

Taking control of gene expression with light-activated oligonucleotides

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The recent development of caged oligonucleotides that are efficiently activated by ultraviolet (UV) light creates opportunities for regulating gene expression with very high spatial and temporal resolution. By selectively modulating gene activity, these photochemical tools will facilitate efforts to elucidate gene function and may eventually serve therapeutic aims. We demonstrate how the incorporation of a photocleavable blocking group within a DNA duplex can transiently arrest DNA polymerase activity. Indeed, caged oligonucleotides make it possible to control many different protein-oligonucleotide interactions. In related experiments, hybridization of a reverse complementary (antisense) oligodeoxynucleotide to target mRNA can inhibit translation by recruiting endogenous RNases or sterically blocking the ribosome. Our laboratory recently synthesized caged antisense oligonucleotides composed of phosphorothioated DNA or peptide nucleic acid (PNA). The antisense oligonucleotide, which was attached to a complementary blocking oligonucleotide strand by a photocleavable linker, was blocked from binding target mRNA. This provided a useful method for photomodulating hybridization of the antisense strand to target mRNA. Caged DNA and PNA oligonucleotides have proven effective at photoregulating gene expression in cells and zebrafish embryos.

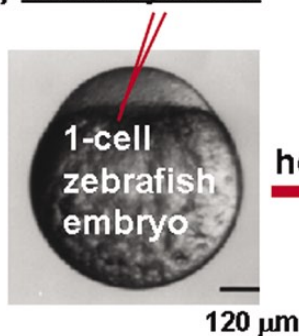
Introduction

A typical vertebrate animal contains approximately twenty thousand unique protein-coding genes (1). This is a small number when one considers the enormity of the tasks required for creating, growing, and sustaining life. To visualize this complexity, imagine a town with 20,000 human inhabitants, each person representing a different gene. Communication occurs between inhabitants and with others in surrounding communities. These signals help to coordinate activities at different locations and times. Through proper organization, the town is able to thrive and create remarkable structures. The existence today of giant pyramids and cathedrals attests to the power of synchronous, well-coordinated human activity.

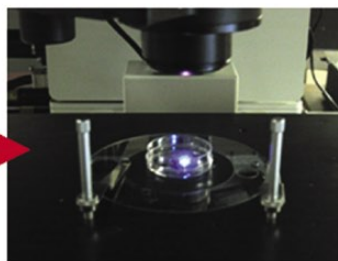
Returning to gene expression in the vertebrate animal—

it is frustratingly difficult to visualize the complex network of signals that is orchestrated at the molecular level, both intra- and extracellularly. The spatial and temporal role of each protein is important, and protein functions can vary by concentration, cell localization, and activation state (i.e., posttranslational modification). Based on the large number of interacting molecules and their complex environment, it is virtually impossible at the present time to identify all of the interacting partners (i.e., protein-protein interactions) for a given cellular process. We still know relatively little at the molecular genetic level about how cells divide, how cell types are specified and are sometimes re-specified at different stages of life, and how animals achieve and maintain a predetermined size. And, it appears likely that the focus in biochemistry on protein

1) Microinjection



2) Laser Activation at ROI



3) Fluorescent Cells (24 hpf)

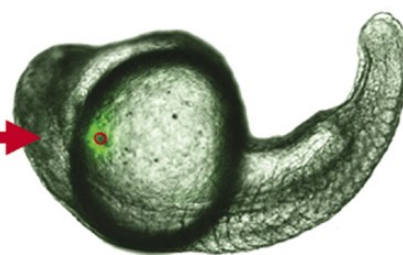


Figure 1. Strategy for photoactivating caged oligonucleotides within embryos of zebrafish and other developing organisms. Nanograms of material are microinjected into zebrafish embryos at the one-cell stage; embryos are cultured at 28°C for a number of hours and subsequently irradiated by an ultraviolet (UV) laser in a region of interest (ROI). To generate this figure, embryos were injected with 5-carboxymethoxy-2-nitrobenzyl (CMNB)-caged fluorescein (Invitrogen, Carlsbad, CA, USA), irradiated by UV confocal laser scanning microscope (Olympus Fluoview™ FV1000), and imaged at 24 h postfertilization (hpf). Green fluorescence from the uncaged fluorescein only appears in the small area of the embryo that is delineated by the ROI (red circle).

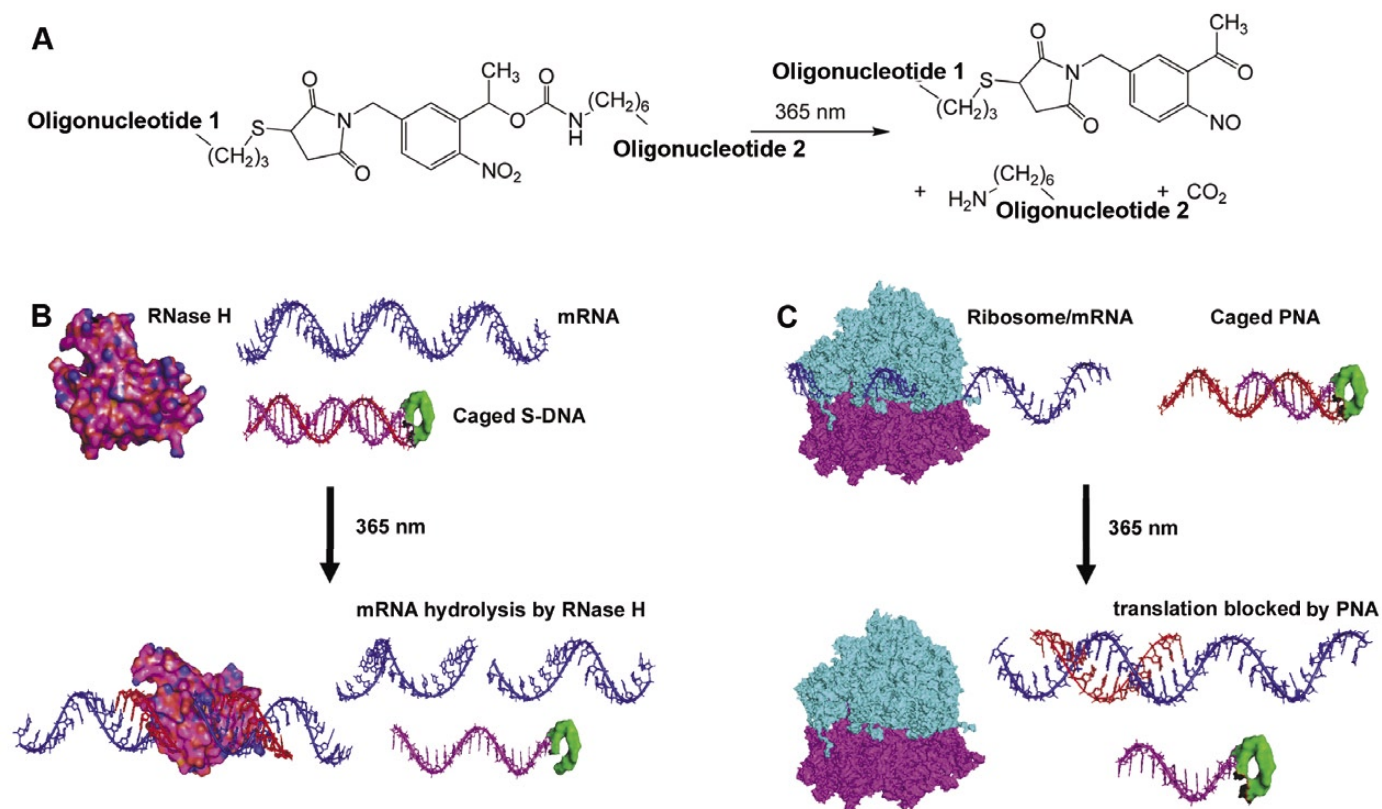


Figure 3. Two approaches for regulating gene expression with light-activated oligonucleotides. (A) Conjugates are composed of complementary thiol- and amine-terminated oligonucleotides linked by a heterobifunctional photocleavable linker. Oligonucleotides 1 and 2 can vary in length and be DNA, PNA, RNA, or other oligonucleotide variant. Ultraviolet (UV) photolysis generates a much less stable duplex, thereby activating the antisense molecule, typically the amine-terminated oligonucleotide 2. (B) Caged S-DNA conjugates are stable until UV activation promotes S-DNA/mRNA duplex formation, which recruits RNase H. In this way, RNase H hydrolyzes target mRNA, thereby reducing gene expression. (C) The ribosome large and small subunits combine to synthesize protein from an mRNA template. Caged PNA does not interfere with this process, but upon UV activation, the antisense peptide nucleic acid (PNA) strand binds target mRNA and blocks protein synthesis.

peptide nucleic acids (PNAs) in order to photoregulate gene expression in living cells and embryos.

Development of Caged Fluorescent Oligonucleotides

Our work began a few years ago with the goal of photoregulating DNA function while minimally perturbing DNA structure (40,41). This related to controlling DNA-mRNA and mRNA-rRNA interactions and thus was an important step toward regulating gene expression with light. To create a self-reporting caged fluorescent oligodeoxynucleotide, we incorporated aminoethyl-deoxycytidine and fluorescein-labeled deoxycytidine at adjacent positions in the middle of a 25-mer DNA oligonucleotide (40). The aminoethyl functionality was subsequently modified with a dimethyl-amino-azobenzenesulfonyl (DABSYL) quencher attached via a 1-[5-(aminomethyl)-2-nitrophenyl]ethanol photocleavable linker, as shown in Figure 2A.

In a duplex formed between this caged 25-mer oligodeoxynucleotide and a shorter (12-mer) complementary

DNA primer, the photoactive DABSYL moiety served to block DNA polymerase (Klenow fragment) from extending the primer (41). In biochemical assays, photocleavage of DABSYL was concomitant with a greater than 50-fold increase in signal from fluorescein (Figure 2B, $\lambda_{em} = 520 \text{ nm}$). Monitored by fluorescence, photo-uncaging resulted in a 25-fold increase in the yield of fully extended primer. Interestingly, oligonucleotides containing either the DABSYL-modified cytidine or fluorescein-modified cytidine showed no effect on the polymerase reaction. Thus, the fluorophore-quencher pair acted synergistically to create a blockade of sufficient size to arrest the enzyme's procession along DNA. This example illustrated the potential of sterically bulky leaving groups to regulate protein-oligonucleotide interactions, which has been validated in other biochemical systems (42–46). The fluorescent reporter is useful for real-time monitoring of photo-uncaging in biological systems, as we demonstrated in living zebrafish embryos by ultraviolet (UV) confocal microscopy (40).

We also investigated the possibility of using the large photocleavable DABSYL moiety to regulate DNA hybridization.

The caged oligonucleotide hybridized to a complementary 25-mer DNA formed a duplex whose melting temperature (T_m) was lowered by 9°C, relative to unmodified DNA. However, only 1°C of thermal stability was restored after photocleavage of the DABSYL moiety. In this example, the DABSYL cleaving group pointed into the DNA major groove, and thus did little to destabilize base stacking. This corroborated studies showing the difficulty of controlling DNA hybridization by attaching photolabile, sterically bulky, moieties to the nucleobases or phosphate backbone (44,47–50). Previous efforts to photoregulate oligonucleotide structure and function *in vivo* required the use of multiple blocking groups, which reduced the yield of fully active compound and resulted in modest biological effects (50–52). DNA-DNA, DNA-RNA, and RNA-RNA duplexes found in biology contain myriad hydrogen bonds and base-pairing interactions, which impart tremendous specificity and stability. Thus, we required a more directed strategy for conditionally regulating hybridization events important in controlling gene expression.

Development of Caged Antisense Oligonucleotides

Our laboratory recently developed caged antisense oligonucleotides of composition sense oligonucleotide-photocleavable linker-antisense oligonucleotide (Figure 3A) (53). This simple caging strategy presents tremendous opportunities for controlling hybridization in many biological systems. The hairpin-like conjugates differ in structure and down-regulate gene expression in cells and embryos by two parallel routes: (i) phosphorothioated DNA (S-DNA) oligonucleotides that degrade cellular mRNA by recruiting RNase H (Figure 3B); and (ii) PNAs that sterically block ribosomal protein synthesis within developing zebrafish embryos (Figure 3C). Extended (20–35 mer) phosphorothioated antisense oligodeoxynucleotides were shown previously to be stable in cells, bind target mRNA, and promote mRNA hydrolysis by RNase H (54). Likewise, PNAs (7) as well as morpholinos (9) bind mRNA sequence specifically at translation initiation sites and block protein synthesis in many biological systems (8,55,56). We recently achieved light-dependent activation of antisense S-DNA and PNA molecules in cells and zebrafish embryos.

Caging strategies were developed for both antisense approaches (Figure 3) by manipulating thermodynamics to create an inactive, very stable hairpin-like conjugate ($T_m > 75^\circ\text{C}$), which, upon photoactivation, generates the much less stable active duplex ($T_m = 35^\circ\text{--}55^\circ\text{C}$). With proper design, it is possible to tune the difference in stability by as much as several kcal/mol, $\Delta T_{m(\text{duplex-conjugate})} \approx -40^\circ\text{C}$. In active duplex form, the antisense strand is liberated to bind target mRNA. In order to achieve the desired biological

effect and compensate for the presence of the competing sense strand, slightly higher concentrations of the caged oligonucleotide can be employed.

Synthesis of caged S-DNA and PNA conjugates follows recently published protocols (57) that detail the incorporation of a photocleavable linker (PL), 1-(5-(N-maleimido-methyl)-2-nitrophenyl)ethanol N-hydroxysuccinimide ester. Amine- and thiol-terminated oligonucleotides that react with opposite ends of the heterobifunctional PL are synthesized in-house or purchased from commercial vendors. Synthesis of the PL and conjugates occurs in good yields and all caged oligonucleotides are purified by reverse-phase or ion-exchange high-performance liquid chromatography (HPLC). Because only a single photocleavable linker is required to generate the caged antisense oligonucleotide, conjugates are activated with high quantum yields by UV light. Purity and photocleavage efficiency can be tested by gel electrophoresis using fluorescent or ^{32}P -labeled conjugates.

Cycles of conjugate synthesis, measurement of thermodynamic parameters, and redesign are readily achieved using DNA oligonucleotides. Upon refinement, phosphorothioated versions can be synthesized for cell studies, and PNA-based oligonucleotides for zebrafish studies. In our experience with caged oligonucleotides, biological experiments present additional challenges, such as sample toxicity, loss of activity due to nucleases, cell compartmentalization, or other undetermined factors *in vivo*, poor solubility at the concentrations required for microinjection, and activation by ambient light during sample handling. Additional problems encountered with *in vivo* experiments are that target mRNAs are typically of varying concentration, much longer, more stable, and generally less accessible to antisense molecules. Care must be taken to ensure that the UV light used for experiments is nontoxic to cells and animals. Cells that are difficult to grow in culture and whose viability is compromised by nucleofection of genetic material, are prone to cell death from long exposures to UV light. Fortunately, with most specimens we have not observed UV light toxicity at modest exposure levels (5–50 mW/cm², $\tau < 10$ min).

Most recently, our laboratory has developed caged PNAs whose activity can be readily modulated with light inside developing zebrafish embryos, with no toxic side effects. These constructs are introduced at the one-cell stage and can be subsequently uncaged with UV light at a specific time (Figure 1), typically several hours postfertilization (hpf). The laboratory is initiating experiments that involve laser-uncaging using a UV confocal microscope. Such experiments should allow unprecedented spatial and temporal control over gene expression within a developing embryo, and make it possible, for example, to reveal the molecular mechanisms by which zebrafish and other

teleosts are able to regenerate limbs and organs.

Acknowledgments

Support for this work came from a Camille and Henry Dreyfus New Faculty Award and the University of Pennsylvania Institutes of Genomics and Medicine and Engineering. Eric Weinberg, Shingo Maegawa, Alan Gewirtz, and Jyothishmathi Swaminathan contributed to the biological studies. We thank Jeffery Saven and Jim Eberwine for access to equipment. Adam Peritz synthesized modified oligonucleotides that were used to make the conjugates described in this article. Julia Richards also made important contributions to this work.

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