Electron micrographic studies of transport of oligodeoxynucleotides across eukaryotic cell membranes

(cell entry/oligonucleotides/autoradiography)

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ABSTRACT   Unmodified oligodeoxynucleotides (ODNs) were synthesized and tested for their ability to cross external eukaryotic cell membranes and to enter the cytosol and nucleus in tissue cultures. The ODNs were labeled with high-specific-activity [^{3}H]thymidine ($\geq$100 Ci/mmol), or [α-^{32}P]ATP or [γ-^{32}P]ATP (300–1000 Ci/mmol; 1 Ci = 37 GBq), and the label was either in the central portion of the molecule or at the 3' or 5' end. The cells employed were for the most part 3T6 murine fibroblasts, grown in monolayers, either semiconfluent or confluent, but some experiments were carried out with chicken embryo fibroblasts or human HeLa cells. Parallel wells in the same experiment were prepared for electron microscopy or for cell fractionation and radioactivity assays. Electron microscopic autoradiography indicated that ODNs cross the external cell membrane, traverse the cytosol, and begin to enter the cell nucleus within a few seconds to 5 min at 37°C in Dulbecco's medium without added serum. After 30–60 min of incubation with ODNs, abundant silver grains were observed at or just inside the nuclear membrane or well distributed across the nucleus, particularly in association with euchromatin. There was a paucity of silver grains associated with nucleoli. Cell entry of oligomer was related to cell cycling events and was energy dependent. Degradation of oligomer to monomers, with reincorporation into DNA, does not appear to explain these results. No sequestration of labeled oligomer in cytoplasmic vesicles en route from the exterior of the cell to the nucleus was observed. The observations are more suggestive of internalization of oligonucleotide by a mechanism as yet unclear or, alternatively, by a caveolar, potocytotic mechanism rather than by endocytosis.

It was shown in 1978 (1, 2) that Rous sarcoma virus replication could be inhibited in chicken embryo fibroblasts (CEFs) grown in tissue culture, by addition to the culture medium of a 13-base oligodeoxynucleotide (ODN) complementary to the reiterated 3' and 5' ends of the linear RNA viral genome strand. In addition, transformation of normal fibroblasts to sarcoma cells could be prevented by the oligomer. Blocking of the 3' and 5' ends of the oligonucleotide as the isourea derivatives enhanced the specific inhibitory activity, presumably by increasing intracellular nuclease resistance. The results strongly suggested that the oligomer crossed the external cell membrane to accomplish these inhibitory functions. No direct proof of this point of view was presented at that time, although other explanations seemed unlikely.

In the intervening years, numerous studies (see general references within refs. 3–6 plus selected references in refs. 7–12) have strengthened the conclusion that externally added oligonucleotides do enter a variety of eukaryotic cells. To examine in more detail parameters of cell entry, we have labeled ODNs with [^{3}H]thymidine, either in the interior of the

molecules, at the 5' end, or as an oligo [^{3}H]thymidine tail on the 3' end, at a high specific activity. Mouse 3T6, CEF and human HeLa cells have been grown with sterile precautions in tissue culture in the presence of the labeled ODNs for times ranging from 0 to 60 min at 37°C. The contents of some of the wells (1 cm^2 in size) have been prepared for electron microscopy. Parallel wells have been used for cell fractionation and radioactivity assay. Unmodified ODNs of 12–20 bases rapidly enter these eukaryotic cells in tissue culture and quickly pass through the cytosol to the nucleus by an energy-dependent mechanism, as evidenced by dinitrophenol inhibition. Cell entry of oligomers is most effective in a phase of the cell cycle (G$_1$/S) in preparation for DNA synthesis.

In control experiments, [^{3}H]thymidine uptake into 3T6 cells was studied. The distribution of the silver grains within the nucleus was more clustered and medusoid than in the case of the ^{3}H-labeled-oligomer, suggesting that DNA synthesis was occurring at discrete sites within the nucleus, in a single-chain progressive way, in contrast to the wide distribution of single grains observed in the nucleus in the case of the ^{3}H-labeled-oligomer.

MATERIALS AND METHODS

Synthesis and Labeling of ODNs. ODNs were synthesized on an automated synthesizer (Biosearch 8700; Milligen). The 3'-end labeling was performed by enzymatic addition of [^{3}H]dTTP to the oligomer by terminal deoxynucleotidyltransferase. Chain extension was restricted to 20 nucleotides or less by limiting the amount of dTTP in the reaction. Purification was carried out by polyacrylamide gel electrophoresis, and the labeled band was eluted. Internal labeling was achieved by extending a primer with the Klenow fragment of *Escherichia coli* DNA polymerase I. The primer was an octamer (5'-TTCCACAC-3') that hybridized internally next to a run of 10 adenosines in a template containing 35 bases (3'-CCCCCCCCCACC<u>AAGGTGG</u>AAAAAAAAAACT-GCGTT-5'). The sequence was chosen simply to avoid extensive complementarity or hairpin formation. The reaction was first pulsed with [^{3}H]dTTP and then chased with all four unlabeled deoxynucleoside triphosphates. This gave a single product 25 bases long, labeled internally in the dT$_{10}$ sequence. Because the primer hybridized to an internal sequence in the template, the product was 10 bases shorter than the template, and so these were resolved by electrophoresis in a preparative 15% polyacrylamide gel. The specific activity of both the internally labeled and the 3'-end-labeled oligomers varied in different preparations from the 300–1000 Ci/mmol for the ^{32}P-labeled ODN used for uptake studies to between 70 and 330 Ci/mmol for [^{3}H]dTTP-labeled oligomers for separate electron microscopy experiments.

Tissue Culture Studies. 3T6, CEF, and HeLa cells were grown routinely in 75-cm^2 Falcon flasks and were fed with Dulbecco's modified Eagle's medium (DMEM) supplemented

with 10% fetal bovine serum, penicillin/streptomycin, 1 mM glutamine, and 1 mM pyruvate. The cells at confluence were trypsinized, counted with a Coulter Counter, and explanted into four-well (1-cm^2 surface area per well) plastic tissue culture dishes. Typically, around 60,000 cells were added per well, in 0.5 ml of the above medium, and were incubated overnight at 37°C in 5% CO_2/95% air. The next morning the cells were well attached as a fibroblastic monolayer of ≈100,000 cells per well, as determined by Coulter Counter, nearly but not completely confluent.

The growth medium was removed carefully by pipette, so as not to disturb the cell network fabric, and was frozen immediately at −70°C. The wells were washed twice with 500 μl of DMEM each time and then 150 μl of DMEM without serum was added. Labeled oligomer (10 μl) was added with gentle stirring to give a final concentration of ≈0.6 μM and incubation was started with the four-well dish in contact with the floor of the 37°C incubator for better immediate temperature equilibration. For zero time points, the incubation medium was removed directly after addition of the labeled oligomer and frozen at −70°C, and the duplicate wells were washed quickly with 200 ml of ice cold DMEM each, added carefully from a small beaker in generous aliquots and then poured off, for a total of 10 times. The dishes were then inverted on blotting paper briefly. Then to the two duplicate wells was added either 0.2 ml of 2% glutaraldehyde buffered to pH 7.4, for fixation prior to electron microscopy, or 0.2 ml of 1 M formic acid, to permeabilize cell membranes and release soluble cellular contents.

In initial experiments, aliquots of incubation medium were assayed for radioactivity after each 1-ml wash. No radioactivity above background was found after the washing procedure described above. The cells were observed under substage visual microscopy after the washing procedure. The fabric of the monolayer was found to be intact, and the cell detail to be grossly unchanged. After the addition of 1 M formic acid, cell boundaries were more readily visualized but the cell fabric was not noticeably changed, nor were the nuclei, as observed by substage polarized light microscopy, up to a magnification of ×400. Nevertheless, the plasma membrane, and probably the nuclear envelope, had become permeabilized, as judged by the presence of radioactive oligomer in the 1 M formic acid extract (15-min extraction at 22°C) (7). After removal of the supernatant solution of 0.15 ml of 1 M formic acid containing soluble cell contents, which was frozen immediately at −70°C, 0.15 ml of 2.5% sodium dodecyl sulfate (SDS) was added to the cells destined for metabolic studies. Following a 15-min extraction at room temperature, with occasional mild shaking of the four-welled dishes, microscopy revealed no cell fabric or gross cell particulates remaining, all cell components being either dissolved or suspended finely in the SDS extract. This extract was frozen immediately at −70°C.

The wells prepared for electron microscopy were washed quickly three times at the chosen time points, with 1 ml of DMEM each time, and then 0.2 ml of 2% glutaraldehyde was added.

Cell Cycle Synchronization. It was determined both by Coulter cell counting and by radioactive labeling of DNA with [^{3}H]thymidine that the doubling time of 3T6 cells in tissue culture under our conditions was 16 hr. As a result of a double thymidine block (13), carried out over a 2-day period, the blocked cells did not increase in number, whereas the unblocked cells in companion wells did so. Thus the unblocked cells grew rapidly, whereas the thymidine-blocked cells remained static.

An unexpected feature of the autoradiography of unsynchronized cells following addition of ^{3}H-labeled oligomers to the growth medium for 30 or 60 min at 37°C was that not all cells developed silver grains either within or above the

nucleus or cytoplasm. Approximately half of the cells did so, in the monolayered semiconfluent fabric. We therefore endeavored to synchronize cell growth and to determine the effect on oligomer uptake. The double thymidine block procedure was used (13). A test of the effectiveness of the double thymidine blockade of cells at the G_1/S boundary was performed by addition of 20 μCi of [^{3}H]thymidine in 3 ml of DMEM without serum to cells for 60 min at 37°C. Release from the G_1/S boundary block was achieved by addition to the cells of 5 ml of DMEM without serum after the cells had been washed twice with DMEM without serum. [^{3}H]Thymidine incorporation into DNA was inhibited 95% by the thymidine blockade 0–30 min after release of the blockade, when compared with unblocked cells.

Treated 3T6 cell cultures were fixed *in situ* with 2% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.4) at 22°C for 2 hr. They were then given three 10-min washes in the same buffer, postfixed with 2% osmium tetroxide in 100 mM cacodylate buffer (pH 7.4) for 1 hr at 22°C, and washed again as above. Dehydration through a graded ethanol series included an overnight stain *en bloc* with 0.5% uranyl acetate in 70% ethanol at 4°C in the dark. The cultures were infiltrated with Poly/Bed 812 (Polyscience).

The polymerized blocks were broken out of the multiwell culture dishes and the areas with the highest density of cells were cut out and re-embedded with the cells oriented face to face so that the two layers of cells could be sectioned simultaneously. The final plane of sectioning was perpendicular to the plane of cell culture.

Thin sections were cut and either picked up on 150- or 200-mesh Formvar/carbon-coated copper grids or picked up with a loop and placed on Formvar-coated microscope slides. Slides with sections were lightly coated with carbon (5–10 nm) and overlaid with Ilford L4 Nuclear Research Emulsion by the "dip" method (14). Sections on grids were coated with Ilford L4 nuclear emulsion by the "loop" method (15). Emulsion-coated specimens were stored in the dark at 4°C. After various times, grids were developed and examined by electron microscopy to determine number and distribution of silver grains. Specimens were examined with a Philips CM10 transmission electron microscope and an accelerating voltage of 80 kV.

Determination of Cell Volume and Number. The total number of 3T6 cells was determined by duplicate Coulter counting in parallel wells. The volume of an individual 3T6 cell was established by cytocrit measurements of large numbers of 3T6 cells after centrifugation. The average volume per cell was 3.7 pl for 3T6 cells as measured in suspension.

RESULTS AND DISCUSSION

The extent of uptake (15 min) into CEF cells of a 21-mer ODN labeled internally by ^{32}P is shown in Fig. 1. At 10 μM external concentration of oligomer, 7.5% had entered the cytoplasm by 15 min at 37°C. Fig. 2*A* depicts the rate of uptake of the oligomer shown in Fig. 1, at 36 μM external concentration, into the soluble fraction of CEF in tissue culture over a 10-min period at 37°C. The uptake was rapid and linear. The 10-min uptake in Fig. 2*A* corresponds to 3.7% of the external concentration. Fig. 2*B* shows the progressive association of the oligomer with formic acid-insoluble internal components of the CEFs, during the same period of time, in the same cells as those shown in Fig. 2*A*. As shown in the electron microscopy experiments described below, the exterior membrane of the cells had been extensively washed, and very little oligomer was associated therewith.

Degradation and Metabolism of ^{3}H-Labeled Oligomers in the Incubation Medium, Soluble Cell Extract, and Cell Membrane and Particulate Fractions of 3T6 Cells After Incubation at 37°C. The integrity of ODN following incubation with cells
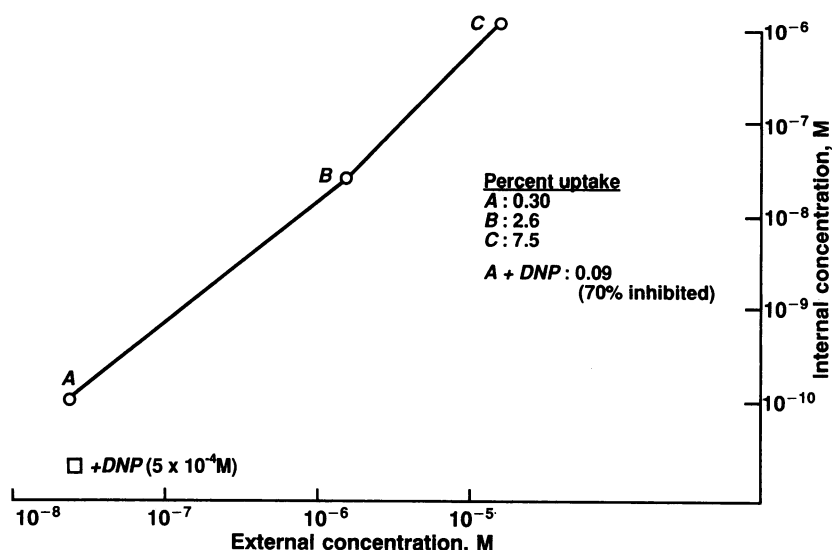
Percent uptake
A : 0.30
B : 2.6
C : 7.5

A + DNP : 0.09
(70% inhibited)

□ +*DNP* (5 x 10⁻⁴M)

FIG. 1. Plot of internalization of ODN (TAGTCTCAATp*GGGCTTGATAA) in confluent CEFs, showing direct relationship of internal concentration of oligomer to external concentration in incubation medium, as measured by 1 M formic acid extraction of soluble components of washed cell monolayer. Dinitrophenol (DNP) at 0.5 mM inhibited uptake into cells by 70% during incubation for 15 min at 37°C.

was monitored by thin-layer chromatography (TLC) on silica plates in 2-propanol/concentrated ammonium hydroxide/water, 60:10:30 (vol/vol). ³H-containing spots were located with a TLC plate scanner, and markers of unlabeled dTTP, dTMP and thymidine were located by UV illumination. Comparison of the starting ODN and the supernatant from the cells after incubation for 30 min at 37°C showed no degradation of the ODN. Material extracted from cells at this time by formic acid showed largely undergraded ODN as well as smaller amounts of degraded material.

Autoradiography of Cells. The "zero time" points represented passage of 10–20 sec at room temperature after addition of ODN, followed by immediate washing of the cells. Nevertheless, after this time occasional silver grains were observed in nuclei as well as at cell margins (Fig. 3A). At 5, 10, and 15 min after incubation, increasing nuclear labeling was found, indicating a progressive passage of oligomer from the incubation medium through the cytoplasm into nuclei (Fig. 3B). No sequestration of ODNs in cytoplasmic vesicles was seen at any time, consistent with uptake by potocytosis (16, 17) rather than by endocytosis. Wells kept in parallel at 0°C showed minimal nuclear labeling. Wells incubated at 37°C for 30 min in the presence of 0.5 mM dinitrophenol in various experiments showed 70–80% inhibition of cell entry of oligomer, as measured in the formic acid extract of cell contents (see Fig. 1) after a 30-min incubation.

An undiminished number of silver grains was found within the cell nuclei when the labeled oligomer was added for 15 min at 37°C just after release of the thymidine block of DNA synthesis (Fig. 3C). This observation provided evidence that the radioactive label found in the nuclei did not represent DNA synthesis from extensive oligomer degradation to monomeric thymidine or dTMP units, followed by incorporation of the latter into nascent DNA. At the time of the thymidine block, there was also excess thymidine still present intracellularly, which would be expected to dilute the specific radioactivity of dTTP formed as a result of ODN degradation. In addition, at the end of the 30- to 60-min experiments, we found intracellular labeled oligomer in large amounts with much lesser amounts of labeled thymidine, dTMP, dTDP, and dTTP.

Of 120 nuclei in the synchronized cells that were assayed for presence or absence of silver grains, 110 were labeled and 10 were unlabeled, a 92% labeling. When the cells were unsynchronized, of 222 nuclei assayed, 119 were labeled and

103 were unlabeled, a 54% labeling. Fig. 3C shows multiple nuclear silver grains over two nuclei in thymidine-blocked cells, as well as several silver grains over the cytoplasm. Fig. 3D shows in unsynchronized cells several nuclei with abundant silver grains and adjacent nuclei with few or no silver grains. In both thymidine-blocked and unblocked conditions, some nuclei showed a concentration of silver grains just inside the nuclear envelope (Fig. 3E). Silver grains consistently appeared to be associated with euchromatin. There was, however, a general paucity of silver grains associated with nucleoli (Fig. 3F). Very few silver grains were seen outside of cells, while at the same time an abundance was present within a nucleus, verifying the cell-washing procedure (Fig. 3C). The "zero time" point also showed few or no grains outside the external cell membrane, although one grain may be seen already apparently associated with the nucleus (Fig. 3A). In unsynchronized cells in which there were no nuclear silver grains, there were also no grains in the cytoplasm, suggesting that the blockade to cell entry was at the
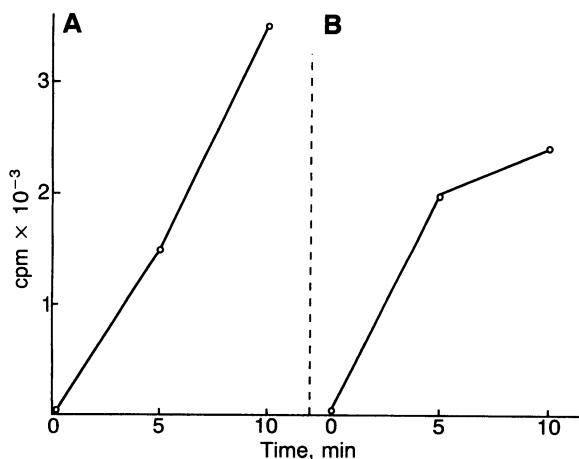

FIG. 2. (A) Linear uptake of 12-mer ODN (5'-end labeled, 36 µM external concentration) at 25°C into soluble intracellular components of confluent CEFs, shown by extraction with 1 M formic acid. Calculated uptake at 10 min was 3.4%. (B) Association of oligomer with cell components remaining after formic acid extraction, but soluble in 2.5% SDS. Plateauing begins at 5 min and continues thereafter, consistent with progressive saturation of intracellular ODN binding sites.
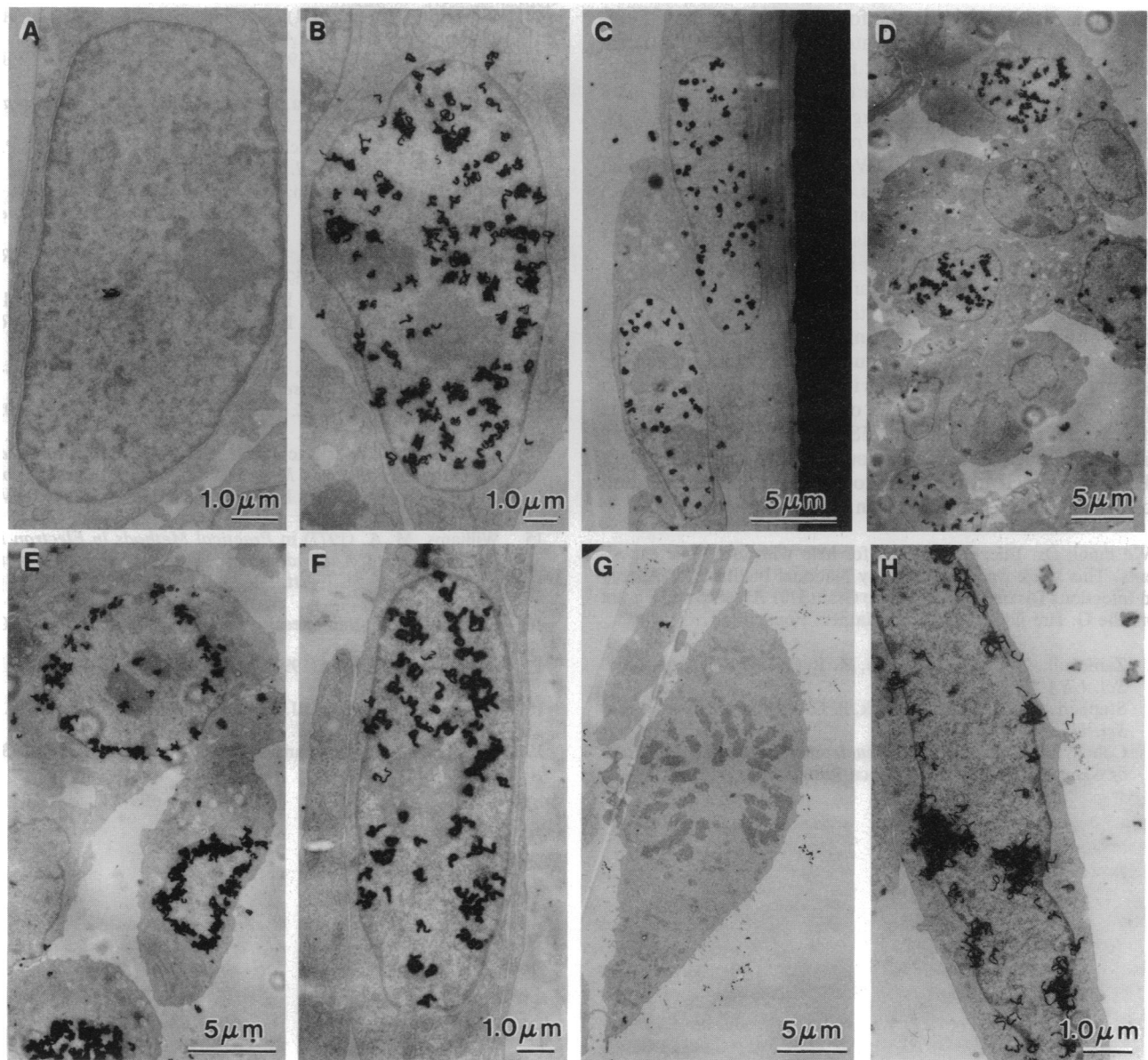
FIG. 3.   Uptake of ³H-labeled ODN (*A–G*) or [³H]thymidine (*H*). (*A*) HeLa cell "zero time," showing one silver grain already in nucleus, none in cytoplasm, and none outside the cell. (*B*) 3T6 cells in unsynchronized culture after 15 min of incubation at 37°C, showing one silver grain on external cell membrane, several grains separately located in the cytoplasm, and multiple grains in the nucleus. (*C*) Silver grains localized over the nuclei in synchronized 3T6 cells. Ninety-two percent of nuclei are labeled after 30 min of incubation at 37°C. (*D*) Unsynchronized 3T6 cells after 15 min incubation at 37°C. Approximately half of the nuclei are labeled. (*E*) Silver grains located just inside the cell nucleus in synchronized 3T6 cells after 15 min of incubation at 37°C. A similar distribution was found in unsynchronized cells (data not shown). (*F*) Exclusion of radioactivity from the nucleolus in labeled 3T6 cells after 15 min of incubation with ³H-labeled oligomer at 37°C. (*G*) Silver grains were consistently absent from the nuclei of dividing cells, seen here after 15 min of incubation with ³H-labeled oligomer at 37°C. (*H*) Discrete medusoid distribution of silver grains over nuclei of 3T6 cells after 15 min of incubation with [³H]thymidine at 37°C. Compare with diffuse distribution for ³H-labeled oligomer in *F*.

external cell membrane and related to the stage of the cell cycle. In cells undergoing mitosis, no labeling of condensed chromatin was seen (Fig. 3*G*).

Comparison of Rates of Uptake into 3T6 Cells of [³H]Thymidine, [¹⁴C]dTMP, and ³H-Labeled 11- to 17-mer ODN. The concentration of the above labeled compounds was adjusted to an incubation medium level of 0.67 μM. The rate of uptake into 3T6 cells grown to semiconfluence was determined in two duplicate wells for each compound at 0, 30, and 60 min of incubation at 37°C in 150 μl of DMEM without serum in 5% CO_2/95% air, in four-well Falcon dishes (1 cm² per well). In one experiment, an average of 92,000 cells per well was determined by Coulter counting of two duplicate wells. There was no detectable uptake of dTMP. The uptake of the

³H-labeled oligomer into the soluble cell extract of the 3T6 cells was comparable with that of the [³H]thymidine at the same molar concentration at 30 and 60 min, a surprising finding. In an hour's incubation at 37°C in an atmosphere of 5% CO_2/95% air, in DMEM without serum, and with an external medium concentration of ³H-oligomer of 0.67 μM, the internal concentration rose to 25–45% of the external concentration. The results demonstrate a rapid uptake of undegraded ODNs into cells, with a low residence time in the cytoplasm and a fast movement into the nucleus. The cell entry is energy-dependent (7) and consistent with a potocytotic mechanism (16, 17), although there may be other explanations. It is particularly active in cells traversing the S phase of the cell cycle.

Our data also give rise to the following suggestions. First, that for unmodified ODNs the rate of cell entry and maximal cell concentration are sufficient for a complementary base-pairing antisense mechanism to occur, without addition of further agents to facilitate passage through the external cell membrane. Second, ODNs pass rapidly out of as well as into cells (P.Z., unpublished data). We have previously detected the presence of oligonucleotides of unknown function, with rapid turnover of their phosphate groups, in hamster lung fibroblasts and tetrahymena cells, and have speculated that they may play a regulatory role in intracellular metabolism and may travel from one cell to another in a similar role (18). In this connection, it has recently been found (reviewed in ref. 19) that naturally occurring oligonucleotides play a regulatory role in the timing sequence and turnoff of genetic readout of developmental events in the worm *Caenorhabditis elegans*. Thus, oligonucleotides of varied functions, such as antisense, sense, tRNA, and 5S RNA for example, may conceivably be synthesized in one type of animal or plant cell, traverse cell membranes to another type of cell, and modify regulatory events therein.

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