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Point of VIEW

Yin–Yang Ways of Controlling Gene Expression Are Now in Our Hands

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ABSTRACT Molecular biologists have long dreamed of switching genes on and off at will in any part of the body during embryonic development. Their dream is now coming true thanks to the transparency of the embryonic body of the zebrafish (*Danio rerio*), which has made gene manipulation by photoillumination possible.

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At least partial recovery of messenger RNA (mRNA) translational activity has already been achieved by the temporally and spatially restricted illumination by long-wavelength UV light of zebrafish embryos that were injected with mRNA modified (caged) *in vitro* with photocleavable groups at the one-cell stage (Figure 1, panel a) (*1–4*). Now, the technology has emerged to make the dream come true: repression of gene activity by photoactivation of caged antisense oligonucleotides in zebrafish embryos (*5, 6*).

The pioneering work of Monroe *et al.* (*7*) reported that a DNA plasmid modified with multiple 1-(4,5-dimethoxy-2-nitrophenyl) ethyl (DMNPE) groups could be reactivated by removing these groups with photoillumination in HeLa cells. Later, Ando *et al.* (*1–4*) labeled mRNA approximately once every 35 bases with a 6-bromo-7-hydroxycoumarin-4-ylmethyl (Bhc) protecting group. They showed that caged mRNA enabled the photomodulation of gene expression (Figure 1, panel b) and used this technique to study Eng2a and Lhx2 in zebrafish brain growth. In both cases, the inactivation of mRNA or DNA depended on reversible and stochastic conjugation of the photocleavable blocking groups to the phosphate moieties of mRNA or DNA.

As a natural extension of this work, interest has increased in applying similar technology to the temporally and spatially controlled repression of specific genes by the photoreactivation of antisense oligonucleotides. However, the use of Bhc to modify antisense oligonucleotides has been hampered by several of its inherent chemical characteristics. First, although antisense morpholino oligonucleotides (MOs) are the most widely used for repression of gene activity in zebrafish embryos, Bhc cannot bind to MOs because they have no phosphodiester linkages (Figure 2, panel a) (*8*). They are replaced with phosphorodiamidate intersubunit linkages. Recently, a new type of DNA mimic, negatively charged peptide nucleic acids (ncPNAs), composed of alternating phosphonate PNA analogues and *trans*-4-hydroxy-L-proline PNA analogues (HypNA-pPNA), has become available as an alternative choice to MOs (Figure 2, panel b) (*9*). These ncPNAs do have the phosphate moieties that could be theoretically bound by Bhc. However, the conjugation of more than one Bhc molecule per ncPNA molecule would make the modified ncPNA almost water-insoluble because of the highly hydrophobic nature of Bhc.

The recent collaboration of Tang and Dmochowski, chemists, and Maegawa and Weinberg, zebrafish developmental biologists, has overcome these difficulties (*5*). They blocked the ability of ncPNA to hybridize with mRNA with a complementary 2′-OMe-RNA strand (Figure 2, panel c), which is attached to ncPNA *via* a single 1-(5-(*N*-maleimidomethyl)-2-nitrophenyl) ethanol *N*-hydroxysuccimide ester photocleavable linker (PL) (Figure 2, panel d). The drastic reduction in the stability of the heteroduplex of ncPNA and small RNA (sRNA) by $\Delta T_m >$ 20 °C after photocleavage of PL enables

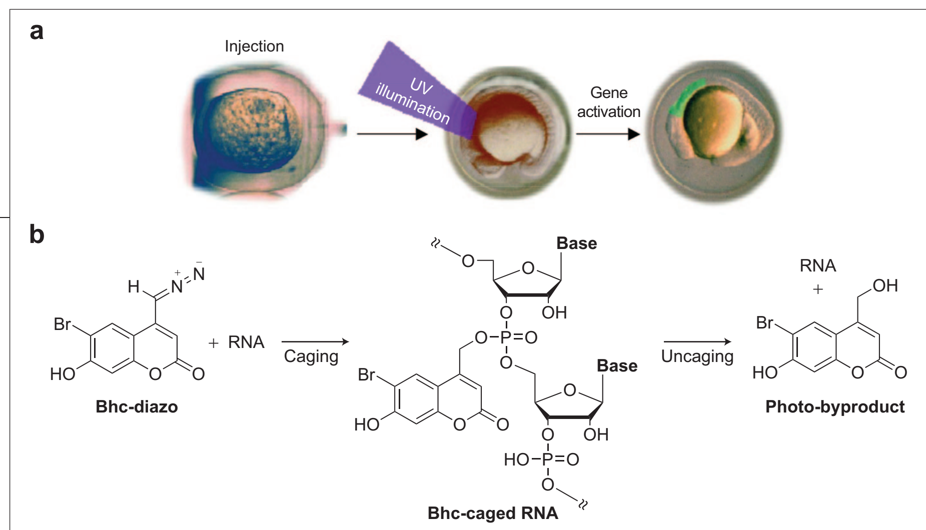

Figure 1. Activation of caged mRNA by photoillumination. a) Injection and activation of caged mRNA in zebrafish embryos. b) Inactivation of mRNA by caging with Bhc and its reactivation by UV illumination.

ncPNA to bind to complementary mRNA and block translation. Unlike when various protecting groups are bound to antisense oligonucleotides by stochastic conjugation of the protecting groups to the phosphate moieties of the oligonucleotides, the high degree of structural homogeneity of this caged ncPNA is expected to facilitate more coherent reaction of molecules to the photocleavage treatment by UV illumination.

The proper design of relatively short sense 2′-OMe-RNA, which significantly reduces the T_m of the ncPNA/sRNA heteroduplex after uncaging, appears to be essential to successfully activate ncPNA *in vivo*. Tang *et al*. obtained the best result when they attached the 18-mer ncPNA against *bozozok* (*boz*) mRNA to an 8-mer sense 2′-OMe-RNA *via* PL. This caged PNA-boz, T_m = 80 °C, is much more stable than the uncaged PNA-boz, T_m = 39 °C (Figure 2, panel c). Embryos injected with this caged ncPNA-boz showed no abnormality when reared in the dark, but almost all of the injected embryos showed the typical *bozozok*-null phenotype when they were irradiated for 8 min at 2 h postfertilization with 365-nm UV light at the peak intensity of 9 mW/cm^2. A severe reduction occurred in the size of the region expressing *goosecoid* mRNA, the specific marker for the dorsal organizer, which is regulated downstream of *bozozok*.

Compared with the uncaging of Bhc-modified nucleotides, which requires relatively low levels of energy (~100 mJ/cm^2 in total), the activation of caged ncPNA needs >40 times more energy, exposing these embryos to longer durations of UV light (*1, 5*). Therefore, potential damage to the developmental process of embryos by this long UV exposure must be considered when the results of the experiments are interpreted.

The uncaging of Bhc-modified mRNA could enhance translational activity by ~5-fold more than the caged mRNA. However, it could recover its translational activity at best up to 15% of the level achieved by intact mRNA (*1*). Tang *et al*. (*5*) estimated by using ncPNA against another gene, *chordin*, that equivalent phenotypes were reached by injection of 5-fold less native ncPNA than injection of caged ncPNA followed by UV irradiation, suggesting 20% recovery of caged ncPNA. Although the efficiency in the recovery by photoactivation does not appear very different between these two methods, caged ncPNA has a great advantage over stochastic modification of mRNA with Bhc. Its activity before UV irradiation can be drastically reduced to almost nothing when the homogeneity of the structure is exploited, as long as the sequences of the complementary ncPNA and sRNA are properly designed (*5*). This could never be achieved when Bhc is used to modify the activity of mRNA; with 15% recovery potential of the Bhc-caged mRNA maintained, ~3% of the intact activity would remain (*1*).

Therefore, in addition to the different ways they act on gene activation, modification of mRNA with Bhc and synthesis of caged ncPNA have both pros and cons. The reactions to conjugate mRNA with Bhc are relatively simple. However, meticulous titrations of reactions are essential to minimize

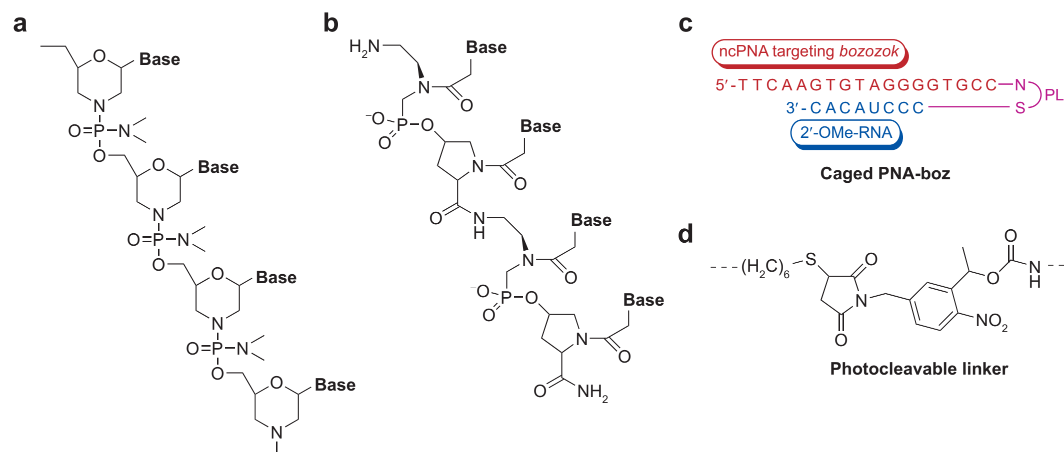

Figure 2. Structure of MO- and PNA-based gene expression regulators. a) Morpholino oligonucleotides. b) Negatively charged PNA. c) Caged PNA-boz. d) Photocleavable linker.

basal leaky expression and maximize the photoactivated expression from the Bhc-caged mRNA. On the other hand, although the reactions to synthesize caged ncPNA are relatively more complicated, the careful design of the nucleotide sequences to maximize the reduction in the melting temperature of the ncPNA/sRNA after uncaging can ensure the maximum effects of photoactivation.

The negative effects of uncaged ncPNA on gene expression could be made positive by targeting it against mRNA encoding the repressor of gene expression (*10*), or removing floxed genes by activating Bhc-caged mRNA for Cre recombinase might also reverse the effect of uncaging Bhc-modified mRNA (*11*). Now that we have yin–yang (positive and negative) ways of controlling gene expression in zebrafish embryos, we have more freedom to study the regulatory networks of gene expression during embryonic development.

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