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Inducible control of tissue-specific transgene expression in *Xenopus tropicalis* transgenic lines

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Abstract

Analysis of gene function in vertebrates is facilitated by gain-of-function studies, such as injection of synthetic mRNA in amphibian embryos. This approach is hampered by lack of spatial and temporal control of expression of the introduced gene product. An additional level of control is obtained by nuclear-transfer-mediated transgenesis, but functional analyses are complicated by variability and background abnormalities in primary transgenic embryos. The GAL4/UAS system permits establishment of stable lines and elimination of nuclear-transfer-associated abnormalities, through generation of separate UAS-'effector' and GAL4 'transactivator' transgenic lines. When the GAL4 DNA-binding domain is combined with a steroid hormone ligand-binding domain, this system allows full temporal regulation of transgene expression by introduction of an exogenous steroid analogue, the progesterone antagonist RU486. We show here that by crossing stable *Xenopus tropicalis* transgenic lines, one bearing a UAS-enhanced cyan fluorescent protein (ECFP) reporter construct, and the other with a GAL4–progesterone receptor fusion driven by a retina-specific promoter, reporter expression in the resulting embryos can be induced with RU486 in a tissue-specific manner. These results suggest that the inducible binary system, in which the target gene expression can be controlled in a stage- and tissue-specific pattern, should be readily applicable for gene function studies at all stages of development.

Keywords: *Xenopus tropicalis*; GAL4/UAS system; Transgenic line; Inducible gene expression

1. Introduction

In *Xenopus* studies, over- or misexpression of a gene product by mRNA injection is the most common way to analyze the function of a cloned gene (e.g. Smith and Harland, 1991). However, misexpression-associated pleiotropy or lethality often masks analysis of later developmental processes. The recently developed frog transgenesis technique (Kroll and Amaya, 1996; Offield et al., 2000) permits the expression of a gene product in a tissue-specific manner by delivering the gene product under the control of a defined promoter (Hartley et al., 2001). Analysis of the consequences of transgene expression in primary transgenics is, however, often complicated by position effects, variability in expression levels, and background abnormalities associated with nuclear transfer. These problems can be circumvented by establishing stable transgenic lines, but only where transgene expression is non-lethal.

To overcome these difficulties, we have applied the GAL4/UAS system extensively utilized in the fly (Brand and Perrimon, 1993). This system divides the target gene (UAS-effector) from transcriptional activation elements (GAL4 transactivator) in two separate transgenic lines. In an effector line the target gene, under the control of GAL4 binding sites (Upstream Activation Sequences) remains silent in the absence of its activator GAL4, ensuring that these transgenic founders are viable. Only in the progeny of a cross of a UAS-effector line to a GAL4 transactivator line is the target gene expressed. This system can also provide an additional level of temporal control of transgene expression by fusing the GAL4 binding domain with a steroid receptor ligand-binding domain (Wang et al., 1994).

In this study, we tested the GAL4/UAS system in the pipid frog *Xenopus tropicalis*, which has emerged as a complementary system to its close relative, *Xenopus laevis* (Amaya et al., 1998). *X. tropicalis* shares many of the advantages of *X. laevis*, but is more suited for multigeneration genetic experiments by virtue of its short generation time and smaller diploid genome.

Here we show that, using the transgenic activator and effector lines we established, the expression of UAS-target

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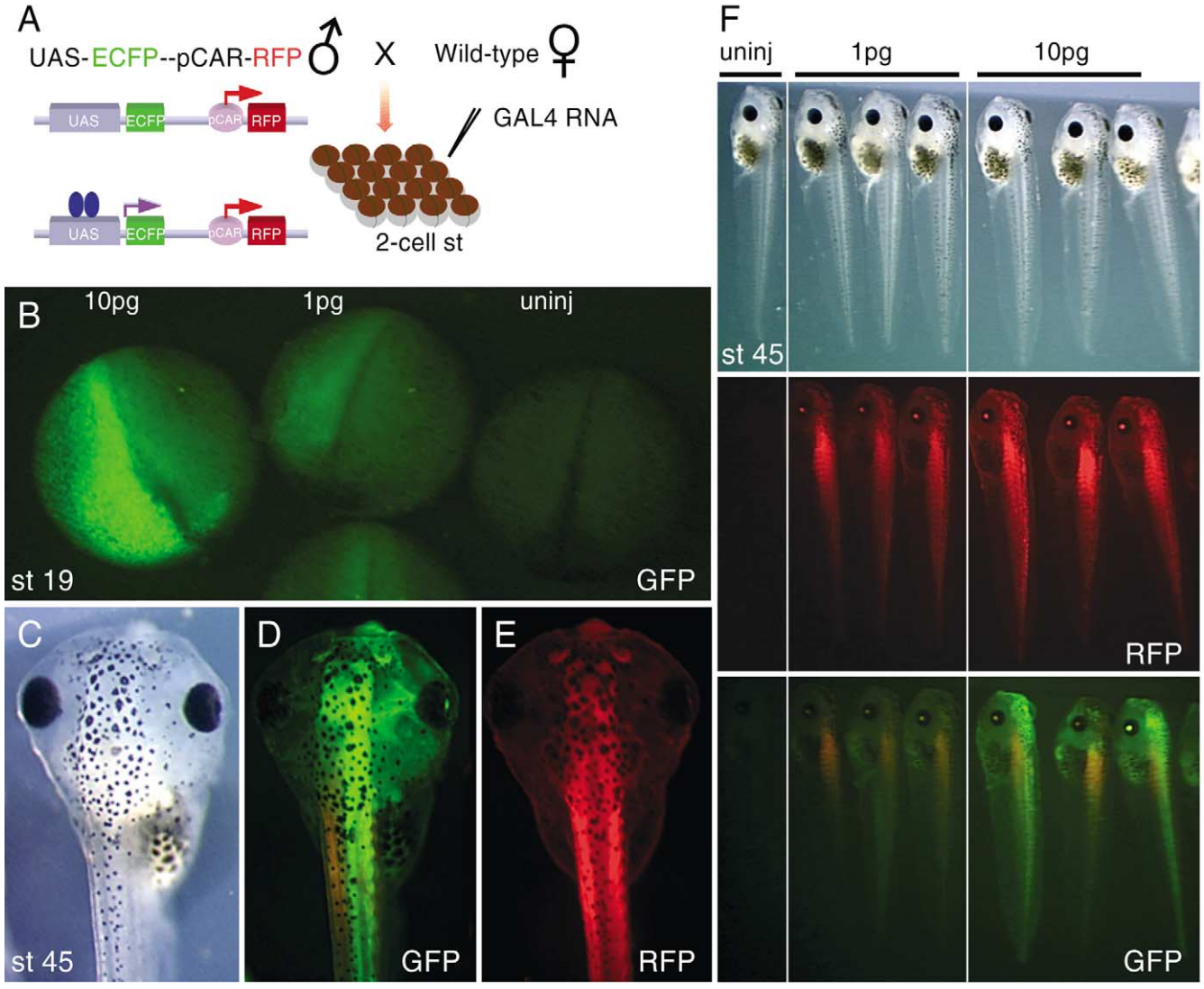

Fig. 1. Injected GAL4 RNA activates the expression of transgenic UAS effector gene. (A) Experimental outline. Injection of GAL4 RNA unilaterally into 2-cell stage UAS–ECFP F1 transgenic embryos. GAL4 protein (blue ovals) binds to UAS sequences, activating transcription of the ECFP reporter (blue arrow). The UAS–ECFP construct carries a second reporter cassette, pCAR–RFP, to drive RFP expression in somitic muscle (red arrow), to delineate transgenic embryos. (B) Dorsal view of stage 19 embryos injected with indicated dose of GAL4. uninj, uninjected non-transgenic sibling control. RFP + siblings which did not receive GAL4 RNA were likewise ECFP − (data not shown). Shown in a higher magnification are bright field (C), GFP (D), and RFP (E) images of dorsal view of embryos injected with 10 pg in (F). Strong ECFP expression is seen on the injected side of embryos. (F) Lateral view of injected sides of stage 45 embryos. Note that some RFP expression remains discernible with the GFP filter used here (D,F).

gene can be efficiently activated by a ligand-inducible GAL4 in response to RU486.

2. Results

2.1. Injected GAL4 RNA activates the transcription of transgenic UAS effector gene

We have generated *X. tropicalis* effector lines carrying a UAS–ECFP construct, in which the gene for a green fluorescent protein (GFP) variant, ECFP (Clontech), is under the control of GAL4 binding motifs. This construct also contains a second reporter cassette (pCAR–RFP) with the cardiac actin promoter driving Red Fluorescent Protein expression in the somites (Kroll and Amaya, 1996; Hartley et al., 2001) to identify embryos carrying the transgene (Fig. 1A).

To establish the utility of the UAS–ECFP line we first tested whether exogenous GAL4 can activate transcription in stable UAS–ECFP transgenic embryos by transient expression assays. A male transgenic founder was crossed with a wild-type female, and resulting embryos were then injected in one blastomere with 1 or 10 pg of synthetic GAL4 mRNA at the two-cell stage (Fig. 1A). A substantial difference in response to the two doses of GAL4 mRNA in

activation of the effector gene was detected by observing ECFP expression in the injected side of embryos at later stages (Fig. 1B,F). This unilateral ECFP expression was particularly obvious in enlarged dorsal view images of stage 45 embryos injected with 10 pg of GAL4 mRNA (Fig. 1C–E). Strong RFP expression in somitic muscle driven by the second cassette at stage 45 (Nieuwkoop and Faber, 1967) enabled us to confirm the stable transmission of the transgene through the germline and to select RFP-positive embryos carrying the UAS-target to assay its activation by injected GAL4 (Fig. 1F). ECFP expression was seen in 55% (21 out of 38) or 95% (19 out of 20) of RFP-positive embryos injected with 1 or 10 pg of GAL4 mRNA, respectively. Uninjected RFP-positive embryos showed no visually-detectable ECFP expression (0 out of 10). No expression was observed following the injection of any dose of GAL4 into RFP-negative embryos that have no target gene to activate (data not shown).

The F1 animals derived from the transgenic UAS–ECFP founder used for GAL4 RNA injections were raised to maturity and found to contain two different UAS–ECFP inserts in subsequent analyses. DNA from F1 animals was analyzed by genomic Southern blotting, and they showed hybridization patterns which indicate that they carry one of two transgenic loci in their founder (data not shown).

Although ECFP was not detected by fluorescence in the untreated UAS lines, the two F1 populations with distinct Southern patterns were further evaluated for background expression of ECFP by Western blotting, and one was found to have detectable background by this method. Each group of F1 animals was outcrossed and resulting embryos were subjected to Western analysis. A correlation between Southern patterns and background expression of ECFP was thereby revealed; one group showed little background while the other group showed a detectable level of background (data not shown). The low background line was used in subsequent experiments.

2.2. *Rx promoter–GFP reporter expression in transgenic X. tropicalis lines*

For the GAL4 transactivator line, we chose to target expression to the retina using the promoter from the homeobox gene *Rx* (Mathers et al., 1997). *X. laevis Rx* has been reported to be expressed primarily in the primordia of the retina. A 2.2 kb 5′-upstream region of the *Rx* gene was obtained by screening a *X. tropicalis* genomic library with a *X. laevis Rx* cDNA probe. In order to confirm that this promoter sequence could direct expression to the developing retina, we generated transgenic lines with GFP fused downstream of the 2.2 kb *Rx* promoter (pRx–GFP) and analyzed the F1 embryos for the GFP expression pattern. Strong GFP expression was seen first in the anterior of neural fold stage embryos, and was subsequently localized to the two fields that give rise to the retina (Fig. 2A,B). At later stages, the retina remains the primary site of GFP expression in the pRx–GFP transgenic embryos (Fig. 2C–E). The same promoter sequence that has the upstream region important for *Rx* expression in the retina was utilized to direct the expression of chimeric GAL4 in the activator lines. An advantage of the use of this eye-specific promoter is the relative ease of assaying the activation of effector in vivo by being able to identify embryos showing fluorescence because it is visible externally.

2.3. *Inducible chimeric GAL4 activates UAS-target in the presence of RU486*

To add the dimension of temporal regulation of target gene expression, we utilized a chimeric transactivator, GALPR, constructed by fusing the GAL4 DNA binding

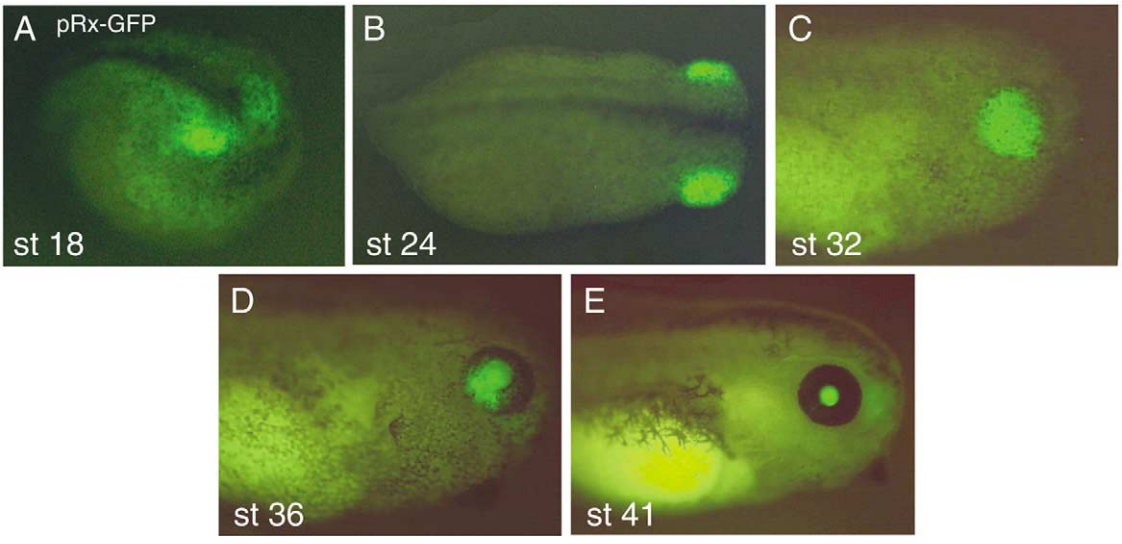

Fig. 2. *Rx* promoter directs GFP reporter expression to the eye in transgenic *X. tropicalis* embryos. A 2.2 kb genomic fragment from the *Rx* gene was fused to GFP (pRx–GFP) and used to establish stable *X. tropicalis* transgenic reporter lines. (A–E) Fluorescent images of pRx–GFP transgenic embryos at indicated stages. The identical promoter sequence was used to drive expression of the chimeric GAL4 transactivator in pRx-GALPR transgenic lines (see Fig. 3).

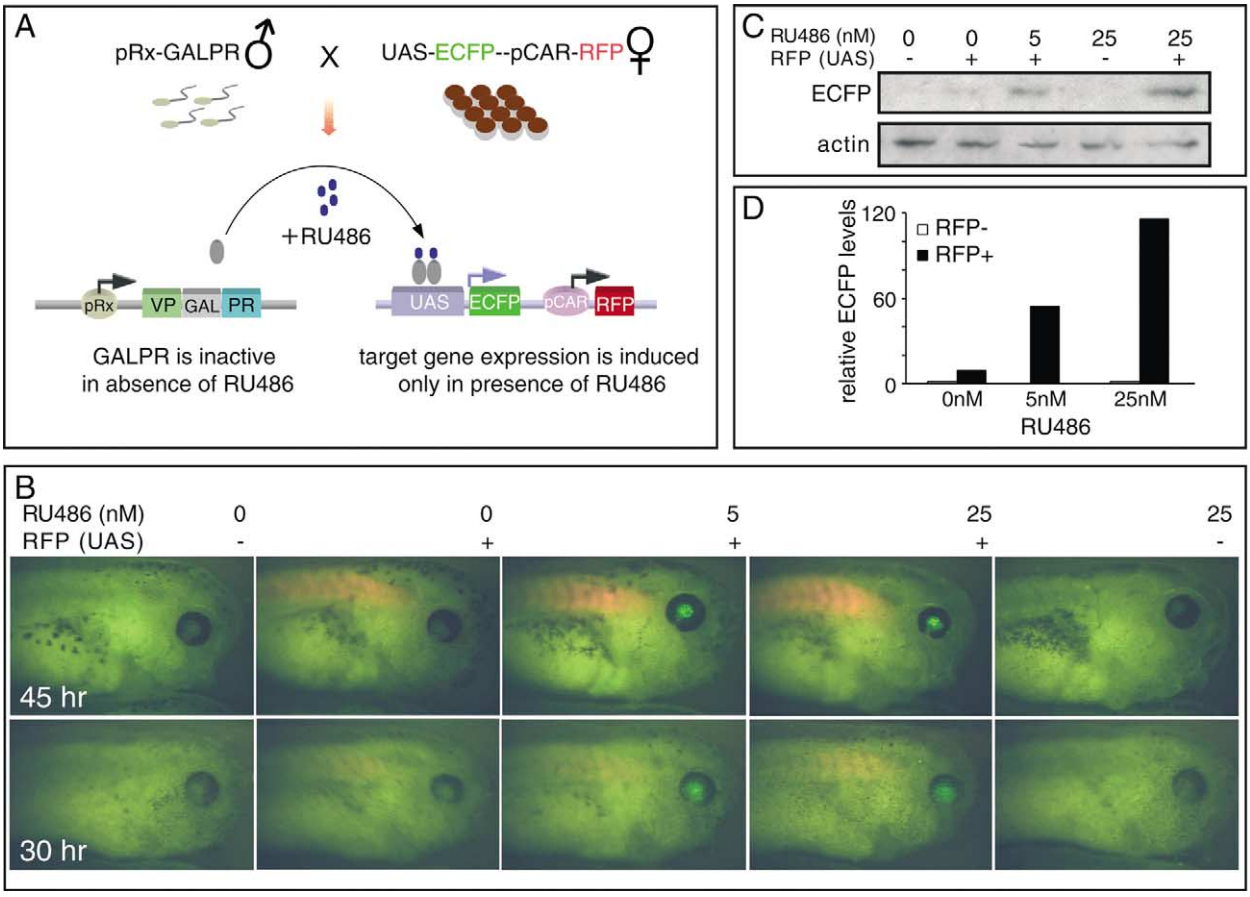

Fig. 3. Inducible GAL4 transgenic construct activates UAS-target gene in response to RU486. (A) A UAS–ECFP line was crossed to a pRx-GALPR line, in which the *Rx* promoter drives a chimeric transactivator gene (GAL4 DNA-binding domain, fused to a herpes virus VP16 transcriptional activation domain, in turn fused to a modified progesterone receptor ligand-binding domain). In the double-transgenic progeny, transcription of the UAS–ECFP reporter cassette is activated only when the synthetic ligand RU486 is present, relieving the cytoplasmic sequestration of the GALPR transactivator protein. (B) Representative embryos from this cross, treated with 0, 5, or 25 nM RU486 from stage 22 onwards for 30 h (bottom panels) or 45 h (top panels). ECFP expression was observed only in RFP-positive embryos. (C) Western blot analysis of eye extract from embryos shown in (B) using EGFP antibody and anti-actin antibody. A pool of 30 RFP-positive or RFP-negative embryos at each dose of RU486 was assayed in each lane. (D) Western blot band intensities are quantified by densitometry, and relative levels of ECFP expression are calculated by normalizing to actin levels.

domain to a mutated human progesterone receptor ligand binding domain and the VP16 transcriptional activation domain (Fig. 3A) (Wang et al., 1994). Upon binding to synthetic progesterone analogue, RU486, this fusion protein escapes cytoplasmic sequestration, travels to the nucleus, and binds UAS sequences, resulting in efficient ligand-inducible transactivation of target genes in cell culture and transgenic mouse models (Wang et al., 1997; Burcin et al., 1998; Zhao et al., 2001). To establish transgenic *X. tropicalis* lines with retina-specific expression of this chimeric transactivator, we generated frogs bearing the *Rx* promoter driving the GALPR construct (pRx-GALPR). Next, we asked whether the chimeric GAL4 activates the transcription of UAS-target in the progeny of the cross of the two lines (Fig. 3A). The pRx–GALPR transactivator line was crossed with UAS–ECFP effector frogs (Fig. 3A) and the progeny cultured with or without RU486 from stage 22 onward (Fig. 3B). Each of the lines in this cross is hemizygous for a transgenic locus, producing four different genotypes: non-transgenic embryos, embryos bearing one transgene (either the effector or transactivator gene), and embryos bearing both effector and transactivator transgenes. Approximately 50% of the progeny expressed RFP in the somites, indicating that they carry a single locus bearing the UAS–effector construct. Effector gene expression was analyzed either by microscopic examination for ECFP fluorescence in the eye or by Western analysis of eye extracts with an EGFP antibody that crossreacts with ECFP (Clontech). The expression of the ECFP in the effector construct is highly localized in the retina and increases in a dose-dependent manner after RU486 treatment in embryos carrying both effector and transactivator genes. This result can be seen in a qualitative way in the series of fluorescent images in Fig. 3B, which illustrates embryos 30 and 45 h after treatment. Table 1 summarizes data from embryos carrying the effector transgene treated with 0, 5 and 25 nM RU486, showing that both the fraction of embryos expressing ECFP, and their level of expression, increases with increasing dose of RU486.

Table 1
Target gene (ECFP) expression in the progeny of the cross of pRx-GALPR and UAS–ECFP animals[a]

	ECFP++	ECFP +	ECFP −
0 nM RU486	0	0	50
5 nM RU486	3	10	37
25 nM RU486	11	8	31

[a] Embryos from the cross of the pRx-GALPR activator line with the UAS–ECFP effector line were incubated with RU486 for 30 h and assayed for ECFP expression. Each of 50 embryos carrying the effector gene (i.e. RFP-positive) cultured in 0 (solvent control), 5, or 25 nM of RU486, respectively was scored as strongly ECFP-positive (++), weakly ECFP-positive (+) or ECFP-negative (−). At the higher dose of RU486, the fraction of embryos expressing ECFP approaches the expected maximum level of 50%, i.e. the fraction of embryos expected to carry both activator and effector transgenes. Effector-negative embryos (i.e. RFP-negative) were also analyzed for ECFP expression in the same way and showed no detectable ECFP expression.

Western blot analysis of dissected eye tissue (Fig. 3C,D) shows that the level of expression of ECFP in treated embryos depends in a quantitative way on the dose of RU486. ECFP expression was minimal in the absence of RU486, as assayed by ECFP fluorescence or Western analysis (Table 1 and Fig. 3B–D, and data not shown), suggesting that this system is not affected by endogenous frog hormones. We also found that elevated ECFP fluorescence 45 h postincubation (Fig. 3B, top panels) returned to baseline in some embryos after RU486 was removed from culture media though others still showed significant fluorescence after 2 days of withdrawal (data not shown). While the decay of the reporter gene activity was slow, it appears that the target gene induction is reversible. We conclude that this inducible regulatory system works efficiently in *X. tropicalis*.

3. Discussion

In this study, we have evaluated the utility of a GAL4/UAS system in *Xenopus tropicalis*. In the stable transgenic activator and effector lines we established, transgenic GAL4 efficiently activates the transcription of a reporter gene in the cells where it is expressed. Development of a GAL4/UAS method for targeted gene expression in the *X. tropicalis* system is desirable for several reasons. First, the significantly shorter generation time of *X. tropicalis*, 3–5 months, allows rapid production and expansion of stable GAL4 and UAS transgenic lines at a relatively low cost. Second, the efficiency of in vivo promoter analysis in transgenic *Xenopus* (Offield et al., 2000) is resulting in an accumulation of defined promoters. These promoters, when combined with GAL4, provide very useful tools for expressing combinations of effector constructs in analysis of a wide variety of developmental processes. Third, the GAL4/UAS system has many potential applications for gene function studies (Phelps and Brand, 1998). For example, this method may be used to produce loss-of-function phenotypes by driving the expression of a gene variant that will antagonize the endogenous gene product. This is highly desirable because currently there is no technique for targeted gene disruption in *Xenopus*.

In addition to showing the utility of a GAL4/UAS system in *Xenopus* for spatial regulation of transgenic constructs, we also have used the construct to achieve the potential for temporal control of gene expression. A ligand-inducible regulatory system based on a mutated progesterone receptor ligand binding domain has been shown to work in adult mouse cells and organs (Wang et al., 1997; Zhao et al., 2001). We show here that this inducible system can be used to study early embryonic development. We demonstrated that this system regulates gene expression in vivo in *X. tropicalis* embryos in response to RU486. We observed that embryos cultured in 25 nM of RU486 showed a strong induction of the target gene with no observable developmental defects (data not shown). We should mention that the induction is possible at concentrations that are significantly lower than the micromolar range required to antagonize progesterone (Wang et al., 1994). Therefore, RU486 treatment is unlikely to affect this endogenous hormone system.

While rapid kinetics of induction are a desirable aspect of any inducible system, we observed a delay in target gene induction, with ECFP fluorescence first detected 17 h postincubation in RU486. However, it has been reported that detectable protein fluorescence lags behind the onset of UAS–GFP gene transcription, possibly due to post-translational modification and folding (Brand, 1995; Phelps and Brand, 1998). Consistent with this, maturation of EGFP (Clontech) takes about 8–9 h in *Xenopus* when tested by microinjection of EGFP mRNA into 2–4 cell embryos (Zaraisky, A., personal communication). Considering that the concentration of mRNA transcribed from the transgene is, in general, lower than that used in microinjection, the time lag for ECFP accumulation to detectable levels would be expected to be considerably longer than 8–9 h. We might expect that induction of other genes used in applications of this system would be substantially faster than that of ECFP shown here.

For the applications usually requiring robust transgene expression such as the gene perturbation assays mentioned earlier, further modulation of induction rapidity and expression level of the effector transgene could be achieved in several ways. First, one can make transgenic lines with very high numbers of inserts (e.g. 30–40 copies) in the *Xenopus* system, either for the effector or transactivator, or both. One could also increase the number of GAL4 binding sites upstream of the target gene (Brand and Perrimon, 1993; Rørth, 1996). In addition, one could insert 5′ or 3′ UTR sequences that would stabilize the transcript from the transgene (Rupp et al., 1994; Turner and Weintraub, 1994). Functional inhibition of some gene products, such as home-

odomain transcription factors including Pax6 and Otx homologs, Crx and SpOtx2, may not require very high levels of overexpression of dominant-negative mutants and therefore the expression level of mutant effector transgene producing the phenotype would be readily reached by the method described here. These genes have been shown to be repressed effectively by dominant-inhibitory variants at concentrations lower than those of wild-type products (Singh et al., 1998; Furukawa et al., 1997; Li et al., 1999).

While culturing embryos with RU486 at stage 22 resulted in 14-fold induction of target gene expression, we also treated with RU486 at stage 41 onward and observed comparable activation of ECFP expression (data not shown). This suggests that this system can overcome a major limitation of RNA expression studies in *Xenopus*, that the instability of RNA prevents analysis of its effects on later stages, and may be used to express effector transgenes at all stages of development, facilitating analysis of genes that function both in early embryogenesis and later developmental processes such as organogenesis.

3.1. Note

While preparing this paper, we learned of relevant studies using fly and *X. laevis* that have now been published by other groups (Osterwalder et al., 2001; Roman et al., 2001; Hartley et al., 2002).

4. Experimental procedures

4.1. DNA constructs and generation of transgenic lines

A 2.2 kb 5′-upstream region of the *Rx* gene was obtained by screening a Lambda FIX-II *Xenopus tropicalis* genomic library (constructed by Lisa Brunet and Richard Harland) with a *Xenopus laevis Rx* cDNA probe containing the 5′ untranslated region. The pRx–GFP construct was generated by subcloning this 2.2 kb region of the *X. tropicalis Rx* promoter into pEGFP-1 vector (Clontech). This construct was linearized with *Afl*II for making transgenic animals. The pRx–GALPR activator construct was generated by deleting an Asp718–*Not*I/blunt fragment containing the EGFP coding sequences in pRx–GFP, and replacing them with an Asp718–*Bam*HI/blunt fragment containing the chimeric GAL4-VP16-mutant progesterone receptor ligand-binding domain isolated from pGL-VP (Wang et al., 1994). To construct the bifunctional UAS–ECFP plasmid, the cardiac actin promoter in pCARGFP (gift of E. Amaya) (Kroll and Amaya, 1996) was removed and cloned into plasmid pDsRed1 (Clontech), creating pCAR–RFP construct. An *Afl*II fragment of the resulting construct containing the CAR–RFP cassette then was inserted into plasmid pECFP-1 (Clontech), resulting in double color reporter pBicolor in which the expression of RFP is driven by the cardiac actin promoter and any promoter can be cloned in front of the ECFP coding sequences. Finally, the *Hin*dIII–*Sma*I fragment containing five copies of the GAL4 consensus binding sites (UAS) linked to the *hsp70* TATA box was isolated from plasmid GUAS-GFP3 (gift from E. Amaya) (Bronchain et al., 1999) derived from pUAST (Brand and Perrimon, 1993) and subcloned into plasmid pBicolor, creating UAS–ECFP (UAS–ECFP/pCAR–RFP). Transgenic lines used in this study were generated as previously described (Offield et al., 2000) with some modifications (see Transgenesis in *X. tropicalis* at *X. tropicalis* website: http://minerva.acc.Virginia.EDU/~develbio/trop/).

4.2. GAL4 mRNA injections

A male transgenic UAS–ECFP founder was outcrossed to a wild-type counterpart. One blastomere of two-cell stage embryos of this cross was injected with 1 or 10 pg of capped synthetic GAL4 RNA transcribed in vitro using SP6 polymerase from a *Not*I-linearized pCS2/GAL4 template (gift of E. Amaya) encoding native GAL4 DNA binding and activation domains. Resulting embryos were then analyzed for GAL4-induced ECFP expression and the expression of RFP in the muscle.

4.3. RU486 treatments and Western analysis

For experiments involving treatment with RU486 (Biomol Research Labs Inc.), embryos expressing activator and effector transgenes were produced by crossing a low background F1 transgenic effector animal (see Section 2) to a GALPR activator founder. Embryos were cultured in 0 (solvent control), 5, or 25 nM RU486 freshly diluted in 0.1× MBS with 0.1% BSA from a stock initially dissolved in DMSO at 25 mg/ml and subsequently diluted to 5.8 μM in 1 M acetic acid. Eye extracts were prepared by sonicating head tissue consisting primarily of eye rudiment dissected from RU486-treated embryos in a standard NP-40 lysis buffer. Protein levels were quantified by Bradford assay (Bio-Rad) and 5 μg of extracted protein was separated on a 12% sodium dodecyl sulfate–polyacrylamide electrophoresis gel and transferred onto a nitrocellulose membrane (Osmonics Inc.). The blot was probed with the Living Colors A.v. Peptide antibody (Clontech) recognizing EGFP derivatives and an Anti-Actin antibody (Sigma) diluted 1:100 or 1:1000, respectively. The signals were visualized by ECL (Amersham Pharmacia). Finally, intensities of resulting bands were determined and calculated using the Kodak Digital Science Image Station 440 CF (NEN).

4.4. Photomicroscopy

Images of transgenic embryos were obtained using a Zeiss Stemi Epi-fluorescence stereoscope with GFP 500 and rhodamine filter sets. Images were captured using a Dage MTI 330 cooled CCD camera.

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