Photo-mediated gene activation using caged RNA/DNA in zebrafish embryos

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We report a new and simple technique for photo-mediated temporal and spatial control of gene activation in zebrafish embryos as an alternative to the gene 'knockdown' approach using antisense, morpholino-modified oligonucleotides (morpholinos). The synthetic compound 6-bromo-4-diazomethyl-7-hydroxycoumarin (Bhc-diazo) forms a covalent bond with the phosphate moiety of the sugar-phosphate backbone of RNA, a process known as caging. The 6-bromo-7-hydroxycoumarin-4-ylmethyl (Bhc) group binds to approximately 30 sites on the phosphate moieties per 1 kb of RNA sequence. Bhc-caged mRNA undergoes photolysis (uncaging) when exposed to long-wave ultraviolet light (350 to 365 nm). We show that Bhc-caged green fluorescent protein (*Gfp*) mRNA has severely reduced translational activity *in vitro*, whereas illumination of Bhc-caged mRNA with ultraviolet light leads to partial recovery of translational activity. Bhc-caged mRNA is highly stable in zebrafish embryos. In embryos injected with Bhc-caged *Gfp* mRNA at the one-cell stage, GFP protein expression and fluorescence is specifically induced by ultraviolet light. We also show that, consistent with results obtained using other methods, uncaging eng2a (which encodes the transcription factor Engrailed2a) in the head region during early development causes a severe reduction in the size of the eye and enhanced development of the midbrain and the midbrain-hindbrain boundary at the expense of the forebrain.

Introduction

Zebrafish (*Danio rerio*) are widely used as model organisms for the investigation of genetic control in early morphogenesis[1] because their transparent bodies make it easy to monitor the development of tissues and neurons *in vivo*[2]. Zebrafish brain development has been well characterized[3,4]. Furthermore, recent advances in systematically compiling information on genomic sequences, expressed sequence tags and expression patterns of individual genes has made the identification of previously unknown genes easier[5]. A 'knockdown' technology using antisense morpholinos has been developed to facilitate direct assignment of functions to such genes[6]. Another powerful approach for studying gene function is gain-of-function by ectopic expression of genes in a temporally- and spatially-specific way. However, there is still no simple and effective system for the temporal and/or spatial control of gene expression in zebrafish. One promising approach is RNA caging, in which RNA is inactivated by the covalent attachment of a photo-removable protecting group (the caging group) and then is reactivated by photo-illumination with light of a specific wavelength. RNA caging was first achieved by the site-specific modification of the 2′-hydroxy nucleophile in the substrate RNA of the hammerhead ribozyme with a caging functionality, the O-(2-nitrobenzyl) caging group[7]. Susceptibility of the substrate RNA to hammerhead-catalyzed cleavage reaction was abolished by this modification, but it recovered rapidly and efficiently after removal of the caging group by photo-illumination (308 nm and 10 J/ cm^2) with excimer laser. Another pioneering attempt to achieve spatio-temporal control of gene expression was made by caging DNA with the 1-(4,5-dimethoxy-2-nitrophenyl)ethyl (DMNPE) group[8]. HeLa cells transfected with a plasmid that encodes GFP and was caged with this agent showed reduced levels (0 to ~25%) of GFP expression compared with cells transfected with intact plasmid, and exposure of these cells to ultraviolet light (365 nm, 0.25 to ~0.5 J/ cm^2) doubled the expression level.

To control gene expression at spatially and temporally high resolution in rapidly developing zebrafish embryos, it is essential to use a caging agent that easily reacts with mRNA *in vitro* and can be removed *in vivo* with a minimum amount of photo-illumination, thereby preventing excessive damage to embryonic tissues. We report here a new RNA-caging system that uses the caging agent 6-bromo-4-diazomethyl-7-hydroxycoumarin (Bhc-diazo)[9]. We originally synthesized Bhc-diazo for other purposes (see Web Note A). We achieved RNA caging with Bhc-diazo by mixing both compounds in dimethylsulfoxide (DMSO), which is important for the efficiency and reproducibility of the reaction. Although we have not completed a structural characterization of the products, Bhc-diazo probably reacts with the free phosphoric acids (non-ionized form) of RNA to yield the 6-bromo-7-hydroxycoumarin-4-ylmethyl ester of the phosphates, as similar phosphoric acid modifications in various nucleotides have been reported[10,11] and most diazoalkanes react with phosphoric acids or carboxylic acids at room temperature without any catalyst[12,13]. This is consistent with our observation that DMSO is a better solvent than aqueous buffer for RNA caging with Bhc-diazo, as the

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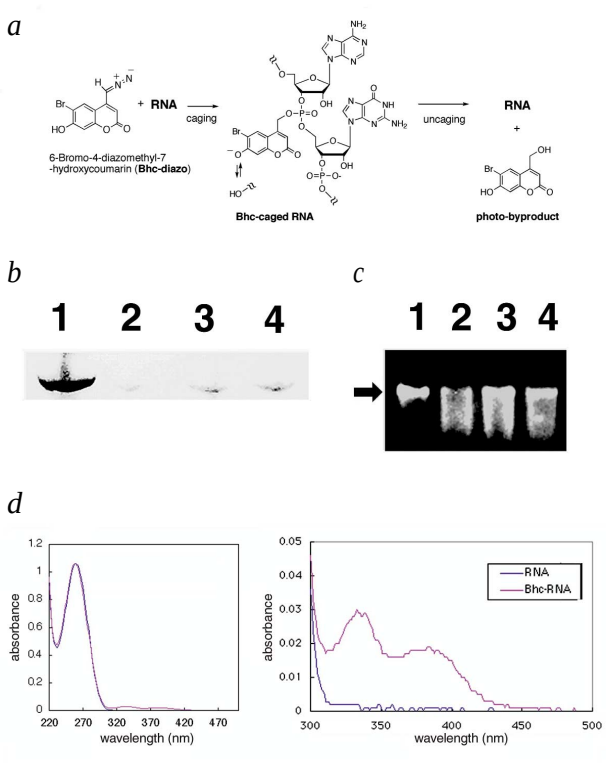

Fig. 1 Inactivation of mRNA by caging with Bhc and reactivation by photo-illumination. ***a***, Reactions for RNA caging by Bhc-diazo and reactivation of the caged RNA by the photolytic removal (uncaging) of the Bhc moiety. Residues in RNA were arbitrarily chosen. Bound Bhc is either protonated or ionized. ***b***, Inactivation of the translational activity of mRNA by caging and its reactivation by uncaging, demonstrated by *in vitro* translation assay. Data compare the translational activities of *in vitro*-synthesized *Gfp* mRNA, intact (lane 1) or Bhc-caged (lane 2), or Bhc-caged and uncaged by exposure to 365 nm light at 10% of the maximum intensity for 10 s (lane 3) or 20 s (lane 4). The densities of individual signals relative to the signal in lane 1 are 4% (lane 2), 18% (lane 3) and 23% (lane 4). ***c***, Shifts in the electrophoretic mobility of mRNA by Bhc caging and uncaging. Corresponding lanes in *b* and *c* represent aliquots of the same mRNA samples. ***d***, Comparison of the absorbance of solutions containing intact (dark blue) and Bhc-caged (red) *GFP* mRNA at the same concentration. Bhc-caged mRNA has additional peaks at 333 nm and 383 nm.

non-ionized form of phosphoric acids might be dominant in DMSO, whereas most of the phosphates are expected to be ionized in aqueous buffer at approximately pH 7.

Bhc-caged mRNA lost almost all translational activity, but illumination with 350–365 nm ultraviolet light removed Bhc from caged mRNA, resulting in a recovery of translational activity. Bhc could be removed from RNA by photo-illumination with a low level of energy (100 mJ/cm^2). This advantage has enabled the precise control of gene expression in live zebrafish embryos by photo-illumination without causing critical damage to the tissue.

Results

Recovery of translational activity of Bhc-caged mRNA after illumination with ultraviolet light *in vitro*

As a first step toward conditional gene expression *in vivo*, we tested the translational efficiency of Bhc-caged mRNA encoding GFP with and without illumination with long-wave ultraviolet light (365±6 nm) using an *in vitro* translation assay. Bhc-caged mRNA has severely reduced translational activity, which is partially recovered by illumination with ultraviolet light (Fig. 1*a*,*b*). Electrophoresis of Bhc-caged mRNA shows a smear of fragments below the main band that coincides in size with that of intact *Gfp* mRNA, and the intensity of the main band is less than that generated by an equal amount of intact mRNA (Fig. 1*c*). In contrast, after illumination with ultraviolet light, the intensity of the main band is restored. This indicates that a substantial portion of the smear below the intact *Gfp* mRNA band is not degradation product, but is full-length mRNA that has undergone various conformational changes because of the covalent attachment of Bhc. We cannot, however, completely rule out the possibility of an increase in RNA cleavage by Bhc-mediated caging, as some smear remains even after uncaging by photo-illumination. The phosphotriester formed by Bhc caging may increase susceptibility to intramolecular attack by the 2′ hydroxyl of ribose, which causes chain cleavage.

Spectrophotometric confirmation of the binding of Bhc-diazo to mRNA

Next, we estimated the caging efficiency of Bhc-diazo by spectrophotometry. Bhc-caged *Gfp* mRNA, which we prepared using standard conditions, produces peaks at 333 nm and 383 nm (Fig. 1*d*) in addition to the main peak at 260 nm. The appearance of the additional peaks was because of the attachment of Bhc, as the Bhc group shows absorbance peaks at 333 nm and 383 nm in protonated and ionized states, respectively. Measurement of the intensity of these additional peaks and the intensity of the main peak for RNA (260 nm) gives an estimated binding of 1 Bhc moiety per 35 bases on average.

Reactivation of caged *Gfp* mRNA by photo-illumination *in vivo*

We then examined whether we could use Bhc-caged mRNA for conditional gene expression *in vivo* (Fig. 2). All embryos (12 of 12) injected with 500 ng/μl intact *Gfp* mRNA showed a homogeneous intense GFP signal at 22 hours (h) after fertilization (Fig. 2*a*,*b*). In contrast, all 12 embryos injected with the same concentration of Bhc-caged *Gfp* mRNA shows severely reduced or no GFP signal (Fig. 2*c*,*d*). However, when the whole bodies of embryos injected with Bhc-caged *Gfp* mRNA are exposed to long-wave ultraviolet light at 2 hours after fertilization (data not shown) or at the shield stage (6 hours after fertilization), the GFP fluorescence signal is substantially recovered by illumination with ultraviolet light (Fig. 2). After exposure to approximately 100 mJ/cm^2 of ultraviolet light by illumination with the 100-W mercury lamp of a microscope at 10% of the maximum intensity for 10 seconds (s), 5 of 12 embryos showed strong GFP fluorescence (Fig. 2 *e*,*f*). After illumination at 10% of the maximum intensity for 20 seconds, 7 of 12 embryos showed strong GFP fluorescence (Fig. 2*g*,*h*), and at 20% of the maximum intensity for 10 seconds, 8 of 12 embryos showed strong GFP fluorescence (Fig. 2*i*,*j*).

We also injected caged β-galactosidase (*lacZ*) mRNA. As with caged *Gfp* mRNA, the activity of β-galactosidase substantially increases after illumination with ultraviolet light (Fig. 2*k*–*m*). We quantified the levels of activation by comparing the enzymatic activities of the extracts from different groups of 50 embryos, each of which had been injected with the same amount of mRNA and then treated in various ways (Fig. 2*n*). Extracts from embryos injected with caged *lacZ* mRNA, but not exposed to ultraviolet light, had only 2.9% the β-galactosidase activity of extracts from embryos injected with intact *lacZ* mRNA. In contrast, extracts from embryos injected with caged *lacZ* mRNA and exposed to ultraviolet light had 13.1% the β-galactosidase activity of extracts from embryos injected with intact *lacZ* mRNA. Therefore, uncaging by illumination with ultraviolet light enhances the β-galactosidase activity 4.5 times. This level of activation roughly matches the degree of translational activation shown above (Fig. 1*b*).

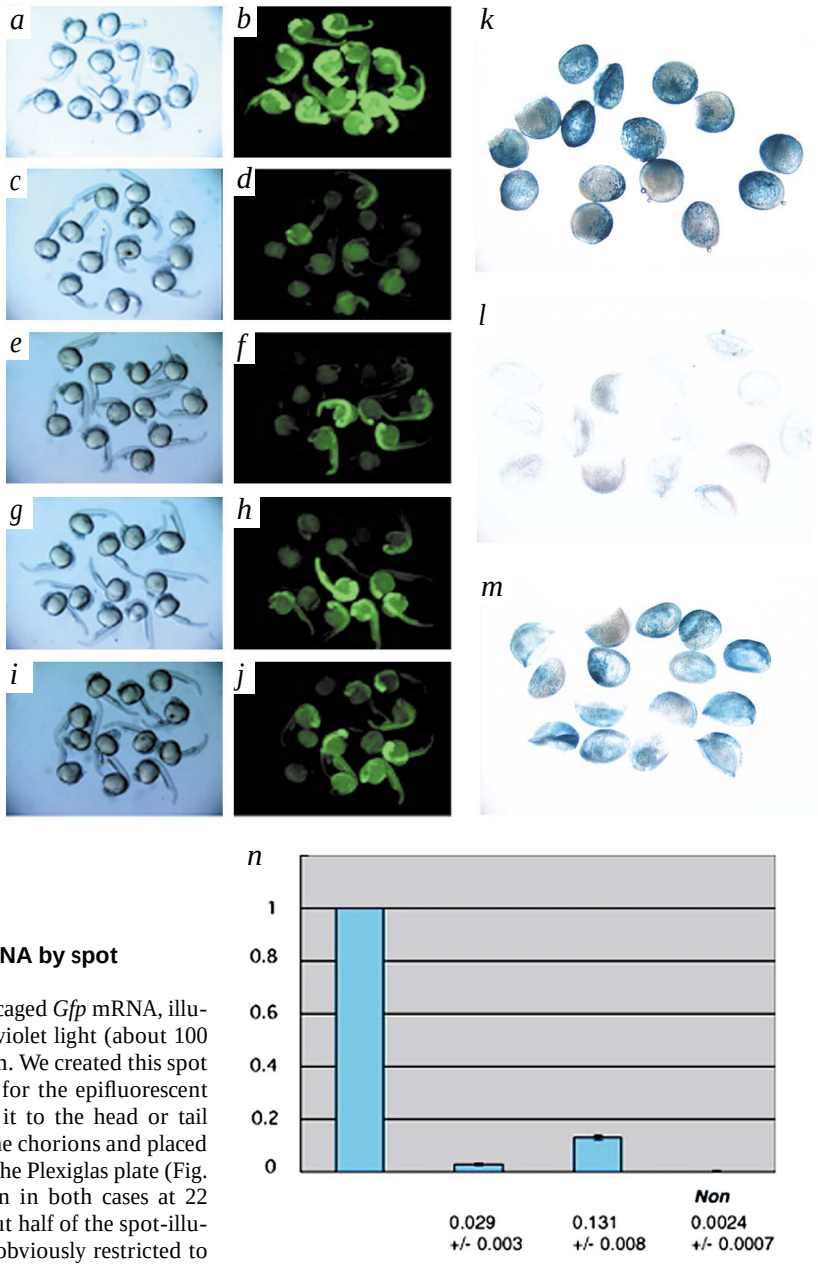

Fig. 2 Recovery of translational activity of Bhc-caged mRNA by illumination of zebrafish embryos with ultraviolet light. ***a–j***, Effects of illumination with ultraviolet light on GFP fluorescence in embryos injected with Bhc-caged *Gfp* mRNA. The entire embryo was exposed to ultraviolet light 6 h after fertilization and observations were made 22 h after fertilization. In all samples, the concentration of the injected mRNA solution was 500 ng/μl. Left, brightfield views; right, fluorescent micrographic views of the GFP signals. Embryos injected with intact *Gfp* mRNA show strong fluorescence (*a*,*b*). Embryos injected with Bhc-caged mRNA but not exposed to ultraviolet show no GFP fluorescence or very weak fluorescence mainly in the yolk (*c*,*d*). In contrast, illumination with ultraviolet light at the shield stage increases the number of embryos showing strong GFP fluorescence in the body. Doses of ultraviolet light: 10% of the maximum intensity for 10 s (*e*,*f*), 10% of the maximum intensity for 20 s (*g*,*h*) and 20% of the maximum intensity for 10 s (*I*,*j*). ***k–m***, Effects of illumination with ultraviolet light on β-galactosidase activity in embryos injected with (500 ng/μl) Bhc-caged or intact *lacZ* mRNA. We histochemically stained embryos for β-galactosidase activity at 6 h after fertilization: intact *lacZ* mRNA (*k*); caged *lacZ* mRNA with no illumination with ultraviolet light (*l*); caged *lacZ* mRNA with illumination with ultraviolet light at 10% of the maximum intensity for 10 s at 2.5 h after fertilization (*m*). The yolks were inadvertently removed. ***n***, Comparison of β-galactosidase activity of the extract from 50 embryos treated as in *k–m*. Fourth sample (Non), non-injected embryos. The scale of activity was normalized so that the activity of extracts from embryos injected with intact *lacZ* mRNA corresponded to 1.0. Each column and the number given below indicate the average and the standard error obtained as a result of three independent experiments.

Localized activation of caged *Gfp* mRNA by spot illumination with ultraviolet light

Next, we used embryos injected with Bhc-caged *Gfp* mRNA, illuminating them with a single spot of ultraviolet light (about 100 μm in diameter) 12 hours after fertilization. We created this spot by closing the luminous field diaphragm for the epifluorescent light as tightly as possible and targeting it to the head or tail region of the embryos, which we kept in the chorions and placed on their backs in the V-shaped grooves of the Plexiglas plate (Fig. 3*a*). We found distinctive GFP expression in both cases at 22 hours after fertilization (Fig. 3*b*,*c*). In about half of the spot-illuminated embryos, GFP fluorescence was obviously restricted to the exposed side of the body. Spot illumination of the head induces GFP fluorescence only in the anterior half of the body (23 of 38 embryos), whereas spot illumination of the tail induces caudally-restricted GFP fluorescence (18 of 39). In many of the remaining embryos, although GFP fluorescence was rather diffusely distributed, the intensity of GFP fluorescence was stronger on the exposed side. Thus, in total, about 70% of the spot-illuminated embryos showed a spatially-restricted pattern of GFP expression on the side of illumination.

To confirm that induction of GFP fluorescence was due to *de novo* synthesis of GFP protein, we stained the embryos that were illuminated on the head with anti-GFP antibody. The same embryo that showed strong GFP fluorescence in the anterior part of the body at 20 hours after fertilization had strong overlapping GFP immunoreactivity (Fig. 3*d*,*e*). These results show that we could control temporal and spatial gene expression by removing Bhc (uncaging) from Bhc-caged mRNA by photo-illumination.

In zebrafish embryos, mRNA injected into the cytoplasm at the one-cell stage tends to be distributed almost ubiquitously in the whole embryonic body (Fig. 3*e*). In spite of the ubiquitous distribution of injected caged *Gfp* mRNA, this embryo (Fig. 3*e*) expressed GFP protein only in the spot-illuminated head region, excluding the possibility that the head-specific expression of GFP protein was due to accidentally biased distribution of injected *Gfp* mRNA into the head region.

Conditional expression of Engrailed2a in zebrafish embryos

We used this mRNA-caging system to examine the effects of ectopic expression of Eng2a[14,15]. Eng2a is expressed mainly in the triangular region around the midbrain-hindbrain boundary (MHB), including the neighboring caudal midbrain and rostral hindbrain, from an early stage of embryonic development (13 hours after fertilization).

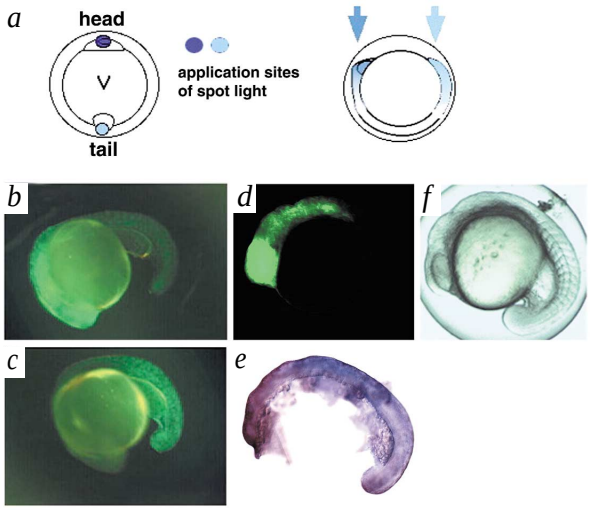

Fig. 3 Ectopic GFP induction by spot illumination with ultraviolet light of embryos injected with Bhc-caged *Gfp* mRNA at 12 h after fertilization. **a**, Application of a spot of ultraviolet light to embryos. Embryos with intact chorions and their backs to the bottom of the grooves curved on the Plexiglas plate received spot illumination with ultraviolet light at 10% of the maximum intensity for 10 s at the target site. Head illumination results in GFP expression in the anterior part of the body; tail illumination induces GFP expression in the posterior half of the body. ***b***,***c***, Embryos 22 h after fertilization subjected to spot illumination with ultraviolet light on the head (*b*) or the tail (*c*). ***d***–***f***, Immunohistochemical staining by anti-GFP monoclonal antibody (4D9) to detect ectopic GFP expression. Head illumination induces fluorescence (*d*) and localized accumulation of GFP (*e*) in an overlapping region in the anterior part of the embryos at 20 h after fertilization. Embryos in *d*–*f* are identical. The embryo in *e* is double- stained for immunohistochemistry (brown) for GFP protein and *in situ* hybridization (purple) for *Gfp* mRNA, showing that *Gfp* mRNA is ubiquitously distributed in the whole embryonic body.

Microinjection of 50 ng/μl intact *eng2a* mRNA into one-cell-stage embryos has a generally mild effect, with a few embryos showing a reduction in eye size (Fig. 4*c*,*d*). In contrast, when embryos injected with 50 ng/μl Bhc-caged *eng2a* mRNA are photo-illuminated at the shield stage, many embryos become completely eyeless (Fig. 4*e*,*f*). None or very few of the embryos injected with Bhc-caged *eng2a* mRNA without photo-illumination show the eyeless phenotype (data not shown; Fig. 4*i*, dark).

The uncaging reaction generates byproducts (Fig. 1*a*), and the triplet-excited state of Bhc might have produced singlet oxygen. However, this could not have caused induction of the eyeless phenotype, as uncaging of other Bhc-caged mRNA, such as caged *Gfp* mRNA, did not cause such an abnormality, as in the activation of caged *eng2a* mRNA.

To more quantitatively compare the effects of injecting intact *eng2a* mRNA versus Bhc-caged *eng2a* mRNA followed by illumination of the head with ultraviolet light at 12 hours after fertilization, we measured eye size at 44 hours after fertilization in these two groups. Whereas the average size of the eye is 215 μm in diameter in non-injected embryos, it is 177 μm in embryos injected with intact *eng2a* mRNA (Fig. 4*i*; nonconditional). In embryos injected with Bhc-caged *eng2a* mRNA without photo-illumination, the average size of the eye is 201 μm (Fig. 4*i*; dark). In contrast, it is only 57 μm in embryos containing the photo-reactivated *eng2a* mRNA (Fig. 4*i*; uncaged at 12 hours after fertilization).

Next, we co-injected Bhc-caged *Gfp* mRNA (500 ng/μl) and Bhc-caged *eng2a* mRNA (50 ng/μl) into one-cell-stage embryos, and spot-illuminated the heads of the embryos with ultraviolet light at 12 hours after fertilization. The affected forebrain region in the eye-

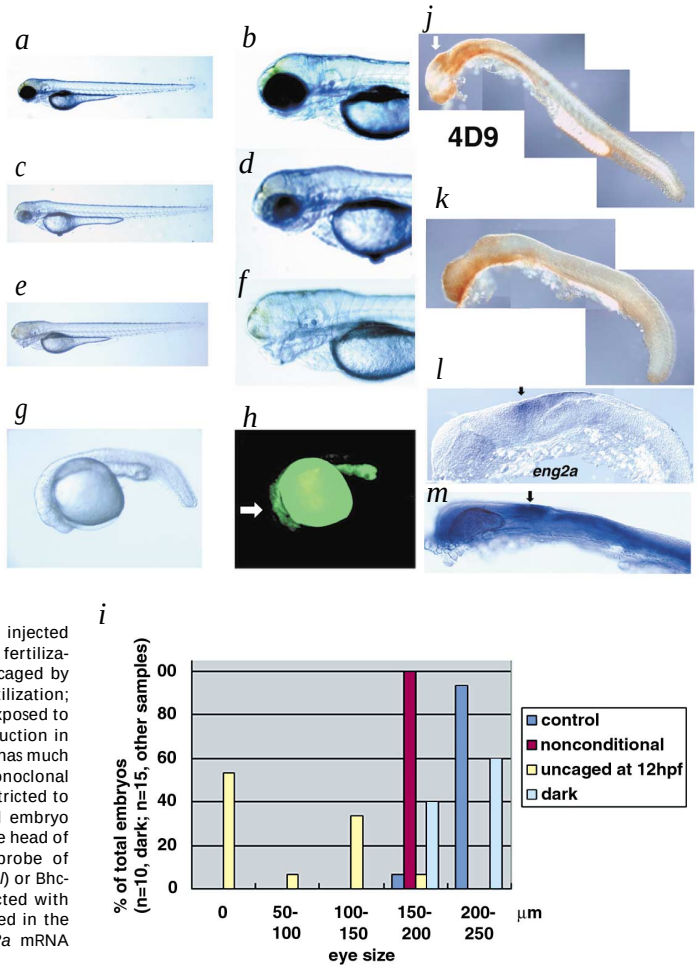

Fig. 4 Effects of Eng2a overexpression by photoactivation of Bhc-caged *eng2a* mRNA. ***a***–***f***, Three-day-old larvae. Right, close-up views of the heads of the embryos shown at left. *a*,*b*, Non-injected control larva. *c*,*d*, Larva injected with intact *eng2a* mRNA. The eyes are slightly reduced in size and shifted ventrally. *e*,*f*, Larva injected with Bhc-caged *eng2a* mRNA and illuminated with ultraviolet light (10% of the maximum intensity for 10 s) at the shield stage. The eyes are significantly reduced in size or totally eliminated. The posterior part of the body looks normal. ***g***,***h***, Embryos co-injected with caged *eng2a* mRNA and caged *Gfp* mRNA 22 h after fertilization and illuminated with ultraviolet light on the head at 12 h after fertilization. *g*, Brightfield view. *h*, Fluorescent micrograph showing GFP fluorescence. The head region (arrow) shows a reduction in the eyes and is positive for GFP fluorescence. ***i***, Quantitative analysis of the effects of uncaging *eng2a* mRNA, measured by changes in eye size at 44 h after fertilization. Control (*n*=15), embryos not injected with mRNA; nonconditional (*n*=15), embryos injected with intact *eng2a* mRNA (40–50 ng/μl); uncaged at 12 hpf (hours past fertilization; *n*=15), embryos injected with Bhc-caged *eng2a* mRNA and uncaged by spot illumination on the head with ultraviolet light at 12 h after fertilization; dark (*n*=10), embryos injected with Bhc-caged *eng2a* mRNA but not exposed to ultraviolet light. Uncaging of caged *eng2a* mRNA causes severe reduction in the eye size, whereas nonconditional overexpression of *eng2a* mRNA has much milder effects. ***j***,***k***, Immunohistochamical staining with anti-En2 monoclonal antibody (4D9) of control (*j*) and uncaged (*k*) embryos. Staining is restricted to the MHB and the mesoderm posterior to the MHB in the control embryo (arrow). In contrast, there is intense staining in the entire region of the head of the uncaged embryo. ***l***,***m***, *In situ* hybridization with an *eng2a* probe of embryos at 22 h after fertilization injected with intact *eng2a* mRNA (*l*) or Bhc-caged *eng2a* mRNA (*m*) and incubated in the dark. In embryos injected with intact *eng2a* mRNA, only expression of endogenous *eng2a* is detected in the MHB (arrow), whereas the embryo injected with Bhc-caged *eng2a* mRNA shows intense *eng2a* signals throughout the whole body.

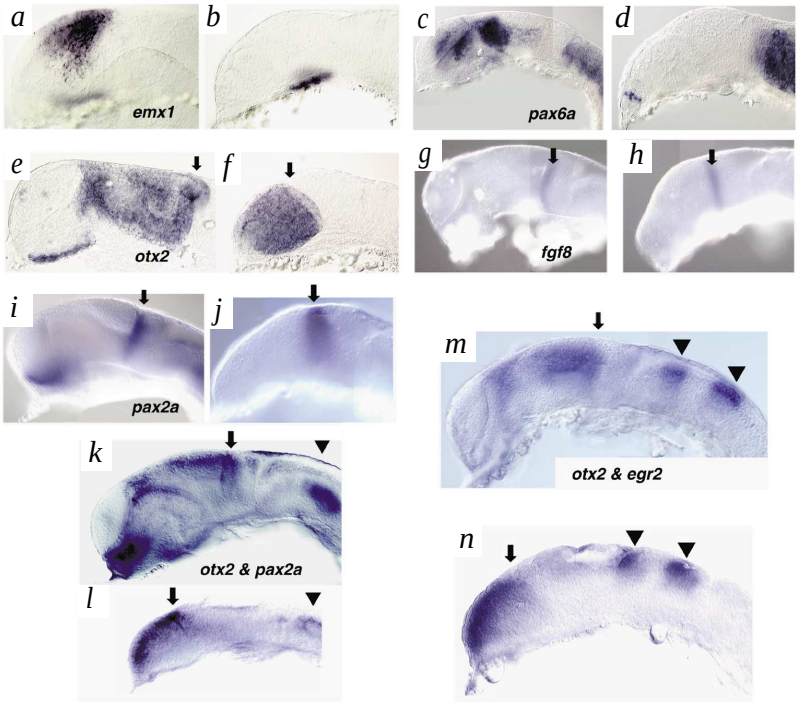

Fig. 5 Changes in the patterns of expression of various region-specific genes at 22 h after fertilization in the embryos injected with Bhc-caged *eng2a* mRNA and spot-illuminated on the head with ultraviolet light at 12 h after fertilization. ***a–j***, Expression patterns of the dorsal telencephalic marker *emx1* (*a* and *b*), the diencephalic marker *pax6a* (*c* and *d*), the midbrain marker *otx2* (*e* and *f*) and the MHB markers *fgf8* (*g* and *h*) and *pax2a* (*i* and *j*) in control normal embryos (*a*,*c*,*e*,*g*,*i*) and in eyeless embryos injected with Bhc-caged *eng2a* mRNA and spot-illuminated with ultraviolet light (*b*,*d*,*f*,*h*,*j*). The *otx2*-positive region is shifted considerably anteriorly (arrow) in the uncaged embryo (*f*). Both *fgf8* and *pax2a* signals (arrows) are detected as sharp stripes in the MHB in the uncaged eyeless embryos (*h*,*j*) as in the normal embryos (*g*,*i*), but are shifted anteriorly. ***k***,***l***, Double labeling by *in situ* hybridization for *otx2* and *pax2a* in control (*k*) and uncaged eyeless embryos (*l*). As in *e*,*f*,*l*,*j*, both markers are shifted anteriorly together in the uncaged eyeless embryo (*l*, arrow). The distance between the *pax2a* signal in the MHB (arrows) and the otic vesicle (arrowheads) is increased in the uncaged eyeless embryo compared with that in the control embryo. ***m***,***n***, Double labeling by *in situ* hybridization for *otx2* and the hindbrain marker *egr2* in control (*m*) and uncaged eyeless (*n*) embryos. The expression pattern of *egr2* (arrowheads) is normal in both control (*m*) and uncaged eyeless (*n*) embryos. The distance between the caudal edge of the *otx2*-positive region (arrows) and the *egr2*-positive regions (arrowheads) is increased in the uncaged eyeless embryo (*m*) compared with the control embryo (*n*).

less phenotype shows GFP fluorescence (Fig. 4*g*,*h*). This result indicates that caged *eng2a* and *Gfp* mRNA are equally distributed and activated simultaneously by spot photo-illumination.

Immunohistochemical staining with an anti-Engrailed monoclonal antibody (4D9; ref. 16) shows that Eng2a is restricted to the region around the MHB in the control embryo (Fig. 4*j*), whereas in the affected eyeless embryo in which *eng2a* mRNA is reactivated by photo-illumination to the head region, we found ectopic overexpression of Eng2a almost ubiquitously in the head region (Fig. 4*k*).

Increase in mRNA stability *in vivo* by Bhc caging

The recovery of efficient translational activity by photo-illumination at 12 hours after fertilization indicates that caged mRNA persists intact to this stage. We were able to reactivate Bhc-caged *eng2a* mRNA by spot illumination with ultraviolet light as late as 17 hours after fertilization, and still found the characteristic phenotypic effects (data not shown). This indicates that caging with Bhc increases the stability of mRNA *in vivo*. In general, intact mRNA injected into one-cell-stage embryos loses almost all activity by 20 hours after fertilization; embryos injected with intact *eng2a* mRNA have only mild retardation of eye development.

To confirm that Bhc caging stabilizes mRNA, we examined the retention of injected mRNA by *in situ* hybridization with an *eng2a* probe in embryos injected with intact or Bhc-caged *eng2a* mRNA 22 hours after fertilization (Fig. 4*l*,*m*). In embryos injected with intact *eng2a* mRNA, *eng2a* mRNA is expressed only in the MHB, where endogenous *eng2a* mRNA is normally expressed (Fig. 4*l*, arrow). In contrast, in embryos injected with Bhc-caged *eng2a* mRNA and incubated in the dark, we detected intense signals throughout the whole body (Fig. 4*m*). This result strongly indicates that Bhc-caged mRNA is much more stable and persists longer than the intact mRNA when injected into one-cell-stage embryos. It also confirms our observation that caged mRNA injected into the one-cell-stage embryos is distributed almost ubiquitously.

Dynamic changes in gene expression patterns in the forebrain caused by ectopic overexpression of *eng2a*

To investigate the mechanism of induction of the eyeless phenotype by conditional overexpression of *eng2a*, we examined the patterns of expression of two marker genes by *in situ* hybridization: paired box gene 6a (*pax6a*)[17,18], a marker for diencephalic development (Fig. 5*c*,*d*), and early growth response 2 (*egr2*, also called *krox20*)[19], a hindbrain marker (Fig. 5*m*,*n*). We spot-illuminated the heads of embryos injected with a 50-ng/μl solution of Bhc-caged *eng2a* mRNA 12 hours after fertilization, and analyzed the affected eyeless embryos by whole-mount *in situ* hybridization. The *pax6a*-positive region in the forebrain shows a considerable reduction in size and is shifted anteriorly. In contrast, the effect of activation of *eng2a* mRNA on the hindbrain is inconspicuous, as *egr2* expression in the hindbrain was not affected in these embryos, although spot illumination of the head with ultraviolet light is likely to have activated caged *eng2a* mRNA in the hindbrain, given the pattern of activation of caged *Gfp* mRNA by the same technique (Figs. 3*b*,*d* and 4*h*). We further examined the expression patterns of four other marker genes: *emx1 (*ref. 20; a dorsal telencephalic marker), *otx2 (*ref. 21; a dorsal midbrain marker whose caudal limit of expression shows a sharp edge near the caudal end of the midbrain), and *pax2a* (refs. 17,18) and *fgf8 (*ref. 22), markers of the MHB (Fig. 5*a–j*). In most of the severely affected eyeless embryos, expression of *emx1* in the telencephalon is completely lost (Fig. 5*a*,*b*). The *otx2*-positive region is shifted rostrally; the anterior limit of this region reaches the most anterior tip of the brain (Fig. 5*e*,*f*). Both *fgf8* and *pax2a* are expressed in the sharp narrow stripes in the MHB, as in normal embryos, but the expression had apparently shifted rostrally (Fig. 5*g–j*). Double *in situ* hybridization for *otx2* and *pax2a* showed that the rostrally shifted *pax2a*-positive signal in the eyeless embryos is still located adjacent to the caudal edge of the *otx2*-positive region, as in the normal embryo (Fig. 5*k*,*l*). However, the considerable increase in the

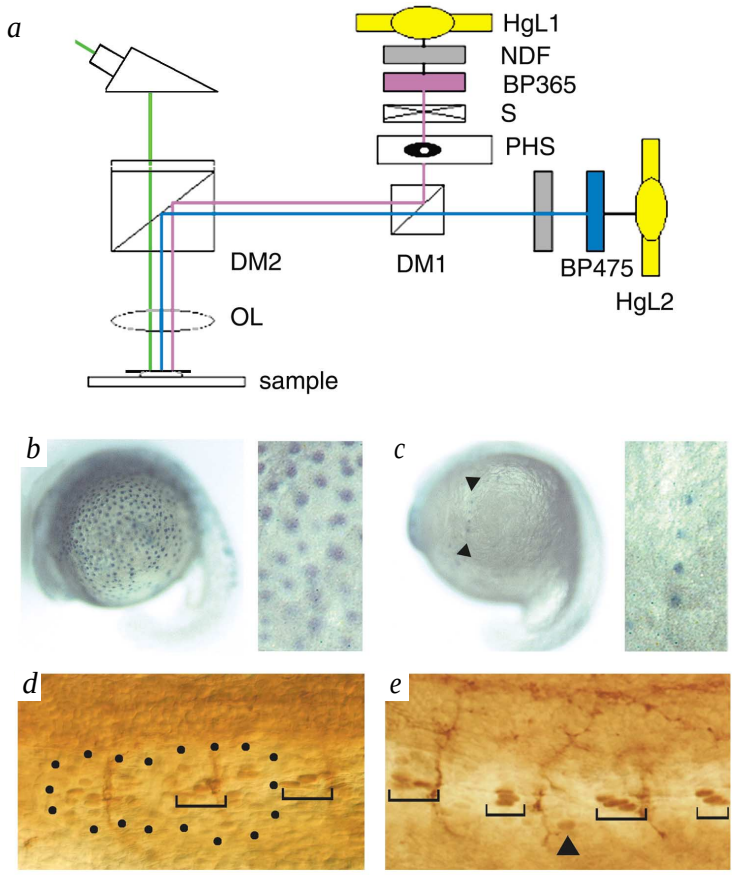

Fig. 6 Localized activation of caged *eng2a* mRNA using a sliding screen with a hole on the confocal plane. ***a***, Optics used for localized activation of caged mRNA. HgL1, mercury lamp for uncaging; HgI2, mercury lamp for observation of GFP signal; NDF, neutral density filter; BP365, band-pass filter (365 nm); BP475, band-pass filter (475 nm); S, electronic shutter; PHS, pinhole sliding screen; DM, dichroic mirror; OL, objective lens. ***b***, Lateral view of embryo 19 h after fertilization injected with intact mRNA for β-galactosidase with the nuclear localizing signal. Right, close-up view of the yolk sac. ***c***, Lateral view of embryo 19 h after fertilization injected with intact mRNA for β-galactosidase with the nuclear localizing signal that received illumination with ultraviolet light on the anterior side of the yolk (arrowhead) at 12 h after fertilization using a confocal sliding screen with a slit. Right, close-up view of the area of the yolk sac where induction of β-galactosidase activity was induced. ***d***,***e***, Side views of embryos at 24 h after fertilization injected with caged *eng2a* mRNA at the one-cell stage that received illumination with ultraviolet light on the somite at 14 h after fertilization with a confocal sliding screen with large (*d*) or small (*e*) pinholes. The nuclei of Eng2a-positive cells in the myotome are stained by 4D9 antibody. Brackets, expression of Eng2a in the muscle 'pioneers'. The area of ectopic induction of Eng2a in nuclei is surrounded by broken line in *d*. A single nucleus ectopically expressing En2 is indicated by an arrowhead in *e*. Axons were stained by znp-1 antibody.

distances between the *pax2a*-positive stripe and the otic vesicle (Fig. 5*k*,*l*) or between the caudal edge of the *otx2*-positive region and the *erg2*-positive stripes (Fig. 5*m*,*n*) indicates expansion of the region including the MHB.

Localized activation of caged mRNA by targeted illumination

To achieve more spatially-restricted activation of caged mRNA, we used another microscope equipped with a special illumination system, in which a sliding screen with a hole of an arbitrary figure could be inserted on the confocal plane of the pathway of the ultraviolet light (Fig. 6*a*). This system enables projection of the same figure on a reduced scale onto the specimen. We adjusted the illumination time to 0.5 s using an electronic shutter.

By using a screen with a slit (50 μm in width and 3 mm in length) on the confocal plane, we illuminated the yolk sacs of the embryos injected with caged mRNA encoding β-galactosidase with the nuclear localizing signal at 12 hours after fertilization. After incubation, we histochemically stained for β-galactosidase activity. We found induction of ectopic expression of β-galactosidase in the nuclei of a group of yolk epithelial cells that are aligned on the illuminated line (Fig. 6*c*).

We also did targeted illumination of the somites of embryos injected with caged *eng2a* mRNA at 14 hours after fertilization using pinholes of different diameters. In the body trunks of normal embryos, Eng2a is expressed only in the muscle pioneers at the dorso–ventral border of the myotomes. Illumination with a larger pinhole (1 mm in diameter) induced ectopic expression of Eng2a in several nuclei of the trunk muscles in the illuminated area (Fig. 6*d*). Ectopic activation of Eng2a in a single cell is also possible using a smaller pinhole (40 μm in diameter; Fig. 6*e*).

DNA caging with Bhc-diazo

Caging of DNA with DMNPE has been reported[8]. To determine whether Bhc-diazo could be used for caging DNA, we added Bhc-diazo to plasmid DNA expressing *Gfp* under control of the muscle-specific enhancer of *acta1*, encoding α-actin[23]. We injected the caged plasmid DNA into one-cell-stage embryos, and incubated them in the dark. At the beginning of gastrulation (6 hours after fertilization), we illuminated the entire region of the animal pole with ultraviolet light for 0.5s using the second illumination system, without inserting a sliding screen, and incubated the treated embryos in the dark again until 24 hours after fertilization. Unlike mRNA, plasmid DNA injected into the one-cell-stage embryos is distributed unevenly in a mosaic manner. However, almost all treated embryos show strong expression of GFP specifically in a random subset of the muscles, whereas we found no or rare GFP expression in control embryos injected with caged DNA without exposure to ultraviolet light (Fig. 7*a*,*b*). These results indicate that transcription of caged DNA is inhibited *in vivo* and can be activated by uncaging.

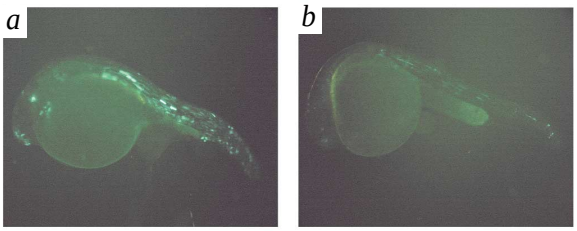

Fig. 7 Photo-activation of Bhc-caged DNA *in vivo*. Embryos 24 h after fertilization injected with caged α-actin–GFP plasmid DNA at the one-cell stage that received illumination with ultraviolet light over the entire region of the animal pole at the beginning of gastrulation (***a***) or did not receive illumination with ultraviolet light (***b***).

Discussion

Injection of a high concentration (250 to ~500 ng/μl) of intact mRNA encoding the *Olyzias latipes* (Japanese medaka fish) ortholog (*Ol-eng2*) of *eng2a* into embryos of the medaka fish also impairs eye development to various degrees and induces formation of an ectopic tectum with an opposite rostrocaudal polarity to that of the original tectum in place of the diencephalon, while keeping the telencephalon intact[24]. However, by the 20-somite stage, when morphological distinction of the tectum and MHB starts to become apparent in medaka embryos, the injected *Ol-eng2* mRNA had been completely degraded, indicating that an early and transient ectopic expression of *Ol-eng2* causes the observed defects by triggering the innate reciprocal inductive signaling cascade[25–27], which promotes development of the tectum- and MHB-like tissues in the diencephalic region. Negative effects of *eng2* expression on diencephalic development were also found in experiments using chick embryos[28]. In contrast, we found that injection of a much lower concentration of caged *eng2a* mRNA (50 ng/μl), followed by reactivation in embryonic development, causes much more robust and consistent defects, such as deletion of the entire forebrain and an anterior shift of the midbrain, rather than a mere duplication of the tectum. Our results show that *eng2a* protects the cells from the fate of the telencephalon or the diencephalon. Injection of intact *eng2a* mRNA at the same low concentration into one-cell-stage embryos induces very little abnormality. A substantial enhancement of the effects of caged *eng2a* mRNA is likely to be due to an increase in its stability, as *in situ* hybridization showes that Bhc-caged *eng2a* mRNA injected into one-cell-stage embryos persists intact until 22 hours after fertilization, but intact *eng2a* mRNA does not. Conformational changes are indicated after binding of Bhc, as the electrophoretic mobility of mRNA is shifted by caging with Bhc and recovered by uncaging. This may have rendered the caged mRNA resistant to enzymatic degradation. The mRNA-stabilizing effect of Bhc may increase considerably the window of opportunity for the induction of genes that function relatively late in development.

The period required to effectively cage mRNA with Bhc-diazo in DMSO varies depending on the nature of mRNA. For example, a reaction time of 1–2 hours is required to achieve nearly complete inactivation of *Gfp* mRNA while retaining its potential for reactivation by photo-illumination. In contrast, 15 minutes is sufficient to inactivate *eng2a* mRNA. One reason for such variation may be that the exceptional stability of GFP causes this protein to persist and accumulate during development and makes it difficult to completely prevent accumulation of GFP protein translated from even a very small amount of mRNA that is not inactivated by the caging reaction.

Here we have presented the first example, to our knowledge, of conditional expression of genes in the zebrafish embryo purely by chemical modification of RNA. This method is simple, rapid and economical compared with other conventional methods involving various genetic manipulations. For example, targeted gene expression using the GAL4-UAS system was accomplished in zebrafish by generating and crossing two stable transgenic lines, one carrying a construct with the given promoter to drive GAL4 expression, and the other carrying the effector gene downstream of UAS[29]. As the DNA-binding activity of GAL4 can be reversibly downregulated by modification of its lysine residues with the amine-reactive caging agent 6-nitro-veratrylchloroformate[30], it may also be possible to establish another system for photo-activating gene expression in zebrafish embryos by overexpressing caged GAL4 in transgenic embryos that carry the effector gene downstream of UAS. Recently, another system was reported that takes advantage of the ability of a caged estradiol to recover the capacity to bind to the estrogen receptor after photo-illumination[31]. However, our method is much simpler in that it does not require generation of any transgenic lines. Our method is also suitable for the simultaneous induction of several genes so as to analyze the functions of genes that may act synergistically.

Caging exogenous mRNA with Bhc-diazo has made it possible to direct the expression of a gene with both spatial and temporal specificity by simple exposure to ultraviolet light. Compared with nitrobenzyl- and DMNPE-caged molecules, Bhc-caged molecules are several-fold more sensitive to illumination with ultraviolet light and ~30-fold more sensitive to photolysis by pairs of coincident infrared photons[32]. Therefore two-photon infrared laser photolysis should permit even better three-dimensional spatial localization, greater penetration into scattering or absorbing tissues and a reduced possibility of photodamage to neighboring cells.

Methods

Nomenclature. We followed the guidelines for nomenclature of zebrafish genes approved by the zebrafish nomenclature committee (http://zfin.org/zf_info/nomen.html#Gene and http://zfin.org/ZFIN/).

Bhc-diazo. We synthesized Bhc-diazo in five steps as described in Web Note A. The construction of 4-diazomethylcoumarin relied on a published procedure with slight modifications[33]. Requests for Bhc-diazo samples should be made to T.F. (furuta@biomol.sci.toho-u.ac.jp). Requests for other technical details of the application of Bhc-diazo for RNA/DNA caging should be made to H.O. (hitoshi@brain.riken.go.jp) and H.A. (andoh@brain.riken.go.jp).

RNA caging. We synthesized mRNA to be caged *in vitro* from full-length cDNA in a pCS2 vector, which encoded GFP, Eng2a, β-galactosidase and β-galactosidase with a nuclear localizing signal (NLS-lacZ), using mMESSAGE mMACHINE (Ambion), then dissolved the mRNA in RNase-free water at a concentration of 1 μg/μl and stored it at –80 °C. For caging mRNA with Bhc-diazo, we precipitated and resuspended a 5-μl aliquot of mRNA in 5 μl DMSO and mixed it with 5 μl Bhc-diazo solution (60 μg/μl for *lacZ* mRNA and *NLZ-lacZ* mRNA, and 120 μg/μl for *Gfp* mRNA). We incubated the reaction mixture for 15–120 min at room temperature depending on the nature of mRNA. After the reaction, we removed free Bhc-diazo from the mixture by column chromatography using Sephadex G50 that had been swollen and equilibrated in DMSO. We precipitated caged mRNA in the initial 500-μl fraction by adding an equal volume of isopropanol and 50μl of 10 M ammonium acetate, rinsed it with 70% ethanol and dried and dissolved it in RNase-free water at a concentration appropriate for microinjection into one-cell-stage embryos. To estimate the concentration, we analyzed 1 μl of the injection solution by gel electrophoresis just before injection. Because of the photosensitivity of Bhc, we treated caged mRNA using a fluorescent lamp laminated with ultraviolet-cut film (YT-57; Mitsubishi Jushi).

DNA caging. We mixed 5 μl plasmid solution (1 μg/μl in DMSO) containing a circular form of the plasmid carrying the α-actin regulatory region and the full-length cDNA sequence of enhanced GFP[23] with 5 μl Bhc-diazo solution (80 μg/μl in DMSO) and incubated it for 40–60 min at room temperature. After column chromatography and precipitation by the same procedure as described for RNA caging, we dissolved the pellet in nuclease-free water to a concentration of 30–50 ng/μl immediately before injection.

Quantification of mRNA activation. We measured the translational activity of mRNA as follows: We spread three 1-μl aliquots of solution containing Bhc-caged *Gfp* mRNA (1 μg/μl) caged for 3 h at RT and another 1-μl aliquot of intact *Gfp* mRNA individually on the bottoms of the wells of a 60-well microtiter plate (Nunclon Delta Surface; NUNC™, Nalge Nunc International). We illuminated two of the Bhc-caged *Gfp* mRNA solutions for either 10 or 20 s using a 10x objective lens (Plan Neofluar; Carl Zeiss) with ultraviolet light at 10% of the maximum intensity that could be achieved by the 100-W mercury lamp of a microscope (Axioscop II; Carl Zeiss). Then we mixed them with the *in vitro* translation solution (TNT Quick Coupled Transcription/Translation System; Promega) to synthesize ^{35}S-labeled GFP. We separated the reaction mixtures containing the products by SDS-PAGE

and analyzed the results by autoradiography and densitometry using the image analyzer (BAS 5000; Fuji Film). We analyzed β-galactosidase activity by measuring the optical density of the chromogenic reaction mixture at 420 nm using o-nitrophenyl-β-D-galactoside as substrate[34].

Spectrophotometric analysis of caged mRNA. We diluted a total of approximately 20 μg Bhc-caged *Gfp* mRNA (caged for 1 h at room temperature) and of intact *Gfp* mRNA in 500 μl of 10 mM Tris–HCl, pH 7.4, and measured absorbances using a spectrophotometer (U-3310; Hitachi). We normalized the absorbance curve for intact *Gfp* mRNA to fit the curve for the caged *Gfp* mRNA, and measured the difference between these two curves at 333 nm and 383 nm. We calculated the average number of nucleotides intercalating the phosphate moieties forming a covalent bond with Bhc as approximately 35 by dividing (absorbance of the caged mRNA at 260 nm, 1.06)/(average molar extinction coefficient of nucleotides, 8,125/cm per M) by [(increase in absorbance at 333 nm, 0.03)/(the molar extinction coefficient of protonated Bhc, 11,100/cm per M)+(increase in absorbance at 383 nm, 0.019)/(the molar extinction coefficient of ionaized Bhc, 18,300/cm per M)]. We calculated the average molar extinction coefficient of nucleotides as follows: 1/cm / (40 x10^{-3} [g/l] / 325 [g/mol, the average molecular weight of nucleotides])=8,125[/cm per M].

Microinjection of mRNA/DNA. We microinjected Bhc-caged mRNA into the cytoplasm of one-cell-stage embryos as described before[23,35], except we eliminated the ultraviolet light from illumination for the dissecting microscope and we injected the mRNA solution using an electronically regulated air-pressure microinjector (IM300; Narishige) to keep the volume of the injected solution constant. We incubated injected embryos at 28.5 °C in the dark.

Illumination of embryos with ultraviolet light. We maintained embryos that had been injected with Bhc-caged mRNA within the chorion, and transferred and positioned them with their backs to the bottom in the V-shaped narrow grooves (1 mm in width and 0.5 mm in depth) curved on the Plexiglas plate. We arranged each embryo in the same orientation. The orientation of the embryos was maintained by the surface tension of the water. We created the spot of ultraviolet light for illuminating the target region by closing the opening of the luminous field diaphragm of the epifluorescent microscope (Axioscop II; Carl Zeiss) to the smallest size and bringing it to the center of the view field. Using conventional brightfield optics with a 20x objective lens (Plan Neofluar; Carl Zeiss), we brought the target tissue into the center of the view field. For illumination using the spot of ultraviolet light, we used the epifluorencent optics with a band-pass filter (365±6 nm; Filter Set 01; Carl Zeiss) inserted into the path of the excitation light. We adjusted the duration of illumination by manually opening and closing the path of the excitation light. To prevent the embryos from drying, we illuminated at most 20 embryos in one cycle of arrangement and illumination of the embryos. We were able to illuminate more than 100 embryos within 30 min. After illumination, we incubated the embryos at 28.5 °C in the dark, until we had made our observations. We measured the intensity of the ultraviolet light with a ultraviolet meter (Model UVA-365; Custom). We placed the ultraviolet-light-detecting probe under the 20x objective lens on its focal plane after removing the condenser unit of the microscope. The intensity of ultraviolet light was approximately 10 mW/cm^2 at 10% of the maximum intensity of the 100-W mercury lamp. For more local activation of caged mRNA, we used a microscope (BX50; Olympus) equipped with an optical system for photolysis of caged compounds as described in the text.

In situ hybridization and histochemistry. We used published procedures for *in situ* hybridization[36], double staining by immunohistochemistry followed by *in situ* hybridization[37] and histochemical staining for β-galactosidase activity[38]. We obtained the anti-GFP antibody used for staining embryos from Santa Cruz Biotechnology.

Imaging. We took pictures with either a microscope with a differential interference contrast optics (Axioplan II; Zeiss) connected to a high-resolution digital camera (DP50; Olympus) or an epifluorescent dissecting microscope (MZFLIII; Leica) connected to a high-sensitivity camera (C5810; Hamamatsu Photonics).

Note: supplementary information is available on the Nature Genetics web site (http://genetics.nature.com/supplementary_info/).

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