Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 3474–3478, May 1989
Biochemistry

Characterization of oligonucleotide transport into living cells

(antisense/flow cytometry/fluorescence)

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Communicated by Henry Taube, December 23, 1988

ABSTRACT **Addition of antisense oligonucleotides to cell cultures has been used to specifically inhibit gene expression. We have investigated the mechanism by which oligonucleotides enter living cells. These compounds are taken up by cells in a saturable, size-dependent manner compatible with receptor-mediated endocytosis. Polynucleotides of any length are competitive inhibitors of oligomer transport, providing they possess a 5′-phosphate moiety. Using oligo(dT)-cellulose for affinity purification, we have identified an 80-kDa surface protein that may mediate transport. Knowledge of the oligonucleotide transport mechanism should facilitate the design of more effective synthetic antisense oligomers as potential clinical agents.**

When oligodeoxynucleotides [oligo(dN)s] complementary to the 5′ region of c-myc mRNA are added to cells in culture, c-myc protein synthesis is specifically inhibited (1–5). Furthermore, addition of antisense oligo(dN)s to cultures inhibits intracellular viral replication (6–8). These data show that exogenous addition to cell cultures of oligomers complementary to portions of mRNA coding for intracellular proteins is an effective technique for specific inhibition of gene expression and dissection of the physiological role of individual gene products. However, these findings contrast with the prevailing view that cells are impermeable to oligo(dN)s. To design more effective synthetic antisense oligo(dN)s, we synthesized oligomers of deoxythymidylic acid [oligo(dT)$_n$] compounds containing 5′-linked acridine and utilized these fluorescent derivatives, in conjunction with flow cytometric analysis, to examine the mechanism of cellular uptake of oligo(dN)s. These compounds, up to 20 bases in length, are taken up by cells in a saturable, size-dependent manner compatible with receptor-mediated endocytosis. Although neither nucleosides nor deoxyribose 5′ phosphate are competitors of uptake, polynucleotides of any length possessing a 5′-phosphate moiety competitively inhibit oligo(dN) transport. Using oligo(dT)-cellulose for affinity purification, we have identified an 80-kDa surface protein that may mediate oligo(dN) transport.

MATERIALS AND METHODS

Cells and Cell Culture. Most experiments were performed with the myeloid cell line HL60. Some investigations were carried out with the human Burkitt lymphoma cell line Daudi, the acute lymphoblastic leukemia T-cell line MOLT-4, and the murine myeloid line DA1. Cells were maintained under 5% CO_2/95% air in RPMI 1640 medium (BRL) containing 10% (vol/vol) fetal bovine serum.

Synthesis of Acridine-Labeled Oligo(dN)s and Quantitation of Intracellular Fluorescence by Flow Cytometry. Oligo(dT)$_n$s were labeled with 6-(phenylthio)-9-(ω-hydroxyalkylamino)-2-methoxyacridine by a modification (9) of the method of Asseline *et al.* (10). These derivatized oligo(dN)s emitted fluorescence at >520 nm when excited at 488 nm by an argon ion laser. Acridine-labeled oligo(dT)$_n$s (final concentration, 12.5 μM) were incubated with HL60 cells at 37°C. Cells were removed from the culture at various times, washed twice in phosphate-buffered saline (PBS), and resuspended in PBS; intracellular fluorescence was assessed by flow cytometric analysis with a FACStar flow cytometer (B. D. FACS Systems, Sunnyvale, CA) using excitation with the 488-nm line of an argon laser (300 mW). The presence of intracellular fluorescence was confirmed by fluorescence microscopy. In general, data are expressed as the median fluorescence of a cell population or as a percent change in the median fluorescence. These data were corrected for cellular autofluorescence and were calculated with software supplied with the FACStar instrument.

Oligo(dT)-Cellulose Affinity Chromatography. HL60 cells were surface-iodinated to a specific activity of 20–40 μCi (1 μCi = 37 kBq) per 10^6 cells by the lactoperoxidase method (11). Cells were lysed in 0.01 M Tris chloride, pH 7.5/0.144 M NaCl/0.5% Nonidet P-40/0.5% sodium dodecyl sulfate/0.1% Trasylol/1 mM phenylmethylsulphonyl fluoride/10 mM iodoacetamide. After 30 min on ice (with mixing every 10 min), the lysates were centrifuged at 10,000 × *g* for 10 min. Lysate prepared in such a manner was incubated with oligo(dT)-cellulose beads (Collaborative Research) and/or Con A-Sepharose beads (Pharmacia) at 4°C for 1 hr. In some cases, the lysates were preincubated with excess phosphorothioate oligo(dT)$_7$ before addition of the beads. After several washes in lysis buffer, the beads were resuspended in 4× Laemmli loading buffer (1) (containing 2-mercaptoethanol), boiled for 5 min, and pelleted. The supernatant was electrophoresed through an 8% polyacrylamide gel, which was then fixed, dried, and autoradiographed.

Determination of the Effect of NaN_3 on Oligo(dN) Internalization. 3′-End-labeled oligo(dN) (random 21-mer labeled with [^{32}P]ATP) was incubated with cells in the presence or absence of 10 mM NaN_3 for 1 hr at 4°C. An aliquot of cells was removed for scintillation counting, and the tubes were placed at 37°C for an additional hour. At this point, another aliquot was removed for determination of total cell-associated radioactivity; the remaining cells were pelleted and washed in 0.2 M glycine (pH 2.8) to strip off membrane-bound oligo(dN) and intracellular radioactivity was determined. That the radioactivity measured was due to labeled oligo(dN) and not exchanged ^{32}P was ascertained by including samples in which a 10,000-fold excess of unlabeled oligo(dN) was added to the incubation mixture. Cell-associated radioactivity obtained with these samples (<10% of the specific radioactivity) was subtracted from the other data.

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Abbreviation: oligo(dN), oligodeoxynucleotide.

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RESULTS

Acridine-Labeled Oligo(dN) Is Accumulated Intracellularly in a Temperature-Dependent Manner. Fig. 1 *Top* depicts a typical fluorescence histogram comparing cells incubated with no oligo(dN) to those incubated for 24 hr with either acridine-labeled oligo(dN) alone or in the presence of excess unlabeled oligo(dN). Intracellular localization of fluorescence was confirmed by fluorescence microscopy of similarly treated cells (Fig. 1 *Middle* and *Bottom*). When we examined the rates of accumulation of variously sized acridine-labeled oligo(dN)s we found that the accumulated intracellular fluorescence after incubation of HL60 cells with 12.5 μM acridine-labeled oligomers [ranging in size from oligo(dT)$_3$ to oligo(dT)$_{20}$] increased gradually, plateauing within $\approx$50 hr after addition of acridine-labeled oligo(dN) to the culture medium (Fig. 2*A*). This is in contrast to the 90 min required for ^{32}P-labeled oligo(dN) to attain maximal membrane binding at 4°C (data not shown). The maximal intracellular concentration of acridine-labeled oligo(dN) achieved was inversely proportional to its length, with oligo(dT)$_3$ > oligo(dT)$_7$ > oligo(dT)$_{15}$ > oligo(dT)$_{20}$. These data suggest that the flow cytometric technique described is monitoring true intracellular oligo(dN) accumulation and not the association of labeled oligo(dN) with the cell surface. A variable amount of acridine-labeled oligo(dN) accumulation was observed in several cell lines, including HL60, Daudi, MOLT-4, and DA1 (Fig. 2*B*). Whereas intracellular accumulation of unconjugated acridine was independent of incubation temperature (data not shown), significant intracellular accumulation of acridine-labeled oligo(dN) was only observed at 37°C (Fig. 2*B*). After 16 hr of culture in the presence of 0.25 μM oligo(dT)$_{12}$, $\approx$11% of the oligo(dN) was internalized. Furthermore, cellular fixation in 1% paraformaldehyde did not affect intracellular accumulation of unconjugated acridine but completely abrogated the accumulation of acridine-labeled oligo(dN) (Fig. 2*C*). Dead cells did not accumulate acridine-labeled oligo(dN) but did accumulate unconjugated acridine (data not shown). These data suggest that cellular uptake of acridine-labeled oligo(dN) is neither a passive process nor a result of intracellular accumulation of free acridine enzymatically cleaved from the oligo(dN).

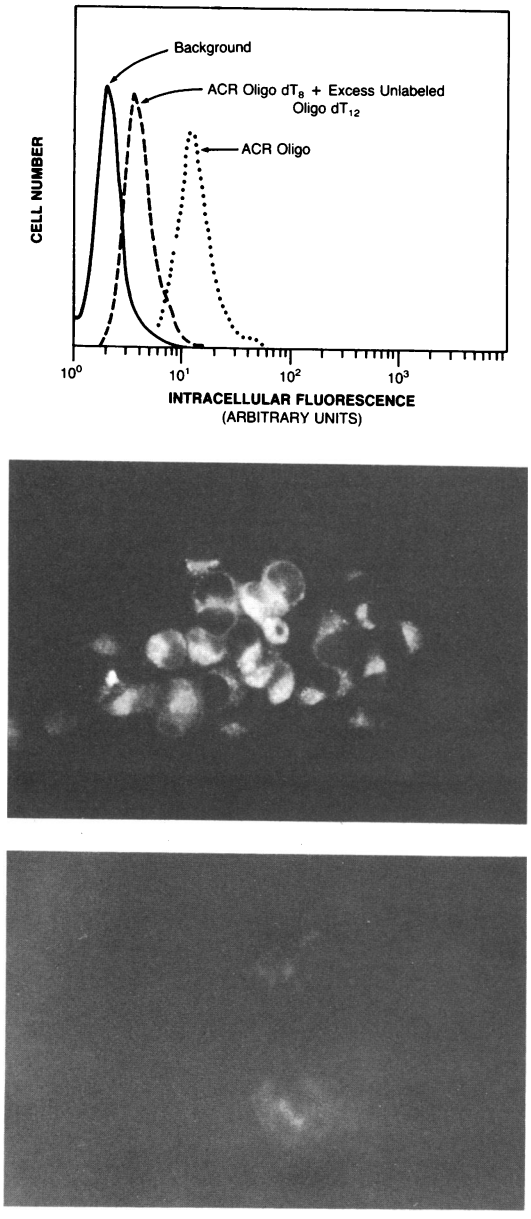

FIG. 1. Uptake of acridine (ACR)-labeled oligo(dN) by HL60 cells. (*Top*) Flow cytometric analysis of acridine-labeled oligo(dN) accumulation by HL60 cells. The *x* axis represents the logarithm of the amplified fluorescence signal. (*Middle* and *Bottom*) Fluorescence microscopy of HL60 cells incubated with 12.5 μM acridine-labeled oligo(dT)$_7$ for 16 hr without (*Middle*) or with (*Bottom*) excess (50 μM) unlabeled oligo(dN).

Cellular Uptake of Oligo(dN)s Is Saturable and Specifically Inhibited. Because incubation of cells with excess unlabeled oligo(dN) reduced the amount of acridine-labeled oligo(dN) accumulated (see Fig. 1 *Top*), we next investigated the saturability and specificity of the uptake process. First, we attempted to block the uptake of 12.5 μM acridine-labeled oligo(dT)$_7$ with various concentrations of unlabeled oligo(dG)$_{12}$, oligo(dA)$_{12}$, oligo(dT)$_{12}$, oligo(dC)$_{12}$, and Myc.1 (a 15-mer c-*myc* antisense oligodeoxynucleotide with 53% G+C content). There was a sequence-independent, concentration-dependent blockade of acridine-labeled oligo(dT)$_7$ cellular accumulation, with 60–80% inhibition in the presence of 25 μM unlabeled oligo(dN) (Fig. 3*A*; confirmed by fluorescence microscopy—see Fig. 1 *Middle* and *Bottom*). However, even 100 μM thymidine (or BrdUrd, data not shown) failed to inhibit acridine-labeled oligo(dT)$_7$ uptake (Fig. 3*A*). Since oligo(dN)s, but not nucleosides, inhibited uptake, we studied the inhibitory effect of ribonucleotides and deoxyribonucleotides (Table 1). All unlabeled deoxyribo- and ribonucleotides blocked intracellular accumulation of acridine-labeled oligo(dT)$_7$ in a concentration-dependent manner. Furthermore, the number of terminal phosphates was unimportant, as deoxynucleotide mono-, di, and triphosphates were equally effective (Table 1 and Fig. 3*B*). Finally, both plasmids (pCD) and yeast tRNA were also effective inhibitors of labeled oligo(dN)s uptake (Table 1).

Structural Specificity of Oligo(dN) Uptake. These data suggest that the phosphate moiety is essential for uptake. Since nucleotides exist as either nucleoside 3′-monophosphates or nucleoside 5′-monophosphates, we tested whether the position of the phosphate linkage was an important determinant of specificity. Nucleotides possessing a 5′-phosphate linkage were much more efficient competitors of oligo(dN) uptake than those with either a 2′- or 3′-phosphate linkage (Fig. 3*B*). Sugar phosphates alone, including deoxyribose 5′-phosphate were ineffective inhibitors, even at 100 μM (Fig. 3*C*). These data show that a single nucleoside 5′-monophosphate moiety is both necessary and sufficient for uptake inhibition.

This structural selectivity of oligo(dN) uptake led us to investigate the consequences of alteration of the phosphate moiety on competitive inhibition. Methylphosphonates are oligo(dN) derivatives in which the phosphate hydroxyl group is replaced by a methyl group (12). These compounds are resistant to nuclease digestion, lack ionizable groups, and possess increased hydrophobicity. Fig. 3*D* shows that a

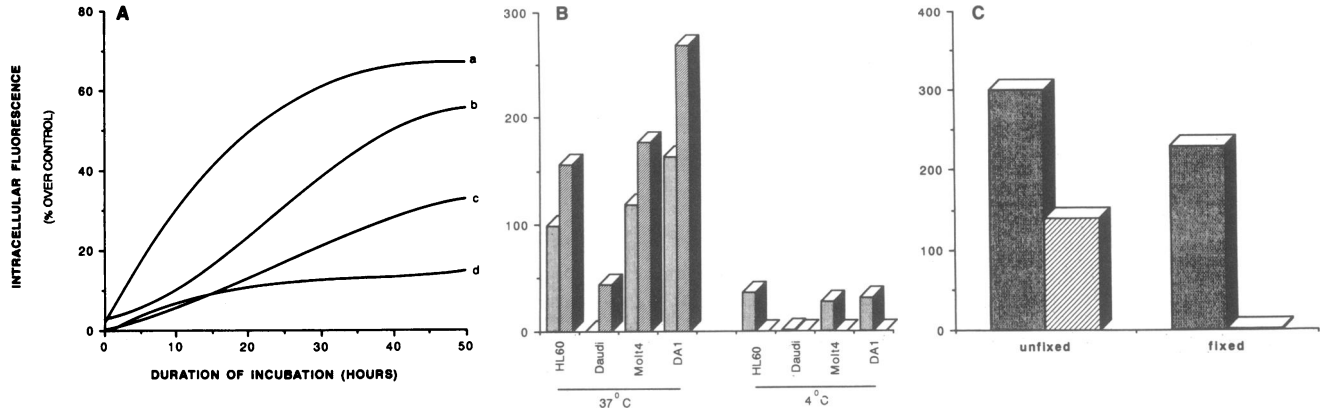

FIG. 2. (*A*) Time course of acridine-labeled oligo(dN) uptake; effect of oligomer length. The data are expressed as the median fluorescence of the population corrected for cellular autofluorescence ("control fluorescence"). Curves: a, cellular accumulation of oligo(dT)$_3$; b, oligo(dT)$_7$; c, oligo(dT)$_{15}$; d, oligo(dT)$_{20}$. (*B*) Oligomer uptake in four hematopoietic cell lines was measured at 4°C and 37°C. Measurements were made at 16 hr (left bar of each pair) and 24 hr (right bar of each pair). (*C*) Accumulation of acridine-labeled oligo(dT)$_7$ in cells fixed with paraformaldehyde. HL60 cells were incubated with 12.5 μM labeled oligo(dN) (hatched bars) or a saturated solution of unconjugated acridine (stippled bars) for 24 hr at 37°C. Where indicated, cells were fixed in 1% paraformaldehyde for 30 min at room temperature before incubation with oligo(dN) or acridine.

methylphosphonate oligo(dT)$_7$ did not block uptake of either acridine-labeled oligo(dT)$_3$ or oligo(dT)$_8$, whereas an unsubstituted oligo(dT)$_{12}$ efficiently inhibited uptake. On the other hand, substitution of one of the nonbridging oxygen atoms in

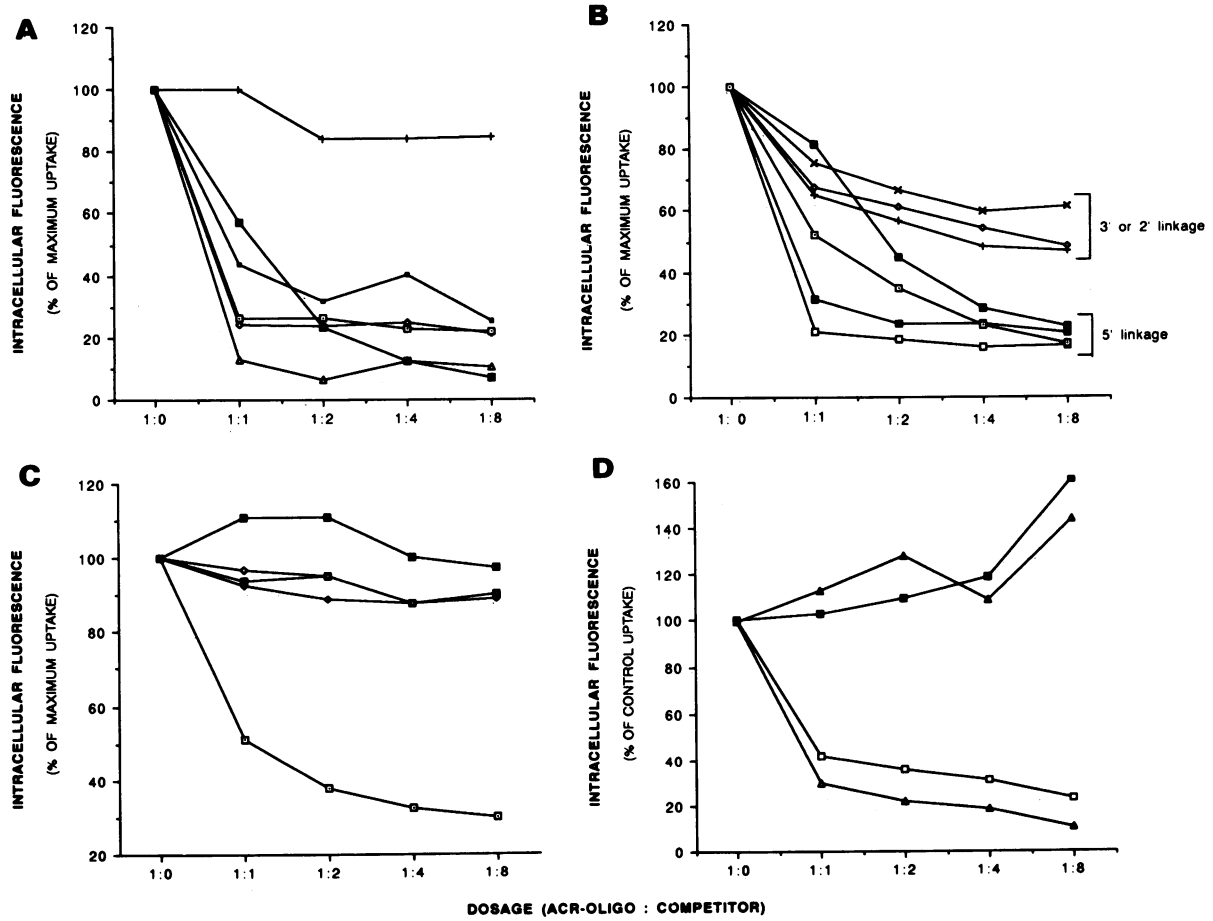

FIG. 3. Inhibition of acridine (ACR)-labeled oligo(dN) uptake. (*A*) Inhibition of oligo(dT)$_7$ uptake by thymidine (+), oligo(dG)$_{12}$ (■), oligo(dT)$_{12}$ (⊡), unlabeled oligo(dA)$_{12}$ (◇), oligo(dC)$_{12}$ (△), or c-*myc* antisense 15-mer (■). Cells were incubated with oligomers for 16 hr, the amount of uptake was determined, and data were expressed as the percentage of maximum uptake [cells incubated with acridine-labeled oligo(dT)$_7$ alone]. (*B*) Inhibition of oligo(dT)$_7$ uptake by ribonucleotides (mono- or diphosphates): 3′-CMP (×), 2′-CMP (◇), 3′-UMP (+), 5′-AMP (■), 5′-ADP (⊡), 5′-UDP (◘), and 5′-CDP (□). The *x* axis shows the ratio of labeled/unlabeled oligo(dN) concentrations. (*C*) Lack of inhibition of oligo(dT)$_8$ uptake by deoxyribose 5′-phosphate (■), mannose 6-phosphate (◇), fructose 6-phosphate (◘), and glucose 6-phosphate (◆). Inhibition by unlabeled oligo(dT)$_{12}$ (⊡) is presented as a control. (*D*) Inhibition by phosphorothioate oligo(dT)$_7$, but not by methylphosphonate oligo(dT)$_7$, of either oligo(dT)$_3$ or oligo(dT)$_8$ uptake: phosphorothioate + oligo(dT)$_3$ (□), phosphorothioate + oligo(dT)$_8$ (▲), methylphosphonate + oligo(dT)$_3$ (■), and methylphosphonate + oligo(dT)$_8$ (▲). The concentration of labeled oligo(dN) was 12.5 μM and the *x* axis represents the micromolar ratio of labeled oligo(dN) to competitor. Data are calculated and expressed as the percentage of control uptake [cells incubated with acridine-labeled oligo(dT)$_3$ or oligo(dT)$_8$ alone].

Table 1. Inhibition of acridine-labeled [oligo(dN)] uptake by nucleotides and related compounds

Test compound	Accumulation of acridine-labeled oligomers at dose ratio, % of control			
	1:1	1:2	1:4	1:8
dATP	71	63	50	49
dCTP	67	57	49	49
dGTP	66	57	52	52
dTTP	64	57	59	49
dAMP	89	75	53	42
dCMP	52	45	44	48
dGMP	75	52	54	50
dTMP	81	54	49	47
ATP	68	68	34	18
CTP	75	75	55	41
GTP	71	70	61	54
TTP	40	20	5	5
Plasmid	77	44	25	22
tRNA	82	77	50	37

Acridine-labeled oligo(dN)s were added to HL60 cells to a final concentration of 12.5 μM. Test compounds were added simultaneously with acridine-labeled oligo(dN)s. The dose ratio is the ratio of micromolar labeled oligo(dN)/inhibitor. The data are expressed as the percentage of accumulation of acridine-labeled oligo(dN) in the absence of test compounds.

each internucleotide phosphate linkage with a sulfur atom [phosphorothioate oligo(dN) (refs. 13 and 14)] did not affect charge and did not abrogate the ability to competitively inhibit oligo(dN) uptake (Fig. 3*D*). Thus, the ionic character of the oligonucleotide phosphate backbone may be critical for oligo(dN) utilization of this uptake mechanism. The fact that methylphosphonates apparently do not use this uptake process is consistent with the assertion of Miller, Ts'o, and colleagues (12) that these compounds are membrane permeable because of their enhanced lipophylic nature and, consequently, are passively transported across the cell membrane.

Identification of a Cell Membrane Oligo(dN)-Binding Protein. The inhibition of oligo(dN) uptake by nucleosides of defined structure suggested that there may be a cell-surface binding protein involved in transport. We used oligo(dT)-cellulose beads as a tool for affinity purification of a putative plasma membrane oligo(dN)-binding protein from surface-iodinated HL60 cells and identified an 80-kDa protein (Fig. 4). In lane 1 is the supernatant obtained from a lysate incubated with Con A-Sepharose beads. Con A did not inhibit oligo(dN) uptake (data not shown) but has been reported to bind the plasma membrane enzyme 5'-nucleotidase, a 130-kDa nucleotide-binding protein that catalyzes the cleavage of phosphate from nucleoside 5'-monophosphates (15). An HL60 cell lysate precleared on Con A-Sepharose was incubated with oligo(dT)-cellulose and analyzed in lane 2. Here only an 80-kDa band was observed. When labeled cell lysate was applied directly to oligo(dT)-cellulose, again only an 80-kDa protein was isolated (Fig. 4, lane 3). When the cell lysate was first preincubated with excess unlabeled phosphorothioate oligo(dT)$_7$ [a competitive inhibitor of oligo(dN) uptake (see Fig. 3*D*)] and then applied to oligo(dT)-cellulose as in lane 3, the 80-kDa protein was no longer detected (Fig. 4, lane 4). These results suggest that the 80-kDa protein identified by its affinity for oligo(dT)-cellulose plays a role in the cellular uptake of oligo(dN)s.

Oligo(dN)s Enter the Cell via Endocytosis. A striking feature of the dynamics of many cell-surface receptors is their involvement in a pathway of either constitutive or ligand-induced endocytosis in which receptors cluster in coated pits, enter the cell via clathrin-coated vesicles, and pass through

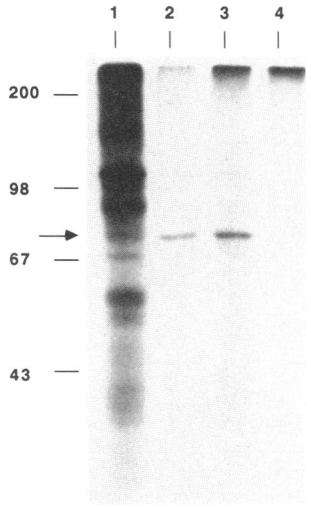

FIG. 4. Identification of a plasma membrane oligo(dN)-binding protein by oligo(dT)-cellulose affinity chromatography. Lanes: 1, material bound to Con A-Sepharose; 2, material passed first over Con A-Sepharose and then over oligo(dT)-cellulose; 3, material applied directly to oligo(dT)-cellulose; 4, same as lane 3 except that the cell lysate was preincubated with excess unlabeled phosphorothioate oligo(dT)$_7$ before incubation with oligo(dT)-cellulose.

an acidified endosomal compartment before either (*i*) fusing with lysomes and being degraded or (*ii*) recycling to the cell surface (16–18). In attempting to determine whether oligo(dN) uptake is an endocytotic process, we used several characteristics of endosomal transport: (*i*) the ability of chloroquine, a lysosomatropic agent, to prevent degradation of endocytosed ligand and receptor (19); (*ii*) the ability of NaN_3 to inhibit internalization of endocytotic vesicles (20); and (*iii*) the fact that fluorescently labeled ligands internalized via endocytosis typically display a punctate pattern of intracellular fluorescence (17). Addition of 80 μM chloroquine to cultures containing 12.5 μM acridine-labeled oligo(dT)$_8$ or oligo(dT)$_{20}$ resulted in markedly increasing intracellular fluorescence when cells were analyzed at 24 hr (Fig. 5*A*). However, chloroquine altered neither the initial rate of oligo(dN) uptake nor the oligo(dN) binding at 4°C (data not shown), consistent with chloroquine-induced inhibition of intracellular oligo(dN) degradation or recycling.

We tested whether NaN_3 inhibited internalization of oligo(dN) and found that, although incubation with NaN_3 did not affect initial oligo(dN) binding at 4°C (data not shown), the total (as well as intracellular) radioactivity measured after 1 hr at 37°C was significantly reduced in the presence of 15 mM NaN_3 (Fig. 5*B*). Cells incubated with NaN_3 contained less intracellular radioactivity (14% of total cell-associated radioactivity was intracellular in NaN_3-treated cells compared with 34% in control cells), again consistent with an endocytotic uptake process. Finally, cells incubated with acridine-labeled oligo(dN) showed a punctate pattern of intracellular fluorescence, blockable by incubation with excess unlabeled oligo(dN) (Fig. 1 *Middle*). These results support the hypothesis that oligo(dN) uptake is an endocytic process. When extracellular oligo(dN) was removed, the level of intracellular oligo(dN) declined (Fig. 5*C*). A similar phenomenon has been reported for the endocytic uptake and recycling of α_2-macroglobulin by NRK cells (21) and transferrin in several cell types (22–24).

DISCUSSION

We have demonstrated that oligo(dN)s are actively transported across the plasma membrane in a temperature-dependent, saturable, and structurally specific manner via an

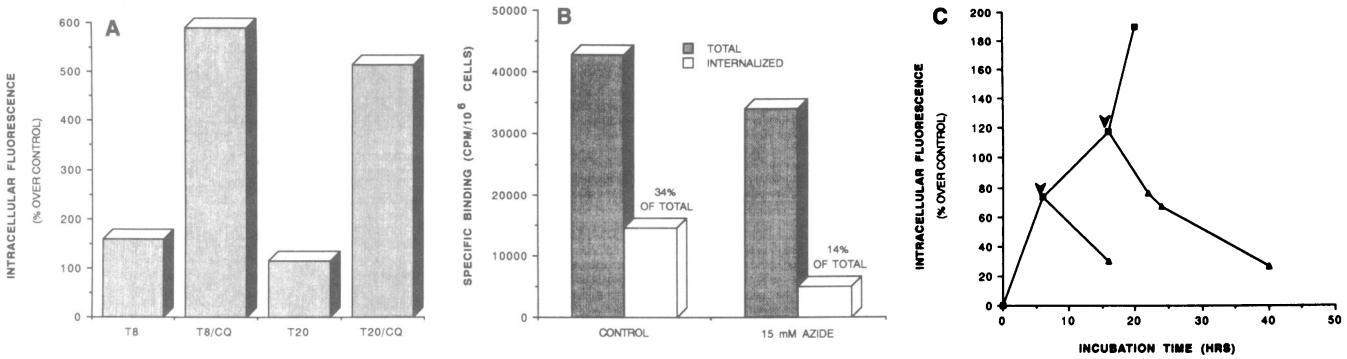

FIG. 5. (*A*) Effect of chloroquine (80 μM) on oligo(dT)$_8$ and oligo(dT)$_{20}$ uptake by HL60 cells. Chloroquine was added with the oligo(dN)s, and intracellular fluorescence was determined 16 hr later. (*B*) NaN_3 inhibits internalization of ^{32}P-labeled oligo(dN), which was incubated with cells in the presence or absence of 15 mM NaN_3. (*C*) Decrease in intracellular concentration of acridine-labeled oligo(dT)$_8$ after its removal from the extracellular medium. The oligo(dN) was removed by washing the cells twice in PBS at the times indicated by arrowheads. The cells were then resuspended at the same concentration in fresh medium, and intracellular fluorescence was monitored at the times indicated. ■, Unwashed samples (controls); △, washed samples.

endocytic process. This transport may involve binding of oligo(dN) to an 80-kDa plasma membrane protein. Other plasma membrane nucleotide-binding proteins have recently been identified. These include a protein of unknown size that transduces a rise in cytoplasmic free calcium level upon ATP binding (25) and a protein of 30 kDa that has been reported to bind high molecular mass (i.e., plasmid) DNA (26). The uptake mechanism described here does not appear to be mediated by either of these proteins. First, although ATP stimulated an increase in cytoplasmic free calcium in HL60 cells, pretreatment of the cells with oligo(dN) did not block the ATP effect, nor did the oligo(dN)s themselves stimulate a change in the cytoplasmic free calcium concentration (data not shown). Furthermore, not all nucleotides tested were effective stimulators of a calcium response, whereas all were equally effective competitive inhibitors of acridine-labeled oligo(dN) uptake. Second, the high molecular weight DNA 30-kDa binding protein described by Bennet and colleagues is not inhibited by either tRNA, oligo(dN)s or mononucleotides, but DNA binding is inhibited by heparin (26). Although the ODN uptake process we have described was inhibited by single nucleotides as well as by tRNA and plasmid DNA, it was not affected by heparin [even at a 10-fold greater concentration than that shown to be maximally effective by Bennet *et al.* (26)]. Most important, the fact that oligo(dN)s do not block the binding of high molecular mass DNA to the 30-kDa protein, even when added at 1000-fold greater concentration (26), strongly suggests that this is not the protein mediating oligo(dN) transport.

Our results clarify how antisense oligo(dN)s added extracellularly can enter cells to specifically inhibit gene expression, although the mechanism by which they are released to the cytosol remains to be determined. Perhaps the majority of the oligo(dN) is in fact degraded in lysosomes with only a small portion escaping to the cytosol. This would be consistent with the tremendous excess of oligo(dN) that must be added extracellularly to achieve inhibition of gene expression. On the other hand, the data in Fig. 5*C* suggest that, like transferrin and α_2-macroglobulin, internalized oligo(dN) may be recycled to the cell surface. Endocytosed ligands that are degraded in lysosomes are dissociated from their receptor at acid pH, while those ligands that are recycled to the cell surface remain associated with their receptor under these conditions (22–24). Studying the binding affinity of oligo(dN) for its receptor at acid pH will help to clarify the intracellular trafficking of this ligand.

Whether there is a physiological role for oligo(dN) transport is unknown. We may, nevertheless, utilize our understanding of the properties of the uptake process to design oligo(dN)s that are transported more efficiently and are more resistant to degradation. To this end, use of acridine-labeled oligo(dN)s, in conjunction with flow cytometric analysis, should prove to be a useful tool for studying, both qualitatively and quantitatively, antisense oligo(dN) uptake by living cells.

This work was supported in part by U.S. Army Medical Research Command Grant DAM17-PP-7801 to J.S.C.

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