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Gene Silencing in Mammalian Cells with Light-Activated Antisense Agents

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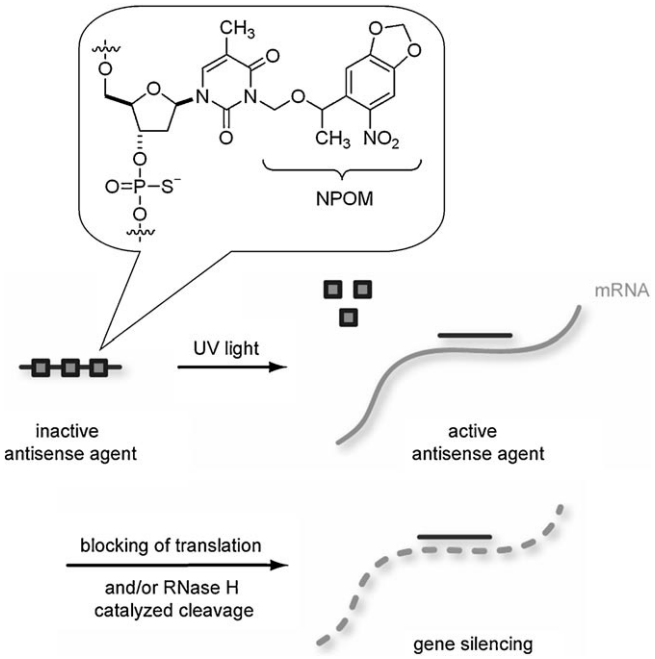
Detailed knowledge of the external regulation of gene function is a fundamental necessity in order to annotate sequenced genomes and to understand biological processes in single cells and multicellular organisms. One of the most widely used approaches for the down-regulation of specific genes is the application of antisense agents. Antisense agents are oligomers that have the ability to hybridize sequence specificslly to mRNAs, inhibiting translation and potentially leading to mRNA degradation through RNAse H recruitment.[1–4] For the investigation of gene function, antisense agents can be transfected into cells in cell culture experiments or injected into the embryos of model organisms at an early point in development. Moreover, antisense oligomers are being investigated as therapeutics, and one reagent, Vitravene (Isis Pharmaceuticals), is available for the treatment of cytomegalovirus retinitis.[5] However, a substantial drawback of current antisense technologies is the inability to regulate their activity with spatial and temporal control. When transfected or injected into cells, antisense agents are instantaneously active and are distributed to all daughter cells in cell culture or a developing multicellular organism. This inhibits the investigation of the spatial and/or temporal regulation of genes. Moreover, genes that are essential in early development cannot be targeted with antisense agents, since they are silenced immediately after injection inducing death in the studied organism. These problems can be solved with light-activated, photocaged, antisense agents.

Photocaging represents an effective means of simultaneously achieving spatial and temporal control over biological functions.[6] The term "caging" refers to the installation of a photoremovable group on a biologically active molecule, thus rendering the molecule inactive. Irradiation with UV light removes the caging group and restores biological activity. Previous research has involved photocaged oligonucleotides,[7–9] peptides,[10] and proteins.[11] We envisioned the photochemical regulation of antisense activity through the incorporation of a caged base into the oligomer (Scheme 1). This strategy is more predictable, synthetically less complex, and less prone to the generation of undesired side products than previous solutions involving the statistical caging of the phosphate backbone or the application of photocleavable inhibitors.[12,13]



Scheme 1. Light-regulation of antisense activity through the incorporation of light-removable groups, which block the hybridization of phosphorothioate DNA oligomers to mRNA. Brief UV irradiation at 365 nm removes the caging groups, enables sequence specific binding to the mRNA, and thus, blocks translation and/or leads to RNase H-catalyzed mRNA degradation.

One of the most commonly employed antisense agents is based on a DNA phosphorothioate (PS DNA) backbone.[14] PS DNA antisense agents have been used in mammalian cell culture and murine models, and have been FDA approved as therapeutic agents in humans. They have been employed in the study and potential therapy of Crohn's disease, Hepatitis C, and various cancers.[15] As in the case of locked nucleic acids (LNA),[4] peptide nucleic acids (PNA),[3] and morpholinos (MO),[2] the phosphorothioate modification conveys intracellular stability to the oligomer. Moreover, it is generally accepted that the formation of PS DNA/RNA duplexes leads to the recruitment of endogenous RNase H, which subsequently hydrolyses the RNA strand in the duplex in a catalytic fashion (an alternative way to achieve RNase H recruitment is through the application of Gapmers).[16] The light regulation of LNA, PNA, MO, and PS DNA antisense agents can be advantageous over siRNAs,[13,17] since some organisms possess an amplification pathway that contains an RNA-dependent RNA polymerase; this leads to a catalytic cascade proliferating the siRNA response.[18] As a result, the ability to tune the silencing event is lost. Moreover,

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in contrast to antisense agents, siRNAs can potentially pass from cell to cell, thus eliciting a systemic effect and impairing the ability to achieve spatial control over gene function.[19] Additionally, LNA, PNA, MO, and PS DNA antisense agents are chemically and physiologically more stable than RNA, affording an easier preparation and delivery of the caged analogues. An advantage of PS DNA over other antisense agents is their ease of synthesis with commercially available monomers and conventional DNA synthesizers. We hypothesized that the hybridization of a PS DNA antisense agent to its corresponding mRNA target can be disrupted through the installation of NPOM (6-nitropiperonyloxymethyl)-caged thymidine[9] residues on the PS DNA (Scheme 1).

Thus, we synthesized a noncaged PS DNA antisense agent, which was previously reported to target the *Renilla* luciferase reporter gene (Table 1).[20] In addition, we synthesized PS DNA antisense agents carrying three or four NPOM-caged thymidine residues under standard DNA synthesis conditions (see the Supporting Information) in conjunction with Beaucage's reagent[21] for the introduction of the phosphorothioate backbone. We selected three and four caging groups, evenly distributed throughout the PS DNA 19-mer, based on our previous experiments on PCR light regulation.[7] Finally, we prepared a control PS DNA sequence that should not induce silencing of the *Renilla* luciferase reporter gene.

Table 1. Synthesized caged and noncaged phosphorothioate DNA oligomers[a].

PS DNA	Sequence	T_m −UV [°C]	T_m +UV [°C]
control	5′-TCCAGAACAAAGGAAACG-3′	n.d.	n.d.
noncaged	5′-CGTTTCCTTTGTTCTGGA-3′	39.5 ± 0.5	39.3 ± 0.8
3-caged	5′-CGTT**T***CCTT**T***GTTC**T***GGA-3′	n.d.	39.2 ± 0.6
4-caged	5′-CGT**T***TCC**T***TTG**T***TC**T***GGA-3′	n.d.	38.9 ± 0.8

[a] Melting temperature of PS DNA/RNA hybrids before and after irradiation (5 min, 365 nm, 23 W). **T*** denotes the caged thymidine. n.d. = not detectable.

In order to assess the effective decaging of the synthesized PS DNA oligomers, we first performed decaging experiments, which were monitored by HPLC (see the Supporting Information). For both the 3-caged and 4-caged oligomers (containing three and four caged thymidines, respectively), 5 min of irradiation with a hand-held UV lamp (23 W) led to the complete disappearance of the caged oligomer and the exclusive detection of the noncaged PS DNA. We then examined the capacity of the three and four caging groups to inhibit hybridization of the caged PS DNA oligomer to the complementary RNA sequence (5′-UCC AGA ACA AAG GAA ACG-3′). We monitored hybridization on a BioRad MyiQ RT-PCR thermocycler by conducting a sequence of three heating and cooling cycles (10 μM of both PS DNA and RNA with 12.5 μL iQ SYBR Green Supermix in a total volume of 25 μL, 30 °C to 80 °C with a 0.5 °C min^{-1} ramp). We detected no hybridization in this temperature range for the control PS DNA or the nonirradiated caged PS DNA oligomers. The noncaged PS DNA/RNA hybrid melted at approximately 39 °C, which agreed with that of similar PS DNAs.[22] Irradiation at 365 nm for 5 min completely restored hybridization for both caged antisense agents (Table 1).

These antisense oligomers were transfected into mouse fibroblast cells (NIH 3T3) together with a dual reporter system encoding *Renilla* luciferase and firefly luciferase as a transfection control. As previously described,[20] the noncaged PS DNA induced a 70% down-regulation of the *Renilla* luciferase signal, and Figure 1 displays all luciferase readouts normalized to that signal. The control PS DNA has no effect on the luciferase signal, as shown by a comparison to cells that had not been transfected with PS DNA. Moreover, the UV irradiation (365 nm, 23 W hand-held UV lamp, 5 min) of 3T3 cells has no effect on the luciferase signal (within the error of the experiment), as shown by comparing the "UV" and "no UV" data for the noncaged and control PS DNA experiments. Gratifyingly, the installation of three caging groups completely inhibits the antisense activity of the PS DNA oligomer, as expected from the hybridization experiments. Moreover, a brief irradiation with UV light quantitatively restores antisense activity to the level of the noncaged antisense agent. The same result was achieved with the PS DNA oligomer containing four caging groups. This clearly demonstrated the ability to regulate gene silencing activity with light through the incorporation of caged monomeric building blocks into phosphorothioate antisense oligomers. The brief UV irradiation did not elicit any toxic effects on 3T3 cells, as demonstrated by a cell viability assay (see the Supporting Information).

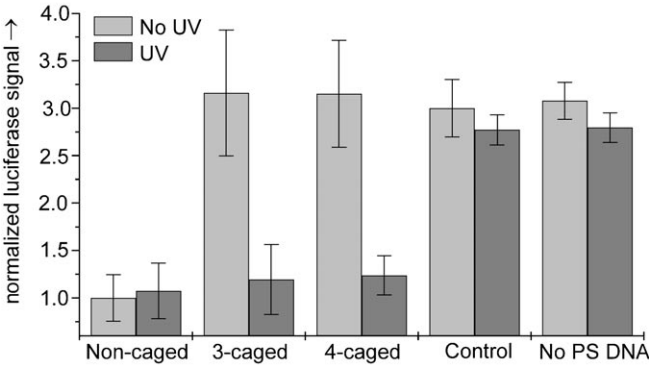

Figure 1. *Renilla* luciferase signal after PS DNA transfection ± UV irradiation (365 nm, 23 W hand-held UV lamp, 5 min), normalized to the transfection of the noncaged PS DNA antisense agent. The error bars represent the standard deviation of three independent experiments.

In order to demonstrate spatial regulation of gene expression with caged PS DNA antisense agents, we transfected 3T3 cells with the *Renilla* luciferase plasmid and with or without PS DNA in a six-well format. After a 4 h incubation, the medium was removed and the cells were irradiated at 365 nm (5 min, 23 W) in a specific location by using a mask. After a further 24 h incubation for luciferase expression to occur, the plate was imaged on a Xenogen Lumina system (Figure 2). A high level of spatial control of antisense activity was achieved, as only the irradiated areas of the cell monolayer transfected with

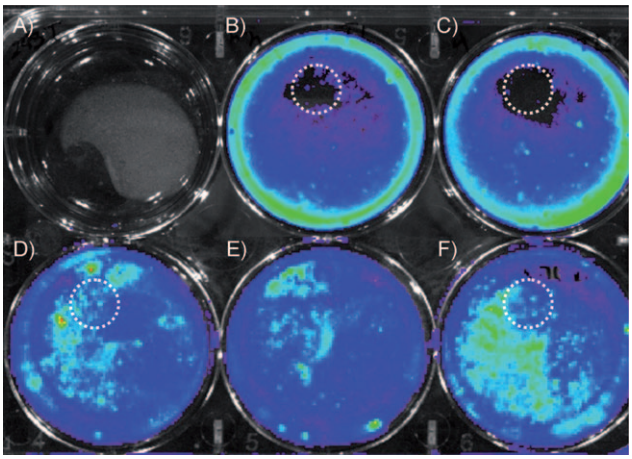

Figure 2. Spatial regulation of *Renilla* luciferase expression with caged PS DNA antisense agents. The cellular monolayer was only irradiated inside the white dashed circle (365 nm, 5 min, 23 W). A) Negative control without luciferase plasmid. B) Transfection with luciferase plasmid and 3-caged PS DNA. C) Transfection with luciferase plasmid and 4-caged PS DNA. D) Positive control without PS DNA. E) Positive control without PS DNA and without irradiation. F) Transfection with luciferase plasmid and inactive control PS DNA.

caged PS DNA agents displayed little to no luciferase expression. In contrast, when wells containing no caged PS DNA were irradiated under identical conditions, no luciferase silencing was observed.

In summary, we have developed an effective light-regulated gene-silencing methodology through the incorporation of a caged thymidine phosphoramidite into phosphorothioate antisense agents under standard DNA synthesis conditions. We have demonstrated the disruption of antisense activity and its restoration with UV irradiation through hybridization studies and in mammalian cell culture with a luciferase reporter gene. Moreover, we obtained precise spatial control of gene expression. Due to its easy synthesis and its excellent light-activation properties, we believe that the developed antisense technology will find widespread application in the investigation and regulation of gene function.

Experimental Section

Luciferase assay. Mouse fibroblast cells (NIH/3T3) were grown at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Hyclone), supplemented with 10% Fetal Bovine serum (FBS, Hyclone) and 10% streptomycin/ampicillin (MP Biomedicals). Cells were passaged into two 96-well plates (200 μL per well, ~1×10^4 cells per well) and grown to ~80% confluence within 24 h. The medium was changed to OPTIMEM (Invitrogen), and the cells were cotransfected with pGL3 (0.43 μg, Promega), pRL-TK (0.043 μg, Promega), and the phosphorothioate DNA (250 pmol) with X-Treme GENE (3:2 reagent/DNA ratio, Invitrogen). The following conditions were used: no phosphorothioate oligomer, a sense-strand control phosphorothioate oligomer, the noncaged *Renilla* luciferase-targeting phosphorothioate, and the phosphorothioate with either three or four caging groups. All transfections were performed in triplicate. Cells were incubated at 37 °C for 6 h, and the transfection medium was removed. One of the 96-well plates was briefly irradiated with a hand-held UV lamp (365 nm, 25 W) for 5 min. The medium was then replaced with standard growth medium, and the cells were incubated for an additional 24 h. After the 24 h incubation, the cells were observed, and no changes in growth or morphology were visible when comparing the irradiated cells with the nonirradiated cells. Following the visible inspection, the medium was removed, and the cells were assayed with the Dual-Luciferase Reporter Assay system (Promega) with a Wallac VICTOR^{3}V luminometer with a measurement time of 1 s and a delay time of 2 s. The ratio of *Renilla* to Firefly luciferase expression was calculated for each of the triplicates, the data were averaged, and standard deviations were calculated by using Microsoft Excel.

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