

Characterization of hybridization between synthetic oligodeoxynucleotides and RNA in living cells

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Received October 10, 1995; Revised and Accepted November 16, 1995

ABSTRACT

Cells internalized synthetic oligonucleotides (oligos) in culture. The hybridization of these molecules to target RNA in the living cell was subsequently detected and characterized after fixation of the cells, with or without previous detergent extraction. Hybridized oligo was distinguished from free oligo in the cell using an *in situ* reverse transcription technique. This assay exploited the ability of the hybridized oligo to prime synthesis of a specific cDNA strand; unhybridized oligo present in the cell could not act as a primer for reverse transcription. Phosphorothioate and fluorochrome-labeled phosphodiester oligo dT were found to enter cells rapidly and hybridize to poly (A) RNA within 30 min. Hybrids containing phosphorothioate oligo dT were detectable in cells after up to 4 h of efflux time. Phosphodiester bonded oligo dT containing covalently-linked fluorochromes appeared more stable in the cell than unmodified phosphodiester oligo dT; hybrids containing these oligos could be detected in cells as long as 18 h after efflux began. The *in situ* transcription assay was also sensitive enough to detect hybridization of anti-actin oligos to actin mRNA in the cell. It is probable, therefore, that this assay can be used to help assess the efficacy of antisense oligos by their hybridization to a target mRNA in cells or tissues; hybridized oligos are more likely to induce a specific antisense effect. Additionally, this assay will help to identify probes that would be useful as stable hybridization tags to follow RNA movement in living cells.

INTRODUCTION

Antisense oligodeoxyribonucleotides (oligos) complementary to particular mRNAs can specifically alter the expression of target mRNAs in a variety of cell types; this is the basis for antisense gene therapy (1). It is thought that the antisense oligomer hybridizes to its target mRNA and prevents translation either by physically blocking ribosome movement along the mRNA or by inducing RNase H cleavage of the mRNA/oligo hybrid, thereby destroying the coding function of the mRNA. Although little direct evidence is available, experiments in which oligos are

microinjected into cells support these hypotheses. For instance, fluorescent energy transfer experiments demonstrate hybridization between exogenous sense and antisense oligos injected into cultured mouse cells (2). Also, anti-*veg-1* oligos injected into *Xenopus* oocytes induce cleavage of *veg-1* mRNA (3). However, microinjected oligos have been shown to accumulate primarily in nuclei, while oligos used for antisense treatments are usually passively taken up by cells or tissues and accumulate in the cytoplasm and sometimes the nucleus (4-6). In some cases, antisense oligos internalized in this way might be sequestered and degraded in lysosomes and thus never gain access to their intracellular targets (7-9). Additionally, although there is ample evidence that antisense oligos inhibit specific protein synthesis in cells, sometimes oligos of various non-complementary sequences also inhibit protein synthesis (7,10,11). This suggests that oligo treatment may effect inhibition in ways other than hybridization to target sequences. For these reasons, it is important to define the mechanism of inhibition by antisense oligos in each experiment. To do this, a method is desirable specifically to detect and measure the amount of hybridized oligo present in cells after antisense treatment.

In situ hybridization studies have shown that some mRNAs are concentrated in discrete regions within the cell (see 12 for review). For example, some maternal mRNAs become localized to particular subcellular regions during embryogenesis (e.g. 13-15), and in somatic cells, β -actin mRNA is found localized to the leading edge of fibroblast lamellipodia (16). However, in these studies, the nature of RNA movement within the cell was surmised after fixation of the cell. Antisense oligos could be used as hybridization tags to follow the movement of target RNAs in living cells. Identification of oligo modifications and hybridization conditions that allow the formation of oligo/RNA hybrids that are stable in the living cell, coupled with the development of imaging methods to track these hybrids *in vivo*, could provide a basis for real time analysis of intracellular RNA mobility.

We describe here the detection and characterization of intracellular hybridization between antisense oligos and their target RNAs after internalization by cultured muscle cells. The poly (A) tail on most mRNAs (17), present at high abundance (10^5 copies per cell), was first used as a homopolymer target for antisense oligo (dT) probes. The low complexity and high concentration of this target provided for direct measurement of *in vivo* hybridization parameters. Additionally, *in vivo* hybridization

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of antisense oligos to a specific mRNA, β -actin mRNA, was detected using this assay. Phosphorothioate-modified oligos (S-oligos), which are more resistant to both external and intracellular nucleases (18), as well as fluorescently labeled phosphodiester-bonded oligos (fl-O-oligos), were used as antisense probes in these experiments.

MATERIALS AND METHODS

Oligodeoxynucleotides

Oligos were synthesized using an Applied Biosystems 394 DNA Synthesizer. Fluorochromes were covalently linked to oligos at specific internal aminated thymidines and fluorochrome-conjugated oligos purified through two Sephadex G-50 columns before use (19). ST30 is a 30 nucleotide (nt) homopolymer of thymidine where sulfur replaces one oxygen at every phosphodiester bond (S-oligo). This homopolymer was labeled at positions 2 and 29 using Texas Red (Molecular Probes, ST30tr). S-oligo dA served as controls for ST30 and ST30tr (designated SA30 and SA30tr respectively). The O-oligo, T43tr, is a 43 nt homopolymer of thymidine with Texas Red covalently linked to positions 2, 12, 22, 32 and 42. This fluorochrome distribution provides the best quantum efficiency and protection against quenching (K. L. Taneja, unpublished). A43tr is the comparable oligo dA control for T43tr; T43f and A43f are identical to T43tr and A43tr, respectively, except that fluorescein (Molecular Probes) is substituted for Texas Red. Unlabeled O-oligos used to test the effect of the fluorochrome moiety included a 40 nt poly dT (T40a) and poly dA (A40a) with an amino group at identical positions to T43f and unmodified 55 nt poly dT (T55) and 55 nt poly dA (A55). Anti-actin oligos used were 18mer phosphorothioates complementary to 3'-UTR sequences unique to β -actin mRNA. These oligos are described in Kislauskis *et al.* (20) as C- and D-. Control oligos were the reverse antisense sequence to C- (C^{rev}, synthesized in the opposite 5' to 3' orientation compared to antisense oligo C- so that it contains the same bases in the reverse order as the antisense oligo) and X, a phosphorothioate oligo of random sequence the same length and GC content as C-, with no known homologies to chicken sequences. Oligo concentrations were calculated assuming 1 OD₂₆₀ unit = 33 μ g/ml.

Cell culture and oligo uptake

L6 rat muscle cells (American Tissue Culture) were cultured using standard techniques in Dulbecco's modified Eagle's media (DMEM) containing 10% fetal bovine serum (FBS, heat-inactivated). Primary cultures of chick embryo fibroblasts (CEFs) were prepared as described (21) and grown in Opti-MEM, 10% FBS. Cells to be treated with oligos were trypsinized and plated at 25 000 cells per 12 mm round glass coverslip (boiled previously in 0.1 N HCl, rinsed and autoclaved, Fisher), in 24-well dishes (Falcon) containing DMEM plus 10% FBS. After growing for 20–28 h at 37°C in 5% CO₂, rat cells were rinsed three times in serum-free DMEM and then incubated with 0.1 μ M oligo in 250 μ l serum-free DMEM for 2 h at 37°C, 5% CO₂ unless otherwise noted. For efflux experiments, oligo-containing media was removed, cells were rinsed three times with serum-free DMEM and then re-incubated with 250 μ l serum-free DMEM without oligo for varying times. Anti-actin oligos were incubated at 8 μ M with CEF cells for 4 h in Opti-MEM plus 10% FBS (20).

Cell extraction and fixation

In some experiments, cells on coverslips were fixed directly in 4% formaldehyde in 1 mM KH₂PO₄, 10 mM Na₂HPO₄, 0.137 M NaCl, 2.7 mM KCl, pH 7.0 (PBS) and 5 mM MgCl₂. For most *in situ* transcription (IST) experiments, cells were permeabilized by Triton extraction before fixing; this procedure also removes soluble molecules (including soluble oligo). Cells were washed in CSK buffer (0.3 M sucrose, 0.1 M KCl, 5 mM MgCl₂, 10 mM PIPES, pH 6.9, 2 mM EGTA, 1 μ g/ml leupeptin (Sigma) and 1 μ g/ml trypsin inhibitor (Sigma) at 4°C and then extracted with 0.5% Triton in CSK buffer for 90–120 s. Extractions were routinely done at 4°C where maximal RNA retention (and hybridization signal) is observed (22). At this temperature, microtubules may dissociate, so cells were also extracted at room temperature and analyzed as described below. No obvious difference in the distribution of signal was observed. After washing with CSK buffer, cells were fixed with 4% formaldehyde, 0.1% glutaraldehyde, 5 mM MgCl₂ in PBS for 15 min at room temperature. Coverslips were then rinsed twice with 70% ethanol and stored in same at 4°C for up to 3 months.

Modified *in situ* transcription (IST)

Cells exposed to various oligos *in vivo* and fixed as described above were washed in PBS for 10 min, and then twice in 150 mM NaCl, 15 mM Na citrate, pH 7.0 (SSC) for 10 min each time. Samples were then treated with AMV reverse transcriptase (Promega) essentially as described (22,23) except that the buffer supplied by Promega was used. Digoxigenin (dig) labeled dUTP (Boehringer-Mannheim) was used at 50 μ M in addition to all four unlabeled deoxynucleotides (Pharmacia) at 250 μ M in the reaction mixture. Label was visualized using either sheep antidigoxigenin antibodies linked to 1 nm gold particles (SAD1, Goldmark Laboratories) followed by silver enhancement (Amersham) (22) or antidigoxigenin Fab fragments linked to alkaline phosphatase followed by bromochloroindolyl phosphate/nitroblue tetrazolium color visualization (24).

In some control experiments, oligos were allowed to hybridize *in situ* to RNA in fixed cells as described (22,23) and then hybridization was detected using IST as described above. Formamide was omitted when S-oligos were used *in situ*, because S-oligo/RNA hybrids have a lower T_m than O-oligo/RNA hybrids (25).

Microscopy and Image Analysis

Coverslips containing cells to be viewed were mounted in Vectashield (Vector Labs) containing 0.1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI). Silver stained or fluorescent cells were viewed and photographed using a Nikon Microphot SA equipped with bright field and fluorescence optics and a 35 mm camera. Fluorescent cells were optically sectioned using a stage equipped with a stepping motor. Images of individual sections captured using a Photometrics CCD camera were corrected to remove field intensity variations and background fluorescence (26). For fluorescence quantitation, 2D images captured with a Photometrics CCD camera were visualized on a monitor and digitized areas of signal individually segregated for quantitation (26). Software used in the above analyses was provided by the Biomedical Imaging Center at University of Massachusetts Medical School. We also used a Discovery™ instrument to automate quantitation

of silver stain and fluorescent signal (Becton Dickinson Cellular Imaging Systems, San Jose, CA). To quantitate silver staining in treated cells, a microscopic field image was captured with a Xillix Microimager 1400 gray-scale camera using a 500 nm interference filter in absorbance mode. Cells were automatically identified using standard image processing routines to segment objects and morphological filters to eliminate artifacts. A field image was captured simultaneously using a fluorescent light source with a DAPI filter set and superimposed on the processed bright field image to verify that segmented objects were cells. Optical density of each silver stained cell was then automatically calculated and images captured for later inspection. Fluorescence in cells containing Texas Red or fluorescein labeled oligos was also measured automatically using the appropriate filters and similar segmentation programs. Raw data (mean fluorescence/cell or A_{500}/cell) was converted to percent maximal signal/cell in any given experiment so that data from separate experiments could be averaged. Error bars represent standard error of the mean and are smaller than the symbols if not shown.

RESULTS

Oligonucleotide uptake

Initial experiments used digital imaging microscopy to characterize the uptake of fluorescently-labeled phosphorothioate oligos (fl-S-oligos) by L6 rat cells. Varying amounts of Texas Red labeled poly dT (ST30tr) or dA (SA30tr) fl-S-oligos were added to cells growing on glass coverslips and cells were incubated for varying amounts of time. Cells were washed, formaldehyde-fixed and intracellular fluorescence analyzed by digitizing the analog microscopic image (see Materials and Methods). Intracellular fluorescence increased with either increasing extracellular oligo concentration or increasing incubation times and approached saturation when incubated at oligo concentrations of 10 μM for 2 h (Fig. 1a and b). Fl-S-oligo dT showed a punctate perinuclear distribution with additional diffuse fluorescence throughout the cell (Fig. 1c). Optical sections showed that the majority of fluorescent signal was internalized and not merely adhering to the outside of the cells (Fig. 1e). Nuclei were labeled more intensely than cytoplasm in ~30% of the cells (e.g. Fig. 1e). When fl-S-oligo dA was incubated with cells under these same conditions, intracellular fluorescence was about half of that observed with fl-S-oligo dT (Fig. 1a and b) but a similar punctate cytoplasmic distribution of label was present (Fig. 1d).

Labeled fl-S-oligos with different fluorochromes (fluorescein or cy3) showed labeling patterns similar to those described above and similar results were obtained in a different cell line (human fibroblasts) exposed to ST30tr or SA30tr (not shown). We conclude that the nature of the fluorochrome label does not appreciably affect the cellular compartments labeled and that S-oligo dT may be sequestered more effectively than S-oligo dA in these cell types (similar results have been obtained in other cell types, see 27–29).

As a result of these experiments, a standard incubation time of 2 h and concentration of 0.1 μM were chosen for further experimentation. Under these conditions, extracellular oligo concentration was at least 1000-fold in excess of the target poly (A) RNA concentration and intracellular fluorescence was reproducible. Cell morphology and growth characteristics appeared normal for at least 24 h after oligo treatment. At high (50 μM)

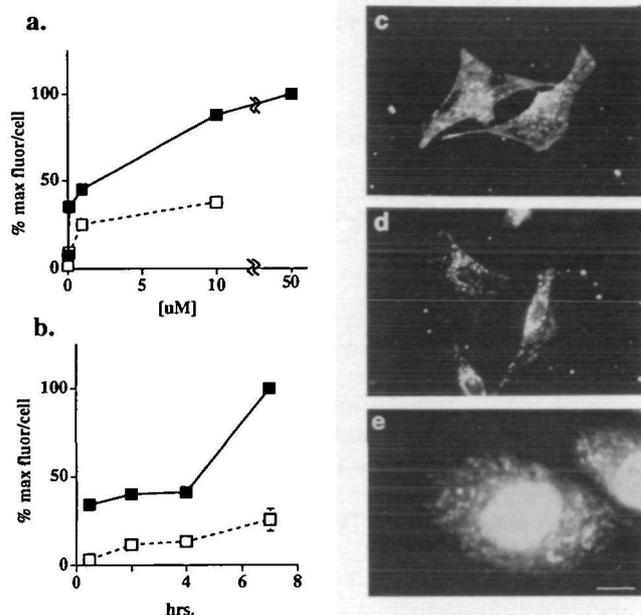


Figure 1. Uptake of fluorescently-labeled S-oligo dT and dA by L6 cells. Cells were incubated with ST30tr (■) or SA30tr (□) in serum-free media, fixed with formaldehyde and analyzed to quantitate intracellular fluorescence (see Materials and Methods). Plots show fluorescence/cell. (a) 0.01–50 μM oligo for 2 h; (b) 0.1 μM oligo for 30 min–7 h; (c) micrograph showing typical distribution of fluorescence after incubation with 0.1 μM ST30tr for 2 h; (d) same as (c) except with SA30tr. (d) was exposed longer than (c) so that labeling patterns could be compared; (e) digitized image of fluorescence in an optical section through the center of an L6 cell grown in 10 μM ST30tr for 4 h. Bar = 20 μm in (c) and (d), 5 μm in (e).

concentrations, vacuolation and cell death sometimes occurred, most frequently with S-oligo dA.

Antisense oligos added to cells *in vivo* prime reverse transcription *in situ*

We postulated that the uptake of the S-oligo dT probe by live cells would result in hybridization to cellular poly (A) *in vivo*. We then reasoned that the resulting oligo-RNA hybrid would be capable of priming elongation by reverse transcriptase (RT) *in situ*. Because RT extends a DNA primer hybridized to an RNA template, only hybridized oligos will prime incorporation of labeled nucleotide into new DNA to give a measurable signal *in situ*. This detection procedure modifies previous *in situ* reverse transcription (IST) assays (22,23,30) by allowing the primer to hybridize *in vivo* first and then assaying the hybridization *in situ* after permeabilization and fixation of the cell. Digoxigenin (dig) labeled nucleotides were used in the RT reaction mixture and after incorporation were detected by SAD1 antibodies linked to 1 nm gold particles. The gold was then enhanced by silver deposition (22).

Figure 2a shows that ST30 taken up by cells *in vivo* can act as a primer for the reverse transcription reaction *in situ*. Cytoplasmic signal was reproducibly observed when cells were incubated with S-oligo dT for 2 h *in vivo*, and then Triton extracted, fixed and subjected to IST. Signal resulting from elongation of the transcripts was detected primarily in the perinuclear region of the cytoplasm, with ~30% of cells also showing nuclear signal. The

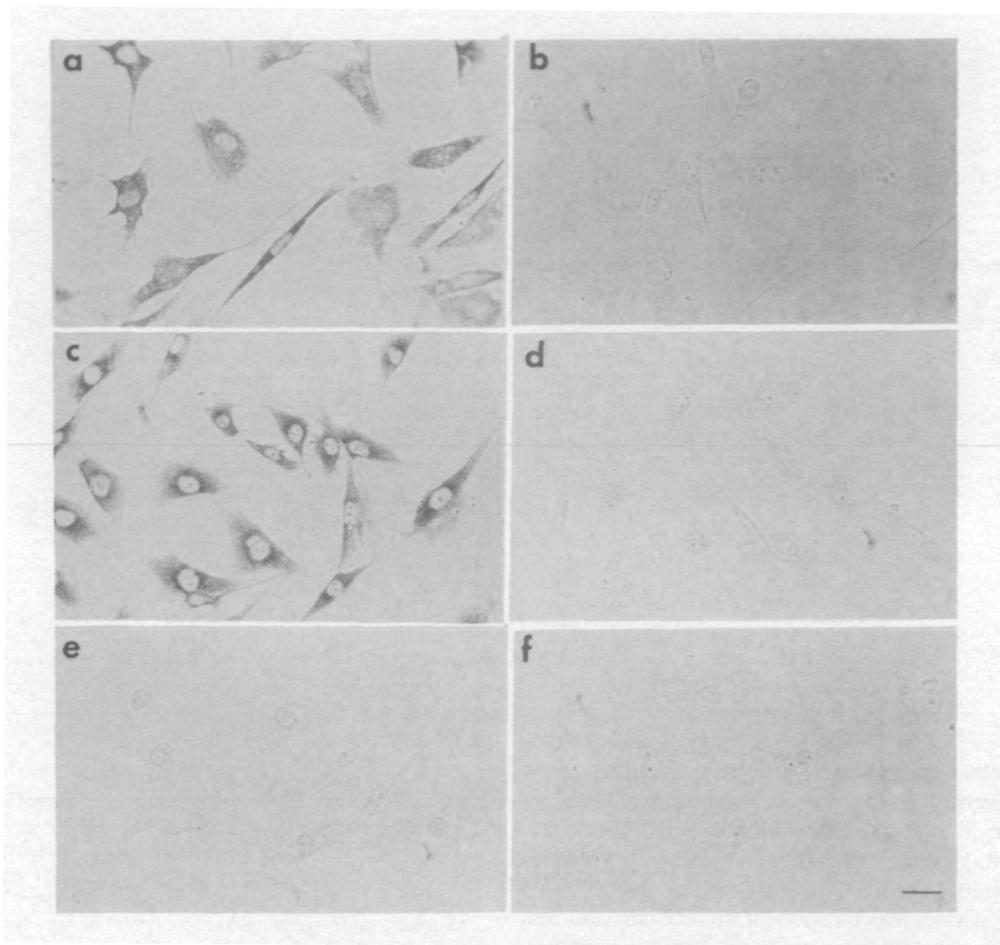


Figure 2. Modified *in situ* transcription (IST) used to detect hybrids formed between S-oligo dT and poly (A) RNA *in vivo*. (a) Cultured cells were incubated in serum-free media containing 0.1 μ M ST30 for 2 h, Triton extracted and fixed in formaldehyde/glutaraldehyde. IST was performed and incorporated dig-dUTP was detected using silver gold enhancement as described in Materials and Methods; (b) same as (a) except SA30 was used; (c) ST30 was hybridized *in situ* to untreated cells after fixation and then detected using IST as above; (d) same as (c) except SA30 was used *in situ*; (e) cells were incubated with ST30 *in vivo* as in (a) but IST was performed with heat-inactivated RT mix; (f) IST treated cells with no added oligo. Phase contrast micrographs. Bar = 20 μ m.

intracellular distribution pattern detected is similar to that obtained when ST30 is instead hybridized *in situ* to untreated cells after fixation and then subjected to IST (Fig. 2c). Controls showed that no reverse transcription was seen in the presence of primer when RT was heat inactivated before IST (Fig. 2e) or in the absence of primer (Fig. 2f). Likewise, use of S-oligo dA (SA30) as a primer, either before (Fig. 2b) or after (Fig. 2d) fixation, did not result in signal. These experiments indicated that ST30 formed hybrids with cellular poly (A) RNA *in vivo* and that these hybrids could be detected specifically by exploiting their ability to prime synthesis of labeled transcripts by RT *in situ*. Similar results were obtained when cells were treated with the fluorescently-labeled analogs of ST30 or SA30 used in Figure 1 (fluorochrome is not linked to the terminal 3' nucleotide so elongation is not inhibited).

Substantial silver stain was also observed when the above experiments were repeated using labeled dCTP, rather than dUTP, as substrate for IST (data not shown), indicating that RT could copy mRNA sequences (containing G residues) upstream of the poly (A) tail. The intracellular distribution of silver stain was similar to the perinuclear distribution of hybridization signal observed using digoxigenin dUTP as label (Fig. 2a).

Hybridization can be detected in unextracted cells

As has been reported previously for IST (22), signal from the modified IST performed here was highest when cells were permeabilized by Triton extraction and then fixed in paraformaldehyde/glutaraldehyde. The majority of poly (A) RNA is strongly associated with the cytoskeleton and is detected in the Triton-insoluble fraction of the cell using IST or *in situ* hybridization (ISH) techniques (22,26). Detection by IST or ISH of poly (A) RNA gives similar patterns in extracted and unextracted cells (22,26). Likewise, priming of the RT reaction was also detected after *in vivo* hybridization in unextracted cells, although the signal was sometimes less intense. Figure 3a shows a field in which cells exposed to ST30tr for 2 h, fixed directly in 4% formaldehyde and subjected to IST as described. Silver stained cells exhibit an intracellular distribution of signal that correlates with the perinuclear distribution pattern observed in cells that were Triton extracted before fixation (e.g. Fig. 2a). Therefore we conclude that Triton extraction did not alter the distribution of poly (A) RNA in a way that affects the oligo hybridization pattern when compared to unextracted cells.

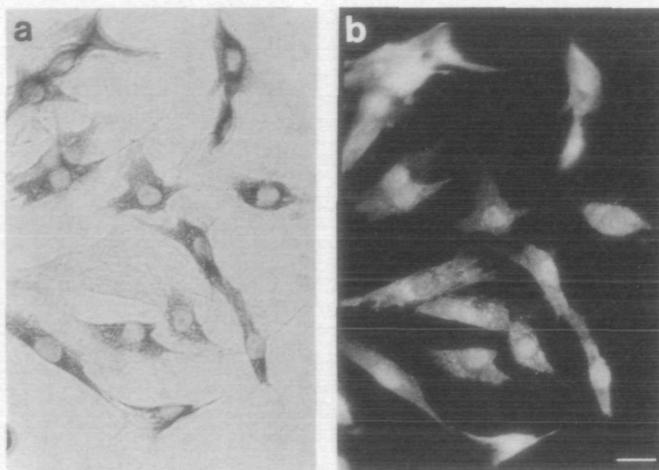


Figure 3. Detection of hybridization in unextracted cells. L6 cells were incubated with 0.1 μM ST30tr for 4 h, formaldehyde-fixed directly and subjected to IST. (a) Cells showing silver stain that represents hybridization of ST30tr to poly (A) RNA; (b) same cells showing fluorescent signal from internalized ST30tr. Bar = 20 μm .

Because these cells were not fixed with glutaraldehyde, which induces autofluorescence, we were able to visualize the fluorescent signal from ST30tr in this same population of cells. This signal represented the cellular distribution of total ST30tr, both hybridized and unhybridized. In general, cytoplasmic fluorescent signal overlaps with the perinuclear location of silver stain and less fluorescent signal or silver stain is seen in the extreme periphery of the cells. However, some cells and/or nuclei that contained oligo (as detected by fluorescence) did not show hybridization signal. (Compare two unlabeled cells in the middle of Figure 3a to the fluorescent pattern in 3b.) It is possible that differences in both plasma and nuclear membrane permeability during the cell cycle affect the ability of IST reagents to diffuse freely into cells and give detectable signal when cells are not detergent treated (see 4,22,31).

Fluorescently-labeled phosphodiester poly dT oligos also hybridize to intracellular RNA *in vivo*

We tested the ability of unmodified O-oligo dT probes to act as primers for IST after their uptake by L6 cells *in vivo*. Low levels or no silver staining was usually observed, even though these oligos were added to cells in serum-free media to reduce nuclease exposure (not shown). These results are consistent with observations that O-oligos are susceptible to intracellular nucleases (1). Interestingly, however, when fl-O-oligo dTs directly conjugated to either Texas Red or FITC at positions internal to the 3' and 5' ends (see Materials and Methods) were tested, these modified O-oligos were found to be as effective as ST30 at priming the reverse transcriptase (see Fig. 4 and below). The intracellular distribution of signal appeared similar to the pattern seen after cells were treated with ST30. We conclude that the covalently-bonded fluorochrome molecules may protect the O-oligos from nuclease digestion. As seen with SA30, cells treated with the fl-O-oligo dA analogues showed very low signal after IST (not shown).

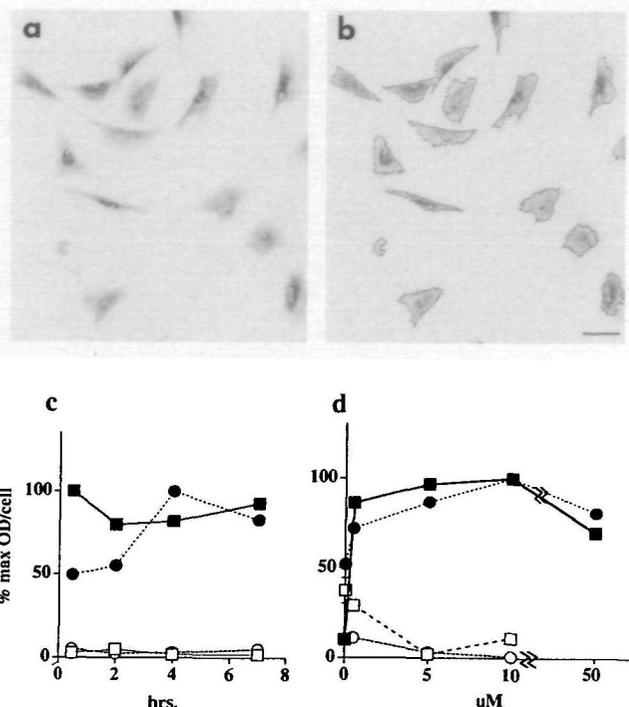


Figure 4. *In vivo* hybridization as a function of external oligo concentration and incubation time. Cells were incubated with oligo for varying times or at varying concentrations of oligo, extracted, fixed and subjected to IST. Silver stain was automated after cells were imaged and segmented from background using an automated image analysis system called Discovery™ (see Materials and Methods). (a) Image of microscopic field showing silver stained T43tr treated cells (0.1 μM for 2 h); (b) same field with red outlines indicating areas automatically segmented for silver stain quantitation. (c) plot showing silver stain absorbance/cell after treatment with 0.1 μM ST30tr (■) or T43tr (●) and their dA analogues (□ and ○ respectively) for 30 min–7 h; (d) same as (c) except incubation time was 2 h and oligo concentrations were varied from 0.01 to 50 μM . Bright field images. Bar = 20 μm .

Kinetics of hybridization *in vivo*

We next quantitated the amount of oligo dT hybridization to poly (A) RNA in live cells as a function of both incubation time and oligo concentration during uptake. Living cells were exposed to varying amounts of either fl-S- or fl-O-oligo dT for varying amounts of time and then extracted and fixed. IST was performed with silver enhanced signal detection as before and average silver stain present per cell was quantitated using a digital imaging workstation, Discovery™ (Fig. 4a and b; see Materials and Methods). Figure 4c shows that maximal levels of intracellular hybridization were reproducibly detected after cells had been incubated with ST30tr for 30 min. Signal resulting from hybridization was detected as early as 5 min after ST30tr oligo treatment began (not shown). These results correlated with intracellular quantitation of fluorescence (see Fig. 1); oligo that entered the cell rapidly (as measured by fluorochrome detection) also hybridized rapidly (represented by silver stain). Fl-O-oligo dT hybridization reached maximal levels by 4 h, later than ST30tr. Figure 4d shows that the maximal detectable hybridization in the cell occurs at external oligo concentrations of about 0.5 μM for both T43tr and ST30tr. Therefore, hybridization appears to saturate at lower concentrations than those required for

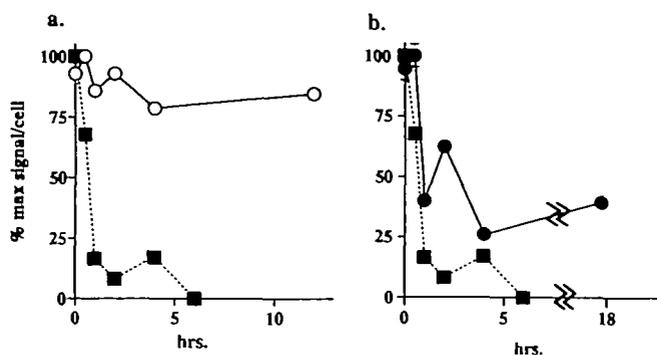


Figure 5. Hybrids between fl-O-oligo dT and intracellular RNA are more stable than hybrids formed with fl-S-oligo dT. Cells were incubated with fl-oligo for 2 h, washed and then allowed to grow in oligo-free media for varying lengths of time. Parallel cell populations were either fixed directly for fluorescence quantitation or Triton extracted, and subjected to IST. Cells were imaged and signal/cell was measured using Discovery™ (see Fig. 4 and Materials and Methods). (a) Plot compares amount of total ST30tr/cell (fluorescence, ○) to hybridization/cell (silver stain, ■) at various efflux times; (b) plot compares T43tr (●) to ST30tr (■) silver stain /cell after various efflux times. Levels of silver stain in cells incubated with control oligo dA were close to zero.

internalized fl-S-oligo to reach a steady state (concentration $>1 \mu\text{M}$; see Fig. 1a). The intracellular distribution of hybridization does not change at the light microscopy level as the concentration or time of incubation increases (data not shown). Control experiments in which A43tr or SA30tr were added to cells showed little hybridization.

Hybrid stability *in vivo*

The amount of fl-S-oligo dT in the cell was measured as a function of efflux time. Cells incubated with ST30tr for 2 h were washed with media and allowed to grow in oligo-free media for varying lengths of time. Fluorescence in the formaldehyde-fixed cells was quantitated using Discovery™. At most, a 20% decrease in the amount of total ST30tr in the cell was seen after 12 h of efflux (Fig. 5a). The intracellular distribution of the fluorescent oligo did not change detectably over the efflux periods examined and resembled that shown in Figure 1c. Levels of SA30tr also remained high after long efflux times. Retention of phosphorothioate oligos in slowly effluxing cellular compartments has been documented previously (28). It was not possible to quantitate accurately fluorescent label in individual cells treated with fl-O-oligo dT or dA because of high levels of extracellular fluorescence. It may be these oligos interact with extracellular components or simply stick to the coverslips. However, diffuse fluorescence remained detectable in both T43tr and A43tr treated cells up to 18 h after efflux.

The amount of hybridized oligo in the cell was next measured after efflux times similar to those described above. After incubation with fl-S- or fl-O-oligo dTs (ST30tr or T43tr) and appropriate efflux times, cells were extracted, fixed and subjected to IST as described above. The amount of silver stain in each cell was quantitated using Discovery™. Hybridization was still detected after a 30 min efflux time in cells treated with either ST30tr or T43tr (Fig. 5b). Cells treated with ST30tr showed no hybridization by 6 h efflux time, even though fluorescent levels of ST30tr remained high (compared in Fig. 5a). It is possible that hybrids are destroyed by RNase H. These results suggest that

antisense phosphorothioates may be retained in cells by interactions other than hybridization to target mRNA.

In contrast with the loss of hybridization signal with ST30tr, signal representing hybridization of T43tr could still be detected in cells after 18 h of efflux time (Fig. 5b). The amount of this hybridization varied from cell to cell, ranging from 10 to 75% of the initial hybridization. This might be expected of an unsynchronized cell population such as used here; cells that have divided would contain less hybridized oligo than undivided cells. Given an 18 h division time, ~50% of maximal signal would be expected. Taken together, these experiments show that both fl-S- or fl-O-oligo dT/poly (A) RNA hybrids can be detected in cells up to 4 h after treatment. Furthermore, fl-O-oligo dT/poly (A) RNA hybrids appear to be stable in cells for as long as 18 h after efflux begins. It may be that the presence of a fluorochrome moiety every tenth base on the oligo prevents RNase H recognition of the hybrid as a substrate.

In vivo hybridization between anti-actin oligos and β -actin mRNA can be detected with modified IST

We hybridized an antisense oligo to a specific mRNA, β -actin mRNA, in the living cell. This mRNA is abundant in chick embryo fibroblasts (CEFs) and its subcellular distribution to the leading edge of lamellipodia is well characterized (16,19). CEFs were incubated with phosphorothioate anti-actin oligos for 4 h and either Triton extracted and fixed, or fixed directly, and then subjected to IST as described. Incorporated dig-dUTP was detected using anti-dig antibodies linked to alkaline phosphatase followed by colorimetric detection. Figure 6 shows that hybridization between the anti-actin oligo and endogenous actin mRNA is detectable in both Triton-extracted cells (Fig. 6a) and in unextracted cells (Fig. 6c). Little or no signal was detected in cells treated with a reverse antisense control oligo (Fig. 6b, same sequence as antisense except in reverse 3' to 5' orientation; see Materials and Methods) or a random sequence antisense oligo of similar size (Fig. 6d, no known homology to chick sequences).

The IST signal obtained in Triton-extracted cells treated with anti-actin oligos was not localized to any particular intracellular areas (Fig. 6a). This has been previously observed using *in situ* hybridization to detergent-extracted cells (unpublished results). However, as would be expected for β -actin mRNA present in formaldehyde-fixed cells, hybridization signal was found localized to the lamellipodia in a significant fraction of the anti-actin treated cells (Fig. 6c, arrows). This localization of the IST signal confirms that the *in vivo* hybridization detected is to the intended target, β -actin mRNA.

DISCUSSION

We have shown directly that synthetic antisense oligonucleotides taken up by living cells hybridize to intracellular RNA. Hybridization *in vivo* was demonstrated by the ability of oligos added to cultured cells to prime reverse transcriptase *in situ*. Both S-oligo dT and fl-O-oligo dT, but not S- or fl-O-oligo dA, acted to prime *in situ* incorporation of labeled deoxyribonucleotide triphosphates by reverse transcriptase. The assay was also sensitive enough to detect hybridization of a synthetic antisense oligo to actin mRNA [present in ~1000 copies/cell (24,31)].

These results provide a direct demonstration that oligos passively internalized by cells hybridize to their intracellular target, an assumption implicit in all antisense experiments. The

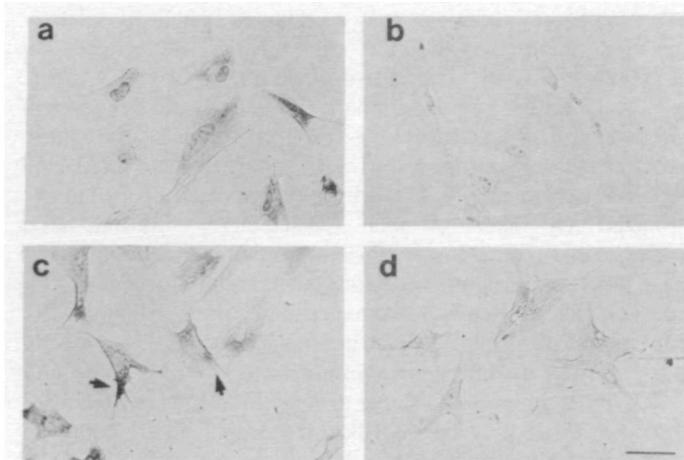


Figure 6. Detection of *in vivo* hybridization between anti-actin oligos and endogenous actin mRNA. (a) Chick embryo fibroblasts were exposed to 8 μ M phosphorothioate anti- β -actin oligo in serum-containing media for 4 h, Triton extracted and fixed in formaldehyde/glutaraldehyde. Hybrids were detected using IST as described previously except that incorporated dig-dUTP was detected using anti-dig Fab fragments linked to alkaline phosphatase (see Materials and Methods); (b) cells were treated exactly as in (a) except that the oligo used was a control 'reverse antisense' oligo (see Materials and Methods); (c) cells were treated as in (a) except that they were fixed directly in formaldehyde and no Triton extraction was done; (d) cells treated as in (c) except with a random sequence phosphorothioate 18mer. Color development for the alkaline phosphatase reaction was three times as long in (c) and (d) as compared with (a) and (b). Phase contrast images. Bar = 30 μ m.

modified IST method described can be used to assess whether cellular changes induced by antisense treatments result from specific hybridization of an oligo to its mRNA target or from other undefined effects. Additionally, efficiency of hybridization and hybrid stability can be evaluated after oligo treatments under various conditions. The assay is sensitive enough to detect oligo hybridization to abundant mRNAs, like β -actin mRNA, present in a few thousand copies per cell. Work will continue to increase the sensitivity of the assay so that it might be used to evaluate hybridization of oligos targeted to mRNAs present even in low abundance in the cell. The value of this approach is the high signal-to-noise ratio obtained, and hence the unequivocal nature of the signal, when two independent events (hybridization and priming) are required. This makes feasible the detection of mRNAs present in very low copy number in the cell.

The ability to elongate a hybridized primer using reverse transcription suggests that RNase H activity (32) in these cells did not immediately digest RNA/DNA hybrids formed with exogenous oligo dT. S-oligo dT/poly rA hybrids formed *in vivo* had an intracellular half life of \sim 30 min, either due to degradation or to dissociation of the hybrid. The T_m of this hybrid is estimated to be 36°C at physiological salt concentrations (25). In contrast with hybrids with S-oligos (labeled or unlabeled) or unlabeled O-oligos, fl-O-oligo dT/poly (A) hybrids can be detected for at least 18 h after oligo treatment. The fluorochrome modifications of the fl-O-oligo thus increased *in vivo* stability. This stability illustrates the suitability of fluorescently-labeled oligos as antisense tags to analyze RNA distribution *in vivo*. Stable hybridization with target RNA *in vivo* would make long-term tracking of tagged intracellular mRNAs possible. Additionally, fluorochrome-labeled O-oligos do not appear to be toxic when

taken up by tissues (8) and may be useful for antisense gene therapy.

Fl-S-oligo dT was detected in nuclei as well as the cytoplasm in \sim 30% of the cells. In these cells, the DNA dye, DAPI, was twice that in unlabeled nuclei (data not shown), suggesting that the nuclear signal could be cell cycle dependent. Oligo uptake is cell cycle dependent in other cell types (4,6) and nuclear envelope permeability changes throughout the cell cycle and varies between quiescent and dividing cell populations (31). When S- or O-oligo dT treated cells were evaluated using *in situ* transcription, about the same fraction (30%) of cells showed silver stained nuclei, indicating hybridization of probe to poly (A) targets. Further work evaluating both cell cycle status and nuclear hybridization in the same cells will resolve this relationship. Nuclear signal was not observed using fl-S-oligo dA. This could be due to differences in transport or to a lack of intranuclear hybridization targets.

These results show that the targeted sequences were available for hybridization *in vivo*. Hybridization occurred despite possible competition with RNA-binding proteins. For instance, poly (A) binding proteins are known to interact with the poly (A) tails of mRNAs (33). Nonetheless, our results show exogenous oligo dTs, even those that form hybrids with lowered T_m due to phosphorothioate modifications (25), hybridized to these sequences *in vivo*. Additionally, intracellular hybrids were detected using anti-actin oligos complementary to the 3'-UTR of β -actin mRNA, a region that probably has proteins bound since it has been implicated in mRNA intracellular transport and/or anchoring (20). Our data are therefore consistent with the idea that antisense oligos have a higher affinity of binding to their target sequences than do the proteins and can interfere with protein/RNA interactions in the cell. Little is known about the relative strengths of these interactions *in vivo*.

Poly (A) RNA is thought to be tightly associated with the cytoskeleton (26). Oligo dT hybridization *in vivo* does not appear to disrupt this association. When cells are exposed to oligo dT *in vivo* and then briefly detergent extracted, oligo dT/poly (A) hybrids were still detected using IST. Although we do not know the extent of oligo dT hybridization along the poly (A) tail, we infer that hybrids large enough to prime RT do not disrupt interactions poly (A) may have with the cytoskeleton (26).

These results support the idea that small oligos might be used as hybridization tags to follow mRNA movement without disrupting the normal cellular activity of the mRNA. In further support of this idea, we have found that anti-actin oligos hybridized to actin mRNA remain localized to the lamellipodia in CEF cells in formaldehyde fixed cells, suggesting again that some antisense oligos can hybridize to target mRNA without interfering with mRNA activity, in this case maintenance of subcellular localization. Choice of antisense oligo will, of course, determine the cellular effect caused by the hybridization; certain oligos can interfere with β -actin mRNA localization and alter the subcellular sites of mRNA expression, while others do not appear to affect localization at all (20). Work is underway to use the modified IST assay to help determine whether phenotypic effects seen after treatment with different antisense oligos can be correlated with intracellular differences in hybrid localization.

Using the *in vivo* hybrid detection method described here, we have found that both phosphorothioate and fluorochrome-labeled phosphodiester oligos hybridize to target mRNA in living cells. Our results indicate that fluorescently-labeled phosphodiester

oligos may be reagents of choice for labelling RNAs *in vivo*, and also may be potential candidates for antisense gene therapy. This work helps define optimal conditions for stable intracellular hybridization between oligos and mRNA and lays the groundwork for the use of fluorescent antisense oligos to study mRNA movement in living cells.

ACKNOWLEDGEMENTS

We are indebted to Shailesh M. Shenoy for help using Discovery™, Edward H. Kislauskis for preparing oligo-treated CEFs, and Doug Bowman and Larry Lipshitz in the Biomedical Imaging Facility at the University of Massachusetts for help with digital imaging. We thank Roy Long and Samuel Politz for a critical reading of the manuscript and helpful discussions. J.C.P. also thanks Chris Powers for support and help learning *in situ* hybridization techniques. This work was supported by NIH-HD18066. JCP is the recipient of a fellowship from the Muscular Dystrophy Association.

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