Up-Regulation of Luciferase Gene Expression with Antisense Oligonucleotides: Implications and Applications in Functional Assay Development†

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ABSTRACT: HeLa Tet-Off cells were transfected transiently as well as stably with a recombinant plasmid (pLuc/705) carrying the luciferase gene interrupted by a mutated human β-globin intron 2 (IVS2-705). The mutation in the intron causes aberrant splicing of luciferase pre-mRNA, preventing translation of luciferase. However, treatment of the cells with a 2′-*O*-methyl-oligoribonucleotide targeted to the aberrant splice sites induces correct splicing, restoring luciferase activity. The effects are sequence-specific, depend on the concentration of the oligonucleotide, and can be modulated by the pretreatment of the cell line, Luc/705, with tetracycline. Thus, the cell line provides, among others, a novel functional assay system superior to other procedures that are based on protein down-regulation. In particular, the system would be ideal in assessing the cellular delivery efficiency of antisense oligonucleotides.

Synthetic oligonucleotides have shown promising therapeutic potentials as antigene or antisense agents (*1*−*5*). However, the development has been greatly hampered by the uncertainties associated with the mechanism of action (*6*), the nonspecific binding of certain oligomers to cellular proteins (*7*), the RNase H-mediated nonantisense mechanism (*8*), and perhaps most importantly by the lack of means to deliver the oligomers at the tissue/organ as well as (sub)-cellular level (*9*−*12*).

The development of effective means to deliver antisense agents to the intracellular target has been slowed in part by the lack of a simple, rapid, and reliable way to evaluate the delivery efficiency. A typical cellular system for assessing antisense activity is based on the inhibition of protein synthesis. Such a system suffers from several drawbacks: low signal-to-noise ratio, delayed response originating from the stability of protein translated from the targeted mRNA, and unknown contribution of cytotoxicity from a given strategy to global protein synthesis. Moreover, the assay does not allow one to ascertain the intracellular site, nuclear versus cytoplasmic, of the antisense activity. Although the latter issue can be addressed by confocal fluorescence microscopy, this technique is far from being unequivocal (*13*). Likewise, it is frequently difficult to determine the influence of delivery-related issues on the overall antisense activity, because the antisense oligonucleotides may be targeted to sequence elements of different accessibility (*14*) or delivered to the cells by different techniques (*15*). Here we report on a novel cellular assay system in which the activity of antisense oligonucleotides results in the up-regulation of the luciferase gene expression and therefore is free of the drawbacks mentioned above.

EXPERIMENTAL PROCEDURES

Materials. LipofectAmine as well as the cell culture media used were from Life Technologies, Inc. (Gaithersburg, MD) unless specified otherwise. The phosphorothioate 2′-*O*-methyl-oligoribonucleotides used in the present study were gifts from Hybridon Inc. (Cambridge, MA) and used as received: ON-705, CCUCUUACCUCAGUUACA; ON-705M, CCUCUUACAUCAGUUACA; ON-119, UGAGACUUCCACACUGAU; and ON-3′C, CAUUAUUGCCCUGAAAG were targeted to respective sequences in the intron 2 of human β-globin gene. As an additional control, a randomized oligonucleotide pool, that is, a preparation synthesized as a library of all possible 18-mers, was used. HeLa Tet-Off cell line, luciferase plasmid pTRE-Luc and hygromycin-resistant plasmid pTK-Hyg were purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Primers used in PCR were custom-synthesized in the Lineberger Comprehensive Cancer Center, the University of North Carolina. Hygromycin was from Boeringer-Mannheim (Indianapolis, IN).

Methods. *Plasmid and Cell Line Construction*. The thalassemic β-globin intron IVS2-705 or its wild-type equivalent IVS2-GL (*16*) was inserted into the luciferase cDNA sequence of the pTRE-Luc plasmid, between nucleotides 1368 and 1369, by PCR-based in vivo recombination (*17*) resulting in plasmids pLuc/705 or pLuc/GL, respectively (Figure 1). The details of the construction are available on request. HeLa Tet-Off cells were transfected with the

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[1] Abbreviations: DMEM, Dulbecco's modified eagle medium; FCS, fetal calf serum; IVS2-705, human β-globin intron 2 with a T-to-G mutation at nucleotide 705; Luc/705, HeLa Tet-Off cells transfected with pLuc/705; ON, oligonucleotide; PCR, polymerase chain reaction; pLuc/705, recombinant luciferase plasmid interrupted by IVS2-705; pLuc/GL, wild-type luciferase plasmid containing normal β-globin intron 2; pTRE-Luc, wild-type luciferase plasmid from Clontech; RT-PCR, reverse-transcription PCR.

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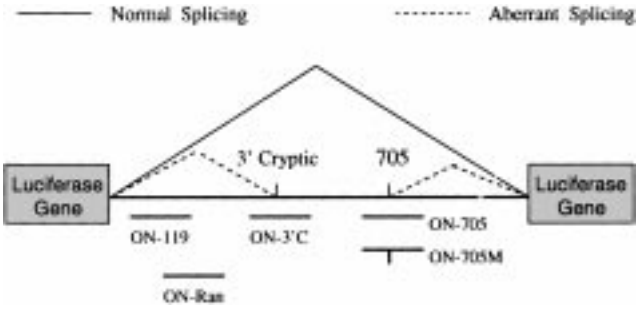

FIGURE 1: Pre-mRNA of modified luciferase gene, pLuc/705. Shaded boxes and solid thick lines represent sequences derived from luciferase cDNA and human β-globin intron 2 carrying a T-to-G mutation at nucleotide 705, respectively. Aberrant splice sites activated by the mutation are indicated. Short bars under the intron indicate phosphorothioate 2′-*O*-methyl-oligoribonucleotides targeted to the aberrant splice sites (ON-705, ON-3′C), an upstream sequence (ON-119), or with a random sequence (ON-Ran). ON-705M hybridizes to its target sequence with a single mismatch. ON-705 and ON-3′C prevent aberrant (dashed line) and restore correct (solid thin line) splicing.

recombinant plasmids using LipofectAmine as a carrier following the supplier's instructions. Stable transfectants were obtained by cotransfecting the cells with the hygromycin-resistant plasmid and one of the above plasmids or the original pLuc plasmid, followed by selection in Dulbecco's modified eagle medium (DMEM) containing hygromycin (200 μg/mL) and 10% fetal calf serum (FCS).

Oligonucleotide Treatment. The cells were treated with a complex of LipofectAmine and oligonucleotides. The complex was prepared as follows. A 0.1-mL aliquot of an oligonucleotide at a given concentration in Opti-MEM was mixed with 0.1 mL of Opti-MEM containing 4 μL of LipofectAmine. After being briefly mixed, the preparation was left at room temperature for 15 min, followed by dilution to 1.0 mL with Opti-MEM. The cells were incubated for 5 h. Subsequently the medium was replaced with DMEM containing 10% FCS, and an additional 18 h later, the protein and the RNA were collected for luciferase (*18*) and RT-PCR (*19*) assays, respectively. Forward and reverse primers (TTGATATGTGGATTTCGAGTCGTC and TGTCAATCAGAGTGCTTTTGGCG, respectively) hybridized to the luciferase sequences flanking the intron.

RESULTS

Inactivation of Luciferase Gene by Insertion of IVS2-705. A single-point mutation at nucleotide 705 in the intron 2 of human β-globin gene, referred to as IVS2-705 in this report, creates an additional 5′ splice site and activates a cryptic 3′ splice site 126 nucleotides upstream. This and similar mutations lead to aberrant splicing which results in the retention of the intron fragment with a stop codon in the coding sequence of the β-globin mRNA and concomitant inhibition of the translation of the full-length β-globin protein. This mechanism is responsible for a large number of cases of a genetic blood disorder, thalassemia (*20*).

Insertion of the IVS2-705 human globin intron into the cDNA of luciferase plasmid pLuc/705 resulted in an aberrantly spliced intron and a generation of the stop codon interfering with the translation of luciferase (Figure 1). Thus, HeLa cells transiently transfected with the pLuc/705 exhibited only background levels of luciferase activity, while those

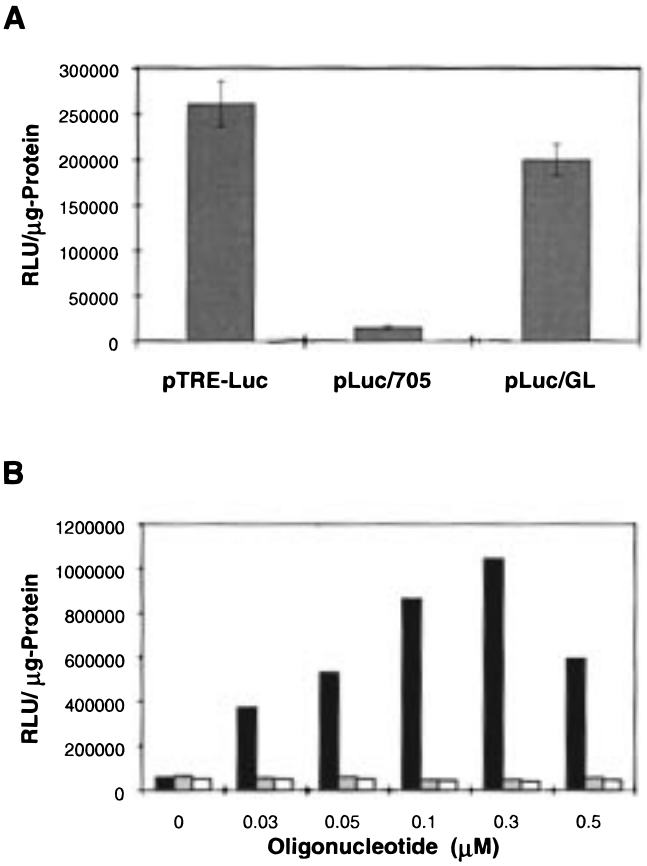

FIGURE 2: Luciferase activity in HeLa cells transiently transfected with plasmids pTRE-Luc, pLuc/705, and pLuc/GL (A), and in transiently transfected HeLa Luc/705 cells treated with oligonucleotides ON-705 (black columns), ON-119 (shaded), and ON-Ran (white) (B). In this and subsequent figures, the activity of luciferase was normalized in total cellular protein and is presented in relative luminescence units (RLU)/μg of protein. Vertical bars in A indicate standard deviations ($n = 3$).

transfected with the vectors carrying either intact luciferase cDNA (pTRE-Luc) or the one with the coding sequence interrupted by the wild-type β-globin intron (pLuc/GL) exhibited 20−25-fold higher activity (Figure 2A). The latter result shows that the wild-type β-globin intron can be spliced out even though it is located in an unnatural sequence context. The difference in the levels of luciferase expression in cells transfected with pTRE-Luc and pLuc/GL is most likely due to differences in the transfection efficiency.

Restoration of Luciferase Activity by ON-705, an Antisense Oligonucleotide Targeted to the Aberrant Splice Site. The cells transiently transfected with pLuc/705 plasmid were subsequently treated with a complex of LipofectAmine and the 18-mer 2′-*O*-methyl-phosphorothioate oligoribonucleotide (ON-705). The latter is targeted to the aberrant 5′ splice site created by the 705 mutation (*16*, *21*). This treatment resulted in a dose-dependent restoration of luciferase activity (Figure 2B), which increased approximately 50-fold over the background at the optimum concentration of the oligonucleotide (0.3 μM). A decline seen at 0.5 μM is due to the improper charge ratio of the cationic lipid and the negatively charged nucleic acid in the complex (*22*). Note that a constant amount of LipofectAmine was used in this and subsequent experiments (see *Methods* section).

The effect was sequence-specific; neither an oligonucleotide targeted to a sequence adjacent to nucleotide 119 (ON-

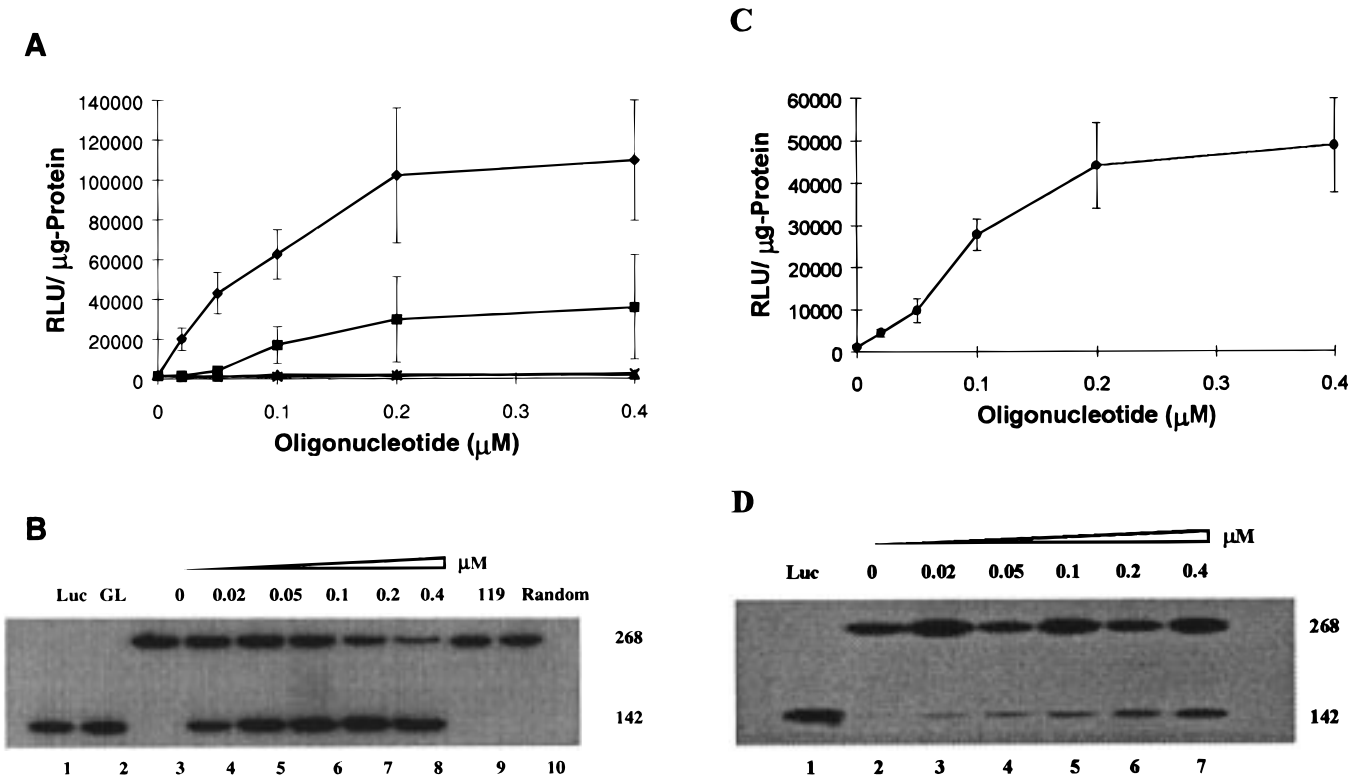

FIGURE 3: Restoration of splicing by antisense oligonucleotides in the stably transfected HeLa Luc/705 cell line. (A) Luciferase activity. The oligonucleotides used were: ON-705 (◆), ON-705M (■), ON-119 (▲), and ON-Ran (×). (B) RT-PCR. Total cellular RNA was subjected to RT-PCR. The 268- and 142-nucleotide bands correspond to the aberrant and correct mRNA, respectively: lanes 1 and 2, control cells expressing the luciferase cDNA (Luc) and the pLuc/GL construct (GL) in which the luciferase cDNA is interrupted by the wild-type β-globin intron, respectively; lanes 3−8, Luc/705 cells treated with ON-705 at varying concentrations; and lanes 9 and 10, Luc/705 cells treated with ON-119 and ON-Ran, respectively. In (C) and (D), the luciferase activity and RT-PCR assays are presented, respectively, for the Luc/705 cells treated with ON-3′C. The latter is targeted to the 3′ splice site activated by the IVS2-705 mutation. All designations are the same as above.

119), which is irrelevant for splicing, nor the one with a random sequence (ON-Ran) showed any effect on luciferase activity (Figure 2B). Furthermore, the luciferase level in cells transfected with pLuc/GL in which the intron is correctly spliced was not affected by the treatment with varying concentrations of the oligonucleotides (data not shown). Taken together, these results indicate that the effects of ON-705 were due solely to its ability to restore correct splicing of the IVS2-705 intron and exclude the possibility of nonspecific activation of the luciferase gene expression (see also below). They clearly show that the concept of an assay system based on protein up-regulation by oligonucleotides works well, with a high signal-to-noise ratio. Note that 2′-*O*-methyl-phosphorothioate oligoribonucleotides do not promote cleavage of the hybridized RNA by cellular RNase H (*23*). This is important because the degradation of the RNA would have led to the removal of the splicing substrate (*24*).

Restoration of Luciferase Activity and Correction of Pre-mRNA Splicing in Stably Transfected Cell Line. The experimental system was further improved by generating a HeLa cell line stably transfected with the pLuc/705 construct. Figure 3A shows that the treatment of the Luc/705 cells with ON-705 generates luciferase activity in a dose-dependent and sequence-specific manner. Control oligonucleotides, ON-119 and ON-Ran, were inactive while a single mismatch in ON-705M resulted in an approximately 70% decrease in the antisense effectiveness of ON-705.

To obtain direct evidence that the generation of luciferase in Luc/705 cells was due to the correction of aberrant splicing of the modified luciferase pre-mRNA, we have subjected the total RNA from the cells treated with the oligonucleotides to quantitative reverse transcription PCR (RT-PCR) (*19*, *25*). The results show that ON-705 indeed induced correction of pre-mRNA splicing. In the untreated cells the only detectable RT-PCR product was 268 nucleotides long. It is the aberrantly spliced luciferase mRNA that retained the 126-nucleotide fragment of the intron (Figure 3B, lane 3). This result indicates that, despite the presence of normal 3′ and 5′ splice sites at the ends of the inserted intron, splicing follows exclusively the aberrant pathway, leading to little or no correctly spliced luciferase mRNA. However, a significant level of the 142-nucleotide product representing correct splicing was already generated at the lowest concentration of the oligonucleotide tested, 0.02 μM (lane 4). Analysis of the data, by densitometry of the autoradiogram and calculation of the ratio of the corrected product to the sum of the corrected and aberrant products, indicated that the correction of splicing increased in a dose dependent manner (lanes 5−7) and reached close to 80% at 0.4 μM oligonucleotide. In agreement with the results obtained by luminometry, 50% correction occurred at approximately 0.1 μM oligonucleotide (Figure 3A).

As expected, the control cell lines, constitutively expressing either the luciferase mRNA transcribed from an intronless construct (pTRE-Luc, lane 1) or the one with a wild-type

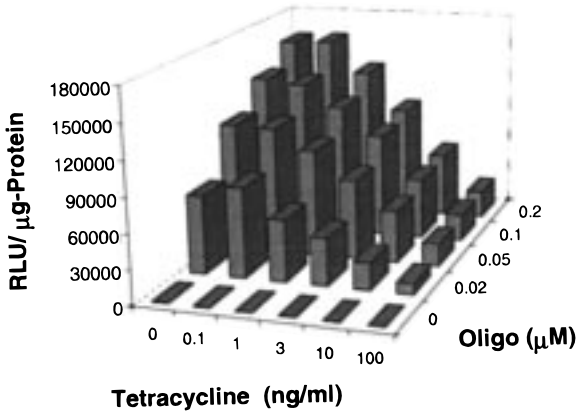

FIGURE 4: Restoration of luciferase activity in Luc/705 cells that had been incubated for 48 h with tetracycline at indicated concentrations prior to subsequent treatment with 0−0.2 μM ON-705.

β-globin intron (lane 2), showed only correctly spliced mRNA. In another series of control experiments, irrelevant oligonucleotides, ON-119 and ON-Ran, were found to be completely ineffective in inducing the cells to correctly splice luciferase mRNA (lanes 9 and 10, respectively). Interestingly, an antisense oligonucleotide targeted to the 3′ cryptic splice site (ON-3′ C), which is activated by the 705 mutation, also resulted in a dose-dependent increase in the luciferase activity (Figure 3C) and concomitant correction of the splicing (Figure 3D). However, this oligonucleotide is less effective and even at 0.4 μM generates no more than 20% of the corrected product. This may reflect its shorter length (17-mer vs 18-mer for ON-705) and/or decreased accessibility of the 3′ splice site (*19*) which interacts with a large number of splicing factors (*26*).

Modulation of Luciferase Gene Expression by Tetracycline and Effects of ON-705. The HeLa cells used in the present study harbor tetracycline-controlled transactivators while the luciferase gene promoter contains a tetracycline-responsive element. In this system, commonly referred to as Tet-Off, the transcription of the luciferase gene is inhibited in the presence of tetracycline (*27*). Thus, the expression of luciferase in the HeLa Luc/705 cell line can be controlled at the level of transcription and/or the RNA processing by the combined activity of tetracycline and the ON-705 oligonucleotide. Indeed, when the cell line was first incubated for 48 h with 0−100 ng/mL of tetracycline and subsequently exposed to 0−0.2 μM ON-705/LipofectAmine complex, the luciferase activity was found to depend on the concentration of both tetracycline and ON-705 (Figure 4). At 0.2 μM ON-705, the luciferase activity decreased approximately 14-fold as tetracycline concentration was increased from 0 to 100 ng/mL. Conversely, at a given concentration of tetracycline, the luciferase activity increased in proportion to the oligonucleotide concentration.

DISCUSSION

The large body of work on antisense oligonucleotides made apparent the importance of the functional assay used for assessing their activity. Although the inhibition of cell growth was frequently equated with evidence for the down-regulation of the target gene, the findings that oligonucleotides may nonspecifically bind to proteins and therefore affect the cell function by unpredictable mechanisms clearly indicated the inadequacy of such an assay system (*7*). Even an assay based on down-regulation of a given protein, although more specific, suffers from several drawbacks described in the Introduction.

In contrast to previous approaches (*28*), the oligonucleotide ON-705 corrects the aberrant splicing of the mutated intron which interrupts the coding sequence of a luciferase gene. The positive readout thus obtained effectively eliminates the flaws associated with a functional assay based on protein down-regulation.

Analysis of a kinetic scheme involving two compartments, one for the mRNA pool and the other for the protein pool (*29*, *30*), can demonstrate that the luciferase activity at a steady state is proportional to the extracellular concentration of ON-705 with rate-limiting cellular entry of ON-705. Assumptions made in deriving this conclusion include the following: the gene is transcribed following a zero-order process (*31*); hybridization occurs rapidly compared with other kinetic processes involved; and the extracellular concentration of ON-705 remains constant during the 5-h incubation of the cells with the oligonucleotide. Since splicing occurs exclusively in the nucleus, the luciferase activity observed reflects only the nuclear concentration of the oligonucleotide. The nuclear envelope embedded with pores of 70 nm (*32*) is, however, expected to allow free passage of oligonucleotides of 15−20-mer in size. Therefore, the results obtained will be inclusive in assessing both cytoplasmic and nuclear delivery of an oligonucelotide. In summary, the present system provides a means of testing and an objective comparison of various cellular delivery strategies as well as other parameters such as the influence of the backbone structure of oligonucleotides.

The luciferase assay is extremely sensitive; for example, approximately 1000-fold more sensitive than the chloramphenicol acetyltransferase assay (*33*). In Tet-Off HeLa cells, the luciferase expression is further amplified and tightly regulated compared with the cells transfected with other expression vectors (*34*). Since the Luc/705 cell line offers an additional 3′ cryptic splice target site that results in a positive readout, two different sequences can be tested simultaneously with one serving as the "internal standard or reference." In theory, this approach should allow separate assessment of the relative contributions of uptake versus hybridization to the target.

The fact that our assay is not applicable to antisense oligonucleotides which promote cleavage of the target RNA by RNase H is not a serious limitation. None of the most promising 2′-*O*-alkyl-oligomers, so-called morpholino-oligomers (*35*), methylphosphonates, and the peptide nucleic acids (*4*) activate RNase H and therefore can be tested in our system. Finally, but not in the least, in the Tet-Off system gene expression is reduced in proportion to the subcytotoxic concentration of tetracycline and shows the highest level in the absence of the antibiotic. The ability to regulate the pre-mRNA level by the antibiotic provides an opportunity to investigate how the concentration of the target affects the requirements of the antisense activity, especially in terms of the kinetics of the cellular delivery of the oligonucleotides. The same subject has been already addressed in the literature for an oligonucleotide which can interfere with the luciferase expression via the activation of RNase H (*36*).

Clearly the HeLa Luc/705 cell line described in the present study represents a versatile assay system that allows easy, rapid, and quantitative analysis of the transport involved in the action of antisense oligonucleotides. The Tet-Off system, in combination with the pLuc/705 construct described here, can be easily established in different cell lines (*34*) extending the range of the assay to a variety of cell types having different uptake or endocytic characteristics.

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