Conditional Expression of a Myocardium-Specific Transgene in Zebrafish Transgenic Lines

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To develop the first heart-specific tetracycline (Tet)-On system in zebrafish, we constructed plasmids in which the cardiac myosin light chain 2 promoter of zebrafish was used to drive the reverse Tet-controlled transactivator (rtTA) and the green fluorescent protein (GFP) reporter gene was preceded by an rtTA-responsive element. In the zebrafish fibroblast cell-line, rtTA-M2, one of rtTA's derivatives, demonstrated the highest increase in luciferase activity upon doxycycline (Dox) induction. We then generated two germ lines of transgenic zebrafish: line T03 was derived from microinjection of a plasmid containing rtTA-M2 and a plasmid containing a responsive reporter gene, whereas line T21 was derived from microinjection of a single dual plasmid. Results showed that line T21 was superior to line T03 in terms of greater GFP intensity after induction and with of minimal leakiness before induction. The photographic images of induced GFP in the heart of F2 larvae showed that the fluorescent level of GFP was dose-responsive. The level of GFP expressed in the F3 3 days postfertilization larvae that were treated with Dox for 1 hr decreased gradually after the withdrawal of the inducer; and the fluorescent signal disappeared after 5 days. The GFP induction and reduction were also tightly controlled by Dox in the F3 adult fish from line T21. This Tet-On system developed in zebrafish shows much promise for the study of the gene function in a specific tissue at the later developmental stage. *Developmental Dynamics* 233:1294–1303, 2005. © 2005 Wiley-Liss, Inc.

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INTRODUCTION

Zebrafish (*Danio rerio*) is an excellent animal model in the study of cardiac research (Poss et al., 2002; Raya et al., 2003; Shu et al., 2003). Zebrafish has a two-chambered heart that is similar in many ways to the three- or four-chambered hearts of higher vertebrates (Fishman and Stainier, 1994; Fishman and Chien, 1997). Transpar-

ent embryos make it possible to observe the dynamic expression of the heart gene in a noninvasive manner. Embryos that receive oxygen through diffusion will result in embryos with cardiac defects, and these embryos can survive for 1 week (Sehnert et al., 2002). Furthermore, many heart mutant forms are available. However, some heart diseases occur at later de-

velopmental stages or even in adulthood. The conventional approaches, i.e., by injection of mRNA for the study of overexpression or by the injection of morpholino for knockdown study, are carried out in one-celled fertilized eggs. This system makes it difficult to generate a heart disease model in some cases. Therefore, we developed a tetracycline (Tet) -depen-

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Fig. 1. Schematic illustration of the plasmid constructs used in this study. One-cell stage fertilized zebrafish eggs were microinjected with the cardiac-specific rtTA plasmid pICML1-rT16-1b, in which rtTA-M2 is driven by a zebrafish *cmlc2* promoter (MLC2) and flanked by AAV-ITR (black vertical bars), and a tetracycline (Tet)-responsive plasmid, in which reporter gene(s) is driven by a Tet-controlled bidirectional promoter with or without flanking AAV-ITR. Silencer, Tet-controlled transcriptional silencer; d2EGFP; unstable enhanced green fluorescent protein (EGFP). The pICMLM2_IBIE and pICMLM2_BILE are single dual plasmids derived from coinjection of pICML1-rT16-1b and pIBIE, or pICML1-rT16-1b and pBILE, respectively.

dent transcriptional activation system in zebrafish, in which the transgene can be induced to express specifically in the heart at any stage of development.

By taking advantage of the regulatory loop of *tet* resistance in prokaryotes, tight control of gene expression can be accomplished by titrating the dose of Tet (Gossen and Bujard, 1992) or its more stable derivative doxycycline (Dox; Gossen et al., 1995). By fusing the Tet repressor (TetR) and activating the transcription domain of the virion protein 16 of the herpes simplex virus, Tet-controlled transactivator (tTA) is generated (Gossen and Bujard, 1992). In the absence of Tet, tTA can stimulate P_{tet-1} , a minimal promoter fused to the *tet* operator (*tetO*), and, thus, can activate downstream genes. This approach is referred to as the Tet-Off system, because the binding affinity between TetR and *tetO* is allosterically affected by Tet. A Tet-On system is developed by mutagenesis, substituting four amino acids in tTA to generate reverse tTA (rtTA; Gossen et al., 1995), which requires Tet for binding to *tetO* and the subsequent activation of the downstream gene. For pharmacokinetic reasons, controlling genes by means of rtTA is of particular advantage

in the study of transgenic animals. Whenever a gene has to be kept silent, the Tet-On system is regarded as more useful than the Tet-Off alternative in developmental biology studies and gene therapy (Jost et al., 1997).

Both tTA and rtTA systems have been used in studies of gene function in various cellular systems (Salucci et al., 2002; Zhu et al., 2002), as well as in whole organisms (Meissner et al., 2001), such as *Toxoplasma gondii* (Nakayama et al., 2000), fungus (Weinmann et al., 1994), plants (Furth et al., 1994), and mice (Passman and Fishman, 1994; Kistner et al., 1996; Yu et al., 1996; Jost et al., 1997). However, these inducible expression systems have not been established in zebrafish.

In the previous study, we demonstrated that a 1.6-kb regulatory region of zebrafish cardiac myosin light chain 2 gene (*cmlc2*) in plasmid pICMLE-(−870/787) specifically drives green fluorescent protein (GFP) expression in the heart of transgenic zebrafish (Huang et al., 2003). In this report, we used this segment of *cmlc2* to direct rtTA in the Tet-On system and also generated germ-line transgenic zebrafish carrying this heart-specific Tet-On

system. The dose-response dependency was analyzed, and the kinetics of Dox-mediated activation of GFP expression was measured. This article is the first report of a zebrafish Tet-On system. We propose that it will be useful for the study of gene function in heart development and for animal modeling of heart disorders.

RESULTS

Efficiency of Various Transactivators in the Cell Line

To evaluate the function of various transactivators in a zebrafish system, the fibroblast cell line was transfected with the DNA constructs expressing various transactivators together with the Tet-regulated luciferase plasmid (Fig. 1). Because the codon usage bias had been proposed as being an obstacle in establishing a Tet-inducible system in *Toxoplasma* (Nakayama et al., 2000), we also compared the transactivation efficiency between tTA, rtTA, rtTA-M2, and rtTA-S2 of prokaryotic origin and the humanized codons rtTA2^S-S2 and rtTA2^S-M2. The results are shown in Table 1. All transactivators displayed a regulated capacity of activating luciferase expression. Among them, rtTA-M2 and the humanized rtTA2^S-S2 mutant had the best performance in terms of increase of luciferase activity after induction. Although rtTA2^S-S2 displayed the least leakiness before induction, the reporter activity was one order of magnitude less than that of the rtTA-M2.

Transient Expression of Induced GFP in Embryos Microinjected With Two Separate Plasmids

To determine the conditional expression of GFP in vivo, we microinjected the zebrafish embryos with the upstream plasmid pICML1-rT16-1b, in which rtTA-M2 was driven by a heart-specific promoter, and the downstream reporter plasmid pIBIE, pBILE, pBILd2, or pITSBIE. Both the background and the induced heart-specific GFP expression were monitored. As shown in Table 2, when

TABLE 1. Doxycycline-Dependent Activation of Luciferase by tTA, rtTA and Its Derivatives in Zebrafish Fibroblast Cells^a

Transactivators	Luciferase activity		Activation folds
	–Dox	+Dox	
tTA	10916.4 ± 1001.2	1571.9 ± 170.3	6.94
rtTA	2630.5 ± 211.8	7979.9 ± 955	3.03
rtTA-S2	2454.8 ± 236.3	17368.1 ± 757.7	7.08
rtTA-M2	2617.4 ± 206.3	22440.7 ± 393.9	8.57
rtTA2 ^s -S2	219.2 ± 33.8	2010.0 ± 101.3	9.17
rtTA2 ^s -M2	904.1 ± 126.2	2787.4 ± 285.7	3.08

^aZebrafish fibroblast cell line ZEM2S was transfected with plasmids expressing their respective transactivators. The upstream plasmids were controlled by a CMV promoter, and the downstream plasmids contained a luciferase reporter driven by various Tet-responsive elements. After transfection, cells were divided in two groups and were grown in the absence or presence of 1 µg/ml of Dox for 40 hr before dual luciferase assays were performed. The values of luciferase activity were arbitrary light units, and fold activation was determined by dividing the values of the induction by the noninduced conditions.

Dox, doxycycline.

TABLE 2. Leakiness, Intensity and Heart-Specificity of GFP in the Transgenic Embryos Before and After Induction*

	GFP expression			
	–Dox (non-specific expression)		+Dox (heart-specific expression)	
	%(Number) ^a	Intensity ^b	%(Number) ^c	Intensity ^b
pICML1-rT16-1b+pIBIE	38(90/235)	***	13(24/186)	*****
pICML1-rT16-1b+pBILE	12(35/287)	**	11(26/247)	****
pICML1-rT16-1b+pBILd2	0(0/244)	–	3(6/173)	*
pICML1-rT16-1b+pITSBIE	24(19/79)	**	10(3/33)	***
pICMLM2_IBIE [#]	35(67/184)	***	23(38/165)	***
pICMLM2_BILE [#]	20(112/554)	*	16(67/415)	****

*Before induction, the transferred 72-hours postfertilization (hpf) embryos were examined for leakiness of green fluorescence. The nonleaky 72-hpf embryos were collected and green fluorescence protein (GFP) expression was induced by adding Dox at a concentration of 10 µg/ml for 72 hr. The level and specific expression of GFP were then observed at 144 hpf.

^aThe number of surviving transgenic embryos expressing GFP either in the heart or in any part of body before induction.

^bThe level of GFP intensity was based on optical observations: *****, the highest expression; *, the lowest expression; –, not detectable.

^cThe number of nonleaky transgenic embryos having heart-specific expression of GFP after induction.

GFP, green fluorescent protein.

pICML1-rT16-1b was co-microinjected with pIBIE, containing EGFP and flanked by AAV-ITR, the embryos showed that the leak rate (38%) and leakiness level (***) reached their highest values. When nonleaky embryos were selected and treated with Dox, we found that the intensity of the heart-specific GFP expression increased dramatically from no expression up to the highest expression level (*****). The heart-specific expression rate after induction for the 186 embryos examined was 13% of 186 nonleaky transgenic embryos.

In the experiment of using pBILE, in which AAV-ITR was absent, the

leak rate was as low as 12% of 287 embryos, which was only one third of the rate for the embryos injected with pIBIE. The Dox-induced heart-specific expression rate of pBILE-injected embryos was 11%, which was quite close to that of the pIBIE-injected embryos. In addition, the level of GFP intensity also increased greatly from no expression up to **** after induction.

When pBILd2, in which enhanced GFP (EGFP) in pBILE was replaced by d2EGFP, was used as the downstream plasmid, there was no leaky expression in the transgenic embryos. However, the rate of heart-

specific induced GFP was as low as 3% for 173 injected embryos, and the intensity of the induced fluorescent signal was relatively faint after induction.

When pICML1-rT16-1b was co-microinjected with plasmid pITSBIE, in which a transcriptional silencer was included, it was found that the leaky expression was not effectively suppressed. In addition, we noticed that only as few as 10% of embryos exhibited heart-specific induced fluorescence and a moderate increase in GFP intensity after induction. In conclusion, co-microinjection of plasmids pICML1-rT16-1b and pIBIE or

Fig. 2. The doxycycline (Dox)-induced green fluorescent protein (GFP) expression in the zebrafish F2 larvae derived from transgenic lines. **A,B:** The photographic images of GFP intensity were taken at 12-hr intervals after the 3-days postfertilization (dpf) larvae from lines T03 (A) and T21 (B) were exposed to 10 μ g/ml Dox. **C:** Dose-response and kinetics of GFP induction occurred in line T03 were analyzed. The mean values of GFP intensity after induction with 10,000, 1,000, 100, and 10 ng/ml Dox were analyzed at 12-hr intervals until the end of 96 hr. A detailed analysis of the GFP intensity is described in the Experimental Procedures section. R^2 represents either logarithm or linear regression coefficient. Scale bars = 0.1 mm in A,B

Fig. 3. Detection of the transcripts of rTA and green fluorescent protein (GFP) genes in the F3 embryos by using semiquantitative reverse transcription-polymerase chain reaction. **A:** Detection the existence of rTA-M2 transcripts every 2 hr starting from 10 hr postfertilization (hpf) embryos before induction. The rTA-M2 transcripts were detectable from 12-hpf, and the signals reached a normal level at 18-hpf. Detection of β -actin transcripts served as an internal control. **B:** Detection of the existence of GFP transcripts before and after the 3-dpf embryos were treated with doxycycline (Dox). The rTA-M2 transcripts were detectable, whereas the GFP transcripts were detectable 2 hr later after Dox induction.

pBILE was the most effective construct in this study in terms of lower leakiness before induction and higher fluorescent intensity after induction.

Transient Expression of Induced GFP in Embryos Microinjected With a Single Dual Plasmid

The highest competence of transactivation was obtained from embryos microinjected with pICML1-rT16-1b and a combination of pIBIE or pBILE (Table 2). Thus, we constructed the plasmids pICMLM2_IBIE and pICMLM2_BILE, which were the cointegrated forms of the upstream plasmid pICML1-rT16-1b and the downstream plasmid pIBIE or pBILE, respectively.

When comparing the transactivation potency of embryos microinjected with the separated forms pICML1-rT16-1b and pIBIE with that of the embryos injected with the cointegrated form pICMLM2_IBIE, it was found that the leak rate and intensity were similar (Table 2). After Dox induction, the intensity of the heart-specific GFP expression in the embryos injected with the cointegrated form of plasmid was much less than that in those injected with the separated forms, despite that the induction rate was as high as 23% of 165 embryos (Table 2). Meanwhile, the leak rate of pICMLM2_BILE-injected embryos was higher than that of the embryos injected with pICML1-rT16-1b and pBILE, but with low leak intensity. After induction, both the heart-specific rate and the intensity of transactivated GFP in pICMLM2_BILE-injected embryos were the same as those in embryos microinjected with the separated forms of plasmid (Table 2). Thus, we can conclude that the intensity of heart-specific GFP expression in the embryos microinjected with a single dual plasmid was not enhanced compared with the embryos microinjected with two plasmids, although the rate of heart-specific expression was slightly increased after Dox induction.

Fig. 4.

Fig. 5.

Induced GFP Expression in the F2 Larvae Is Dependent on Dose and Duration of Dox Exposure

We generated two transgenic lines: T03 and T21. Line T03 was derived from embryos microinjected with plasmid pICML1-rt16-1b and plasmid pI-BIE, whereas line T21 was derived from microinjection of the single dual pICMLM2_IBIE. The GFP expression was induced specifically in the heart of approximately 50% of siblings when the F2 3-days postfertilization (dpf) larvae derived from both line T03 and line T21 were treated with Dox in a concentration of 10 μ g/ml for 12 hr (Fig. 2A,B). Generally, the induced GFP expression was only slightly different among individuals of transgenic F2 siblings. There was no silencing of the transgene.

The dose-response and kinetics of Dox-mediated gene expression were measured by monitoring the time course of the GFP intensity. As shown in Figure 2C, Dox was ineffective for

induction at a concentration of less than 10 ng/ml and caused a weak GFP response at a concentration of 100 ng/ml. However, the GFP intensity increased steadily when 1 and then 10 μ g/ml Dox were used for induction. Of those two concentrations, 10 μ g/ml was the most effective dose for induction. In addition, the intensity of induced GFP expression became stronger with the increased induction time of the 10 μ g/ml Dox treatment, from 12 to 96 hr starting in 3-dpf larvae (Fig. 2A–C).

Transcriptions for GFP and rtTA-M2 Were Detected by Reverse Transcription-Polymerase Chain Reaction

The mRNA transcripts of rtTA-M2 driven by a *cmlc2* promoter were detectable by using semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) at the 12 hr postfertilization (hpf) onward in F3 embryos derived from both line T03 and line T21 before induction (Fig. 3A). The RT-PCR signal reached a normal transcriptional level at 18 hpf (Fig. 3A). Despite the presence of rtTA-M2 mRNA, the GFP transcripts were not detectable in the 3-dpf embryos (Fig. 3B). However, when these embryos were treated with Dox, a faint signal of GFP mRNA was detectable at 2 hr after induction (Fig. 3B). This signal became clearly visible at 6 hr after induction and reached a high expression level at 12 hr (Fig. 3B).

Dox Exposure Time Required to Initiate GFP Expression

F3 embryos were monitored using a fluorescence microscope at 2-hr intervals from 10 hpf until induced GFP expression was observed. When 24-hpf F3 embryos were exposed to and kept in the presence of Dox, the earliest Dox-dependent GFP expression was observed at 31 hpf. Interestingly, when embryos younger than 24 hpf, such as 10, 12, 14, 16, 18, 20, and 22 hpf, were treated with Dox, GFP expression was not observable until at 31 hpf. However, the time required for GFP to appear decreased if embryos were dechorionated and then were treated with Dox. The GFP could be

seen as early as 25-hpf when 18-hpf dechorionated embryos were treated with Dox. Meanwhile, when 72-hpf embryos were induced by Dox, the GFP was visible at 79 hpf. Therefore, we concluded that the duration of exposure required for detecting the reporter GFP fluorescence was 7 hr after induction. Unexpectedly, when a wild-type female was immersed in Dox for 2 days before mating with an F2 homozygotic male, GFP expression was visible at 25 hpf in these F3 embryos.

Dox Withdrawal Switches Off Induced GFP Expression in F3 Larvae and Adults

Whereas a high level of GFP expression in the absence of Dox was apparent in some 5-month-old adults derived from transgenic line T03, no such GFP background signal was found in any of the noninduced 13-dpf larvae or 5-month-old adult fish derived from the transgenic line T21. Therefore, we chose F3 larvae and adults derived from line T21 for performing the Dox withdrawal experiment.

To turn off the induced GFP expression in Dox-induced larvae, we stopped adding Dox to the culture water. If we treated the F3 3-dpf larvae derived of germ line T21 with Dox for only 1 hr, then within 5 days after Dox was removed from the water, the GFP signal was shut off (Fig. 4). However, the longer the Dox exposure, the more time (days) was needed for GFP expression to fade. For example, the heart still maintained induced GFP expression even 25 days after removal of Dox if the F3 3-dpf larvae had been treated with Dox for 96 hr, although the expression level of GFP was reduced slightly (Fig. 4). We also observed that the GFP signal was not reduced immediately when the Dox was removed. Instead, the intensity of the GFP fluorescence remained at a high level or even increased for 2 or 3 days after removal. We conclude that the time required to switch off the reporter gene in the induced larvae totally depended upon the length of exposure to Dox (Fig. 4).

After F3, 2-month-old adult fish derived from the transgenic line T21 were treated with Dox, the hearts were isolated, and photographs of GFP expression were taken every 24 hr. Results showed that the GFP re-

Fig. 4. Green fluorescent protein (GFP) expression in heterozygotic F3 larvae derived from transgenic line T21 after withdrawing the inducer doxycycline (Dox). The intensity of GFP signal was expressed differently in 3-days postfertilization larvae exposed to 10 μ g/ml of Dox for 1, 7, 24, or 96 hr, as indicated. The number of days after Dox withdrawal is shown in the left corner on each panel. The GFP intensity increased continually for 2 or 3 days after Dox was withdrawn, then the level of the signal started to decrease. The greater the Dox exposure time, the higher the GFP expression level, and consequently, the more time needed to drop to the background level. Scale bar = 0.1 mm.

Fig. 5. The photographic images of green fluorescent protein (GFP) appeared in the F3 adult fish after doxycycline (Dox) was added and then was withdrawn. After the 2-month-old adult fish derived from F3 of the transgenic line T21 were treated with Dox (+Dox) for the time indicated, the hearts were isolated and photographs of GFP expression were taken every 24 hr. After adult fish were treated with Dox for 96 hr, the fish were maintained in the absence of Dox and photographs of GFP expression were taken at the number of days indicated. Unexpectedly, the GFP expression increased for 10 days after Dox was withdrawn, and then the signal slowly decreased to the background level. The last panel shows that the 5-month-old adult fish (5-month) derived from the transgenic line T21 (T21) exhibited a relatively low background signal before induction. Scale bar = 0.5 mm, except 0.1 mm for the last panel.

porter gene was induced and expressed specifically in heart after exposure to Dox for 24 hr (Fig. 5). The intensity of the GFP signal increased substantially after induction for 48 hr (Fig. 5). On the other hand, when we withdrew Dox from the solution after the adult zebrafish had been treated with Dox for 96 hr, the GFP expression increased steadily for another 10 days after Dox withdrawal, and the GFP signal stayed at a relatively high level even 40 days after withdrawing Dox (Fig. 5). It took more than 150 days for GFP expression levels to return to background levels in the adults (Fig. 5). In conclusion, Dox treatment followed by Dox withdrawal demonstrated that GFP expression was tightly controlled by Dox also in adult fish: length of time of induction and the amount of time after Dox was withdrawal to reverse repression were much longer in adults.

DISCUSSION

In this study, we compared the luciferase activity of various transactivators, such as tTA, rtTA, rtTA-S2, rtTA-M2, rtTA2^S-S2, and rtTA2^S-M2, in zebrafish fibroblast cell lines (Table 1). Of the transactivators used in this study, tTA acts as a Tet-Off system, and the rest act as Tet-On systems. Unlike a previous study that demonstrated that the Tet-Off system is more effective than the Tet-On system in regulating transgene expression (Mizuguchi and Hayakawa, 2002), we found that rtTA-S2, rtTA-M2, and rtTA2^S-S2 all have higher-fold induction than tTA. A further look at our results shows that rtTA-M2 and the humanized rtTA2^S-S2 mutants have the best performance in terms of increase in luciferase activity after induction, but that rtTA-M2 can reach dramatically higher levels than rtTA2^S-S2. These data suggest that prokaryotic codons of rtTA are favorable to human codons in zebrafish. This result is consistent with the Tet-On system as reported in *Drosophila* (Stebbins et al., 2001) and with the unicellular parasite *T. gondii* (Nakayama et al., 2000). However, this is not the case for HeLa cell line (Urlinger et al., 2000). The factors involved in this inconsistency remain to be determined in future studies. Be-

cause rtTA-M2 displays the best inducible transactivator in the zebrafish cell line, we used this construct as an upstream plasmid to drive various rtTA-dependent downstream plasmids in a transgenic study.

To enhance the expression of an induced gene in the heart, we constructed plasmids in which the structural gene is flanked on both sides by AAV-ITR (Fu et al., 1998; Fig. 1). However, there is little difference after induction in terms of heart-specific rate and intensity of GFP expression in embryos microinjected either with or without the presence of AAV-ITR in the downstream reporter plasmids pIBIE and pBILE. This evidence suggests that AAV-ITR is not the most effective factor for enhancing the inducible expression in this system.

We attempted to reduce the leakiness as much as possible, while keeping the induced GFP intensity as high as possible. The transcription silencer tTS can be used to reduce the background level of the luciferase and for improving the regulation in the Tet-On system (Mizuguchi and Hayakawa, 2001). We constructed a downstream reporter plasmid pITSBIE, in which a transcriptional silencer is included (Fig. 1). Unfortunately, results showed that both the leak intensity and the induction intensity were reduced. Compared with embryos injected with pBILE, the induced GFP intensity was relatively faint in the embryos injected with pBILd2 (Table 2A). This finding may be explained by the fact that EGFP is more stable than d2EGFP, because the half-life of GFP is approximately 26 hr (Corish and Tyler-Smith, 1999) and that of d2EGFP is approximately 2 hr (Li et al., 1998). As a reporter gene, enhanced GFP (EGFP) is easier to monitor than d2EGFP.

In the transient expression assay, we found that the heart-specific GFP expression rate of the embryos injected with the cointegrated form pICMLM2_IBIE was higher than that of the embryos co-microinjected with the two separate plasmids pICML1-rT16-1b and pIBIE (Table 2). Based on the genetic inheritance analysis of the F2 offspring derived from line T03, we conclude that there is only a single insertion site for pICML1-rT16-1b and pIBIE in the host chromosome.

Concatamerization of transferred DNA fragments has been reported in transgenic fish (Yang et al., 1999; Hsiao et al., 2001). We speculate that concatamerization may occur between these two plasmids before integration into the chromosome of the host cells. Unlike the cointegrated form of plasmid pICMLM2_IBIE, the ratio of rtTA-M2 gene to IBIE gene in the concatamer of pICML1-rT16-1b and pIBIE is unclear.

Because it is rather difficult with the naked eye to discern intensity when the signal strength of the GFP is high, we used a digital camera with KODAK 1D Image Analysis Software (software for analyzing the results of gel electrophoresis) to quantify the GFP signal shown on the photographic images. For accurate quantification of fluorescent signals in living cells, we considered both the fluorescence and the structural properties of the GFP, following the basic photophysical characteristics for determining brightness (Patterson et al., 1997). As all the photographs were taken under the same conditions, the structural properties exerted by the GFP, being uniform for each sample, thus could be disregarded. The images, therefore, can be compared directly to assess the GFP content. The higher the image intensity we observed, the higher the GFP content we obtained. Using this software, we took the average intensity of the pixels of the defined area (mean intensity) as an index to quantify the amount of fluorescent signals occurring in the heart after induction, as shown in Figure 2. The quantification is useful for making a comparison of the GFP expression in similar developmental stages.

As shown in the RT-PCR experiment (Fig. 3A), the transcript of rtTA-M2 is detectable at 12 hpf, which is 4 hr earlier than previously reported by whole-mount in situ hybridization (Huang et al., 2003). The time course of Dox induction was investigated by examining the images of the GFP. Of interest, we found that, no matter what stage before 18 hpf of the dechorionized embryos being induced, the transactivated GFP cannot be imaged until 25 hpf. On the other hand, when Dox was added to any stage after 18 hpf, GFP became visible after

induction for 7 hr. This result is consistent with embryos originating from parental females that are treated with Dox for 3 days. On the basis of the semiquantitative RT-PCR results shown in Figure 3A, the rtTA-M2 mRNA level increases dramatically at 18 hpf. Therefore, it is reasonable to conclude that the molecules of rtTA-M2 are not transcribed sufficiently to transactivate the downstream GFP gene until 18 hpf. We also note that the GFP is not visible until 31 hpf if the induction is carried out on embryo whose chorion has not been removed. We speculate that the chorion may be a barrier for transferring Dox, resulting in a delay of induction. In fact, the GFP mRNA is detectable by RT-PCR after being induced for 2 hr (Fig. 3B), although the GFP cannot yet be observed until 7 hr after induction. We conclude that the time required to transactivate the GFP reporter in this system is 7 hr after induction but that the strength of the promoter and the existence of the chorion affect the efficiency of the zebrafish Tet-On system.

In a study of the tTA system in the heart of transgenic mice, the reporter luciferase gene was nonspecifically apparent in the kidney, lung, and tongue (Yu et al., 1996). However, in the system we developed, the GFP reporter gene is transactivated specifically in the heart of the heterozygous F3 individuals derived of line T21 without showing any leaky expression of GFP. Urlinger et al. (2000) reported that rtTA has a distinct residual affinity toward *tetO* and produces background activity of P_{tet} -1 when rtTA is present in relatively high intracellular concentrations. The transgenic lines in this study, T03 and T21, present different levels of background GFP expression, indicating that the microinjection of a cointegrated form of plasmid is superior to the microinjection of dual plasmids in terms of minimal leakiness of the GFP.

When the Tet-On system is carried out in cell lines, the inducer is immersed in the medium and enters directly into the cells. The activation of a transgene can reach a maximum level after induction for 24 hr (Gossen et al., 1995). However, when the Tet-On system is carried out on transgenic fish, the fluorescent signal in the

derived larvae cannot reach the maximal level, even after induction for 96 hr (Fig. 2C). In adult fish, this time course is extended further, not only in the length of time of Dox induction but also in that of Dox withdrawal. The longer time that is needed indicates that the inducer needs to be transported to the heart of the fish through the circulatory system, rather than through simple diffusion.

It takes more than 25 days (Fig. 4) or 100 days (Fig. 5) to abolish the GFP expressed in induced larvae and adult fish, respectively, after Dox withdrawal. One of the possible reasons is that the concentration of Dox located at the inter- and intracellular level remains at a rather high level, even after the Dox is withdrawn. The GFP expression in embryos generated by crossing the Dox-incubated wild-type female with the transgenic T03 male provides evidence to support the idea that the GFP signal appears in these embryos at 25 hpf without any Dox treatment. The image is similar to that of the Dox-treated and dechorionated embryos. Nevertheless, this feature could be beneficial in some cases, such as in an animal model of disease in which a transgenic product that lasts longer after induction is desirable.

Currently, zebrafish has become an important vertebrate model organism, because this species can be used to study human diseases (Hsieh and Liao, 2002; Shin and Fishman, 2002), physiology (Farber et al., 2001), and pharmacology (Langherinrich, 2003). We successfully developed a Tet-On system in zebrafish that enables the transgene to be expressed specifically in the heart after induction. This system may be applied in the investigation of the mechanism of the function and development of the heart, in the model of cardiovascular disease, and in the screening of drugs for the treatment of heart disease.

EXPERIMENTAL PROCEDURES

Cell Line and Luciferase Assay

The zebrafish fibroblast cell-line ZEM2S (ATCC, CRL-2147) was cultured with L-15 complete medium: 50

% L-15 medium (ATCC 30-2008) supplemented with 35 % DMEM (high glucose, GIBCO 12100), 15 % F12 medium (GIBCO 21700), 0.18 g/L sodium bicarbonate, 15 mM HEPES, and 10 % heat-inactivated fetal bovine serum. The explants were incubated at 28°C, without extracting CO₂. The cells were cotransfected with the plasmid containing a transactivator, such as tTA, rtTA, rtTA-S2, rtTA-M2, rtTA2^S-S2, or rtTA2^S-M2 (Urlinger et al., 2000), controlled by the CMV promoter and the plasmid pUHC13-3 (Gossen and Bujard, 1992), containing a luciferase reporter gene driven by the Tet-responsive element (TRE). Cells were grown in the absence or the presence of 1 µg/ml Dox for 40 hr and then harvested for dual luciferase assays (Promega), which were carried out according to the recommendations of the manufacturer. Luciferase activity was measured from the cell extracts and normalized to *Renila* luciferase activities expressed from pRL-TK included in the transfection mixture.

Plasmid Constructs

The six constructs used in this study are illustrated schematically in Figure 1. The cardiac-specific rtTA construct pICML1-rtT16-1b was generated by modifying the previously described pICMLE-(−870/787) plasmid (Huang et al., 2003), in which the upstream region (−870/−1), exon 1, intron 1, and a part of exon 2 of zebrafish *cmlc2* were included. The EGFP segment of pICMLE-(−870/787) was replaced by rtTA-M2. The resulting pICML1-rtT16-1b consisted of the regulatory region of *cmlc2*, rtTA-M2, and AAV-ITR. The rtTA-dependent bidirectional *tetO* (Clontech) was cloned into pIEGFP (Chou et al., 2001) to generate pIBIE, resulting in TRE being located upstream of the EGFP gene. Plasmid pITSBIE followed a similar construction to that of pIBIE, except that a Tet-controlled transcriptional silencer (Clontech) was included. Plasmids pBILE and pBILd2 were generated by cloning EGFP and d2EGFP cDNA into pBIL (Clontech), respectively. Luciferase and EGFP were engineered downstream on each side of bidirectional *tetO* in the absence of AAV-ITR.

Another strategy of plasmid construction used in this study was the cointegrated form of a plasmid containing the cardiac-specific rtTA and a plasmid containing the rtTA-dependent GFP reporter gene. Plasmid pICML1-rtT16-1b was fused with plasmid pIBIE or pBILE to generate a single dual plasmid of pICMLM2_IBIE or pICMLM2_BILE, respectively.

Experimental Fish and GFP Observations

Zebrafish of the AB strain were cultured and maintained according to the procedures described by Westerfield (1995). The developmental stages were determined according to criteria described by Kimmel et al. (1995). One-cell stage fertilized eggs were collected and microinjected with linearized constructs (Wang et al., 2002). A total of 15 µg/ml of each plasmid was microinjected. Injected embryos were incubated at 28.5°C. Transgenic embryos were observed under a stereodissecting microscope (Leica MZFIII) equipped with a fluorescent module and an enhanced EGFP filter cube (Kramer Scientific). We categorized the level of GFP intensity by using stars: the highest expression was designated *****, whereas the lowest was designated *.

Transgenic Germ Lines, Induction, and GFP Detection

The embryos microinjected with pICML1-rtT16-1b and pIBIE were cultured under a 14-hr light and 10-hr dark photoperiod at 28.5°C. Pairs of mature fish were kept together in tanks, and their eggs were collected for study. Transgenic embryos were monitored for GFP expression after induction with 10 µg/ml of Dox (Sigma) for 3 days, after which the GFP-positive progeny were bred.

The F2 embryos derived from the transgenic germ lines were kept in water until 72 hpf, then they were treated with 10 µg/ml, 1 µg/ml, 100 ng/ml, or 10 ng/ml of Dox. Individual larvae, six larvae from each concentration, were kept in individual wells of 24-well culture plate. After induction, GFP expression was observed under the fluorescence microscope.

Fish were orientated ventrally and tilted at 45 degrees when taking photos using a Coolpix 990 digital camera (Nikon) with 10-sec exposure time at ISO 400. The photographic image of the GFP intensity was analyzed by using KODAK 1D Image Analysis Software. An area for analysis was defined by using the Free Form Tool in the ROI Toolbar. The mean intensity of a selected area from the ROI information window was recorded by quantifying the strength of the GFP fluorescent signal. Fluorescence intensity was processed and quantified using by Microsoft Excel. For every larva, one set of photos was taken at 12-hr intervals, from before Dox exposure until 96-hr after Dox treatment. If the fish died before the 96-hr period was up, the photos were not used in the analysis.

Strategies for Dox Induction

Three methods were used to induce GFP expression after the 10 µg/ml Dox treatment. (1) Immersion of a female: a wild-type female was immersed in Dox for 2 days and crossed with an F2 homozygotic male. (2) Immersion of embryos: F3 embryos were incubated with Dox at different developmental stages at 2-hr intervals from 10 hpf to 24 hpf. (3) Immersion of dechorionated embryos: same procedure as for method 2 except that the chorion of the embryo was removed. The GFP expression in the embryos was monitored by using a fluorescence microscope at 2-hr intervals until GFP was observed.

RT-PCR

The RNA of the embryos at different stages was isolated using Trizol (Pro-Tech) and treated with RNase-free DNase (Promega). RT was carried out using Moloney-murine leukemia virus (M-MLV; Promega) reverse transcriptase with 2 µg of total RNA in a volume of 25 µl. PCR was then performed using 1 U of VioTag (Viogene) and 1 µl of the RT product per 50-µl reaction for 35 cycles at an annealing temperature of 62°C for EGFP detection, and at 54°C for rtTA M2 detection. Primers used for detecting EGFP gene were EGFP-404F, ACGACGGCAAC-TACAAGACCCGC, and EGFP-814R,

ACTTGTACAGCTCGTCCATGCCGA, whereas primers for detecting rtTA-M2 gene were rT16-1F4, AT-TACGGGTCTACCATCGAGGGC, and rT16-1R5, CCCTACCCACCGTACT-CGTACAAT. Primers used for detecting the β-actin gene, which acted as an internal control, were BA-F, CG-GATTCGCTGGAGATGATGC, and BA-R, CGGAGGATGGCATGGGGCA. The PCR reaction was carried out at an annealing temperature of 66°C for 25 cycles. The sizes of the expected PCR products were 410, 340, and 473 bp for genes of EGFP, rtTA-M2, and β-actin, respectively.

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