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Antisense oligonucleotides reach mRNA targets via the RNA matrix: downregulation of the 5-HT1A receptor

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Abstract

Successful application of antisense oligonucleotides (ODNs) in cell biology and therapy will depend on the ease of design, efficiency of (intra)cellular delivery, ODN stability, and target specificity. Equally essential is a detailed understanding of the mechanism of antisense action. To address these issues, we employed phosphorothioate ODNs directed against specific regions of the mRNA of the serotonin 5HT1A receptor, governed by sequence and structure. We demonstrate that rather than various intracellular factors, the gene sequence per se primarily determines the antisense effect, since 5HT1a autoreceptors expressed in RN46A cells, postsynaptic receptors expressed in SN48 cells, and receptors overexpressed in LLP-K1 cells are all efficiently downregulated following ODN delivery via a cationic lipid delivery system. The data also reveal that the delivery system as such is a relevant parameter in ODN delivery. Antisense ODNs bound extensively to the RNA matrix in the cell nuclei, thereby interacting with target mRNA and causing its subsequent degradation. Antisense delivery effectively diminished the mRNA pool, thus resulting in downregulation of newly synthesized 5HT1A proteins, without the appearance of truncated protein fragments. In conjunction with the selected mRNA target sequences of the ODNs, the latter data indicated that effective degradation rather than a steric blockage of the mRNA impedes protein expression. The specificity of the antisense approach, as described in this study, is reflected by the effective functional downregulation of the 5-HT1A receptor.
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Introduction

Antisense oligonucleotides (ODNs) offer a great potential as sequence-specific modulators of gene expression, which may be exploited for therapeutic and cell biological purposes. In fact, recent evidence suggests that mammalian cells do use natural antisense transcripts to regulate gene expression [1,2].

Work carried out thus far indicates that ODNs per se poorly permeate across cellular membranes. As transport vehicles, cationic lipids strongly promote cellular entry, which appears to proceed along the pathway of endocytosis [3]. Physical parameters of the complex, in particular a lamellar-to hexagonal phase transition of the lipid phase [4], subsequently determine the ability of the ODNs to translocate across the endosomal membrane and to acquire access to the nucleus, reflected by the specific accummulation of fluorescently tagged ODNs [5,6]. Once in the nucleus, antisense ODNs may form compact nuclear bodies or longitudinal rodlets in a concentration-dependent manner [7], but the implication to the antisense effect remains unclear.

Several mechanisms have been proposed as to how gene expression is inhibited [8], including antisense ODN hybridization with target mRNA, thereby providing a steric

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block for the translation machinery [9,10] or an effective target for RNase H, resulting in mRNA degradation [11,12]. Furthermore, to reliably attribute the effect of ODNs to a specific antisense effect, it is equally essential to demonstrate a direct link between target specificity and functional consequences, resulting from the downregulation of protein expression [13–17].

To obtain further insight into the mechanism by which ODNs cause their antisense effect, we have investigated the effect of ODNs directed against the serotonin receptor 5-HT1A as a target gene. Since this receptor is involved in diseases like depression and anxiety, the receptor is of obvious neurological interest as a potential therapeutic target. In addition, these receptors are widely distributed in the CNS, being expressed on different cell types, yet genetically and structurally indistinguishable. This provided an opportunity to investigate cell type-dependent antisense effects involving the same protein receptor, i.e., the 5-HT1A receptor as naturally expressed in raphe neuronal cells (RN46A) and septal neuronal cells (SN48) and overexpressed in mouse LLP-K1 cells. Here we show a direct correlation between mRNA downregulation and an inhibition of both expression and functioning of a membrane receptor, 5-HT1A. Our data support a mechanism involving antisense ODN-induced degradation of the target mRNA, triggered via binding to the RNA matrix in the nucleus.

Materials and methods

Materials and ODNs

SAINT-2 was synthesized as described in detail elsewhere [18]. Lipofectamine 2000 was purchased from Invitrogen. All other chemicals were from Sigma (St. Louis, MO, USA), unless stated otherwise. Two antisense PS-ODNs, complementary to rat 5-HT1A mRNA (for sequence see GeneBank Accession No. J05276), targeted to bp 115–128 (antisense 1, AS1) and bp 885–902 (antisense 2, AS2) with the sequences ATC CAT GCC TGC CT and ACT ACC TGG CTG TCC GTT, respectively, as well as two mismatched randomized ODNs with the sequences TCC TCT TCG ACT GCT CTC (MAS1) and ACC TAC GTG ACT ACG T (MAS2), and a fluorescein-labeled random-ized-sequence, ACT ACT ACA CTA GAC TAG, were designed and manufactured by Biognostik (Göttingen, Germany). All ODNs were thioated and purified by HPLC, cross-flow dialysis, and ultrafiltration. Biotin-labeled phosphorothiate ODNs were synthesized by Invitrogen Ltd. (Breda, The Netherlands).

Cell culture

RN46A and SN48 cells were kindly provided by Dr. Paul Albert, University of Ottawa, Ottawa, Canada [19,20]. RN46A cells were maintained in Dulbecco's modified Ea-

gle's medium (DMEM, Life technologies, Paisley, UK) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin (50 U/ml)/ streptomycin (50 U/ml) at a permissive temperature of 33°C in 5% CO_2. RN46A cells were differentiated by substituting serum for a mixture of 1% bovine serum albumin in DMEM, containing 1 μg/ml transferrin, 5 μg/ml insulin, 6.3 μg/ml progesterone, and 16.1 μg/ml putrescine at 39°C [21]. SN48 cells were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin (50 U/ml)/ streptomycin (50 U/ml) at 37°C in 5% CO_2. After transfection, SN48 cells were differentiated by a reduction of the fetal calf serum concentration to 1% and the addition of 1 μM retinoic acid [20]. LLP-K1 cells stably transfected with the 5-HT1A receptors (LLP-5-HT1A) were kindly provided by Dr. Michèle Darmon, Boulevard de I'Hôpital, Paris, France [22]. LLP-5-HT1A cells were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin (50 U/ml)/streptomycin (50 U/ml) at 37°C in 5% CO_2 under the selection of 1 mg/ml geneticin.

Preparation of antisense complexes and their incubation with cells

Antisense ODNs were complexed with sonicated cationic lipid vesicles consisting of SAINT-2 and DOPE (1:1) as described in detail elsewhere [6]. In preliminary work, Lipofectamine 2000, purchased from Invitrogen, was also employed, and antisense ODN complexes were prepared essentially as instructed by the manufacturer. Where indicated, 0.5 mol% *N*-(lissamine rhodamine sulfonyl)-PE (N-Rh-PE, Avanti Polar Lipids Inc) was included in the lipid mixture to monitor the fate of the lipid complex by confocal fluorescence microscopy. Antisense/cationic lipid complexes were prepared as follows: 15 nmol SAINT-2/DOPE vesicles or 1–3 μl Lipofectamine 2000, diluted in 500 μl DMEM, was gently mixed (4–5 min) with 0.1 nmol ODNs, also diluted in 500 μl DMEM. The complexes were allowed to equilibrate for 20 min at room temperature, after which time they were immediately added to the cell cultures. After 4 h incubation with antisense ODN complexes, the cells were washed with Hanks' balanced salt solution (HBSS, Life Technologies), and analyzed directly with a TCS Leica SP2 confocal laser scanning microscope (Wetzlar, Germany). To quantify cellular binding and uptake of FITC-ODNs, cells were rinsed with PBS, trypsinized, and resuspended in medium prior to quantifying fluorescence by FACS. FITC-labeled beads were used as a standard.

Analysis of newly synthesized 5-HT1A after antisense treatment

Complexes (100 nM ODNs), prepared as described above, were incubated with 70–80% confluent cultures of LLP-5-HT1A cells, or SN48 cells and RN46A cells for 6 h, respectively. After the cells were washed, methionine-free

DMEM containing 5% fetal calf serum was added, and the incubation was continued for 1 h. Metabolic labeling of newly synthesized protein was then carried out with 100 μCi/ml [^{35}S]methionine[^{35}S]cysteine (PRO-MIX L-[^{35}S] in vitro Cell Labelling Mix, Amersham Pharmacia Biotech, Buckinghamshire, UK) in methionine-free medium containing 5% fetal calf serum or differentiation supplement. During metabolic labeling, RN46A and SN48 cells were incubated under differentiation culture conditions to enhance 5-HT1A expression. After labeling for 16 h, medium was removed and RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS. 10 μl/ml RIPA) was added at 4°C. After 10 min the cells were disrupted by repeated aspiration through a 25-gauge needle, and the supernatant was collected by centrifugation at 10,000g for 10 min. 5-HT-1A antibodies (Santa Cruz) were conjugated to Sepharose (protein A Sepharose CL-4B, Amersham Pharmacia Biotech, Uppsala, Sweden) for 8 h at 4°C. Equal amounts of the supernatant fraction and antibody–Sepharose conjugate were then incubated for 16 h at 4°C. Immunoprecipitates were washed, collected by centrifugation, and analyzed by SDS–PAGE (Bio-Rad, Hercules, CA, USA). Proteins were autoradiographed for 16 h at −80°C. The relative protein amount was analyzed by the software Scion Image on the film.

mRNA analysis by Northern blot after antisense treatment

The pharmacological effect of antisense ODNs was evaluated by examining the level of 5-HT1A mRNA in LLP-5-HT1A cells treated with SAINT-2/DOPE/100 nM antisense ODN complex. LLP-5-HT1A cells were grown to 70–80% confluency and treated with PS-ODN/SAINT-2/DOPE complexes prepared as described above. After 16 h, total RNA was extracted from 6×10^6 cells using the Qiagen RNeasy kit (Hilden, Germany). The quantity and purity of the RNA preparation were determined by recording the absorbance at 260 and 280 nm. Equal amounts of RNA were run on a 1.2% agarose gel containing 6.7% formaldehyde, and transferred to a nylon membrane by vacuum transfer. Total RNAs were fixed onto the membrane by heating for 1 h at 80°C. The RNA was intact without any degradation, as indicated by 18S and 28S bands on the membrane, visualized by methanol blue staining. 5-HT1A and β-actin probes were prepared by amplifying 5-HT1A cDNA by RT-PCR from total RNA in LLP-5-HT1A cells. The 5-HT1A and β-actin primers were CCA AAG AGC ACC TTC CTC TG/TAC CAC CAC CAT CAT CAT CA and AAC ACC CCA GCC ATG TAC/ATG TCA CGC ACG ATT TCC, respectively. PCR was performed using oligo(dT)-primed cDNAs (synthesized with reverse transcriptase, Boehringer Mannheim, Germany) as a template under the following conditions: 30 cycles, 94°C 30 s, 55°C 30 s, 72°C 45 s, and a final 72°C extension of 5 min). The

probes from the PCR products were gel purified (QIAEXII Gel Extraction Kit, Germany) and labeled with [α^{32}P] ATP using a random priming kit (Gibco-BRL, Breda, the Netherlands). The level of 5-HT1A mRNA was probed by the cDNA probe for the 5-HT1A gene, and visualized by autoradiography. To normalize the level of RNA loading, the 5-HT1A probe was removed by stripping the membrane in boiling SDS solution (0.5%) for 10 min. The membrane was then probed with β-actin probe. The relative amount of mRNA was analyzed by the software of Scion Image on the film.

Removal of nuclear protein, chromatin, and RNA matrix

RN46 cells were grown on coverslips in a 12-well plate and treated with FITC–ODN–SAINT–2/DOPE complexes for 6 h. Cells were either fixed directly or subjected to RNase digestions. Nuclear RNA was removed by digesting cells with 1 mg/ml RNase A (Roche, Indianapolis, IN, USA, boiled for 10 min to destroy residual DNase) and 0.1% Triton X-100 in HBSS for 10 min at room temperature. After digestion, cells were fixed in 3% PFA or directly examined by confocal laser microscopy.

Nuclear localization of ODNs by electron microscopy

RN46 cells were grown to 70% confluency and treated with 100 nM biotin-labeled ODN/cationic lipid complexes as described above. After 6 h, the complexes were removed by washing with PBS. The cells were fixed with 4% paraformaldehyde in PBS for 15 min, and permeabilized with 0.1% Triton X-100 for 30 min. Subsequently, the cells were incubated with FluoroNanogold–streptavidin conjugate (1.4-nm gold particles, attached to streptavidin; Nanoprobes Inc., Stony Brook, NY, USA) for 2 h, washed three times with PBS, and immediately examined by epifluorescence microscopy. After examination, the cells were washed with 0.2 M sodium citrate, and the silver enhancement procedure was performed according to the manufacturer's instructions (Nanoprobes Inc.). The cells were rinsed with PBS and postfixed with 1% glutardialdehyde for 10 min. Finally, the samples were embedded in Epon, serially sectioned (60-nm thin sections), and examined at 60 kV with a Philips EM-201 or CM-100 transmission electron microscope (FEI Electron Optics, Eindhoven, The Netherlands).

Binding of antisense to target mRNA

To determine the intracellular binding of antisense ODNs to target mRNA, 1 nmol ODN was labeled with 10 μl [γ-^{32}P] ATP (3000 Ci/mmol, 10 μCi/μl) with Ready-To-Go T4 polynucleotide kinase. Nonincorporated nucleotide was removed, using MicroSpin G-25 columns (Amersham Pharmacia Biotech, Buckinghamshire, UK). LLP-5-HT1A cells were seeded on 75-cm^2 flasks, and after reaching approx 70% confluency, the cells were incubated

for 16 h with 5 ml lipoplexes containing either 100 nM ^{32}P-labeled antisense or mismatched sequences. The cells were lysed and total RNA was isolated with the Qiagen RNeasy kit (Hilden, Germany). To exclude that antisense hybrization to target mRNA had occurred during or after RNA extraction, the same amount of labeled antisense was added to untreated cells during RNA isolation. Then RNAs were separated on a 1.2% agarose gel containing 6.7% formaldehyde, and transferred to a nylon membrane by vacuum transfer. A film was exposed to the membrane for 3 days. To determine the position of the antisense-bound mRNA on the developed film, experiments were done in parallel with unlabeled ODNs directed against 5-HT1A mRNA. 5-HT1A mRNA was probed with ^{32}P-labeled 5-HT1A probes by Northern blot, as described above.

Analysis of the total 5-HT1A pool after antisense treatment

Protein levels of 5-HT1A were evaluated by Western immunoblot. An aliquot of 10^6 SN48 cells were seeded in 10-cm dishes and grown to 50% confluency. Cells were treated with 100 nM antisense ODN complexes for 6 h. After this interval the complexes were removed and fresh medium was added. Cells (the medium was refreshed daily) were harvested 5 days after the initial treatment and lysed. Samples (40 μg of protein) were then analyzed by 12.5% SDS–PAGE (Bio-Rad), blotted on a pure nitrocellulose membrane (Trans-Blot transfer medium, Bio-Rad), and probed with rabbit anti-rat SR1A antibodies (1:500, Santa Cruz), followed by horseradish peroxidase conjugate anti-rabbit antibody (1:5000, Nenox, Japan). The blot was processed with ECL (Amersham Pharmacia Biotech, Buckingham, UK) according to the manufacturer's protocol. For the second blotting, the blot was washed with PBST (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, and 150 mM NaCl, pH 7.4, 0.3% Tween 20), and then stripped with stripping buffer (Restore Western Blot Stripping Buffer, Pierce, Rockford, IL, USA). The membrane was probed with mouse anti-β-actin antibody (1:1000, Sigma), followed by alkaline phosphatase-conjugated sheep anti-mouse antibody (1:3000, Chemicon, Temecula, CA, USA). The blot was color processed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl. The relative amounts of protein were analyzed by Scion Image software.

Functional assay for 5-HT1A receptor activity in SN48 cells

The function of 5-HT1A in SN48 cells was validated using a cytosensor, which measures the metabolic change of the cell as reflected by pH changes on a challenge with an agonist. The cells were plated on cytosensor membranes and

grown for 1 day before the measurements. Cells were either challenged with the agonist flesinoxan for 2 min at indicated concentrations from 0.003 to 0.1 mM or blocked with the antagonist WAY100635 at 0.1 mM for 10 min, before challenge with flesinoxan (0.1 mM for 2 min). To determine the effect of antisense treatment on 5-HT1A receptor activity, SN48 cells were treated with antisense ODNs as described above, after which they were plated on cytosensor membranes, and further grown for 16 h. To normalize the amount of cells on each membrane, the cells were first challenged with acetylcholine, which was followed by a challenge with flesinoxan for 2 min at the concentrations indicated. The cells were brought back to base level for 5 min between challenges.

Results

Parameters affecting uptake of antisense ODNs

When phophorothioate ODNs were incubated with LLP-5-HT1A, RN46A, or SN48 cells at a relatively high concentration of 2 μM, they were endocytosed and observed as punctuate structures within the cells. However, in either cells type, accumulation of ODNs in the nucleus was not apparent (Figs. 1A–C). By contrast, when complexed with cationic liposomes, not only was uptake of ODNs by either cell type significantly enhanced, but also their translocation across the endosomal compartments was evident, as reflected by their nuclear accumulation (Figs. 1D–F, G–I). Since the eventual effect of the antisense ODN is likely dose-dependent, and conceivably depends on nuclear uptake, we compared two types of cationic liposome formulations as delivery vehicles to accomplish the most optimal delivery efficiency. As shown in Fig. 1 (G–I vs D–F), in all three cell lines, the formulation based on SAINT-2/DOPE was substantially more potent in facilitating the uptake and translocation of ODNs than Lipofectamine 2000. Indeed, FACS analysis revealed that net uptake of the Lipofectamine 2000 lipoplexes was two- to sixfold lower than that observed for the SAINT lipoplexes. In fact, cell type-dependent differences in uptake efficiency were also seen when employing Lipofectamine 2000, whereas in the case of SAINT-2-containing complexes, the delivery was approximately equally effective in either cell type (Fig. 1, numbers in upper left corner in G–I). Elsewhere, we [5,6] and others [7] have reported that the antisense efficiency correlates well with the cellular uptake of ODNs. Indeed, in line with the distinctions in uptake, SAINT-2/DOPE is approximately two to three times more potent than Lipofectamine 2000 in eliciting an antisense effect and in transfecting cells, when monitored by a GFP reporter plamid (transfection efficiency 25–30% (LF2000) vs 50–70% (SAINT-2/DOPE); data not shown). Accordingly, in the following experiments, SAINT-2/DOPE was applied as ODN vector. The antisense uptake was not dependent on the
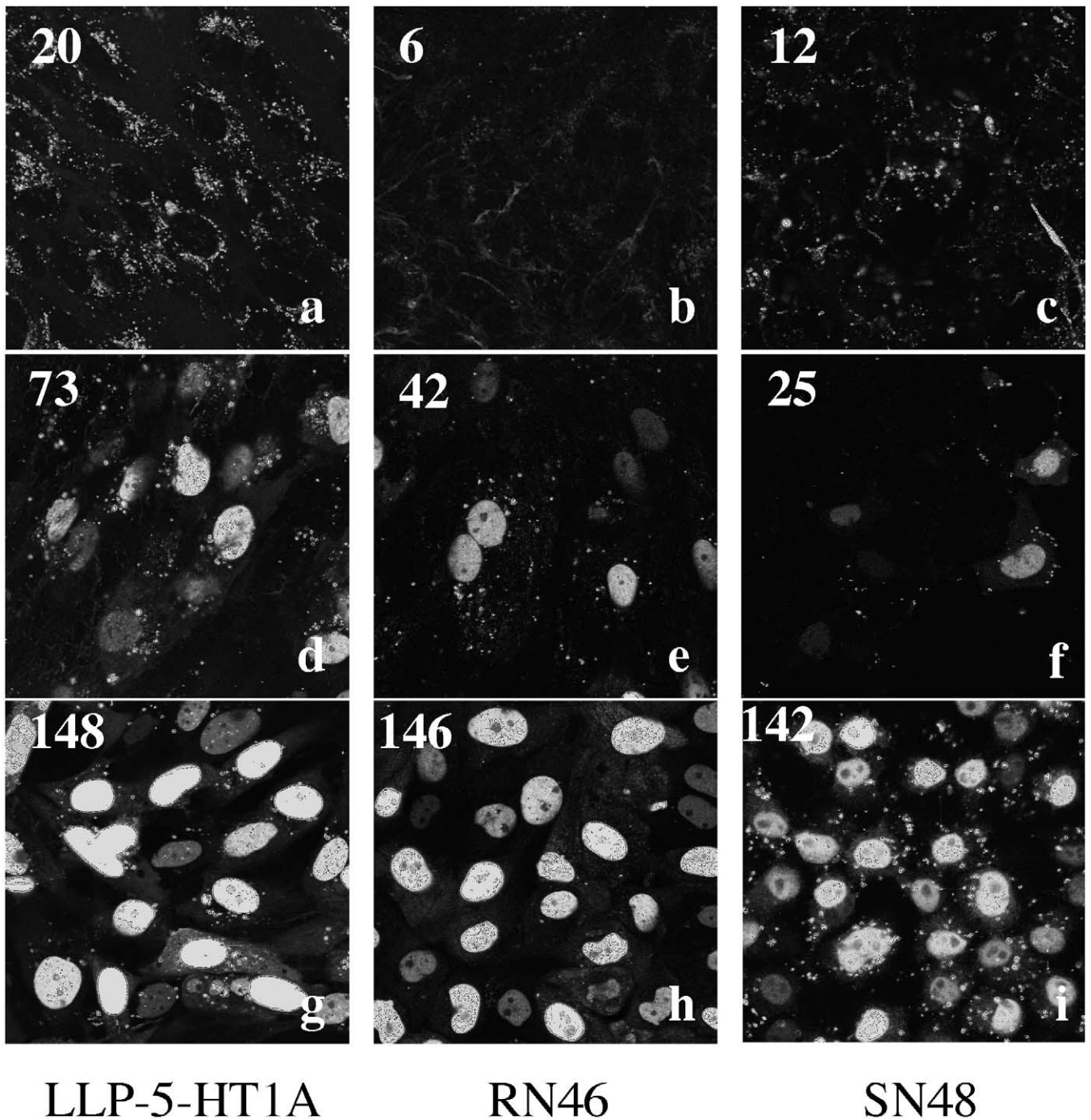
Fig. 1. Intracellular uptake of FITC-labeled PS-ODNs in free form and when complexed with cationic lipid complexes. PS-ODNs alone, PS-ODN/ Lipofectamine 2000 complexes, and PS-ODN/SAINT-2/DOPE complexes were incubated with LLP-5-HT1A (A, D, G), RN46A (B, E, H), and SN48 cells (C, F, I). The concentration of free ODNs was 2 μM, and the incubation was carried out for 24 h. Note the punctate appearance in intracellular compartments in the cytosol (A–C). 100 nM PS-ODN complexed with Lipofectamine 2000 (D–F) and SAINT-2/DOPE (G–I) was incubated with the cells for 4 h. Note the differences in efficiency of uptake and the nuclear accumulation of PS-ODNs in this case. The efficiency of ODN uptake in each case is indicated by the relative cell-associated fluorescence, indicated in the left upper corner.

sequence, since all sequences used in the present work, including fluorescent and biotinylated ODNs, showed essentially the same amount of uptake (not shown). In addition, none of the sequences applied caused significant cytotoxicity, as verified by morphological criteria and the MTT assay (not shown). We next examined whether the internalized antisense ODN in either cell type effectively interfered with expression of the target 5-HT1A receptor.
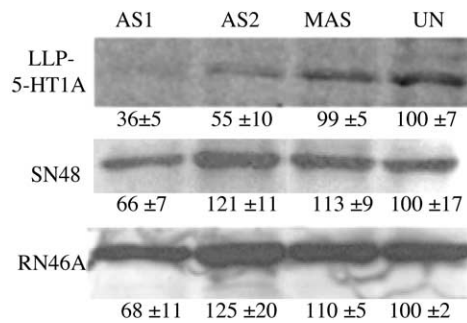
Fig. 2. Antisense treatment efficiently downregulates newly synthesized 5-HT1A target receptors. LLP-5-HT1A cells, SN48 cells, and RN46A cells were treated with 100 nM antisense ODN sequence 1 (AS1), sequence 2 (AS2), and a mismatched sequence (MAS), complexed with SAINT-2/DOPE. The newly synthesized 5-HT1A was monitored by metabolic labeling, immunoprecipitated and separated by PAGE, as described. Numbers under the bands indicate the amount of protein (%) relative to the amount in untreated cells (UN), as obtained in two independent experiments.

Downregulation of newly synthesized 5-HT1A receptor

To reveal an antisense effect, we monitored the effect on the pool of newly synthesized proteins, since the existing pool is not affected other than by metabolic turnover. Two antisense sequences (AS1 and AS2, see Materials and Methods), selected by computer-facilitated analysis of 5-HT1A mRNA, were examined. Throughout this work, the cells were treated with 100 nM antisense or mismatched ODN complexed with SAINT-2/DOPE (1:1). In preliminary, concentration-dependent experiments, carried out as described in detail previously [5], it was established that at this concentration the antisense effect is optimal, without significant cell cytotoxic effects. After antisense treatment for 6 h, metabolic labeling was carried out for 18 h in differentiation medium to enhance 5-HT1A expression. Subsequently, the cells were lysed, and 5-HT1A was immunoprecipitated and analyzed by PAGE, as described under Materials and Methods. The data, shown in Fig. 2, reveal that the mismatched sequence had no significant effect on the target (lane 3 vs lane 4, 99, 113, and 110% relative to untreated cells in LLP-5-HT1A, SN48, and RN46A, respectively). However, the two antisense sequences display differences in potency, the AS1 sequence being the most effective one (lanes 1 and 2 vs lane 4). Thus, following AS1 antisense treatment the expression of newly synthesized receptor in LLP-5-HT1A, SN48, and RN46A was inhibited by 64, 34, and 32%, respectively. In the 5-HT1A overexpressed LLP-K1 cells, AS2 inhibited receptor expression by approx 45%. It is of interest to note that AS1 was directed to a region in the mRNA at the beginning of the translation site (bp 115–128), whereas AS2 was directed to a region localized in the middle of the translation sequence (bp 885–902). Accordingly, in the latter case, truncated protein fragments of the receptor should have been produced if the antisense effect relies on steric interference with mRNA translation. How-

ever, such truncated protein fragments were never detected in the gel. Remarkably, in contrast to AS1, AS2 was essentially without effect on the newly synthesized protein fraction in neuronal SN48 and RN46A cells. However, in line with the strongly diminished activity of AS1 in LLP-5-HT1A cells, these observations might suggest a cell- type-dependent effect of antisense efficiency, discriminating a natural target (as in SN48 and RN46A) from one that is overexpressed (LLP-5HT1A).

Antisense ODN treatment reduces the expression of target mRNA

As noted above, steric interference of the antisense ODN with translation is less likely the cause of the decrease in protein expression. Rather, the data may imply mRNA degradation. At steady state the mRNA levels of 5-HT1A differ markedly in a cell-dependent manner, being most pronounced in the overexpressing cells (data not shown). For quantitative reasons, we therefore employed LLP-5HT1A cells. In line with the diminished expression at the protein level of the receptor in LLP-5-HT1A cells (Fig. 2, LLP-5-HT1A), a similar decrease in mRNA levels was observed following treatment with AS1 (Fig. 3A, 75% inhibition; lane 1). Evidently, AS2 was less effective in decreasing the protein level (Fig. 2, lane 2), and consistently, the decrease in mRNA expression was less pronounced (approx 60% inhibition). Note that the effect of the mismatched sequences (MAS1 and MAS2) was essentially negligible (lanes 3 and 4 vs lane 5, 98 and 106% compared with untreated cells). Given the close correlation between the inhibition of protein expression and the reduction of mRNA levels, and the absence of detectable truncated protein fragments, the data suggest that the consequences of antisense treatment might be due to the rapid and efficient degradation of mRNA.

To obtain further insight into the pathway by which the ODNs gain nuclear access and the potential correlation of
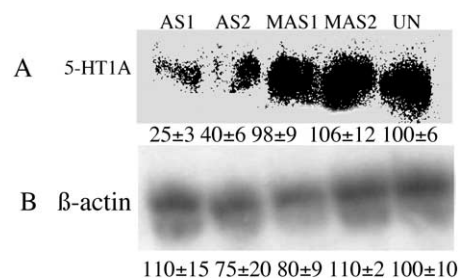

Fig. 3. Downregulation of antisense ODN to 5-HT1A mRNA. (A) LLP-5-HT1A cells were treated with 100 nM antisense ODN sequence 1 (AS1), sequence 2 (AS2), and mismatched sequences (MAS1 and MAS2), complexed with SAINT-2/DOPE. Total RNA was isolated and 5-HT1A was probed with ^{32}P-radiolabeled 5-HT1A cDNA. (B). The same blot was probed with ^{32}P-radiolabeled β-actin cDNA probe. Numbers under the bands indicate the amount of mRNA (%) relative to the amount in untreated cells (UN), as obtained in two independent experiments.
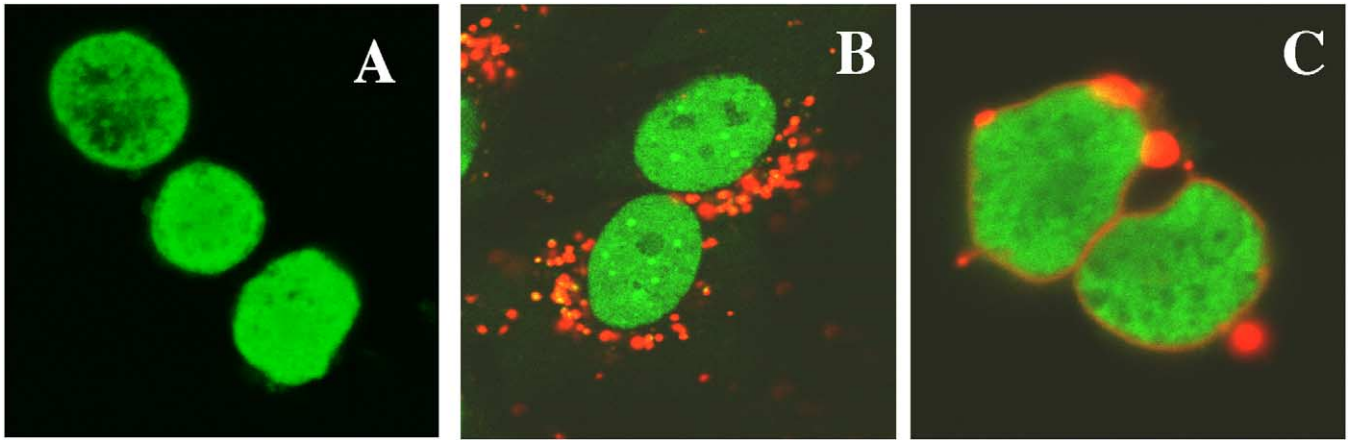
Fig. 4. Accumulation of PS-ODNs in the nuclei. When FITC labeled PS-ODNs (100 nM) were incubated with isolated nuclei (30 min), they readily accumulated in the nuclei (A). Note that for intranuclear accumulation neither cytosolic proteins nor cationic lipid complexes are needed. When cationic lipids/PS-ODNs were incubated with intact cells. PS-ODNs (FITC-labeled, green) also accumulated in cell nuclei, while the cationic lipids (Rh-label, red) remained in the endosomal compartments (B). When cationic lipids/PS-ODNs were incubated with isolated nuclei. PS-ODNs (FITC-label, green) also accumulated in cell nuclei, and cationic lipids (with Rh-label, red) partly fused with nuclear membranes The intense red staining represents clustered lipoplexes (C).

this localization with the eventual cytosolic degradation of mRNA, the next experiments were carried out.

Nuclear delivery and interaction of antisense ODNs with the RNA nuclear matrix

It is generally assumed that once in the cytosol, ODNs may gain rapid access to the nucleus via diffusion through the nuclear pores. Some studies indicated that antisense ODN may bind to intracellular proteins [17], but whether such proteins are instrumental in nuclear homing remains to be determined. It is also unclear whether cationic lipids are needed or may facilitate this event, other than their functioning in effectively translocating ODNs across endosomal membranes [23]. To investigate these issues, we isolated

nuclei, which were then incubated with free and complexed FITC-labeled ODNs. As shown in Fig. 4, free ODNs rapidly and efficiently acquired nuclear access (Fig. 4A), irrespective of the presence of cytosol, implying that proteins were not needed to accomplish efficient nuclear accumulation of the ODNs. Similarly, when whole cells or nuclei were incubated with ODN complexes, which also contained the fluorescent lipid analogue N-Rh-PE as a marker of the cationic lipid phase, the net extent of nuclear accumulation of the ODNs in either case was virtually indistinguishable. Note that when added to whole cells (Fig. 4B), lipid-derived fluorescence (red) remains localized in the endosomal/lysosomal compartments [5], without any (detectable) appearance at or in the nucleus. The ODNs show a largely random and diffuse distribution throughout the nucleus (green flu-
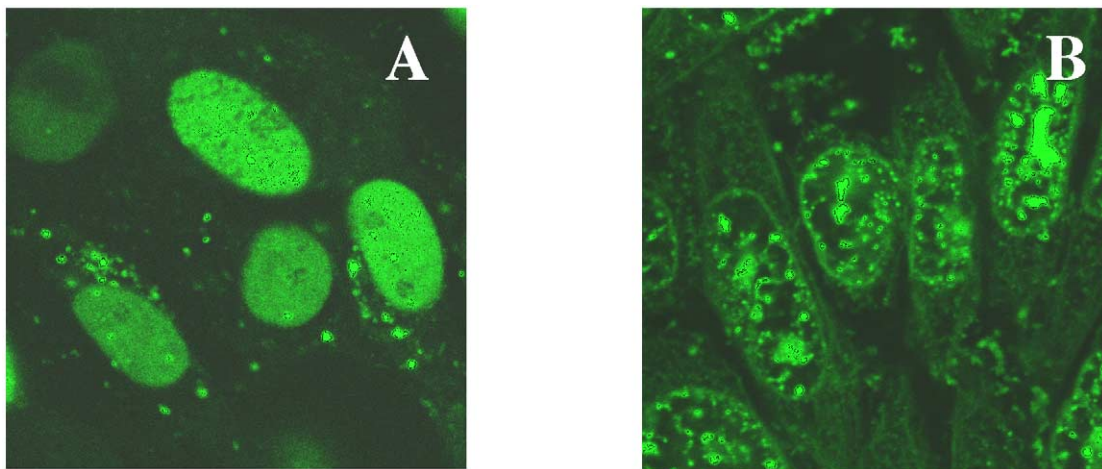

Fig. 5. Intranuclear PS-ODNs associate with the nuclear matrix. When cationic lipids/PS-ODNs were incubated with cells, PS-ODNs (FITC labeled) accumulated in the cell nuclei (A). Following digestion with RNase A, the PS-ODNs were largely removed from the nuclei, and only PS-ODN nuclear bodies remained in the nuclei. Also, some diffused fluorescence was now apparent in the cytoplasm (B).
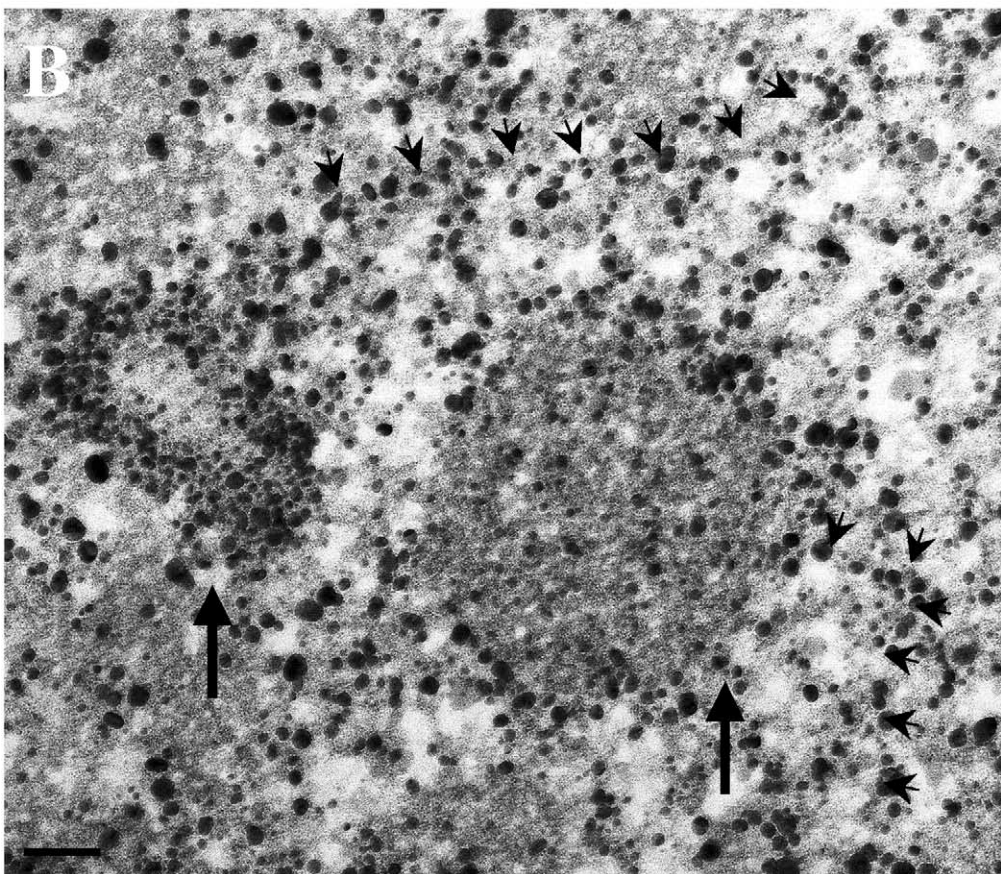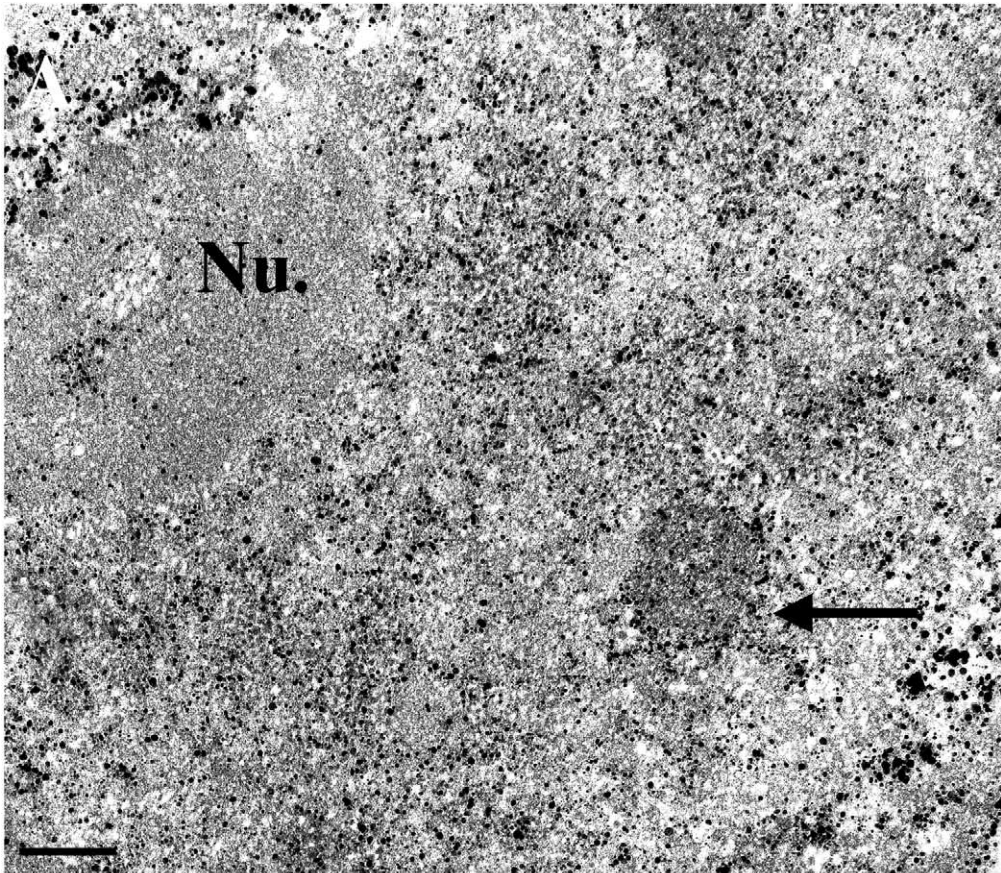
orescence) except for the nucleolus (dark round spheres), and occasionally form concentrated ODN bodies (bright green dots) (Fig. 4B). When incubating ODN/lipid complexes with isolated nuclei, delivery of ODNs within the nucleus occurs (green fluorescence). Concomitant entry of the lipids is not seen, as they associate largely with the nuclear membrane as attached clustered complexes (intense red dots, Fig. 4C) or become laterally diffused within the plane of the membrane (red ring, Fig. 4C). Hence, these data indicate that neither (cytosolic) proteins nor entry of the cationic lipid (complex) into the cytosol is required for effective nuclear delivery of ODNs.

To obtain further insight into a potential correlation between intranuclear access and antisense mechanism, we subsequently determined the intranuclear fate of the ODNs, following the treatment of intact cells with FITC-labeled ODN complexes. First, nuclear proteins were extracted and nuclear chromatins were digested with RNase-free DNase. Following this treatment, no significant alteration in intranuclear ODN distribution was apparent, when compared with the distribution in control cells (Fig. 5A). Interestingly, when nuclear RNA was digested with RNase A to remove the RNA matrix in the nucleus, most of the ODNs disappeared and only ODNs assembled into so-called phosphorothioate ODN (PS [7]) bodies remained (Fig. 5B). As a control, when the cells were treated with the same buffer without RNase, no effect on ODN distribution was seen (data not shown). Furthermore, ODNs are not seen in the nucleolus, where rRNAs are synthesized and processed and ribosome subunits are assembled. Identical results were seen for LLP-5HT1A and SN48 cells (data not shown). From these experiments, we conclude that following nuclear entry, ODNs associate largely with the nuclear RNA matrix. To obtain further support, the intranuclear localization of ODNs was further examined by EM. RN46 cells were incubated with biotin-labeled ODN cationic lipid complexes, and then the ODNs were visualized by subsequent labeling with a streptavidin–gold conjugate, as described under Materials and Methods. Consistent with the fluorescence distribution, the gold labeling was seen throughout nucleoplasm, with little if any labeling in the nucleolus (Fig. 6A, Nu.). Dense gold labeling was seen on the ODN bodies, identified as bright dots by fluorescence microscopy, especially at the periphery (as indicated by arrows in Figs. 6A and B). At higher magnification, gold labeling of ODNs in the nucleoplasm is apparent along fibers of the internal nuclear matrix (arrowheads in Fig. 6B [24]), visible as a diffuse distribution by fluorescence microscopy. As a control, labeling was also carried out with unlabeled ODNs, and was followed by the addition of streptavidin–gold particles.

Labeling was not detectable under those conditions (data not shown).

In conjunction with the observed mRNA degradation as shown in Fig. 3B, these data would be consistent with the notion that the antisense ODNs might act on mRNA or hnRNA in the nucleus. The next experiments were aimed at elucidating how sequence selectivity is expressed in terms of antisense activity following its binding to the RNA matrix.

Intracellular affinity of antisense ODNs for target mRNA

Although antisense has been shown to bind to isolated target mRNA or mRNA fragments in a test tube, direct evidence of the association of antisense with mRNA within cells has not been provided thus far. The very small amounts of target mRNA, as observed here in the RN46 and SN48 cells, and the potential rapid degradation of mRNA in the cells may constitute a limitation in resolution in that regard. However, the abundance of 5-HT1A mRNA in LLP-5-HT1A cells evidently provided an opportunity for further investigation of this issue. Lipoplexes containing 100 nM ^{32}P-labeled antisense (Fig. 7A. AS)or mismatched (Fig. 7A, MAS) sequences were incubated with LLP-5-HT1A cells for 16 h. To exclude that antisense hybrization to target mRNA might have occurred during or after extraction, the same amount of ^{32}P-labeled antisense was added to untreated cells during RNA isolation (Fig. 7, UN/AS+). Following total RNA extraction and its concentration by ethanol precipitation, RNA was separated on a Northern gel and blotted on a nylon membrane. The potential binding of antisense to mRNA was then visualized by autoradiography. As shown in Fig. 7, antisense binding to mRNA in LLP-5-HT1A cells could be readily revealed even though the signal was rather low (Fig. 7A, AS). The relatively low signal may well be related to mRNA degradation, triggered by antisense binding, as seen in Fig. 7B (AS vs MAS and UN/AS+). The mismatched (Fig. 7A, MAS) or antisense (Fig. 7, US/AS+) sequences added during RNA isolation failed to bind to the mRNA. These data are consistent with the notion that the antisense effect is elicited via binding of ODNs to the nuclear matrix in the nucleus, the antisense specificity being conveyed through high binding affinity to target mRNA. Having established the efficiency of the antisense ODNs in the reduction of newly synthesized 5-HT1A and target mRNA, we finally examined the ultimate goal of antisense treatment, i.e., whether and under what conditions the total pool of the receptor could be modulated to accomplish a biological effect.

Fig. 6. EM visualization of ODNs, localized in the nucleus. One hundred nanomolar biotin-labeled ODNs, complexed with SAINT-2/DOPE, were incubated with RN46 cells. The ODNs were visualized with FluoroNanogold–streptavidin conjugate. Note that gold labeling is seen throughout the nucleoplasm (black dots), with little if any labeling in the nucleolus (A, Nu,). Dense gold labeling is apparent on the ODN bodies, especially at the periphery of the bodies (A and B, arrows). At higher magnification (B), gold labeling of ODNs in the nucleoplasm can be seen associated along the fibers of the nuclear matrix (arrowheads) Bars = 344 nm and (A), 100 nm (B).
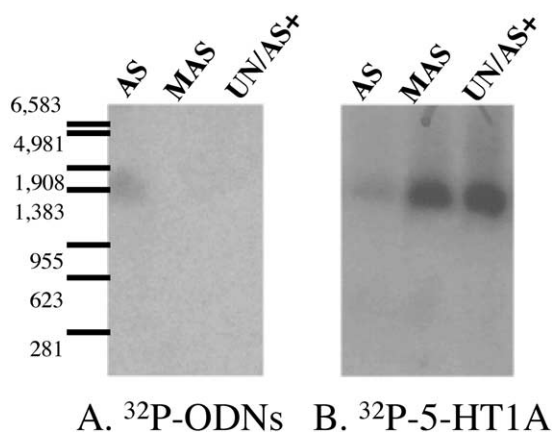
Fig. 7. Antisense ODNs bind to intracellular mRNA targets. One hundred nanomolar ^{32}P-labeled antisense ODNs (AS) or mismatched sequences (MAS), complexed with cationic lipid vectors, were incubated with LLP-5-HT1A cells, and total RNA was isolated. Alternatively, 100 nM ^{32}P-labeled antisense ODNs were added to untreated cells during the RNA isolation (UN/AS+). The RNAs were separated on agarose gel and ODN–mRNA binding was determined by autoradiography (A). The location of 5-HT1A mRNA on the gel in (A) was determined from that in (B). LLP-5-HT1A cells were treated as in (A), except that the ODNs were not labeled. RNA was isolated and separated on the gel. The 5-HT1A mRNA was then probed with ^{32}P-labeled 5-HT1A cDNA probe (B). The specific antisense binding to 5-HT1A mRNA could be identified (A, AS), as it locates at the same position as the 5-HT1A mRNA (B, AS). The binding to 5-HT1A mRNA was not seen with mismatched sequences (A, MAS) or on addition of antisense ODNs during RNA isolation (A, UN/AS+).

Functional downregulation of 5-HT1A receptor activity following antisense treatment

The three cell types were treated over a period of 5 days with antisense complexes or control, mismatched complexes. Each treatment was carried out by incubating the ODN complexes with the cells for 6 h, after which they were washed and grown in medium for another 48 h. This protocol was repeated twice and on the sixth day the cells were harvested and analyzed by Western immunoblot. As shown in Fig. 8A for SN48 cells, over the period of treatment, the total pool of 5-HT1A can be effectively downregulated by approx 67%, following treatment with AS1. Similar results were obtained for the treatment of RN46A cells, resulting in a downregulation of 64% with AS1. In both cell lines, AS2 was less effective. In LLP-5-HT1A cells, AS1 and AS2 showed 68 and 51% downregulation of 5-HT1A. Mismatched sequences (MAS1 and MAS2) did not alter 5-HT1A expression, and expression of the internal control, β-actin, was not affected by any treatment (Fig. 8A). In addition, no alterations were seen in total protein expression when analyzed by PAGE for control and ODN-treated cells, further emphasizing the specificity on the 5-HT1A receptor. The functional consequence of the downregulation of the receptor was examined with a cytosensor, which measures the metabolic change of the cell on challenge with an agonist. The procedure was validated by challenging SN48 cells with flesinoxan, an agonist to

5-HT1A. The cells responded to this treatment in a concentration-dependent manner, and the activation could be blocked by preincubation with WAY 100635, an antagonist to 5-HT1A (data not shown). Next, the cells were treated for 5 days with 100 nM antisense AS1 complexes, as described above. The treated cells were plated onto the cytosensor membrane 16 h before the measurements. To normalize the activity for the number of cells on each membrane, the cells were first challenged with acetylcholine, which is a ligand for choline receptors expressed on SN48 cells. Then, the cells were challenged with increasing concentrations of flesinoxan. To further support specificity, the 5-HT1A was subsequently blocked with WAY 1000635, and the cells were challenged once more with flesinoxan. As shown in Fig. 8B, flesinoxan activated control cells and cell treated with mismatched ODNs in a concentration-dependent manner, which could be blocked by WAY 100635. In contrast, activation of the antisense-treated (AS1) cells was completely abolished. Note that the negative value is due to acidification on addition of flesinoxan, an effect that is normally overcome on activation of the 5-HT1A receptor. Antisense sequence 2 (AS2) did not alter the function of 5-HT1A receptor (data not shown). This is consistent with the observation that a change was not seen in protein level following AS 2 treatment (Fig. 8A). Hence the data indicate that following antisense treatment, the function of the 5-HT1A receptors can be inhibited in a highly efficient manner.

Discussion

For an antisense treatment to be effective, it is essential to design a sequence that effectively matches the secondary and/or tertiary structure of target mRNA. Since studies on mRNA secondary and tertiary structure are still scarce, these structures are usually predicted by computer-facilitated analysis. Thus, antisense sequences can be selected by prediction of the mRNA structure [25], although empirical design and experimental mRNA mapping are also used [26–28]. Here, sequences were applied that relied on computer-aided antisense sequence design and selection technology, called RADAR (Rational Algorithmic Design of ANTISENSE Reagents), which avoids costly, time-consuming, and laborious screening for appropriate antisense sequences [29–34].

The two antisense sequences produced in this manner and applied in the present work showed different potencies but, interestingly, did show a cell type-dependent effect when comparing naturally expressed and overexpressed receptors. Whether this distinction is related to structural differences of the overexpressed mRNA in LLP-5HT1A cells remains to be determined. Yet, one of the sequences (AS1) effectively downregulated the target expression in all three cell types studied, suggesting an antisense sequence-dependent effect as well.
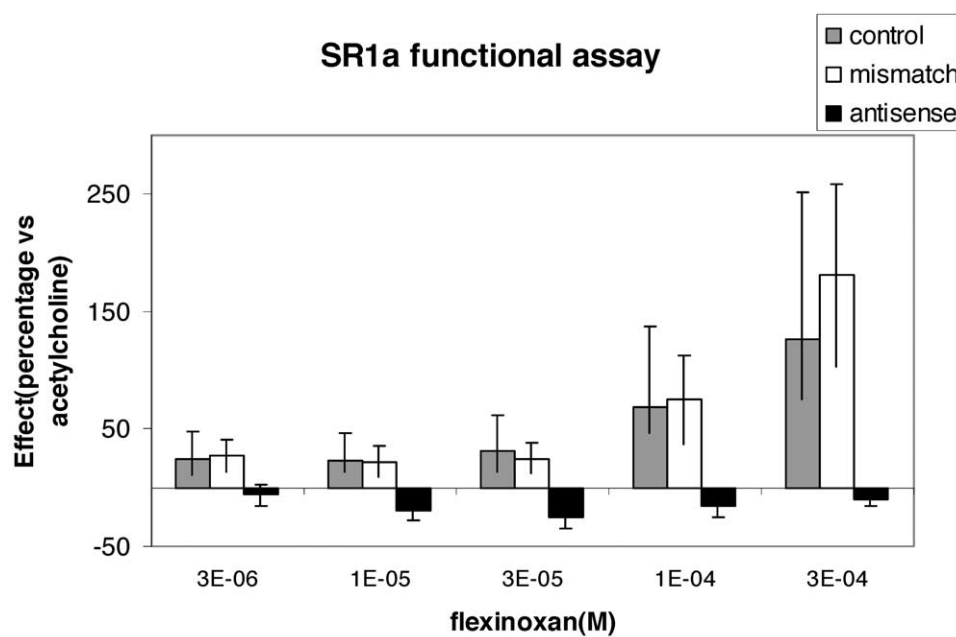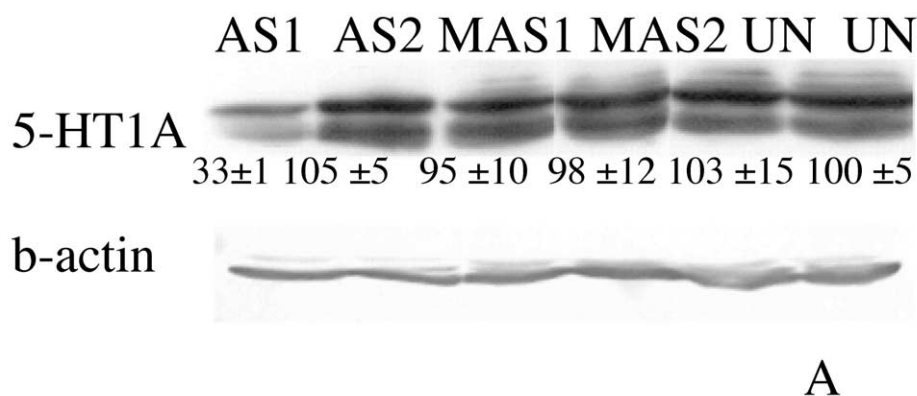
Fig. 8. (A). Downregulation of the total 5-HT1A receptor pool in SN48 cells. The cells were treated according to a protocol as described for 5 days with 100 nM antisense ODN sequence 1 (AS1), sequence 2 (AS2), and mismatched sequences (MAS1 and MAS2), complexed with SAINT-2/DOPE. The presence of 5-HT1A was analyzed by Western immunoblot. β-Actin was used as an internal control. The numbers under the bands indicate the amount of 5-HT1A (%) relative to untreated cells (UN) from two experiments. (B) Functional downregulation of 5-HT1A receptor activity after antisense treatment. Control activity and receptor activity in 100 nM antisense (AS1), and mismatch ODN complex-treated SN48 was determined using a cytosensor approach, as described under Materials and methods. The cells were challenged with acetylcholine and the 5-HT1A agonist flesinoxan. Flesinoxan activation was plotted as a percentage relative to acetylcholine activation. Flesinoxan activated 5-HT1A control cells in a concentration-dependent manner (hatched bars). Mismatched sequence did not show a significant change (open bars) relative to control. Note that flesinoxan activation of the 5-HT1A receptor was abolished in cells treated with antisense AS1 ODN under the same conditions (black bars). Also, no change was observed, relative to control, when cells were treated with AS 2 ODNs (data not shown). The data are expressed relative to the acetylcholine effect, which was taken for calibration (100%), and flesinoxan activation was plotted relative to acetylcholine activation.

To successfully apply antisense ODNs, insight into their optimal design, mechanism of internalization, and mechanism of action is crucial. As shown here, for effective nuclear delivery, a vector is needed to facilitate effective translocation of the ODNs from the endosome into the cytosol [5], rather than a delivery vehicle required for entry into the nucleus per se (Fig. 4). Indeed, elsewhere we have provided evidence that SAINT-2/DOPE-mediated delivery of plasmids relies on entry of these complexes via clathrin-mediated endocytosis [3]. Similarly, ODN internalization via the same vector localizes to endosomal/lysosomal compartments [5,6] and its uptake is inhibited on energy or potassium depletion, which inhibits clathrin-mediated endocytosis (data not shown).

In permeabilized cells, it has been reported that ODNs bind extensively to intermediate filaments in the cytosol, with a preference for cytokeratin intermediate filaments [35]. In the same study, ODNs localized in the nucleus interacted with nuclear lamina and caused the decompaction of chromatin [35]. Such phenomena were not seen on microinjection of ODNs into the cytosol [7,36,37] or following cationic lipid-mediated ODN delivery [5,7,38]. Possibly, in permeabilized cells, a rapid and high degree of saturation of potential intracellular binding sites might be accomplished on exogenous addition of ODNs. Obviously, such conditions are not readily achieved on vector-mediated delivery, either in vitro or in vivo. Furthermore, actin filaments are less likely encountered when ODNs are released from the endosomal compartment, and the data as shown here (Fig. 4) and elsewhere [5,7,38] indicate a rapid transfer of ODNs into the nucleus under such conditions. Nuclear access requires neither cationic lipids nor cytosolic proteins (Fig. 4).

The exact subnuclear localization of ODNs is still not well defined. The eukaryotic nucleus consists of chromatin, RNA, and proteins. Interestingly, we observed that the ODNs almost exclusively bind to the nuclear matrix, which consists largely of hnRNA and a large family of proteins. This is consistent with previous findings reported in an elegant study by Lorenz et al. [7]. Others have found a high degree of complex formation between oligonucleotides and nuclear proteins, as revealed by a gel shift assay [39]. However, no protein has been identified thus far.

Interference with a biological function is evidently the final goal of antisense technology, a misleading factor for proper interpretation often being the specificity of the antisense effect. Clearly, in all three cell types we observed a substantial decrease in newly synthesized protein, the first effect to become apparent, following ODN treatment. Also, a specific diminishment in mRNA level of the target protein was observed, supporting the specificity of the observed antisense effect in the present work. Since truncated protein fragments were not observed, our data support an efficient degradation of target mRNA by RNase H as a leading mechanism in directing ODN antisense efficiency, and in the present work we provided direct evidence for the intracellular association of antisense ODN and target mRNA. Given the intranuclear localization of RNase H [40,41], the need for a nuclear localization of antisense to accomplish its effect can thus be readily rationalized.

Taken together, here we have shown that with appropriate sequences, and in a cell type-dependent manner a functional antisense effect can be accomplished, which originated from a nuclear association of the ODN with target mRNA via binding to the nuclear matrix. Our data indicate that such an interaction causes degradation of the target RNA and, consequently, downregulation of the newly synthesized serotonin receptor 5-HT1A. In conjunction with metabolic turnover, the pool of the receptor can then be specifically diminished to a level that results in complete abolition of its biological activity as well (Fig. 8B). Both cell biological studies and therapeutic ex vivo treatments may benefit from such an approach.

Acknowledgments

References

[1] C. Vanhee-Brossollet, C. Vaquero, Do natural antisense transcripts make sense in eukaryotes? Gene 211 (1998) 1–9.
[2] B. Lehner, G. Williams, R.D. Campbell, C.M. Sanderson, Antisense transcripts in the human genome, Trends Genet. 18 (2002) 63–65.
[3] I.S. Zuhorn, R. Kalicharan, D. Hoekstra, Lipoplex-mediated transfection of mammalian cells occurs through the cholesterol-dependent clathrin-mediated pathway of endocytosis, J. Biol. Chem. 277 (2002) 18021–18028.
[4] J. Smisterova, A. Wagenaar, M.C. Stuart, E. Polushkin, G.T. Brinke, R. Hulst, J.B. Engberts, D. Hoekstra, Molecular shape of the cationic lipid controls the structure of cationic lipid/dioleylphosphatidylethanolamine–DNA complexes and the efficiency of gene delivery, J. Biol. Chem. 276 (2001) 47615–47622.
[5] F. Shi, A. Nomden, V. Oberle, J.B. Engberts, D. Hoekstra, Efficient cationic lipid-mediated delivery of antisense oligonucleotides into eukaryotic cells: down-regulation of the corticotropin-releasing factor receptor, Nucleic Acids Res. 29 (2001) 2079–2087.
[6] F. Shi, L. Wasungu, A. Nomden, M.C.A. Stuart, E. Polushkin, J.B. Engberts, F.N.D. Hoekstra, et al., Interference of poly(ethylene glycol)–lipid analogues with cationic-lipid-mediated delivery of oligonucleotides: role of lipid exchangeability and non-lamellar transitions, Biochem. J. 366 (2002) 333–341.
[7] P. Lorenz, B.F. Baker, C.F. Bennett, D.L. Spector, Phosphorothioate antisense oligonucleotides induce the formation of nuclear bodies, Mol. Biol. Cell 9 (1998) 1007–1023.
[8] S.T. Crooke, Molecular mechanisms of action of antisense drugs, Biochim. Biophys. Acta 1489 (1999) 31–44.
[9] N. Dias, S. Dheur, P.E. Nielsen, S. Gryaznov, A.V. Aerschot, P. Herdewijn, C. Helene, S.T.E. Behmoaras, Antisense PNA tridecamers targeted to the coding region of Ha-ras mRNA arrest polypeptide chain elongation, J. Mol. Biol. 294 (1999) 403–416.
[10] I. Robbins, B. Lebleu, Vesicular stomatitis virus as model system for studies of antisense oligonucleotide translation arrest, Methods Enzymol. 313 (2000) 189–203.
[11] G.J. Veal, S. Agrawal, R.A. Byrn, Sequence-specific RNase H cleavage of gag mRNA from HIV-1 infected cells by an antisense oligonucleotide in vitro, Nucleic. Acids Res. 26 (1998) 5670–5675.
[12] C. Boiziau, N.T. Thuong, J.J. Toulme, Mechanisms of the inhibition of reverse transcription by antisense oligonucleotides, Proc. Natl. Acad. Sci. USA 89 (1992) 768–772.
[13] I. Lebedeva, L. Benimetskaya, C.A. Stein, M. Vilenchik, Cellular delivery of antisense oligonucleotides, Eur. J. Pharm. Biopharm. 50 (2000) 101–119.
[14] P. Rockwell, W.J. O'Connor, K. King, N.I. Goldstein, L.M. Zhang, C.A. Stein, W.J. O'Connor, K. King, N.I. Goldstein, L.M. Zhang, C.A. Stein, Cell-surface perturbations of the epidermal growth factor

and vascular endothelial growth factor receptors by phosphorothioate oligodeoxynucleotides, Proc. Natl. Acad. Sci. USA 94 (1997) 6523–6528.

[15] W. Wang, H.J. Chen, A. Schwartz, P.J. Cannon, C.A. Stein, L.E. Rabbani, Sequence-independent inhibition of in vitro vascular smooth muscle cell proliferation, migration, and in vivo neointimal formation by phosphorothioate oligodeoxynucleotides, J. Clin. Invest. 98 (1996) 443–450.

[16] A. Anselmet, E. Mayat, S. Wietek, P.G. Layer, B. Payrastre, J. Massoulie, Non-antisense cellular responses to oligonucleotides, FEBS Lett. 510 (2002) 175–180.

[17] I. Brukner, G.A. Tremblay, Cellular proteins prevent antisense phosphorothioate oligonucleotide (SdT18) to target sense RNA (rA18): development of a new in vitro assay, Biochemistry 39 (2000) 11463–11466.

[18] A.A.P. Meekel, A. Wagenaar, J. Smisterova, J. Kroeze, P. Haadsma, B. Bosgraaf, M.C.A. Stuart, A. Brisson, M.H.J. Ruiters, D. Hoekstra, Synthesis of pyrimidinium amphiphiles used for transfection and some characteristics of amphiphile/DNA complex formation, Eur. J. Org. Chem. 2000 (2000) 665–673.

[19] X.M. Ou, H.J. Nejad, J.M. Storring, J.H. Meng, S. Lemonde, P.R. Albert, Novel dual repressor elements for neuronal cell-specific transcription of the rat 5-HT1A receptor gene, J. Biol. Chem. 275 (2000) 8161–8168.

[20] X.M. Ou, J.M. Storring, N. Kushwaha, P.R. Albert, Heterodimerization of mineralocorticoid and glucocorticoid receptors at a novel negative response element of the 5-HT1A receptor gene, J. Biol. Chem. 276 (2001) 14299–14307.

[21] P.L. Andersen, C.A. Webber, S.R. Whittemore, D.J. Schreyer, Divergent regulation of GAP-43 expression and CNS neurite outgrowth by cyclic AMP, J. Neurosci. Res. 61 (2000) 626–635.

[22] X. Langlois, S.E. Mestikawy, M. Arpin, A. Triller, M. Hamon, M. Darmon, Differential addressing of 5-HT1A and 5-HT1B receptors in transfected LLC-PK1 epithelial cells: a model of receptor targeting in neurons, Neuroscience 74 (1996) 297–302.

[23] I.S. Zuhorn, V. Oberle, W.H. Visser, J.B. Engberts, U. Bakowsky, E. Polushkin, D. Hoekstra, Phase behavior of cationic amphiphiles and their mixtures with helper lipid influences lipoplex shape, DNA translocation, and transfection efficiency, Biophys. J. 83 (2002) 2096–2108.

[24] J.A. Nickerson, G. Krockmalnic, K.M. Wan, S. Penman, The nuclear matrix revealed by eluting chromatin from a cross-linked nucleus, Proc. Natl. Acad. Sci. USA 94 (1997) 4446–4450.

[25] V. Patzel, G. Sczakiel, Theoretical design of antisense RNA structures substantially improves annealing kinetics and efficacy in human cells, Nat. Biotechnol. 16 (1998) 64–68.

[26] S.P. Ho, D.H. Britton, B.A. Stone, D.L. Behrens, L.M. Leffet, F.W. Hobbs, J.A. Miller, G.L. Trainor, Potent antisense oligonucleotides to the human multidrug resistance-1 mRNA are rationally selected by mapping RNA-accessible sites with oligonucleotide libraries, Nucleic Acids Res. 24 (1996) 1901–1907.

[27] M. Sohail, H. Hochegger, A. Klotzbucher, R.L. Guellec, T. Hunt, E.M. Southern, Antisense oligonucleotides selected by hybridisation to scanning arrays are effective reagents in vivo, Nucleic Acids Res. 29 (2001) 2041–2051.

[28] N. Milner, K.U. Mir, E.M. Southern, Selecting effective antisense reagents on combinatorial oligonucleotide arrays, Nat. Biotechnol. 15 (1997) 537–541.

[29] H.C. Welch, W.J. Coadwell, C.D. Ellson, G.J. Ferguson, S.R. Andrews, H. Erdjument-Bromage, P. Tempst, P.T. Hawkins, L.R. Stephens, P-Rex1, a PtdIns (3,4,5) P3- and G-beta-gamma-regulated guanine-nucleotide exchange factor for Rac, Cell 108 (2002) 809–821.

[30] V.L. Raghavendra Rao, K.K. Bowen, V.K. Dhodda, G. Song, J.L. Franklin, N.R. Gavva, R.J. Dempsey, Gene expression analysis of spontaneously hypertensive rat cerebral cortex following transient focal cerebral ischemia, J. Neurochem. 83 (2002) 1072–1086.

[31] Y. Kubota, T. Tanaka, A. Kitanaka, H. Ohnishi, Y. Okutani, M. Waki, T. Ishida, H. Kamano, Src transduces erythropoietin-induced differentiation signals through phosphatidylinositol 3-kinase, EMBO J. 20 (2001) 5666–5677.

[32] Y. Kashima, T. Miki, T. Shibasaki, N. Ozaki, M. Miyazaki, H. Yano, S. Seino, Critical role of cAMP-GEF11–Rim2 complex in incretin-potentiated insulin secretion, J. Biol. Chem. 276 (2001) 46046–46053.

[33] A. Von Eckardstein, C. Langer, T. Engel, I. Schaukal, A. Cignarella, J. Reinhardt, S. Lorkowski, Z. Li, X. Zhou, P. Cullen, G. Assmann, ATP binding cassette transporter ABCA1 modulates the secretion of apolipoprotein E from human monocyte-derived macrophages, FASEB J. 15 (2001) 1555–1561.

[34] J. Fang, Y. Shing, D. Wiederschain, L. Yan, C. Butterfield, G. Jackson, J. Harper, G. Tamvakopoulos, M.A. Moses, Matrix metalloproteinase-2 is required for the switch to the angiogenic phenotype in a tumor model, Proc. Natl. Acad. Sci. USA 97 (2000) 3884–3889.

[35] R.L. Shoeman, R. Hartig, Y. Huang, S. Grub, P. Traub, Fluorescence microscopic comparison of the binding of phosphodiester and phosphorothioate (antisense) oligodeoxyribonucleotides to subcellular structures, including intermediate filaments, the endoplasmic reticulum, and the nuclear interior, Antisense Nucleic Acid Drug Dev. 7 (1997) 291–308.

[36] J.P. Leonetti, N. Mechti, G. Degols, C. Gagnor, B. Lebleu, Intracellular distribution of microinjected antisense oligonucleotides, Proc. Natl. Acad. Sci. USA 88 (1991) 2702–2706.

[37] T.L. Fisher, T. Terhorst, X. Cao, R.W. Wagner, Intracellular disposition and metabolism of fluorescently-labeled unmodified and modified oligonucleotides microinjected into mammalian cells, Nucleic Acids Res. 21 (1993) 3857–3865.

[38] Y. Xu, F.C. Szoka Jr., Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection, Biochemistry 35 (1996) 5616–5623.

[39] D.A. Brown, S.H. Kang, S.M. Gryaznov, L. DeDionisio, S. Heidenreich, S. Sullivan, X. Xu, M.I. Nerenberg, Effect of phosphorothioate modification of oligodeoxynucleotides on specific protein binding, J. Biol. Chem. 269 (1994) 26801–26805.

[40] C. Crum, J.D. Johnson, A. Nelson, D. Roth, Complementary oligodeoxynucleotide mediated inhibition of tobacco mosaic virus RNA translation in vitro, Nucleic Acids Res. 16 (1988) 4569–4581.

[41] R.J. Crouch, M.L. Dirksen, Ribonucleases H, in: S.M. Linn, R.J. Roberts (Eds.), Nucleases, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1985, pp. 211–241.