



In situ visualization of messenger RNA for basic fibroblast growth factor in living cells

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Abstract

We examined whether messenger RNA for basic fibroblast growth factor (bFGF) could be visualized specifically by a fluorescent probe in living cells. A 15-nucleotide-long antisense or sense sequence for human bFGF was sandwiched between two complementary 5-nucleotide-long arm sequences. A fluorophore, 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS), was joined to the 5'-terminal phosphate, while 4-(4'-dimethylaminophenylazo)benzoic acid, quencher for EDANS, was joined to the 3'-terminal hydroxyl group. The probe emitted blue fluorescence only upon hybridization with the complementary 18-nucleotide-long sequence under ultraviolet light. The antisense or sense probe carried with liposome was delivered to human cells, trabecular cells of the eye, in a glass-bottom culture dish placed on the stage of an inverted microscope. Cells with the antisense probe did, but not with the sense probe, show blue fluorescence under ultraviolet light. The present study opens a way to measure the changing levels of a specific messenger RNA in living cells. © 1998 Elsevier Science B.V.

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1. Introduction

Regulation of the level of messenger RNA for a certain protein is a key part in cellular response to external stimuli. To monitor in real time the changing levels of specific messenger RNA in living cells, a stable probe with the complementary sequence which shows fluorescence only upon hybridization with the target messenger RNA, as a calcium-binding fluorophore, has to be developed and delivered to cells without disturbing cellular function. Recently, Tyagi and Kramer have described such a probe that fluoresces upon hybridization, and named it as a molecu-

lar beacon [1]. Their probe consists of a stem-and-loop structure of oligodeoxynucleotides with the loop containing a probe sequence. A fluorophore, 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS), is joined to the 5'-terminal phosphate, while 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL), quencher for EDANS, is joined to the 3'-terminal hydroxyl group. The stem keeps the fluorophore and its quencher in close proximity to each other, resulting in absorption of the fluorescence emitted by the fluorophore. When the loop sequence hybridizes to the target sequence, the stem opens and the fluorophore at one end of the probe becomes apart from the quencher at the other end, leading to emittance of the fluorescence. In the present study, we designed a probe for messenger RNA of basic

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fibroblast growth factor (FGF) based on this structure, and delivered it to living human cells in culture, trabecular cells of the eye, with liposome as a carrier. We chose trabecular cells as a model because they are known to produce basic FGF [2,3].

2. Materials and methods

2.1. Synthesis of oligodeoxynucleotides coupled with EDANS and DABCYL [1]

A 25-nucleotide-long oligodeoxynucleotide consisting of 15-nucleotide-long probe (sense or antisense) sequence sandwiched by complementary 5-nucleotide-long arm sequences was covalently linked to DABCYL at the 3'-end and then to EDANS at the 5'-end with specific spacers in two consecutive reactions. The sequence of the antisense probe was 5'-EDANS-(CH₂)₆-S-CH₂-CO-GCGAG-GACACAAC-TCCTCTCTCT-CTCGC-(CH₂)₇-NH-DABCYL-3', while that of the sense probe was 5'-EDANS-(CH₂)₆-S-CH₂-CO-GCGAG-AGAGAGAGGAGTTGTGTC-CTCGC-(CH₂)₇-NH-DABCYL-3'. The sequence was reported previously to be used successfully as a primer for polymerase chain reaction [4,5].

Oligodeoxynucleotides were synthesized from adenine, guanine, cytosine, and thymine phosphoramidates (Perkin Elmer Japan, Urayasu) upon 3'-amino-modifier C7-CPG, 1-dimethoxytrityloxy-3-fluorenylmethoxycarbonylamino-hexane-2-methylsuccinoyl-long chain alkylamino-controlled pore glass (PerSeptive Biosystems Japan, Tokyo) by a DNA automatic synthesizer (model 394, Perkin Elmer), and a trityl-hexylthiol linker, (*S*-trityl-6-mercaptohexyl)-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidate (PerSeptive Biosystems) was linked to their 5' end. After treatment with 28% ammonium at 55°C for 6 h to remove the CPG support and protective moieties at amino groups and phosphate groups except for a trityl moiety at the 5'-sulfhydryl end, oligodeoxynucleotides were purified by a reverse phase cartridge column (Waters Sep-Pak C18, Millipore, Milford, MA), and then fractionated by high-pressure liquid chromatography (HPLC) with a Capcellpak C18 column (4.6 × 250 mm: Shiseido, Tokyo, Japan) with a linear gradient of 5–40% acetonitrile dissolved in 0.05 M triethylammonium acetate (pH 7.0) run for

30 min at a flow rate of 1.0 ml/min at 40°C and detected at 254 nm.

The dried oligodeoxynucleotides were dissolved in 100 μl water and mixed with 50 μl of 1.0 M sodium bicarbonate (pH 9.0). The mixture was then incubated with 1.6 mg DABCYL succinimidyl ester (Molecular Probe, Eugene, OR) dissolved in 200 μl dimethylformamide for 3 days at room temperature under dark to link the DABCYL moiety to the 3'-amino group. The reaction mixture was passed through a Sephadex-G25 gel column (Pharmacia Biotech Japan, Tokyo) to remove free DABCYL, and the oligodeoxynucleotides coupled with DABCYL were purified by HPLC. The absorption by DABCYL was confirmed by spectrophotometry.

To remove a trityl moiety from the sulfhydryl group at the 5'-end, the dried DABCYL-coupled oligodeoxynucleotides were dissolved in 100 μl of 0.1 M triethylammonium acetate (pH 7.0), incubated with 15 μl of 1.0 M silver nitrate for 30 min at room temperature, and incubated further with 20 μl of 1.0 M dithiothreitol for 5 min. The supernatant was obtained by centrifugation to remove a precipitate formed from silver and dithiothreitol, and then passed through a Sephadex-G25 gel column to remove dithiothreitol. The supernatant containing oligodeoxynucleotides with DABCYL at the 3'-end and a sulfhydryl group at the 5'-end was mixed with 1.6 mg of 1,5-iodoacetylated EDANS (Molecular Probe) dissolved in 50 μl of 1.0 M sodium bicarbonate (pH 9.0), and incubated at room temperature for one day under dark to link the EDANS moiety to the 5'-sulfhydryl group. The EDANS-coupled oligodeoxynucleotides were purified by HPLC.

2.2. Culture of human trabecular cells [6–10]

Human trabecular tissues excised during trabeculectomy in patients with primary open-angle glaucoma were placed in wells of a 24-well multiplate (Corning Coster Japan, Tokyo) with Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum (FCS), 100 mg/1 ampicillin, and 100 mg/1 streptomycin, and incubated at 37°C under a humidified atmosphere of 5% carbon dioxide and 95% air. Trabecular cells which usually grew out of the explants after 2–4 weeks were passaged to larger dishes. Morphological characteristics

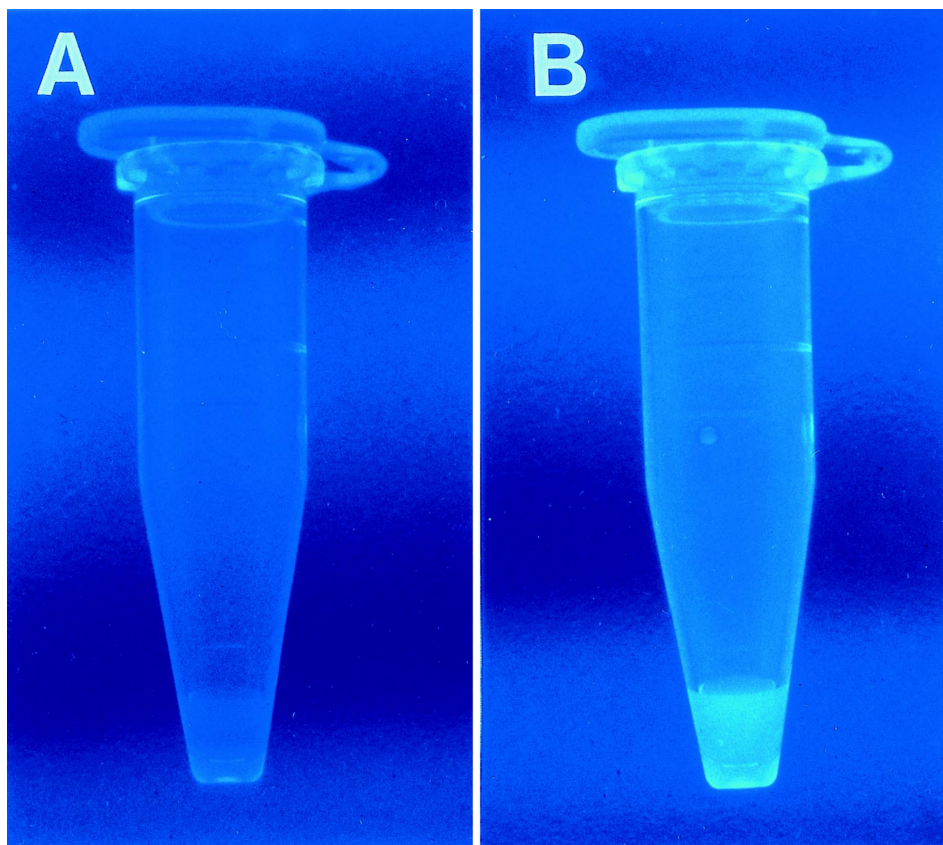


Fig. 1. Fluorescence of the probe in the absence (a) or presence (b) of the complementary target. Under ultraviolet light, the probe with the antisense sequence for human basic FGF shows blue fluorescence only when hybridizes with the target sequence. The probe with the sense sequence used in this study has the same feature. Photographed with ASA 100 film for 8 s without any filter.

of the cells were confirmed the same as those of human trabecular cells described previously [6–10].

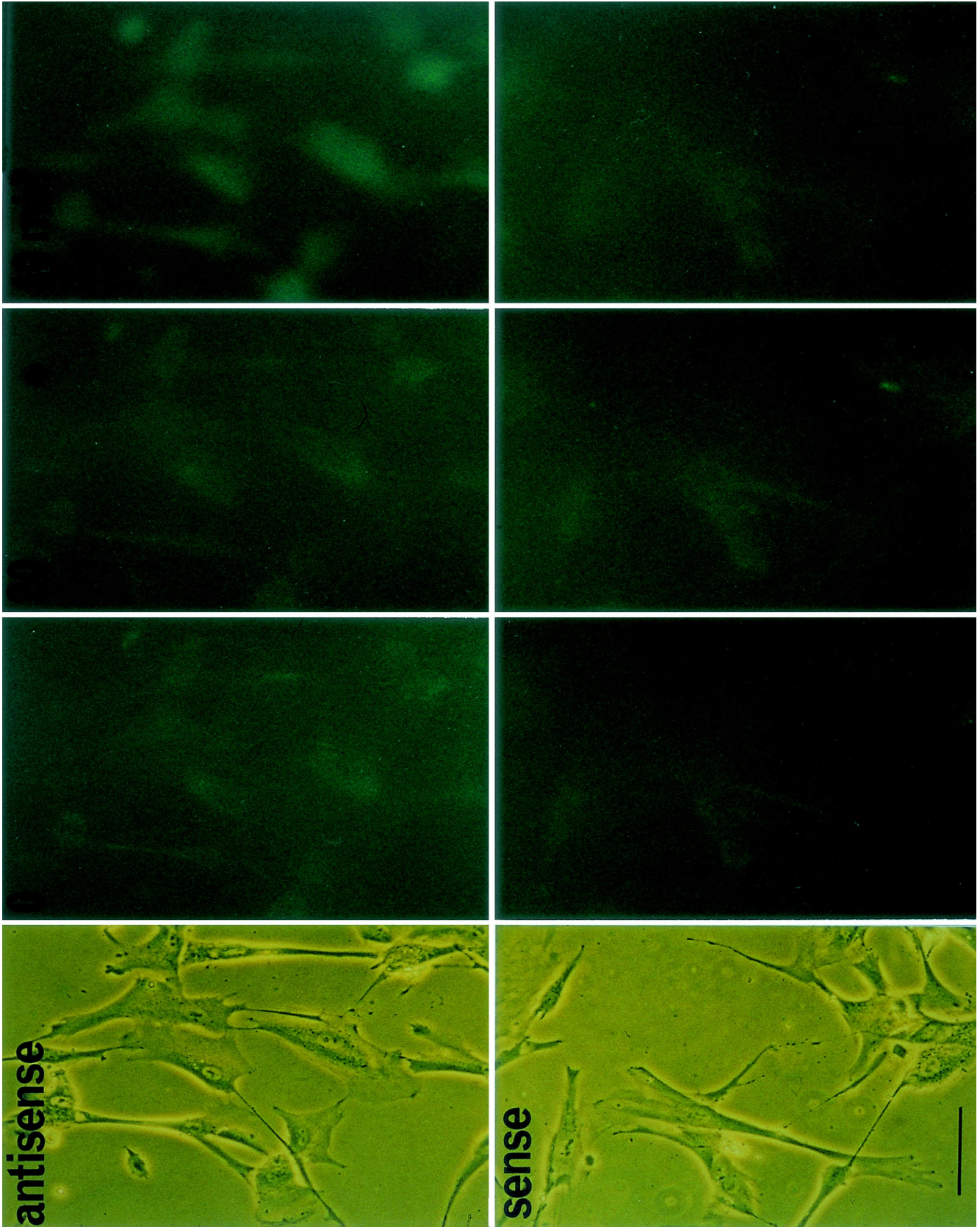
2.3. Delivery of fluorescent probes to cells and their visualization

2 μ g of the fluorescent probe dissolved in 2 μ l of water were mixed with 9 μ l of liposomes consisting of 0.4 mg of *N,N,N',N'*-tetramethyl-*N-N'*-bis(2-hydroxyethyl)-2,3-dioleoyloxy-1,4-butanediammonium iodide and 0.3 mg of *L*-dioleoyl phosphatidylethanolamine suspended in 400 μ l of nuclease-free water (Tfx™-50 Reagent, Promega, Madison, WI, USA), vortexed, and incubated for 10 min.

The liposomes were scaled up to 0.8 ml with HEPES buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, 10 mM HEPES, pH 7.4), and added to trabecular cells in a 35 mm glass-bottom culture dish (MatTek, USA) which was placed on the stage of an inverted microscope (Axiovert 405M, Zeiss, Germany). For a longer period of observation, cells were incubated with DMEM and 15% FCS after 1 h uptake of the liposome.

Ultraviolet light through a filter (UG1, Leitz, Germany) from a 100 W mercury lamp (HBO 100 W, Zeiss) was projected to cells through a 20 \times objective lens (UVFL20, Olympus, Tokyo, Japan), and blue fluorescence was observed with a dichroic mirror (FT460, Zeiss) and a filter (LP470, Zeiss).

Fig. 2. Levels of fluorescence in living human trabecular cells after the addition of liposome-carried probes with the antisense or sense sequence for basic FGF. Cells with the antisense probe show growing fluorescence on the negligible background of autofluorescence. Photographed with ASA 400 film for 15 s. Bar = 100 μ m.



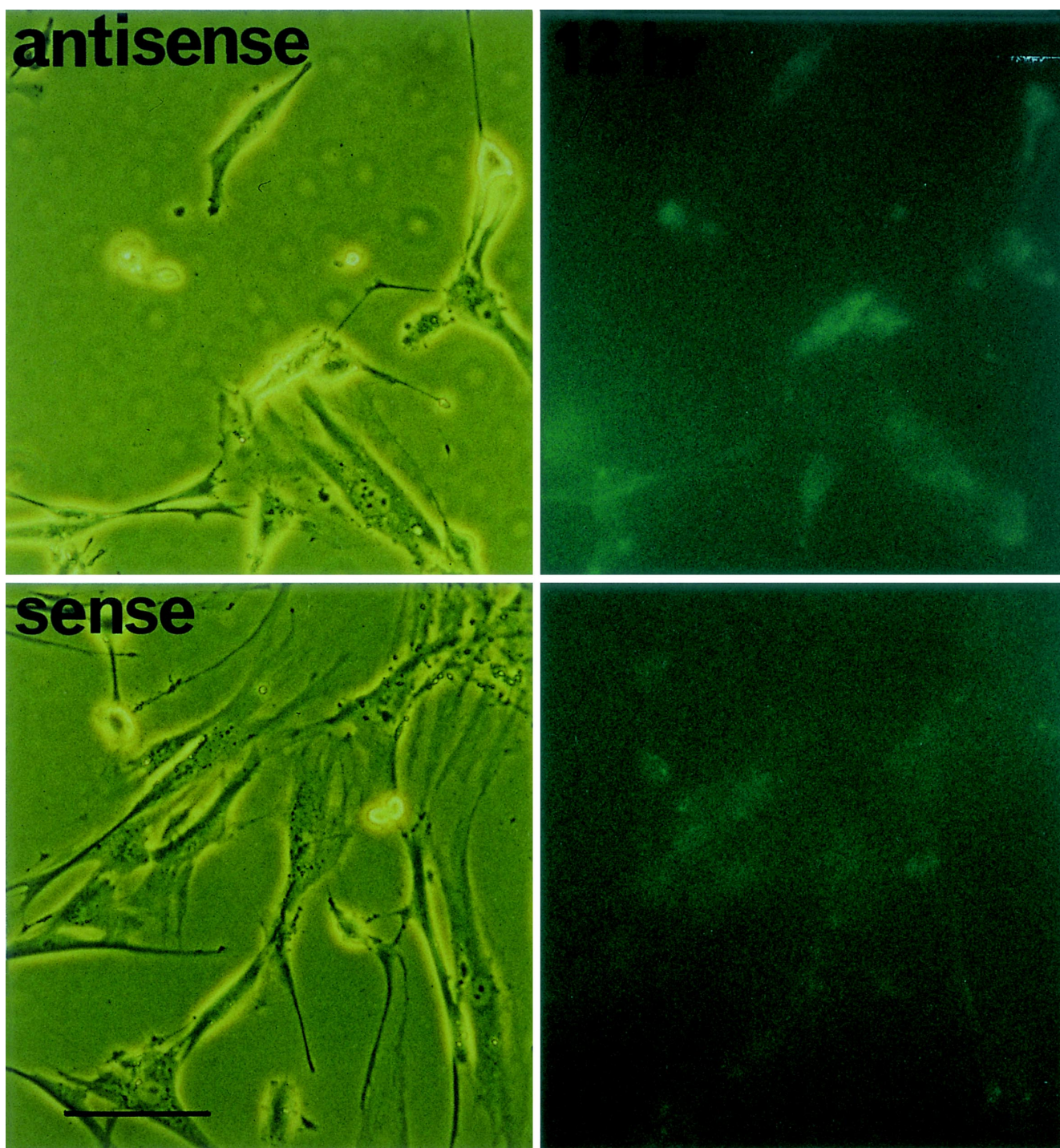


Fig. 3. Levels of fluorescence in living human trabecular cells 12 h after uptake of liposomes with the antisense or sense probe. Cells with the antisense probe show a higher level of fluorescence than cells with the sense probe. Photographed with ASA 400 film for 15 s. Bar = 100 μ m.

3. Results and discussion

Probes with the antisense or sense 15-nucleotide-long sequence were confirmed to emit fluorescence by excitation with ultraviolet light only when they hybridized with the complementary oligodeoxynucleotides with the same length (Fig. 1).

Trabecular cells in a glass-bottom culture dish showed a negligible background of autofluorescence under ultraviolet light, and were incubated with liposomes carrying either probe with the antisense or sense sequence. The cells began to show fluorescence around 30–40 min after the incubation only with the antisense probe, but not with the sense probe (Fig. 2). The fluorescence of cells with the antisense probe remained stationary in its intensity for the additional 2 h as observed, while cells with the sense probe also showed a weaker level of fluorescence after 1 h. Cells with the antisense probe had a higher level of fluorescence than cells with the sense probe even 12 h after uptake of the liposomes (Fig. 3).

For a control experiment, cells in dishes were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min and incubated with the antisense or sense probe in HEPES buffer for 1 h. After a single wash with HEPES buffer, cells with the antisense probe showed fluorescence, in contrast with no fluorescence by cells with the sense probe. The same level of fluorescence as that in living cells could only be obtained in the fixed cells at 10 times as high a concentration of the probe as used for living cells.

Oligodeoxynucleotides containing phosphodiester linkages delivered to cells are prone to intracellular degradation, and their half-life is around 20 min [11–14]. Degradation of the probes used in this study would release a fluorophore, EDANS, and its quencher, DACYL, and make these compounds apart in the cells, resulting in emission of fluorescence by EDANS. Under the circumstances, fluorescence emitted by free EDANS would be mixed with that by EDANS linked to oligodeoxynucleotides which hybridize with target messenger RNA. To avoid such ambiguity, we focused on the initial phase of uptake of the probes by cells within 1 h, and clearly demonstrated the presence of growing fluorescence with the antisense probe, in contrast with its persistent absence with the sense probe.

The release of free fluorophores by degradation of the probe would contribute to the fluorescence in cells with the sense probe observed after 1 h. The absence of fluorescence by the sense probe on the fixed cells suggests that degradation of the probe does not take place in living cells. It should be noted that cells with the antisense probe showed a higher level of fluorescence than cells with the sense probe even 12 h after uptake of the liposomes. This could be ascribed to the fact that oligodeoxynucleotides which hybridize with the target messenger RNA become more resistant to degradation by cellular enzymes than unhybridized oligodeoxynucleotides. In addition, the molecular beacons are perhaps more stable than simple oligodeoxynucleotides because they have a hairpin conformation and both of their ends are protected by the chromophores. Free fluorophores such as fluorescein are known to be expelled rapidly from cells [11–14], which might underlie the absence of a higher level of fluorescence in cells with the sense probe in spite of its possible degradation.

The final purpose for visualizing a specific messenger RNA in living cells is to observe the changing levels of a messenger RNA in response to stimuli in real time. To this end, oligodeoxynucleotides have to be made less prone to intracellular degradation by introducing, for example, phosphorothioate linkages.

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