Regulating Gene Expression in Zebrafish Embryos Using Light-Activated, Negatively Charged Peptide Nucleic Acids

ARTICLE *in* JOURNAL OF THE AMERICAN CHEMICAL SOCIETY · OCTOBER 2007

Impact Factor: 12.11 · DOI: 10.1021/ja073723s · Source: PubMed

4 AUTHORS, INCLUDING:

Xinjing Tang
Peking University
45 PUBLICATIONS **649** CITATIONS
SEE PROFILE

Shingo Maegawa
Kyoto University
33 PUBLICATIONS **1,012** CITATIONS
SEE PROFILE

Ivan J Dmochowski
University of Pennsylvania
80 PUBLICATIONS **1,778** CITATIONS
SEE PROFILE

J|A|C|S COMMUNICATIONS

Published on Web 08/21/2007

Regulating Gene Expression in Zebrafish Embryos Using Light-Activated, Negatively Charged Peptide Nucleic Acids

XinJing Tang, Shingo Maegawa, Eric S. Weinberg, and Ivan J. Dmochowski*

Departments of Chemistry and Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received May 23, 2007; E-mail: ivandmo@sas.upenn.edu

Achieving a molecular understanding of biological processes will require quantitative tools for regulating gene activity with high spatial and temporal resolution. Such methods will allow, for example, real-time functional studies of cellular proteins. One promising approach involves reverse complementary "antisense" oligonucleotides, such as "mopholinos"[1] and negatively charged peptide nucleic acids (ncPNAs),[2,3] which down-regulate target genes in model organisms. Short ncPNAs bind tightly and sequence specifically to complementary mRNA, possess a nuclease-resistant pseudopeptide backbone, and, when targeted to translation initiation sites, block ribosomal protein synthesis. Thus, the application of light-activated "caging" strategies[4] to ncPNA would provide an "on → off" switch for controlling gene expression (Figure 1A). It was shown previously that attaching sterically bulky photolabile groups to oligonucleotides has modest effects on duplex formation.[5] Herein, we report the synthesis, characterization, and in vivo application of two light-activated ncPNAs whose hybridization to mRNA is conditionally blocked by a complementary 2′-OMe RNA strand (Figure 1B).

Efforts to photomodulate oligonucleotide function within cells and embryos have met with limited success.[6−9] Monroe and co-workers demonstrated the photoregulation of GFP expression in HeLa cells by labeling a DNA plasmid with multiple 1-(4,5-dimethoxy-2-nitrophenyl)ethyl (DMNPE) groups.[6] In other pioneering work, Ando et al. labeled mRNA and DNA constructs approximately once every 35 bases with a 6-bromo-7-hydroxycoumarin-4-ylmethyl (Bhc) protecting group.[7] Caged mRNA and DNA constructs enabled the photomodulation of GFP expression and subsequent study of Lhx2 in zebrafish forebrain growth.[8] Using a similar statistical labeling approach, Friedman et al. reported a light-activated small interfering RNA (siRNA) that modulated GFP expression 2-fold in HeLa cells.[9] In these examples, heterogeneous mixtures of molecules labeled with multiple blocking groups required greater exposure to UV light and resulted in poor spatiotemporal resolution and photoconversion. To address these problems, we recently developed sensitive methods for regulating oligonucleotide function using a single photolabile blocking group.[10]

In the present study, we attached an amine-terminated, 18-mer antisense ncPNA (Active Motif, Carlsbad, CA) targeting the Kozak sequence and start codon of zebrafish *chordin* mRNA to a thiol-terminated 12-mer 2′-OMe-RNA sense strand (sRNA) via a 1-(5-(*N*-maleimidomethyl)-2-nitrophenyl)ethanol *N*-hydroxysuccinimide ester photocleavable linker (PL). Chordin plays several important roles during zebrafish embryonic development, including dorsal-ventral patterning.[11] UV cleavage of caged PNA-chd (T_m > 90 °C) yielded the less stable PNA-chd/sRNA duplex (T_m = 70 °C). In this form, it was anticipated that PNA-chd would bind complementary *chordin* mRNA and block protein synthesis.

Control experiments showed no differences between UV-irradiated and non-irradiated zebrafish embryos (Figure 2A).[12] Ninety-five percent of embryos injected with native PNA-chd showed a shortened tail and reduced brain characteristic of the *chordino* (no chordin) phenotype[15] at 24 hpf (average of three

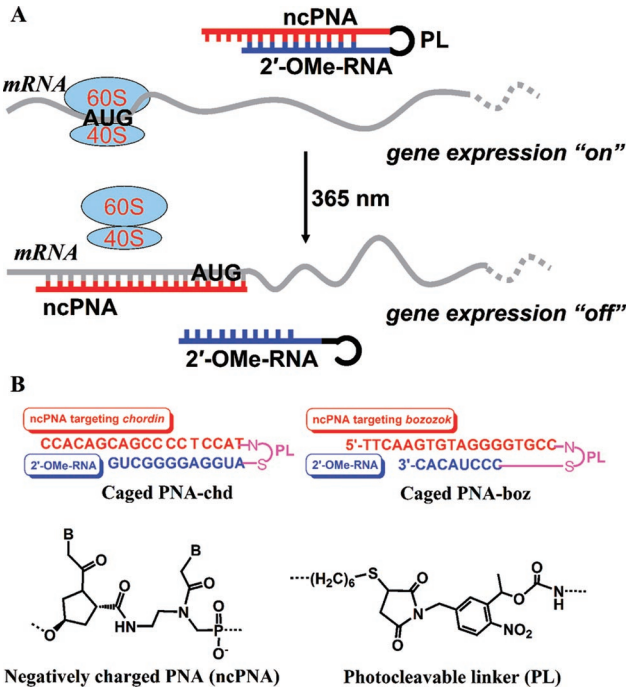

Figure 1. (A) Controlling gene expression using negatively charged peptide nucleic acid (ncPNA) attached to complementary 2′-OMe RNA via a photocleavable linker (PL). Photolysis promoted ncPNA binding to mRNA, thereby blocking protein synthesis in zebrafish embryos. (B) Structures of caged PNA-chd, caged PNA-boz, ncPNA, and PL.

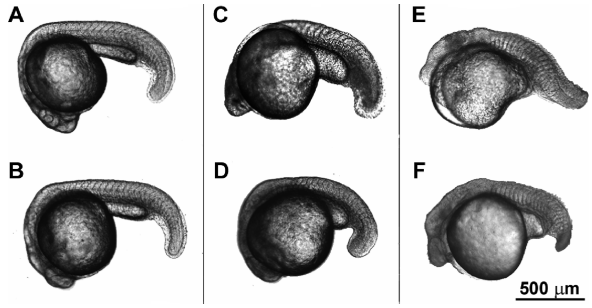

Figure 2. Transmitted light images of representative 24 hpf zebrafish embryos. (A) Uninjected embryos UV-irradiated at 3 hpf for 8 min developed normally. (B) Injection with 0.5 mM caged PNA-chd or caged PNA-boz had no effect in the dark. Embryo shown was injected with caged PNA-chd; an embryo injected with caged PNA-boz is shown in Figure S7. (C) Embryos microinjected with 0.1 mM PNA-chd exhibited "no chordin" phenotype. (D) Injection with 0.5 mM caged PNA-chd and UV irradiation at 3 hpf gave the "no chordin" phenotype. (E) Embryos microinjected with 0.5 mM PNA-boz exhibited "no bozozok" phenotype. (F) Injection with 0.5 mM caged PNA-boz and 8 min UV irradiation at 2 hpf gave the "no bozozok" phenotype.

experiments, n = 90; Figure 2C). Lower than 100% antisense activity was due to small variations in microinjection volume.

10.1021/ja073723s CCC: $37.00

Embryos injected[13] with caged PNA-chd (average of three experiments, $n = 150$) were divided, with one dish kept in the dark, the other irradiated for 8 min;[12] 85% of embryos that remained in the dark appeared completely normal at 24 hpf (Figure 2B), indicating that the conjugated sRNA strand blocked binding of PNA-chd to target mRNA.[14] The other 15% of embryos showed a mild *chordino* phenotype at 24 hpf. This was likely caused by features of the caged PNA-chd, such as rRNA quadruplex structure, which reduced the blocking effects. Trace amounts of PNA-chd may also have contributed to this result. Photoactivation of caged PNA-chd[12] produced the *chordino* phenotype in 81% of embryos (Figure 2D). As expected when chordin is down-regulated,[16] these embryos also showed greatly reduced expression of the gene *otx2* (Figure S1). The high thermal stability of the PNA-chd/sRNA duplex contributed to the lack of a *chordino* phenotype in 19% of the irradiated embryos. Photocleavage occurring within 8 min in buffer and zebrafish was confirmed by gel electrophoresis using fluorescently labeled PNA conjugates.

We investigated phenotypic differences between embryos containing uncaged PNA-chd or native PNA-chd. Photoactivation of caged PNA-chd gave an equilibrium between PNA-chd/sRNA (inactive) and PNA-chd/mRNA (active) duplex forms, which was assayed in a molecular beacon experiment (Supporting Information). These results were validated with zebrafish dose−response experiments which showed that phenotypes were equalized by injecting 5-fold less native PNA-chd (0.1 mM, Figure 2C) than caged PNA-chd (0.5 mM solution), followed by UV irradiation (Figure 2D). Caged PNA-chd blocked chordin expression in a light- and concentration-dependent manner.

To test the specificity of caged ncPNA, we targeted a second gene, *bozozok*, with important roles in organizer formation during early zebrafish development.[17] An 18-mer ncPNA was previously identified for *bozozok* that produced definitive gene knockdown.[3,18] This PNA-boz sequence was attached via PL to an 8-mer sense 2′-OMe-RNA (Figure 1B). The shorter blocking strand was employed to promote efficient PNA-boz/mRNA duplex formation after in vivo photoactivation. Melting temperature experiments indicated that caged PNA-boz, $T_m = 80$ °C, was much more stable than uncaged PNA-boz, $T_m = 39$ °C. The large ΔT_m of −41 °C validated the caged ncPNA strategy and facilitated removal of unreacted ncPNA/sRNA from caged PNA-boz by HPLC.

Embryos injected[13] with caged PNA-boz and left in the dark developed normally (Figure S7), whereas embryos injected with native PNA-boz (Figure 2E) or injected with caged PNA-boz then irradiated for 8 min[12] at 2 hpf showed the typical bozozok null ("no bozozok") phenotype at 24 hpf with block-shaped somites, neurectodermal defects, and absence of axial mesodermal tissues (Figure 2F).[3,17,18] Some embryos were fixed at 5−6 hpf at the early gastrula stage and visualized by in situ hybridization to detect the expression of *goosecoid* (*gsc*), which is a marker of the dorsal organizer known to be dependent on bozozok. Whereas uninjected embryos showed *gsc* expression in the dorsal organizer (Figure 3A), PNA-boz injection caused complete loss of *gsc* in 90% of embryos (Figure 3B), as expected for loss of *bozozok* function.[17] Of embryos injected with caged PNA-boz, 95% showed no effect on *gsc* expression at 6 hpf (Figure 3C). In contrast, irradiation[12] of these embryos at 2 hpf greatly reduced *gsc* in 85% of embryos (Figure 3D). These data highlight the power of caged ncPNAs to control complex gene networks.

In summary, we succeeded in regulating two developmentally important genes in zebrafish embryos using caged negatively charged PNAs. UV light efficiently restored antisense activity. By this strategy of conditional repression, we plan to vary protein levels quantitatively throughout the embryo by UV laser.

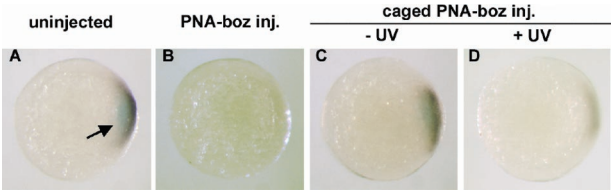

Figure 3. Effect of PNA-boz on *goosecoid* mRNA levels in the dorsal organizer at 6 hpf. (A) Uninjected wild-type embryos showed *gsc* in the organizer (black stain, identified with arrow) in the organizer (identified with arrow). (B) Injection of PNA-boz resulted in loss of *gsc*. (C) Injection of caged PNA-boz did not affect *gsc* expression. (D) UV activation of caged PNA-boz at 2 hpf caused reduction of *gsc* mRNA levels.

Acknowledgment. Support to I.J.D. came from a Camille and Henry Dreyfus Teacher-Scholar Award and UPenn Institutes of Genomics and Medicine and Engineering. E.S.W. was supported by NIH grants R01 HD39272 and R01 DC03080. Jeffery Saven provided a fluorometer. Adam Peritz synthesized modified 2′-OMe-RNA.

Supporting Information Available: Sequences, synthesis, characterization, and use of caged ncPNA. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Summerton, J. *Biochim. Biophys. Acta* **1999**, *1489*, 141−158. (b) Nasevicius, A.; Ekker, S. C. *Nat. Genet.* **2000**, *26*, 216−220.
(2) (a) Hanvey, J. C.; Peffer, N. J.; Bisi, J. E.; Thomson, S. A.; Cadilla, R.; Josey, J. A.; Ricca, D. J.; Hassman, C. F.; Bonham, M. A.; Au, K. G.; Carter, S. G.; Bruckenstein, D. A.; Boyd, A. L.; Noble, S. A.; Babiss, L. E. *Science* **1992**, *258*, 1481−1485. (b) Wickstrom, E.; Urtishak, K. A.; Choob, M.; Farber, S. A. *Methods Cell Biol.* **2004**, *77*, 137−158.
(3) Urtishak, K. A.; Choob, M.; Tian, X.; Sternheim, N.; Talbot, W. S.; Wickstrom, E.; Farber, S. A. *Dev. Dyn.* **2003**, *228*, 405−413.
(4) (a) Mayer, G.; Heckel, A. *Angew. Chem., Int. Ed.* **2006**, *45*, 4900−4921. (b) Tang, X.; Dmochowski, I. J. *Mol. BioSystems* **2007**, *3*, 100−110.
(5) (a) Tang, X.; Dmochowski, I. J. *Org. Lett.* **2005**, *7*, 279−282. (b) Ghosn, B.; Haselton, F. R.; Gee, K. R.; Monroe, W. T. *Photochem. Photobiol.* **2005**, *81*, 953−959. (c) Kroeck, L.; Heckel, A. *Angew. Chem., Int. Ed.* **2005**, *44*, 471−473.
(6) Monroe, W. T.; McQuain, M. M.; Chang, M. S.; Alexander, J. S.; Haselton, F. R. *J. Biol. Chem.* **1999**, *274*, 20895−20900.
(7) Ando, H.; Furuta, T.; Tsien, R. Y.; Okamoto, H. *Nat. Genet.* **2001**, *28*, 317−325.
(8) Ando, H.; Kobayashi, M.; Tsubokawa, T.; Uyemura, K.; Furuta, T.; Okamoto, H. *Dev. Biol.* **2005**, *287*, 456−468.
(9) Shah, S.; Rangarajan, S.; Friedman, S. H. *Angew. Chem., Int. Ed.* **2005**, *44*, 1328−1332.
(10) (a) Tang, X.; Richards, J. L.; Peritz, A. E.; Dmochowski, I. J. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 5303−5306. (b) Tang, X.; Dmochowski, I. J. *Angew. Chem., Int. Ed.* **2006**, *45*, 3523−3526.
(11) Miller-Bertoglio, V. E.; Fisher, S.; Sánchez, A.; Mullins, M. C.; Halpern, M. E. *Dev. Biol.* **1997**, *192*, 537−550.
(12) Photolysis conditions: UV transilluminator (Spectronics Corporation, cat. no. TL-365R), 9 mW/cm^2 at peak intensity, 365 nm.
(13) Microinjection solutions were typically 0.5 mM, 10 nL per embryo, at the one- or two-cell stage. Embryos were cultured at 28 °C.
(14) Background activity was reduced by putting a 400 nm long-pass filter (Chroma) before the sample during microinjection. 2′-OMe-RNA served as an effective blocking strand, however, the analogous ncPNA−PL−sDNA conjugate was very toxic to developing zebrafish.
(15) Schulte-Merker, S.; Lee, K. J.; McMahon, A. P.; Hammerschmidt, M. *Nature* **1997**, *387*, 862−863.
(16) Fisher, S.; Amacher, S. L.; Halpern, M. E. *Development* **1997**, *124*, 1301−1311.
(17) (a) Koos, D. S.; Ho, R. K. *Dev. Biol.* **1999**, *215*, 190−207. (b) Fekany, K.; Yamanaka, Y.; Leung, T.; Sirotkin, H. I.; Topczewski, J.; Gates, M. A.; Hibi, M.; Renucci, A.; Stemple, D.; Radbill, A.; Schier, A. F.; Driever, W.; Hirano, T.; Talbot, W. S.; Solnica-Krezel, L. *Development* **1999**, *126*, 1427−1438.
(18) Maegawa, S.; Varga, M.; Weinberg, E. S. *Development* **2006**, *133*, 3265−3276.

JA073723S