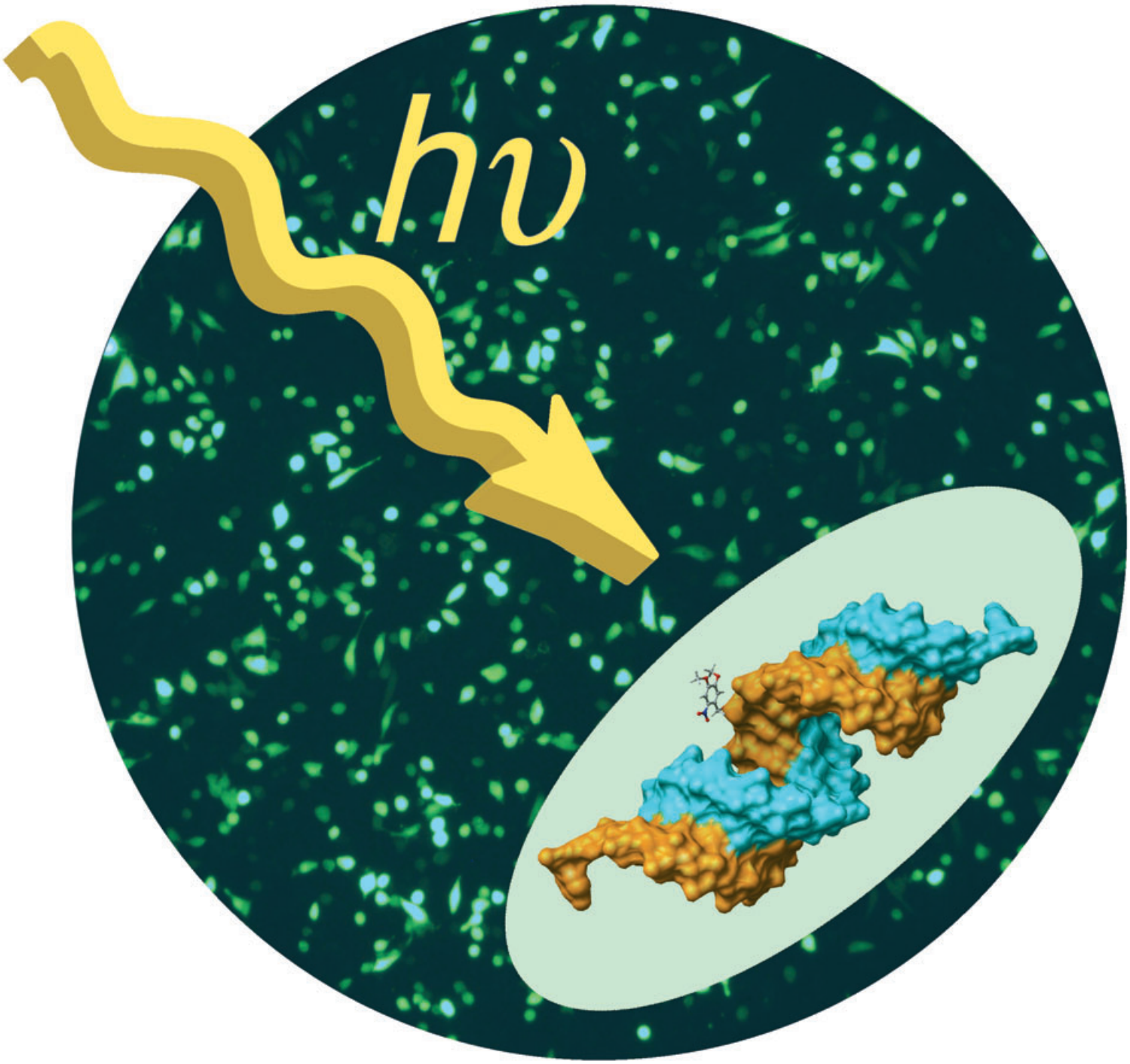
# Communications




The process of RNA interference can be controlled with light. On the following pages, S. H. Friedman and co-workers demonstrate that siRNAs modified with photolabile groups have reduced ability to effect RNA interference until irradiation deprotects them and releases fully active siRNA.

  DOI: 10.1002/anie.200461458 

*Gene Expression*

# Light-Activated RNA Interference**

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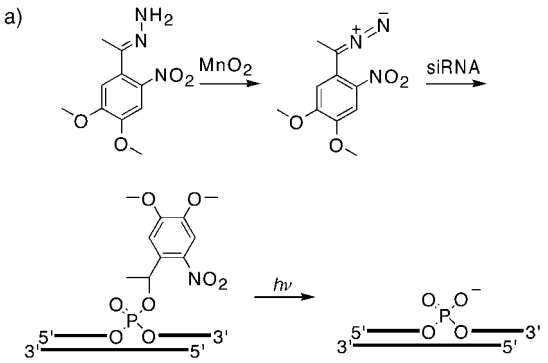
Small interfering RNA (siRNA) molecules are 21–23 nucleotide-long duplex RNAs that are able to control gene expression through the process of RNA interference.[1–4] RNA interference involves siRNA binding to the RNA-induced silencing complex (RISC), which then directs the destruction of messenger RNA (mRNA) that is complementary to the antisense strand of the siRNA. RNA interference directed by siRNA has proven to be a broadly applicable method for controlling gene expression and has therefore become a central tool in investigating the role of specific genes in cellular processes.

The aim of the work described herein is to develop a method to control the process of RNA interference with light (light-activated RNA interference, (LARI)). This will allow the spacing, timing, and degree of gene expression to be controlled by irradiation. This added level of control to the widely applied method of RNA interference should be particularly beneficial for the study of cell development, which is tied closely to the location, timing, and degree of expression of key proteins (i.e., morphogens). Our rationale involves the covalent attachment of a photolabile group to an siRNA duplex to sterically block the initial siRNA–RISC interaction, thus preventing the degradation of its target mRNA. Only upon irradiation would active siRNA be released. The photolabile moiety that we used for this study is the 4,5-dimethoxy-2-nitrophenylethyl (DMNPE) group previously used by Monroe, Haselton, and co-workers to cage plasmids.[5] Caging is effected by reaction of the precursor hydrazone to create the corresponding diazo compound, which can react with phosphate groups (Scheme 1a). We have used the GFP-targeting siRNA (GFP = green fluorescent protein) developed by Chiu and Rana.[6] This allows quantitation of gene expression through the fluorescent signal generated by GFP. A further advantage of this system is that it has been well-characterized. Therefore, potential complicating factors such as an antisense effect have been ruled out.




b)

Target: 5'GCAGCACGACUUCUUCAAGdTdT 3'
3'dTdTCGUCGUGCUGAAGAAGUUC

Control: 5'GUGGGAGCGCGUGAUGAACdTdT 3'
3'dTdTCACCCUCGCGCACUACUUG

***Scheme 1.*** a) Reactions to introduce photolabile groups into siRNA (see Experimental Section. b) Target and control sequences of siRNA used in the study.

To analyze the effect of caging, we used two siRNA sequences (Scheme 1b): the target siRNA that directs the degradation of GFP mRNA, and a control siRNA that has been shown to have no effect on GFP expression.[7] RNA oligonucleotides were deprotected after automated synthesis and annealed to form duplex siRNA. Target and control duplexes (1 nmol each) were photoprotected by using a 100-fold excess of diazo reagent relative to RNA duplex. The extent of photocaging was determined with the technique of Monroe, Haselton, and co-workers by using the absorbance of the sample at $\lambda = 355$ nm.[5] Under these conditions we obtained 1.4 photocaging groups per duplex. This 3% caging efficiency is similar to the results found in two other systems.[5,8] Melting temperature ($T_m$) determination was used to characterize further the target caged and uncaged siRNA duplexes ($T_m = 65$ and 54 °C, respectively). Finally, in an analysis of the modified duplex through ESI MS, we were able to observe sense and antisense strands, both with and without a photolabile group (Figure 1).

Gene expression was analyzed by using the system developed by Rana and Chiu which examines GFP expression in HeLa cells.[6] We have modified this approach for use with 96-well plates. This allows us to quantitate GFP expression in vivo with a scanning fluorescence microplate reader equipped with a 485-nm excitation filter and a 535-nm emission filter. It also allows us to average larger numbers of experimental observations. HeLa cells were seeded in 96-well cell-culture plates at 70% confluency and allowed to grow over a period of 18–20 h. These cells were then transfected with the pEGFP-C1 plasmid and the appropriate siRNA by using lipofectamine. GFP controls were transfected with plasmid only, and mock transfection controls had neither plasmid nor siRNA. After a transfection period of 6 h, the cell culture media was removed and replaced with fresh media. The wells were then either exposed to light or masked.

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[**] We thank Dr. Dhananjay Pal and Professor Ashim K. Mitra for access to cell culture facilities. We also thank Dr. Scott Carter and Professor Paramjit Arora for useful discussions. Molecular graphics images were produced in part with the UCSF Chimera package from the Computer Graphics Laboratory, University of California, San Francisco (supported by NIH P41 RR-01081).[14]

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  

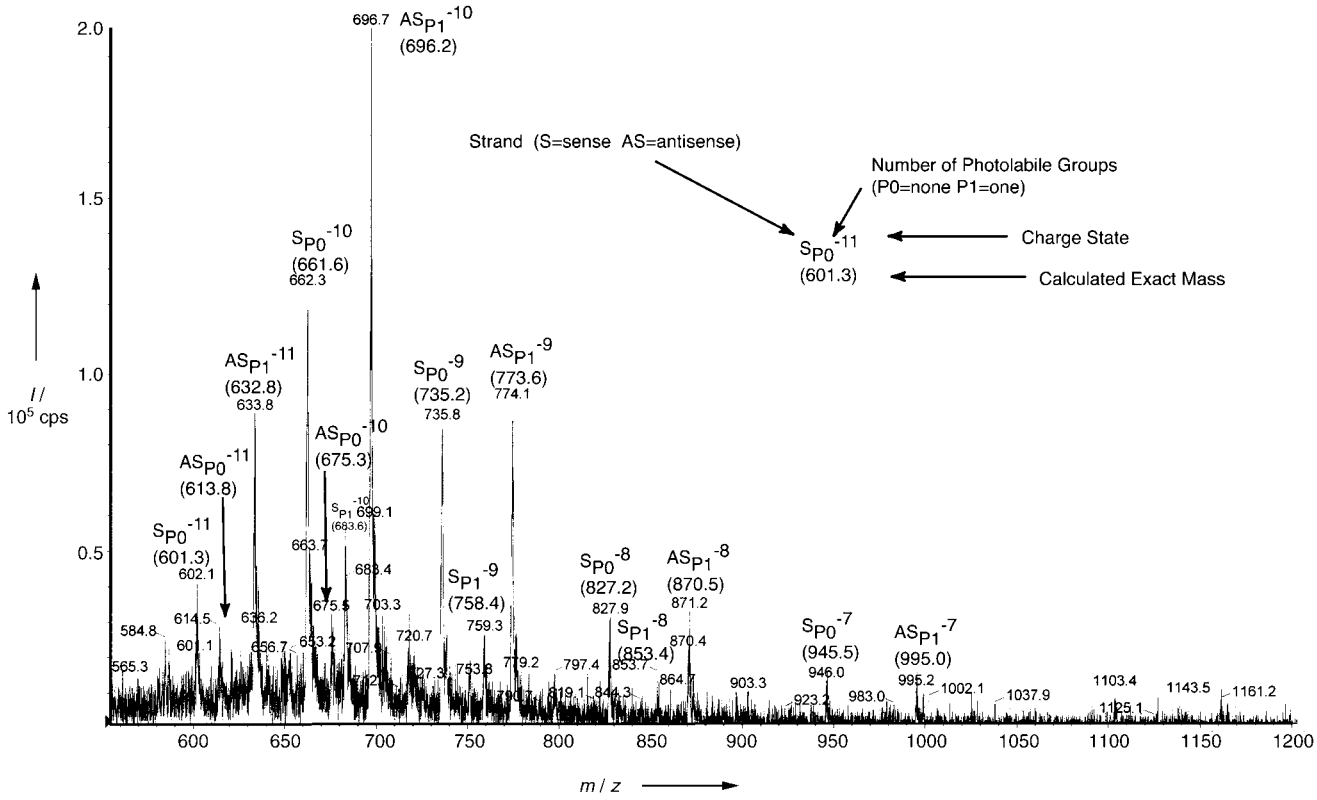


***Figure 1.*** ESI mass spectrum of modified siRNA. Peaks are annotated to indicate the strand (AS = antisense; S = sense), the number of photolabile groups (P0 = none, P1 = one group, etc.), and the charge state (e.g. −10). Below the annotation is the expected *m*/*z* ratio in parentheses which is positioned above the actual *m*/*z* ratio. cps = counts $s^{-1}$.

A WG-320 longpass filter (Edmund Industrial Optics) permitted transmission of the frequencies needed for deprotection ($\lambda > 320$ nm) but blocked shorter and more-toxic wavelengths. The samples were exposed for 12 min with a Blak-Ray fluorescent UV lamp (XX-15 L, 15 W) at a distance of 10 cm. After exposure to light, the culture media were changed again, and the cells were allowed to culture for an additional 42 h. This is the period of time found by Rana and Chiu to produce an optimal decrease in GFP expression in this system.[6] The GFP signal was then quantitated in vivo by using microplate fluorescence. We have not found it necessary to normalize for transfection efficiency with an internal standard. To accommodate variations in transfection efficiency, each experimental point represents an average of five wells. Furthermore, all the points represented in a figure come from a single 96-well plate, another factor that contributes to more consistent transfection efficiencies. This consistency is evidenced by the relatively low standard errors observed. Cells that were exposed to mock transfection (transfection agent without GFP plasmid) show auto-fluorescence signals of $<4\%$ of the GFP control signal. This value does not change with irradiation of the cells.

The results are summarized in Figure 2. The only cells that had a significant difference in GFP expression upon exposure to light were those that had been treated with the caged target siRNA ($p < 0.005$). Selective phototoxicity can be eliminated as a mechanism of action, because light had no significant effect on GFP signal in any of the other samples. Light-induced damage of the RISC can also be eliminated, because the non-photoprotected target siRNA gave equivalent suppression of GFP signal with and without light. Nonspecific reduction in overall gene expression as a result of nucleic acid

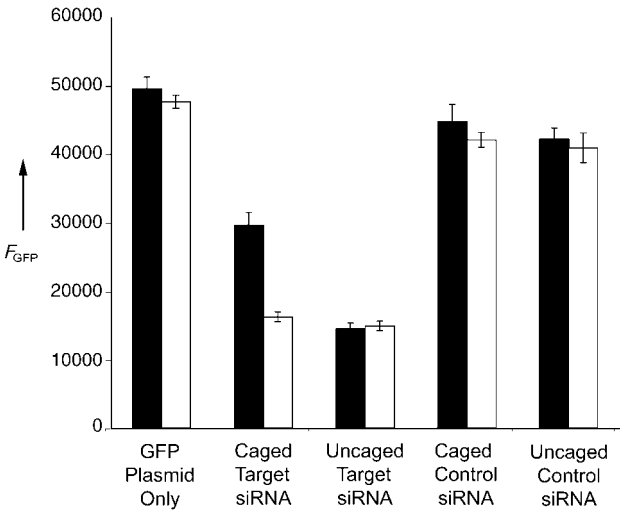


***Figure 2.*** Influence of modified siRNA on GFP expression in HeLa cells upon exposure to and in the absence of light. Dark bars indicate signals from nonirradiated samples. Light bars indicate signals from irradiated samples. Only caged target siRNA shows a significant decrease upon irradiation ($p < 0.005$). $F_{GFP}$ = GFP fluorescence signal.

  

transfection can be eliminated as a mechanism of action, because the control siRNA at identical concentration to target siRNA produced no significant change in GFP expression. Finally, selective toxicity of the released DMNPE group can be eliminated as a mechanism of action, because the caged control siRNA gave equivalent GFP signal with and without light. The most reasonable interpretation of the results is that the DMNPE group blocks RNA interference and that exposure to light releases active siRNA.

The lack of light-dependent changes in GFP expression in the presence of photoprotected control siRNA minimizes the possibility that the light-dependent changes observed with target siRNA are nonspecific. To further support the conclusion that the changes stem from the release of active siRNA and subsequent RNA interference, a similar experiment was performed with GFP expression normalized to red fluorescent protein (RFP) expression in which RFP-expressing plasmid was cotransfected with GFP-expressing plasmid. Again, photoprotected siRNA that targets GFP produced a light-dependent decrease in GFP signal normalized to RFP signal. Likewise, photoprotected siRNA that targets RFP produced a light-dependent decrease in RFP signal normalized to GFP signal (Figure 3).

We have also explored the ability of caged siRNA to modulate the degree of RNA interference. This is an additional potential benefit of caging, as it allows the variation of the amount of expression of a given gene in target tissue. We therefore examined the expression of GFP in cells that had been treated as before with caged target siRNA. Exposure to light during deprotection with DMNPE was varied from 0 to 12 min. Figure 4 summarizes the results of this experiment. Increasing the time of light exposure gave a gradual increase

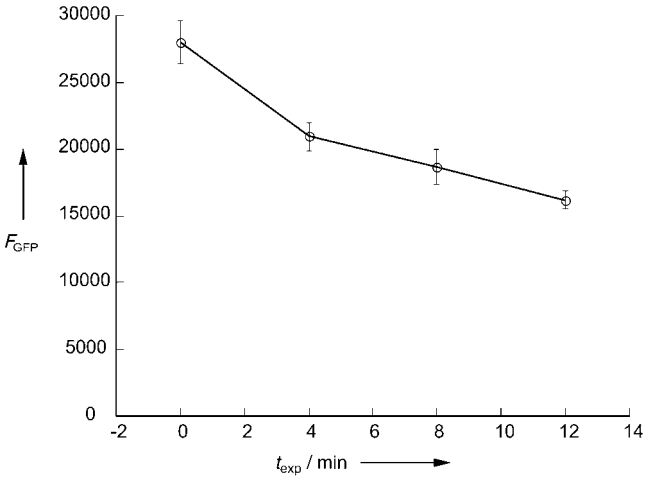


***Figure 4.*** Decrease in GFP signal with increased time of exposure to light in cells treated with caged target siRNA.

in the RNA interference effect between the two limits established previously. In this experiment, eight wells were averaged for each time point.

The results of the experiments described herein show that RNA interference can be brought under the control of light through the use of photolabile groups. It is clear that the caging of the siRNA does not completely eradicate RNA interference. A likely reason for this is the 3% caging efficiency, which corresponds to an average of 1.4 phosphate groups modified per duplex. It is probable that some duplexes are completely unmodified, while others are modified in positions that are unable to block the siRNA–RISC interaction. A potential solution to this problem is to increase the substitution level, which would produce a larger proportion of the siRNA duplexes that have blocked positions.

We have examined this potential solution by systematically increasing the number of DMNPE equivalents we used during caging. For this experiment we used 175, 875, and 1750 equivalents of caging (DMNPE) compound relative to duplex to modify three different samples of target siRNA. The experiments resulted in 4.8%, 10.8%, and 15.2%, respectively, of phosphate groups modified. These modified siRNAs were then transfected in a manner identical to the previously described experiment, and their effect on GFP expression determined in the presence and absence of light exposure. The results are shown in Figure 5. Again, the only samples that showed a statistically significant difference in GFP signal upon irradiation were those treated with modi-

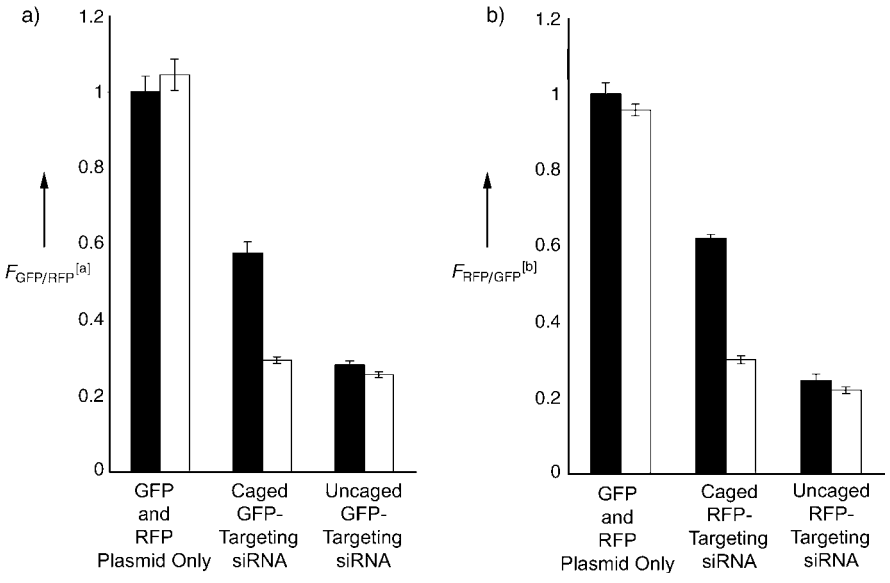


***Figure 3.*** Normalized GFP and RFP expression in the presence and absence of modified siRNA. Dark bars indicate signals from nonirradiated samples. Light bars indicate signals from irradiated samples. a) For each set of experimental conditions, the GFP/RFP ratio was normalized to the same ratio in the nonirradiated, plasmid-only sample (far left bar). [a] $F_{GFP/RFP}$ = GFP fluorescence normalized to that of RFP. b) The same approach was taken using the RFP/GFP ratio. [b] $F_{RFP/GFP}$ = RFP fluorescence normalized to that of GFP. All signals were corrected for autofluorescence, which is typically $<4\%$ of the total signal.

  

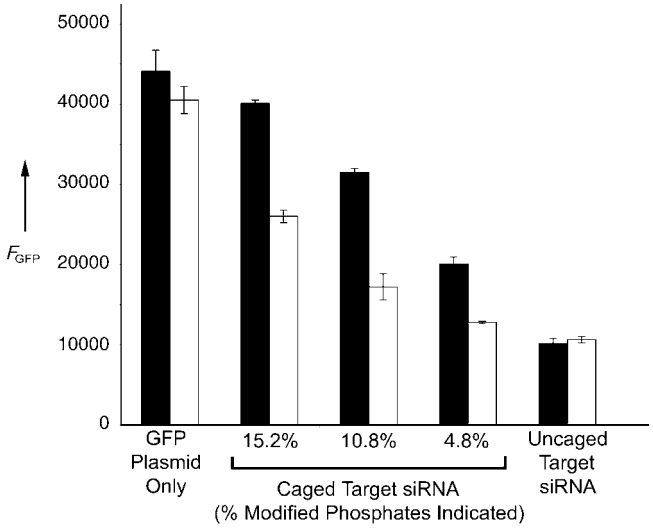


***Figure 5.*** Influence of increasing amounts of caging compound upon blocking and release of siRNA. Dark bars indicate signal from nonirradiated samples. Light bars indicate signals from irradiated samples. Higher amounts of caging compound result in greater blocking of RNA interference before irradiation and less RNA interference upon irradiation.

fied siRNAs ($p < 0.05$). With increasing amounts of siRNA modification, there is an increased blocking of RNA interference before irradiation. This is paralleled by a greater resistance to complete release of fully active siRNA after irradiation. In theory, increasing the time of irradiation could lead to a complete release of active siRNA. We have found, however, that increasing irradiation beyond 10–12 min leads to a decrease in control GFP expression that *is* significant in comparison with cells that are not irradiated, which is likely a result of phototoxicity. Modification of some positions on the duplex should be more effective at blocking RNA interference than others, and we are exploring this as a possible route to complete caging of the siRNA.

These results indicate that light-mediated control of RNA interference is possible. We have demonstrated that the modification of siRNA with a photolabile group results in a species that is blocked from full RNA interference until irradiated, upon which it is as active as unprotected siRNA. We have further shown that this effect can be modulated by varying the time of photodeprotection. Finally, we have shown that increasing the number of equivalents of caging compound to siRNA increases the extent to which RNA interference is blocked, but also results in incomplete RNA interference after irradiation. Based on the growing understanding of the structural features required for effective RNA interference, we are currently exploring the positional and steric factors that may allow complete caging of siRNA.[6,9–11] Previous efforts at light-mediated control of gene expression include the caging of hormones,[12] mRNA,[8] whole plasmids,[5] and ribozymes.[13] A potential advantage of our approach is that it utilizes the generality found in RNA interference. This allows the specific targeting of endogenous genes, something that plasmid and mRNA caging cannot do. We anticipate that this approach may be useful for a range of biological studies, in particular studies of development, in which the spacing, timing, and amount of gene expression are key factors in determining developmental outcome.

## Experimental Section

siRNA caging with DMNPE: $MnO_2$ (10 mg, 0.115 mmol) was added to 4,5-dimethoxy-2-nitroacetophenone hydrazone (2.5 mg, 0.011 mmol) (Molecular Probes, Inc., Eugene, OR) in DMSO (250 μL) and gently agitated for 45 min at room temperature. The solution was then filtered through Celite (Molecular Probes) supported by glass wool in a borosilicate glass pipet to remove $MnO_2$ from the activated caging compound. The filter pad was washed with DMSO (750 μL) to extract the activated caging compound. A volume of this filtrate appropriate to generate the required molar ratio described in the text was gently agitated with siRNA (50 μL, 20 μM) in tris-acetate EDTA buffer for 24 h at room temperature, protected from light. To remove the excess caging compound from the reaction, the caged siRNA was precipitated from EtOH in the presence of glycogen, followed by two extractions with $CHCl_3$. The caged siRNA was stored in RNase-free, diethylpyrocarbonate-treated water (United States Biochemical) at −20 °C, protected from light. The extent of modification of the siRNA was determined through the method of Monroe, Haselton, and coworkers by using the extinction coefficient for DMNPE–ATP and absorbance at $\lambda = 355$ nm. [5] The stability of the caged duplex was confirmed by melting studies on the double-stranded siRNA molecule (1 °C min$^{-1}$). The duplex was further characterized by ESI MS, which showed sense and antisense strands, with and without caging groups. See Supporting Information for detailed procedures used for cell culture, duplex formation, mass spectrometry, and normalized expression studies.

Received: July 28, 2004
Published online: January 11, 2005

**Keywords:** gene expression · oligonucleotides · photochemistry · photolysis · RNA recognition

[1] T. Tuschl, *ChemBioChem* **2001**, *2*, 239.
[2] D. M. Dykxhoorn, C. D. Novina, P. A. Sharp, *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 457.
[3] S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, *Nature* **2001**, *411*, 494.
[4] S. M. Elbashir, W. Lendeckel, T. Tuschl, *Genes Dev.* **2001**, *15*, 188.
[5] W. T. Monroe, M. M. McQuain, M. S. Chang, J. S. Alexander, F. R. Haselton, *J. Biol. Chem.* **1999**, *274*, 20895.
[6] Y.-L. Chiu, T. M. Rana, *Mol. Cell* **2002**, *10*, 549.
[7] Y.-L. Chiu, T. M. Rana, *RNA* **2003**, *9*, 1034.
[8] H. Ando, T. Furuta, R. Y. Tsien, H. Okamoto, *Nat. Genet.* **2001**, *28*, 317.
[9] A. Reynolds, D. Leake, Q. Boese, S. Scaringe, W. S. Marshall, A. Khvorova, *Nat. Biotechnol.* **2004**, *22*, 326.
[10] M. Amarzguioui, T. Holen, E. Babaie, H. Prydz, *Nucleic Acids Res.* **2003**, *31*, 589.
[11] F. Czauderna, M. Fechtner, S. Dames, H. Aygun, A. Klippel, G. J. Pronk, K. Giese, J. Kaufmann, *Nucleic Acids Res.* **2003**, *31*, 2705.
[12] F. G. Cruz, J. T. Koh, K. H. Link, *J. Am. Chem. Soc.* **2000**, *122*, 8777.
[13] S. G. Chaulk, A. M. MacMillan, *Nucleic Acids Res.* **1998**, *26*, 3173.
[14] E. F. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng, T. E. Ferrin, *J. Comput. Chem.* **2004**, *25*, 1605.

  