniques for cell fractionation [21, 43, 44], all of which reported significant oligonucleotide levels in the nucleus following incubation of the cells in oligonucleotide-containing medium. Relative nuclear levels of the three DNA analogs (MP, PS, PO-NH_2) were similar, approximately 25% of total cellular uptake after 8 hr of incubation. In contrast, 2OM and PNA analogs had significantly lower percentages. The results would suggest that intracellular trafficking is similar for the DNA analogs but differs to some degree for 2OM and PNA. Oligonucleotides rapidly accumulate in the nucleus following release from endosomes or direct microinjection, a process that appears to involve passive diffusion through nuclear pores and binding to various nuclear proteins [21, 45–47]. MP has been reported to accumulate in the nucleus to a lesser extent than charged oligonucleotides following microinjection [46], but we found no difference in the distribution of MP, PS, and PO-NH_2. 2OM and PNA have not been investigated for nuclear binding, but the fact that we saw decreased nuclear accumulations for these two analogs suggests a reduced affinity for nuclear proteins.

Physicochemical methods rather than microscopy were employed in these experiments to separate various cellular compartments—intracellular from extracellular, nuclear from cytoplasmic. This approach has clear advantages in quantitation and sampling of cell populations. It also allows for the use of a small, internal radioactive label. The small size of the label limits any effects on the behavior of the oligonucleotide while the internal location of the label, as compared to end labeling, limits exonuclease effects. Separation of the intracellular from the extracellular compartment was accomplished by extensive washing, including either a high salt/low pH buffer or trypsin buffer. The washing procedure using a high salt/low pH buffer yielded relatively reliable measurements of intracellular uptake that did not change with increasing washes, an indication that only internalized or very strongly cell-associated oligonucleotide remained in the intracellular compartment samples. This is consistent with previous reports that an acid wash eliminates most membrane-bound, non-internalized oligonucleotide [48]. Moreover, replacing the high salt/low pH buffer with trypsinization of the cells yielded essentially equivalent results, also suggesting that the measurements only included internalized oligonucleotide. The separation of nuclear and cytoplasmic compartments was accomplished by selective lysis of the plasma membrane, a process

verified in this study by light microscopy and in one experiment by electron microscopy. The problem of contamination of the nuclear pellet with endosome/lysosome organelles is a major concern [49], especially considering the high concentration of oligonucleotides in these organelles. However, electron microscopy did not indicate contamination of the nuclear pellet by intact endosomes/lysosomes. Although separation of the nuclear and cytoplasmic compartments can be verified by microscopy, leakage of oligonucleotides from one compartment to another during processing cannot be monitored easily. Even the use of chemical markers is limited in this instance, since the movement of oligonucleotides probably differs substantially from any leakage exhibited by nuclear or cytoplasmic protein markers. Because of the strong tendency for oligonucleotides to accumulate in nuclei, any leakage would presumably lead to increased nuclear levels, overestimating nuclear uptake. Cell fractionation studies [21, 43, 44] and some microscopy studies using fixed cells [37, 50] did in fact, report significant nuclear levels of PO and PS, whereas other microscopy studies found little nuclear localization [23]. Oligonucleotide leakage has been proposed as a factor contributing to differences in fluorescence microscopy results between fixed and live cells [23]. It is not clear whether intercompartmental leaking during processing is the key variable or whether other variables account for the discrepancies. The absence of quantitative data and limited sample numbers from microscopy studies make comparative evaluations between studies more difficult. A combination of approaches seems to be the most appropriate approach at present to elucidate the critical question of nuclear uptake.

Differences among analogs in cellular efflux rates may also contribute to differences in observed rates of uptake. Similar efflux half-lives of 7–10 days were observed for PO and PS oligonucleotides in liposomes, while MP oligonucleotides effluxed with a half-life of 4 days [22]. However, in H9/Hut78 immortalized T cells, much more rapid uptake and efflux half-lives of 40–60 min for PS, and 20 min or less for PO, were reported [37], implying an active cellular efflux pathway. If efflux rates from cells are, in general, comparable to influx rates, and uptake is active, then oligonucleotide levels in cells should eventually equilibrate, with steady-state intracellular oligonucleotide concentrations higher than extracellular concentrations, as reported above.

In studies of oligonucleotide uptake by cells, it is also critical that the radioactive measurements accurately reflect intact or relatively intact oligonucleotide. This requires that the ^{14}C-label remain bound to relatively intact oligonucleotide through the course of the experiment. The analogs were selected, in part, because of their relative biological stability, at least under the conditions of the experiments. Oligonucleotide analyses following incubation in cell culture medium indicated that all analogs remained essentially intact with their radioactive label through 8 hr of incubation. This is consistent with previous reports on the stability of both the analogs [9, 11, 44, 51–54] and the methyl label on the exocyclic amines [35]. The low specific activity of the ^{14}C-labeled oligonucleotides did not permit analysis of the intactness of intracellular oligonucleotides. This primarily concerns PS, PO-NH_2, and 2OM oligonucleotides, which demonstrated some susceptibility to nuclease activity in the cell medium. However, several points suggest that the radioactivity measurements reflect relatively intact intracellular oligonucleotide, at least through much of the 24-hr period. Previous reports have demonstrated that intracellular PS and PO-NH_2 are relatively stable up through approximately 12 hr [23, 44]. Also, the pattern of uptake over time and the relative distribution between cytoplasmic and nuclear compartments were similar for all analogs, both those partially susceptible to nucleases (PS, PO-NH_2, 2OM) and those completely resistant (MP, PNA). Degradation of an oligonucleotide leads to rapid removal of monomers from the cells and results in a discernible alteration in the uptake pattern [47]. Finally, the relative differences in uptake among the various analogs were evident within 1–3 hr and did not change substantially through 24 hr.

Differences in cellular uptake among oligonucleotide analogs clearly contribute to differences in their efficacy as antisense agents. Of the analogs examined in the present study, PS demonstrated the highest uptake, an effect presumably due to its negative charge and high membrane affinity. PS have also shown the most general efficacy as antisense agents among these first generation analogs, despite their nonspecific effects [1]. In contrast, the second generation analogs 2OM and PNA, which hybridize and resist nucleases better than PS, displayed modest and low uptake, respectively. It was encouraging, however, to note that all analogs were taken up more aggressively by transformed cells than by their immortalized parental lines.

Efficacy is controlled by a number of properties including cellular uptake, biological stability, affinity for the target RNA, and ability to stimulate RNase H hydrolysis or some other degradative process. Design and development of third generation antisense agents require, therefore, a quantitative evaluation of how each of these factors, including cellular uptake, is affected by each structural modification.

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