Novel Double Promoter Approach for Identification of Transgenic Animals: A Tool for In Vivo Analysis of Gene Function and Development of Gene-Based Therapies

LIEZHEN FU, DANIEL BUCHHOLZ, AND YUN-BO SHI*
Unit on Molecular Morphogenesis, Laboratory of Gene Regulation and Development, NICHD, NIH, Bethesda, Maryland

ABSTRACT Advances in vertebrate genetics have allowed studies of gene function in developing animals through gene knockout and transgenic analyses. These advances have encouraged the development of gene-based therapies through introduction of exogenous genes to enhance and/or replace dysfunctional or missing genes. However, in vertebrates, such analyses often involve tedious screening for transgenic animals, such as PCR-based genotype determinations. Here, we report the use of double-promoter plasmids carrying the transgene of interest and the crystallin-promotor-driven Green fluorescent protein (GFP) in transgenic *Xenopus laevis* tadpoles. This strategy allows a simple examination for the presence of GFP in the eyes to identify transgenic animals. PCR-based genotyping and functional characterization confirms that all animals expressing GFP in the eyes indeed carry the desired promoter/transgene units. Thus, the use of this and other similar vectors should dramatically improve current transgenesis protocols and reduce the time and cost for identifying transgenic animals. *Mol. Reprod. Dev. 62: 470–476, 2002.*
© 2002 Wiley-Liss, Inc.

Key Words: transgenesis; green fluorescent protein; *Xenopus laevis*; matrix metalloproteinase

INTRODUCTION

With the rapid progress in sequencing of animal and plant genomes and fast expansion in the data banks for expressed sequence tags, the vast majority of the genes encoded by the animal and plant genomes will soon be identified. More and more attention is being shifted toward functional analyses of the thus- identified genes. Studies on gene function and the developmental mechanisms in live animals or plants represent the ultimate step of such endeavors. They have become much more approachable with the advances in genetics in both plants and animals.

Genetic studies of gene function can be roughly classified into two types: gene inactivation and mis-expression. With each type, a major, time-consuming step involves identifying animals or plants that carry the intended changes in the genome. Often identification of transgenic animals or plants requires tedious analysis of the genomic DNA (Henneberger et al., 2000). Green fluorescent protein (GFP) or enhanced GFP (EFGP) has been used as a marker to show that, in principle, identification of transgenic embryos under UV light could work with varying degrees of identification efficiency (Ikawa et al., 1995; Takada et al., 1997; Kato et al., 1999). Limited studies have shown that the use of marker transgenes is potentially useful in transgenesis by somatic cell nuclear transfer (Uhm et al., 2000). However, these approaches did not allow the introduction of a gene of interest, in addition to the GFP marker, to the transgenic animals. Here, we report a simple and reliable method to identify transgenic animals carrying both a gene of interest and the GFP marker. Our approach is to combine in a single piece of DNA, both the desired transgene, under the control of any promoter of interest, and the GFP, driven by the eye-specific, cyrstallin promoter. This strategy allows a simple examination of the eyes under UV light to identify transgenic animals.

To test the validity of the approach, we apply it towards studying gene function during *Xenopus laevis* metamorphosis. Amphibian metamorphosis involves transformation of every organ/tissue of the tadpole and is controlled by thyroid hormone (TH) (Dodd and Dodd, 1976; Shi, 1999). TH is presumed to regulate the expression of genes in target tissues through its nuclear receptors, i.e., TH receptors (TRs). Earlier studies from different laboratories have led to the identification of many TH response genes (Shi, 1999). Of particular interest for TH-dependent tissue remodeling is the gene encoding matrix metalloproteinase (MMP) stromelysin-3 (ST3), which has been implicated in the modification of the extracellular matrix to affect cell fate determination and cell migration in response to TH

Liezhen Fu and Daniel Buchholz contributed equally to this work.

*Correspondence to: Yun-Bo Shi, Laboratory of Gene Regulation and Development, NICHD, NIH, Building 18T, Rm 106, Bethesda, MD, 20892. E-mail:shi@helix.nih.gov

Received 20 December 2001; Accepted 5 February 2002
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/mrd.10137

© 2002 WILEY-LISS, INC.

(Damjanovski et al., 1999; Ishizuya-Oka et al., 2000). To facilitate our functional studies of ST3 and TRs during metamorphosis, we use the double-promoter construct above to generate transgenic tadpoles by using the sperm-mediated gene transfer method of Kroll and Amaya (Kroll and Amaya, 1996). We show here the 100% correlation of GFP expression driven by the crystallin promoter as seen under UV light with the presence of the desired transgene, TR or ST3, by PCR analysis and functional characterization.

MATERIALS AND METHODS

Plasmid Construction

The GFP gene fragment was excised from pS65T-C1 (Clontech, Palo Alto, CA) by double digestion with AflII and EcoRI and inserted into pHS1 (Wheeler et al., 2000) after first digesting it with BamHI, followed by blunt-ending it with *E. coli* DNA polymeraseI Klenow fragment and then digesting it again with EcoRI. The resulting construct, pHG, expresses GFP under the control of a *X. laevis* heat shock inducible promoter. The fragment containing γ-crystallin promoter driving GFP expression was isolated from NotI digested pCG (pCRY-GFP, a gift from Dr. R. Grainger, University of Virginia, Charlottesville, VA; (Damjanovski et al., 2001)) and inserted into the NotI site of pHG to create pCGHG-Not2. One of the two NotI recognition sites was removed by partial digestion with NotI, blunt-ended, then re-ligated with T4 DNA ligase to generate pCGHG, which contains two promoters, the γ-crystallin and heat shock inducible promoters, both driving the expression of GFP.

Stromelysin 3 wild type (ST3w) cDNA was cut out from pCSwG, which expresses ST3w–GFP fusion protein (with GFP fused at the carboxyl terminus of ST3) under the control of CMV promoter (Damjanovski et al., 2001) and inserted into the unique AgeI site of pCGHG to create the construct pCGHSwG (Fig. 1). This construct expresses GFP under the γ-crystallin promoter and ST3w–GFP fusion protein under the heat shock-inducible promoter.

To generate pCGCSwG, which expresses GFP under the γ-crystallin promoter and ST3–GFP under the CMV promoter, the fragment that contains the CMV promoter driving ST3w–GFP was excised from pCSwG by digesting it with AseI. The fragment was blunt-ended and digested with BglII. This fragment was used to replace the fragment that contains the heat shock inducible promoter driving the expression of ST3w–GFP in pCGHSwG after digesting the latter plasmid with SalI followed by filling the ends with *E. coli* DNA polymeraseI Klenow fragment and re-digesting it with BglII.

The construct pCGCG, where both the γ-crystallin and CMV promoters drive the expression of GFP, was generated by removing ST3w fragment of pCGCSwG with AgeI digestion and re-ligation. This and all the above constructs were linearized with NotI for transgenesis.

To produce the double promoter plasmid containing the rat intestinal fatty acid binding protein (IFABP) promoter driving a dominant negative form of *Xenopus* TRαA (dnTRαA) and γ-crystallin promoter driving GFP, the NotI-fragment containing γ-crystallin promoter and GFP from pCG was cloned into the NotI site in a plasmid containing GFP fused to the 3′-end of dnTRα A cDNA driven by the rat IFABP promoter (IFABP:GFP-ΔTRα, gift from Dr. D. D. Brown, Carnegie Institute of Washington, Baltimore, MD). This double promoter plasmid was linearized with SacII for transgenesis.

Transgenesis and Heat Shock Treatment

Transgenic *X. laevis* animals were produced as described (Kroll and Amaya, 1996; Damjanovski et al., 2001). Normally cleaving embryos (receiving one sperm nucleus/egg) were selected at the 4–8 cell stage and again at neurulation (around stage 20). They were reared first overnight in 0.1 × MMR plus 6% Ficoll in petri dishes at room temperature and then transferred to 0.1 × MMR.

Tadpoles/embryos that were generated from fertilization with sperm nuclei containing plasmid bearing the heat shock inducible promoter were heat shocked at

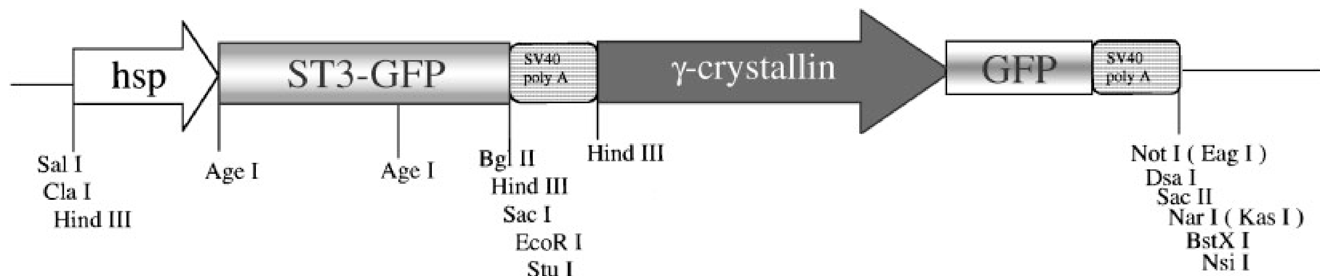

Fig. 1. Schematic diagram of the double promoter construct for transgenesis. The γ-crystallin promoter drives the expression of GFP coding region, which is followed by the SV40 polyadenylation signal. The transgene, ST3 fused to GFP used as an example here, is driven by the *Xenopus* heat shock inducible promoter (Wheeler et al., 2000). In theory, any promoter or transgene can be used in conjunction with the crystallin promoter. The plasmid backbone used here is pBluescript KS(−) (Stratagene, CA) (Wheeler et al., 2000) and can be replaced with any other appropriate vectors.

Nieuwkoop and Faber stage 20 (Nieuwkoop and Faber, 1956) with three cycles of 15 min at 33°C plus 15 min at 18°C as described (Wheeler et al., 2000).

Genotyping of the Transgenic Animals

Upon reaching stage 30 when γ-crystallin is first expressed, embryos were assayed for phenotype and GFP expression using an Olympus fluorescent dissection microscope with a Chroma GFP filter set. A Spot RT camera was used for photography. Tadpole phenotypes and GFP-expression were followed until stage 48. Genotypes were determined by PCR amplification of genomic DNA from the animals with primers specific for transgenes. It was carried out on tail tip or whole animal DNA isolated using DNeasy Tissue Kit (Qiagen) from equal numbers of transgenic animals with green eyes and without green eyes, the latter of which were generated from the same transgenic procedure, but failed to integrate the transgene. The primers used in the PCR genotyping were: 5′GGAATTCATGCATCT-CCTCATCCTTCTACC and 5′TATGCCATGCTTGT-CATCTGCACTAAGGC to yield a 725-bp fragment for stromolysin-3; and 5′CATCCGCGGATGGACCAGAA-TCTCAGCGG and 5′GCTCATCTGAAATTTTGGCT-TGCAGAGTCTTGATCATC to yield a 489 bp fragment for TRαA (data not shown).

RESULTS AND DISCUSSION

We and others have previously shown that the MMP genes ST3, collagenase-3 (Col3), and collagenase-4 (Col4) have distinct spatial and temporal expression profiles during *X. laevis* metamorphosis (Berry et al., 1998a,b; Damjanovski et al., 1999). In particular, the expression of ST3, but not Col3 or Col4 is associated with larval cell death in both the intestine and tail during metamorphosis. To investigate their functions in vivo, we generated transgenic animals that expressed ST3 or Col4 under the control of the ubiquitous promoter CMV (Damjanovski et al., 2001). To facilitate transgene detection, we fused GFP to the C-terminus of ST3 or Col4. Although we were able to generate transgenic animals that expressed the transgenes, all died prior to metamorphosis due to the precocious, ubiquitous expression of the enzymes.

To overcome these embryonic lethal effects, either tissue-specific and/or inducible promoters are needed for studying gene function during postembryonic development. Although ST3–GFP or Col4-GFP fusion proteins under the control of CMV promoter were easily detectable, such detection would be difficult with tissue-specific promoters or inducible promoters prior to the activation of the promoter. In addition, GFP fusion proteins often fluoresce less intensively than GFP alone, making its detection more difficult. Furthermore, in many cases, it is desirable to have transgenes without fusion to GFP as GFP fusion may interfere with the function of the proteins. Thus, it is often necessary to have an independent method to identify the transgenic animals. Traditionally, this is done by genotyping individual animals, which requires rearing each animal separately during the genotyping procedure (Henneberger et al., 2000), making it extremely labor intensive for analysis of large numbers of transgenic animals.

To simplify the identification of transgenic animals, we designed a construct that contained two promoters (Fig. 1). One of them is the γ-crystallin promoter, which drives the expression of GFP. The second one can be any promoter of choice (heat shock inducible promoter as an example here in Fig. 1) and is used to drive the expression of a desired transgene (e.g., ST3–GFP fusion gene). Although, double heat shock-inducible promoter constructs have been used in transgenesis before (Wheeler et al., 2000), the transgenic animals generated using the earlier constructs require heat shock to induce the expression of the GFP marker, a process which is potentially deleterious and also activates the transgene expression, an often-undesirable event for the reasons above. Our use of the γ-crystallin promoter to drive GFP expression allows the animals to be identified without going through any treatment or activating the transgene expression prematurely.

To test the validity of the approach, we generated the construct pCGCG, which contains two promoters, γ-crystallin promoter and CMV promoter, both driving GFP expression. Transgenesis was carried out with pCGCG and pCG, which contains only the γ-crystallin promoter driving GFP expression. Transgenic animals were sorted based on the presence or absence of green eyes under a fluorescent microscope for GFP detection. As shown in Figure 2, green-eyed animals generated from pCG had GFP expression only in the eyes (Fig. 2B), whereas green-eyed animals generated from pCGCG had ubiquitous GFP expression (Fig. 2C). By contrast, non-transgenic animals resulting from the same transgenic procedure, but without integrating the transgene showed no GFP expression (Fig. 2A), indicating that the eye-color correlated with the presence/absence of the plasmid DNA in the genome.

We then carried out transgenesis with the plasmid pCGHG, containing the γ-crystallin and heat shock inducible promoters driving GFP expression. Transgenic (Fig. 3A,B) and non-transgenic (Fig. 3C,D) animals were identified based on the presence of GFP in the eyes. In the absence of heat shock, the transgenic animals had little GFP expression except in the eye (Fig. 3A) and heat shock strongly induced the expression of GFP throughout of the animals (Fig. 3B). In contrast, non-transgenic animals had no GFP expression anywhere regardless of heat shock (Fig. 3C,D).

To show that the heat shock inducible promoter is capable of driving high levels of transgene expression, we compared the expression levels of the GFP under the control of the CMV (pCGCG) and the heat shock inducible (pCGHG) promoter. Transgenic and non-transgenic animals were identified based on the presence of GFP in the eyes. They were photographed under a fluorescent microscope under the same settings. As shown in Figure 4, the heat shock inducible promoter had a low level of GFP expression even in the

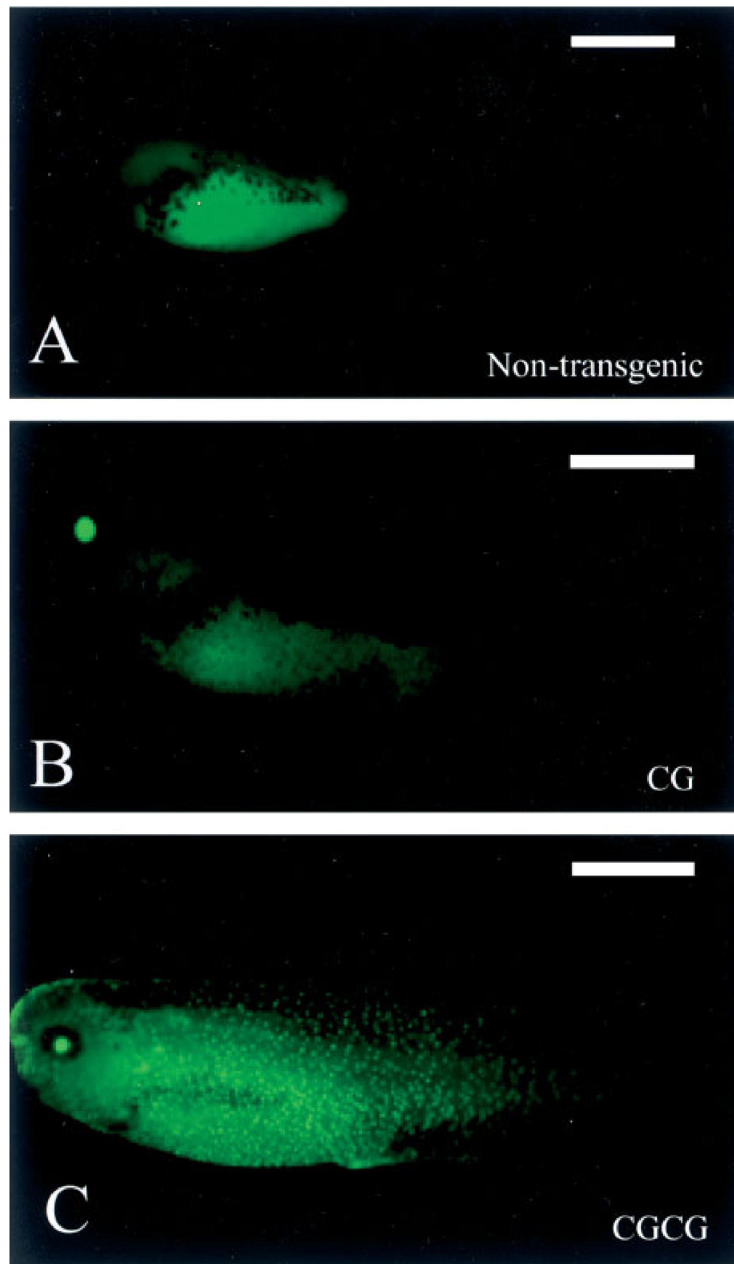

Fig. 2. Transgenic animals were generated with the plasmids pCG (γ-crystallin promoter-GFP) (**B**) or pCGCG (γ-crystallin promoter-GFP and CMV-GFP) (**C**). At stages 38–41, the transgenic and non-transgenic animals (based on the lack of GFP in the eye) were photographed under a fluorescent microscope. **A**: An animal generated from the same transgenesis procedure for (B), but failed to integrate the transgene, thus expressing no GFP. The green color in the abdominal region in all animals was due to auto fluorescence of the yolk; bar: 1 mm.

absence of heat shock (Fig. 4A, compare it to the non-transgenic animal in Fig. 4B), after heat shock (Fig. 4C), GFP was widely expressed to similar levels as that from the CMV promoter (Fig. 4D).

To use the double promoter construct for our studies of MMP function in development, we placed the coding region for *X. laevis* ST3, fused in frame to the 5′-end of the GFP sequence, under the control of the *Xenopus* heat shock inducible promoter in the double promoter construct (pCGHSwG) (Fig. 1). For convenience, we also fused GFP to the C-terminus of ST3. These plasmids were used in the sperm-mediated transgenesis as above. The resulting animals were examined at stages 30–35 when the γ-crystallin promoter was first activated in the animals as the eyes developed. All animals with green eyes under UV light were expected to be transgenic and also carry the heat shock inducible promoter driving ST3–GFP expression. Indeed, when

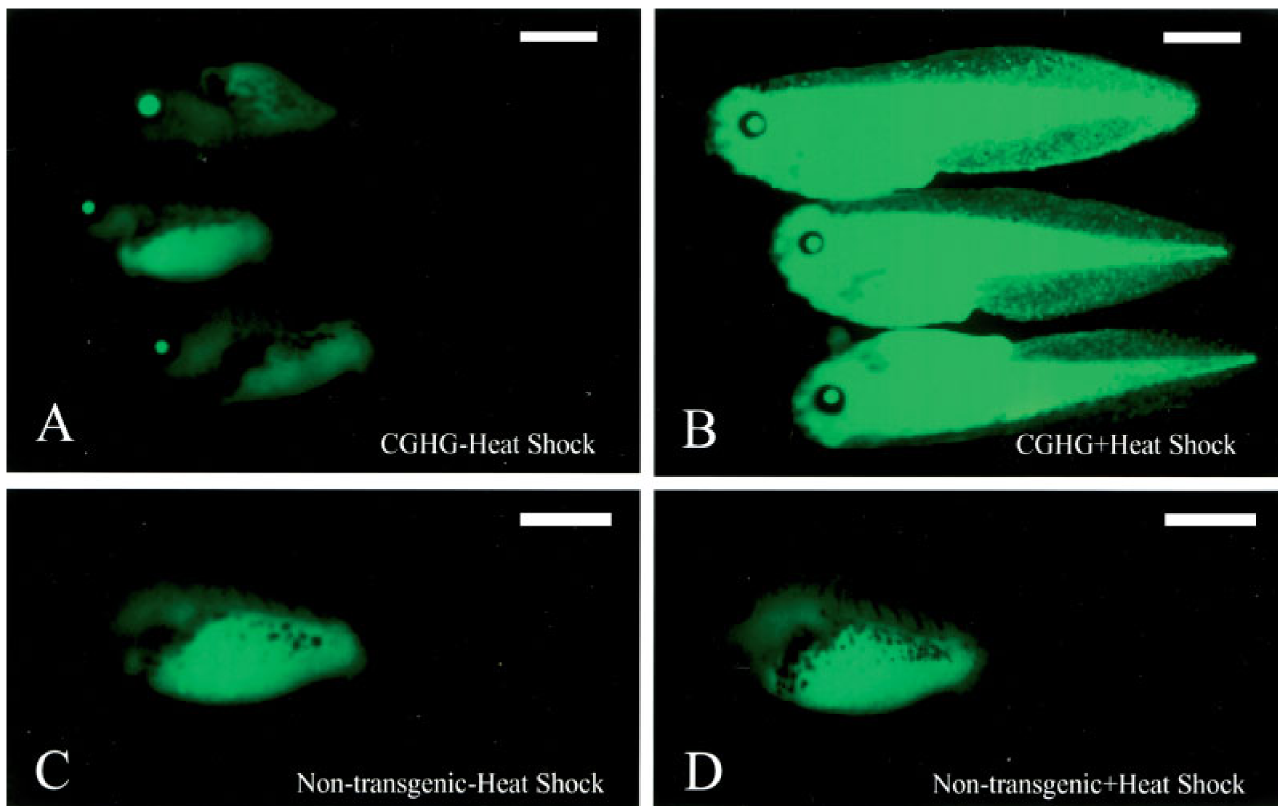

Fig. 3.

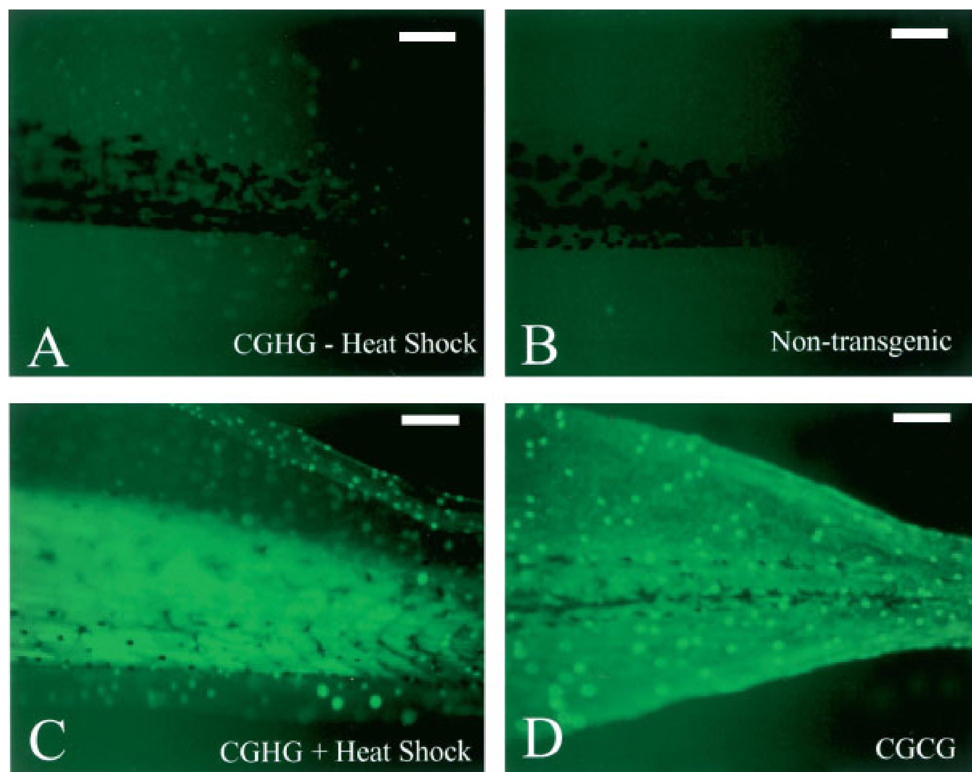

Fig. 4.

the animals were subjected to heat shock, all those with green eyes expressed ST3–GFP as reflected by the green fluorescence (due to ST3–GFP) throughout the animal in addition to the GFP in the eyes (data not shown). In contrast, animals without GFP in the eyes had no GFP anywhere in the animals even after heat shock (not shown). To confirm that animals with green eyes indeed contained the ST3 transgene, PCR-based genotyping was carried out. Whole animal DNA was isolated from animals with or without green eyes and subjected to PCR amplification using primers specific for the ST3 coding region (the primers were in different exons, and thus would only amplify the transgene, but not the endogenous ST3 genomic DNA due to the presence of large introns). All animals with green eyes gave a product of the expected size and animals without GFP in the eyes did not (Fig. 5). Overall, 10 green-eyed animals were assayed and all were positive in PCR amplification of the ST3 gene.

Similar results were obtained using a dominant negative form of *X. laevis* TRαA (dnTRαA) under the control of the rat intestinal fatty acid binding gene (IFABP) promoter, which directs transcription only in the epithelium of the small intestine The double-promoter construct carrying IFABP-promoter driving the expression of GFP fused to the N-terminus of dnTRαA was introduced into *Xenopus* tadpoles, and transgenic animals were selected based on GFP expression in the eyes. Because the transgene dnTRαA-GFP was expressed only in the intestinal epithelium, it was not possible to detect its expression by examining whole animals under UV light. Thus, we carried out PCR genotyping by isolating DNA from tail tips of the animals. Again, all eight green-eyed animals were positive for the presence of TRα cDNA (data not shown), although we have not yet determined if the IFABP promoter functions properly in the animals carrying the double promoter construct.

Results from the four double promoter constructs show that GFP expression under the control of the crystallin promoter faithfully indicates the presence of the transgene regardless of the nature of the transgene or the promoter used to drive its expression. Furthermore, our studies with the heat shock inducible promoter-driving ST3–GFP expression show that both promoters in the construct worked properly, without interfering

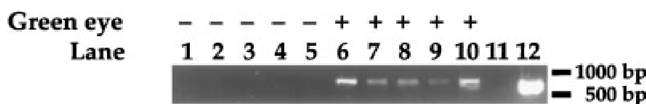

Fig. 5. PCR-based genotyping confirmed that green-eyed animals carried the transgene ST3–GFP (both from a single transgenesis procedure using the plasmid pCGHSwG, i.e., γ-crystallin promoter-GFP and heat inducible promoter-ST3–GFP). Animals were sorted based on the presence (+) or absence (–) of green eyes (GFP expression driven by the crystallin promoter) (**lanes 1–10**). Whole animal DNA was isolated and subjected to PCR amplifications with two primers encompassing 725 bp of ST3 coding region. The resulting PCR products were analyzed on an agarose gel with ethidium bromide staining. **Lane 11** had no added DNA during PCR amplification as a negative PCR control, and **lane 12** had pCGHSwG DNA during PCR amplification as a positive control. Note that all green-eyed animals had the expected PCR band amplified from the transgene.

with each other. Finally, our studies here as well as those published earlier (Damjanovski et al., 2001) have established that GFP expression driven by the γ-crystallin promoter does not interfere with animal development, making it a useful selection marker for developmental studies.

Having shown the feasibility to use the inducible, double promoter system in transgenesis, we then analyzed the developmental effects of ST3–GFP overexpression in transgenic animals generated with the plasmid pCGCSwG and pCGHSwG. The pCGCSwG has the CMV promoter driving the expression of ST3–GFP, thus similar to the construct used in our earlier study (Damjanovski et al., 2001), plus the presence of the GFP under the control of γ-crystallin promoter. As a control, we generated animals with pCGCG, which has both CMV and γ-crystallin promoters driving GFP expression. Animals that were morphologically normal at stage 20 were selected for further analysis, and transgenic and non-transgenic animals were sorted based on the GFP fluorescence from ST3–GFP before stage 30 and GFP fluorescence from the eye after stage 30. As we reported earlier, a wide range of developmental defects were detected in the presence of ST3–GFP (Damjanovski et al., 2001). Quantification of the percentages of morphologically normal animals surviving to various developmental stages indicated that overexpression of ST3–GFP from the pCGCSwG plasmid led to late embryonic/tadpole

Fig. 3. Transgenic animals generated with pCGHG (γ-crystallin promoter-GFP and heat inducible promoter-GFP) had green eyes and upon heat shock, expressed GFP throughout the animals. Animals were sorted based on their eye color under UV and were photographed at stages 38–41. The green-eyed ones (**A**, **B**) expressed little GFP elsewhere without heat shock (A), but high levels of GFP in heat shocked animals (B). In contrast, animals which had no GFP in the eyes (**C**, **D**), had no GFP expression anywhere either before (C) or after (D) heat shock. Note that three animals are present in (A) and (B) to show that different transgenic animals may have different levels of transgene expression (e.g., the animal on the top of each panel had higher levels of GFP), while only a single animal is shown in (C) and (D) as no GFP was expressed anywhere; bar: 1 mm.

Fig. 4. The expression levels of the GFP from the heat shock inducible promoter (**C**) were comparable, upon heat shock, to those from the CMV promoter (**D**). Transgenic animals were generated with pCGHG (**A** and **C**) or pCGCG (D). Animals were sorted based on their eye color under UV. Some transgenic animals from pCGHG were treated with heat shock (C), whereas others were not (A). Animals were then photographed at stages 42–45 with a fluorescent microscope under the same settings to compare the expression levels of the GFP. B: Any animal generated from the same transgenesis procedure for (A), but failed to integrate the transgene, thus expressing no GFP. Such non-transgenic animals and animals generated with pCGCG (D) were photographed without heat shock. Note that the heat shock promoter produced higher levels of GFP in the muscle relative to the CMV promoter; bar: 0.1 mm.

TABLE 1. Percentages and Numbers (in Parenthesis) of Embryos With or Without Transgenic Overexpression of ST3 That Developed Normally to a Given Stage

	CMV:ST3–GFP/CRY:GFP (CGCSwG)		CMV:GFP/CRY:GFP (CGCG)		HSP:ST3–GFP/CRY:GFP (CGHSwG)			
					Heat shock at stage 20		No heat shock	
Stage	Transgenic	Non-transgenic	Transgenic	Non transgenic	Transgenic	Non-transgenic	Transgenic	Non transgenic
20	100% (48)	100% (13)	100% (60)	100% (5)	100% (70)	100% (33)	100% (78)	100% (37)
30/35	35% (17)	62% (8)	80% (48)	60% (3)	47% (33)	52% (17)	72% (56)	46% (17)
40	13% (6)	54% (7)	58% (35)	60% (3)	13% (9)	39% (13)	41% (32)	43% (16)
47/48	6% (3)	46% (6)	33% (20)	40% (2)	13% (9)	30% (10)	36% (28)	35% (13)

Only the embryos that were normal at stage 20 were selected for further analysis.

lethality (Table 1), and the non-transgenic animals generated from the same procedure or with the control plasmid pCGCG had little effect (Table 1, some death occurred due to the transgenic and heat shock procedures themselves). These results are similar to what we reported earlier using a single promoter construct to drive ST3–GFP or GFP expression (Damjanovski et al., 2001). More importantly, when we used the heat shock inducible promoter (pCGHSwG) to drive the expression of ST3–GFP in transgenic animals, we found that without heat shock, animals bearing the transgene survived similarly as the non-transgenic animals and that heat shock alone had little effect on non-transgenic animals (Table 1). In contrast, heat shock treatment of transgenic animals bearing pCGHSwG led to embryonic/tadpole death to a similar extent as the transgenic animals bearing CMV promoter to drive the ST3–GFP expression (Table 1). These results are consistent with the fact after heat shock, the expression from the heat shock inducible promoter is comparable to that from the CMV promoter (Fig. 4) and further show the usefulness of the heat shock inducible promoter in preventing the embryonic effect of proteins of interest for functional studies at later stages.

CONCLUSIONS

We have developed here a novel approach for easy detection of transgenic animals without subjecting the animals to any potentially harmful treatments. Although, we have applied it to generating transgenic *X. laevis* animals, it can be used in any animals as crystallin gene is expressed in the lenses of all animal species. In addition, it can also be used in gene targeting and gene knock-out studies as the crystallin promoter-GFP fragments can be included as part of DNA inserted into the genome. Thus, this method should aid in studies of gene function in vivo in different animal species.

ACKNOWLEDGMENTS

We thank Dr. K.L. Kroll and Dr. M. Wu for advice and help in the transgenesis procedure, and Dr. D.D. Brown, Dr. R. Grainger, Dr. S. Hoppler for the gifts of the plasmids used in the experiments.

REFERENCES

Berry DL, Rose CS, Remo BF, Brown DD. 1998a. The expression pattern of thyroid hormone response genes in remodeling tadpole tissues defines distinct growth and resorption gene expression programs. Dev Biol 203:24–35.

Berry DL, Schwartzman RA, Brown DD. 1998b. The expression pattern of thyroid hormone response genes in the tadpole tail identifies multiple resorption programs. Dev Biol 203:12–23.

Damjanovski S, Amano T, Li Q, Pei D, Shi Y-B. 2001. Overexpression of matrix metalloproteinases leads to lethality in transgenic *Xenopus laevis*: Implications for tissue-dependent functions of matrix metalloproteinases during late embryonic development. Dev Dyn 221:37–47.

Damjanovski S, Ishizuya-Oka A, Shi YB. 1999. Spatial and temporal regulation of collagenases-3, -4, and stromelysin-3 implicates distinct functions in apoptosis and tissue remodeling during frog metamorphosis. Cell Res 9:91–105.

Dodd MHI, Dodd JM. 1976. The biology of metamorphosis. In: Lofts B, editor. The Biology of Metamorphosis. New York: Academic Press. p 467–599.

Henneberger C, Grantyn R, Rothe T. 2000. Rapid genotyping of newborn gene mutant mice. J Neurosci Methods 100:123–126.

Ikawa M, Kominami K, Yoshimura Y, Tanaka K, Nishimune Y, Okabe M. 1995. A rapid and non-invasive selection of transgenic embryos before implantation using green fluorescent protein (GFP). FEBS Lett 375:125–128.

Ishizuya-Oka A, Li Q, Amano T, Damjanovski S, Ueda S, Shi YB. 2000. Requirement for matrix metalloproteinase stromelysin-3 in cell migration and apoptosis during tissue remodeling in *Xenopus laevis*. J Cell Biol 150:1177–1188.

Kato M, Yamanouchi K, Ikawa M, Okabe M, Naito K, Tojo H. 1999. Efficient selection of transgenic mouse embryos using EGFP as a marker gene. Mol Reprod Dev 54:43–48.

Kroll KL, Amaya E. 1996. Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. Development 122:3173–3183.

Nieuwkoop PD, Faber J. 1956. Normal table of *Xenopus laevis*, 1st edition. Amsterdam: North Holland Publishing. 252 p.

Shi Y-B. 1999. Amphibian metamorphosis: From morphology to molecular biology. New York: John Wiley & Sons, Inc. 288 p.

Takada T, Iida K, Awaji T, Itoh K, Takahashi R, Shibui A, Yoshida K, Sugano S, Tsujimoto G. 1997. Selective production of transgenic mice using green fluorescent protein as a marker. Nat Biotechnol 15:458–461.

Uhm SJ, Kim N-H, Kim T, Chung HM, Chung KH, Lee HT, Chung KL. 2000. Expression of enhanced green fluorescent protein (EGFP) and neomycin resistant (Neo-R) genes in porcine embryos following nuclear transfer with porcine fetal fibroblasts transfected by retrovirus vector. Mol Reprod Dev 57:331–337.

Wheeler GN, Hamilton FS, Hoppler S. 2000. Inducible gene expression in transgenic *Xenopus* embryos. Curr Biol 10:849–852.