Chapter 16

Tet-On Binary Systems for Tissue-Specific and Inducible Transgene Expression

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Abstract

Tissue-specific and inducible control of transgene expression is a cornerstone of modern studies in developmental biology. Even though such control of transgene expression has been accomplished in *Xenopus*, no general or widely available set of transgenic lines have been produced akin to those found in mouse and zebrafish. Here, I describe the design and characterization of transgenic lines in *Xenopus* constituting the Tet-On binary transgene expression system comprising two components: (1) rtTA transgenic lines, i.e., lines harboring the doxycycline- (Dox-) dependent transgenic transcription factor rtTA under control of a tissue-specific promoter and (2) transgenic promoter (TRE) transgenic lines, i.e., lines harboring a gene of interest (hereafter called the transgene) under control of a promoter (TRE). In double transgenic animals, i.e., embryos or tadpoles harboring both the rtTA and TRE components, transgene expression remains off the absence of Dox. Addition of Dox to the rearing water causes a conformational change in rtTA allowing it to bind the TRE promoter and induce transgene expression. Tissue specificity of transgene expression is determined by the promoter regulating rtTA expression, and inducibility is determined by the addition of Dox to the rearing water. Deposition of rtTA and TRE transgenic lines enabling tissue-specific inducible control of transgene expression into the *Xenopus* stock center will provide a powerful and flexible resource for studies in developmental biology.

Key words: Frog transgenesis, *Xenopus*, rtTA, Tetracycline response element, Doxycyline

1. Introduction

The simplest and most common transgene expression system involves inserting foreign DNA, consisting of a promoter controlling a transgene, into the genome (1, 2). Using tissue-specific promoters, investigators can examine the role of a transgene in a particular tissue. Commonly, early misexpression of a transgene alters development, precluding study of the role of the transgene later in development and leading to the need to control the timing of transgene expression. Inducible expression of transgenes using a heat shock (3, 4) or metallothionine promoter (5) allows researchers

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to avoid early deleterious effects of a transgene. However, these inducible promoters result in ubiquitous transgene expression throughout the organism, dissallowing inducibility in a tissue-specific manner. Binary transgene expression systems enable tissue-specific and inducible control of transgene expression (6).

Binary transgene expression systems utilize (1) a ubiquitous or tissue-specific promoter controlling a transgenic transcription factor, which binds and regulates (2) a transgenic promoter controlling the transgene. Two such binary methods have been shown to work in frogs, the GAL4/UAS and Tet-On systems (6, 7). A third binary strategy using Cre/lox has been established in frogs, where Cre expressed from one component acts on a separate transgene with appropriately placed lox sites to activate another transgene (8, 9). We focus here on the Tet-On system because of its success to delimit the timing of thyroid hormone influence in hind limb innervation (6), to examine genes important for limb muscle development (10), to reveal gene switching during liver metamorphosis (11), and transdifferentiation of tadpole pancreatic acinar cells to duct cells (12). In contrast, the tissue-specific inducible versions of the Gal4/UAS system in frogs have not gone beyond the proof of principle (7, 13), and tissue-specific and inducible control has not yet been done with the Cre/lox system.

In the Tet-On system (commercialized by Clontech), two components work together, the transgenic transcription factor, rtTA, and the transgenic tetracycline-inducible promoter, TRE. The rtTA is derived from TetR, a tetracycline-inhibited transcriptional repressor from *Escherichia coli*. This protein, when fused to three copies of the minimal viral transactivation domain of VP16 from Herpes simplex virus, forms tTA (tetracycline-inhibited transcriptional activator). Mutations in tTA converted it to rtTA, reverse tetracycline-controlled transcriptional activator, which activates transcription upon addition of tetracycline or the more stable tetracycline mimic doxycycline (Dox). In the absence of Dox, rtTA does not bind DNA or regulate transgenes. In the presence of Dox, a conformational change allows rtTA to bind DNA and induce transgene expression from the TRE. The TRE is composed of a minimal CMV promoter (from cytomegalovirus which does not support transcription by itself) and seven copies of the bacterial tetO operator to which TetR and derivatives bind.

In addition to enabling tissue-specific inducible control of transgene expression, another advantage of Tet-On and other binary expression systems stems from the combinatorial possibilities of having separate rtTA and TRE transgenic lines. As more rtTA lines and more TRE lines are produced, the binary system approach rapidly multiplies the combinations of tissue specificity and

transgenes of interest readily available to researchers. Transgenic lines from previous studies using the Tet-On system (6, 10) are of limited future use because the rtTA and TRE components are not separable, i.e., these components co-integrated into the same chromosomal position. Thus, few transgenic lines using binary expression systems are available that can be flexibly applied to a wide variety of research questions (14), highlighting the need to characterize new Dox-inducible transgenic *Xenopus* lines. The current protocol describes methods to design and characterize Tet-On transgenic rtTA and TRE lines with minimal leakiness and maximal levels of Dox-induced transgene expression.

2. Materials

1. Transgenesis plasmids. An rtTA and a TRE plasmid (Fig. 1) are available to researchers as a template to facilitate engineering their own transgenesis plasmids (see Note 1).
2. Materials for engineering transgenesis plasmids using standard cloning procedures (15).
3. Materials for breeding and rearing *X. laevis* (16) (see Note 6).
4. Transgenic lines harboring rtTA or TRE transgenesis constructs (Fig. 1) for characterizing Tet-On transgenic lines in this protocol (available from the *Xenopus* Stock Center (14)).
5. Materials for transgenesis (see Chapters 11, 12, 13 and 14) (see Note 7).
6. Doxycycline hyclate: 1,000× stock solution of 50 mg/mL in water stored at −20 °C.
7. 60 mm Petri dishes with dechlorinated fresh water ("frog water").
8. Foil for Petri dishes to block light (doxycycline is light sensitive).
9. Fluorescence dissecting microscope with filter sets for visualizing green and red fluorescent proteins.
10. 10% v/v Benzocaine in 100% EtOH for anesthesia.
11. Material and equipment for quantitative reverse transcriptase PCR (17).
12. Material and equipment for tissue sectioning and immunohistochemistry (18).

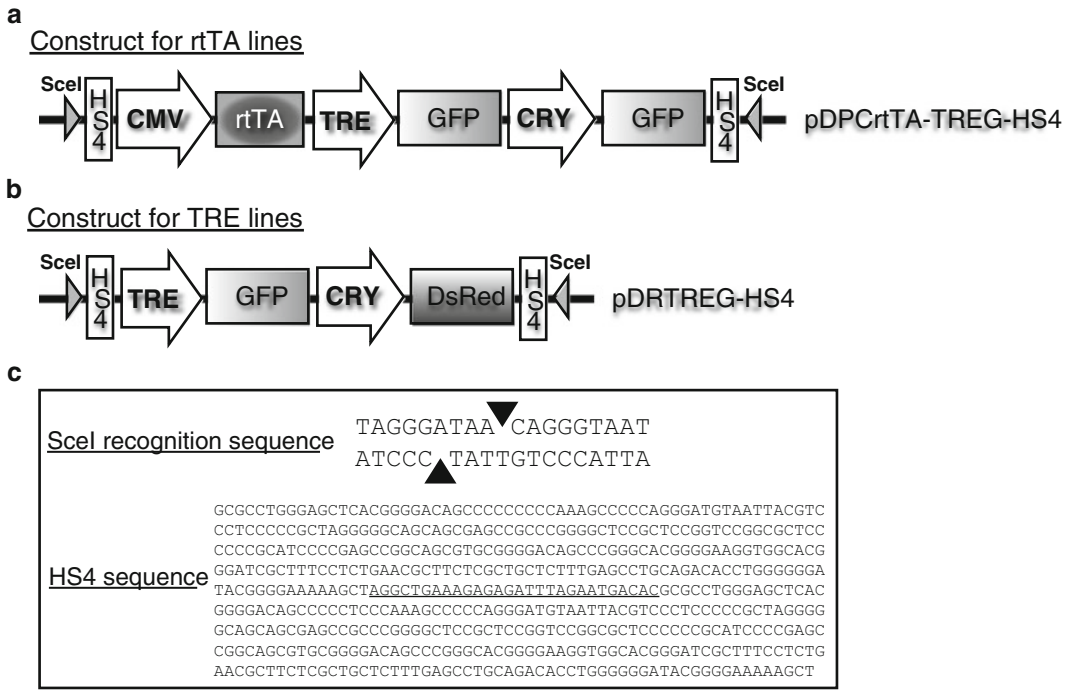

Fig. 1. Diagram of transgenesis plasmids. (**a**) Transgenic lines with this construct have ubiquitous expression of the Dox-activated transgenic transcription factor rtTA via the full length CMV (cytomegalovirus) promoter. Only in the presence of Dox does rtTA bind the transgenic tetracycline responsive promoter, TRE, and induce expression of GFP (green fluorescent protein) all over the body due to the TRE:GFP cassette (14) (see Notes 1 and 2). CRY is 300 base pairs of the *Xenopus* crystallin promoter driving constitutive GFP expression in the lens of the eye enabling identification of transgenic animals in the absence of Dox (25) (see Note 3). (**b**) Transgenic lines with this construct have GFP under control of TRE, such that GFP will be expressed only when Dox is present and only in tissues that express rtTA. The CRY:DsRed cassette drives DsRed expression in the eyes enabling identification of transgenic animals via DsRed expression in the eyes. I propose the convention that GFP be the marker for rtTA lines and DsRed be the marker for TRE lines so that Tet-On transgenic lines can be exchanged by researchers using Stock Centers. (**c**) Sequences for SceI and HS4. For both transgenesis constructs, the SceI recognition sites are required for transgenesis (see Note 4). Also, each HS4 represents two direct repeats of chicken 5' HS4 250 base pair core sequences (see Note 5).

3. Methods

3.1. Engineer Transgenesis Constructs

To suit individual research goals, the available transgenesis constructs (Fig. 1) can be modified using standard cloning techniques by replacing the promoter regulating rtTA expression in construct A and/or by replacing the transgene regulated by the TRE in construct B (15)(see Note 2).

3.2. Production of rtTA and TRE Transgenic Founders

1. Transgenesis (see Chapters 11, 12, 13, and 14), use method of choice (see Note 7).
2. Sort embryos and remove dead and deformed ones daily. Maintain healthy embryos in 60 mm Petri dishes (<50 per dish) in dechlorinated fresh water changed daily.

3. After 5–7 days at room temperature (about NF stage 38–42), select founders by choosing tadpoles that have lens-specific expression of GFP or DsRed in one or both eyes (see Note 8). GFP under control of the lens crystallin promoter is a marker for rtTA plasmids, and DsRed under control of the lens crystallin promoter is a marker for TRE plasmids. For small numbers of tadpoles, unanesthetized individuals may be observed in a droplet of water in a depression slide. For large numbers of tadpoles, 1–2 drops of benzocaine in ethanol added to the 60 mm Petri dish with 30–50 mL of frog water 5 min before viewing may be used.
4. Rear transgenic animals, i.e., tadpoles with GFP or DsRed expression in one or both eyes, to adulthood, about 12 months for *X. laevis* (see Note 9).

3.3. Characterization of rtTA Founders

1. Cross rtTA founders with pDRTREG-HS4 (Fig. 1) transgenic animals by natural mating (16) (see Note 10).
2. Examine eye fluorescence in embryos as above (see Subheading 3.2, step 3). Identify adult founders that have germ-line transgene expression by observing lens-specific GFP fluorescence in the F1 offspring using the GFP filter set. The lens-specific DsRed expression from the pDRTREG-HS4 parent (present in half of the F1 offspring) will not be visible if lens-specific GFP is expressed.
3. For all rtTA germ-line founders, sort F1 double transgenics harboring the rtTA and pDRTREG-HS4 transgenes by identifying tadpoles simultaneously expressing GFP in the eyes using the GFP filter set and DsRed in the eyes using the RFP filter set.
4. Treat F1 double transgenic tadpoles (both green and red fluorescence in eyes) with 50 μg/mL Dox for 1d (or 3d for higher Dox-induced GFP induction) at a stage the promoter controlling rtTA is expected to be strong.
5. Select rtTA germline founders which produce F1 offspring with the highest expressing, promoter-appropriate GFP fluorescence (see Note 11). In cases where Dox-induced GFP is not externally visible, dissection or histological sectioning and staining of tadpoles to check fluorescence will be necessary.
6. Rear F1 offspring from selected rtTA founders to adulthood and deposit some in the *Xenopus* Stock Center (see Note 12).
7. F1 double transgenic tadpoles from this cross can be used for GFP-dependent lineage tracing (Kerney and Buchholz, in prep).

3.4. Characterization of TRE Founders

1. Cross TRE founders with pDPCrtTA-TREG-HS4 (Fig. 1) transgenic animals by natural mating (16) (see Note 13).

2. Examine eye fluorescence in embryos as above (see Subheading 3.2, step 3). Identify adult founders that have germ-line transgene expression by observing lens-specific DsRed fluorescence in the F1 offspring using the red filter set. The lens-specific GFP expression from the pDPCrtTA-TREG-HS4 parent (present in half of the F1 offspring) will not be visible in the red filter set.
3. Treat F1 tadpoles double transgenic for the TRE and pDP-CrtTA-TREG-HS4 transgenes (as indicated by both green and red fluorescence in the eyes) with 0 or 50 μg/mL Dox for 1d at NF40-45.
4. Carry out quantitative reverse transcriptase PCR from whole bodies using TaqMan primer/probe set for the transgene. Follow external protocols (17) for isolating total RNA, synthesizing cDNA, designing and ordering TaqMan primer/probe sets for the gene of interest and a house keeping gene such as rpL8 (19), and carrying out and analyzing the quantitative PCR reaction. It is important to use DNAfree (Ambion) on the RNA sample prior to cDNA synthesis to remove contaminating DNA to avoid amplifying it in the quantitative PCR reaction.
5. Select TRE founders with the lowest leakiness and highest maximal expression by comparing results from quantitative reverse transcriptase PCR in double transgenic animals treated with and without Dox.
6. Rear single transgenic F1 offspring to adulthood from selected TRE founders and deposit some in the *Xenopus* Stock Center (see Note 12).
7. F1 double transgenic tadpoles from this cross can be used for studies where ubiquitous expression of the Dox-induced transgene is desired.

3.5. Crossing the rtTA and TRE Lines of Interest

Because there are so few rtTA and TRE transgenic lines as of now, it is likely that a line expressing rtTA in a particular tissue of interest and TRE regulating a particular transgene of interest will not be available. Thus, researchers will need to produce and characterize their own rtTA and TRE transgenic lines as detailed above. However, once available, any rtTA line can be crossed to any TRE line for specific research purposes.

1. Cross a singly transgenic rtTA line of choice to a singly transgenic TRE line of choice by natural mating (see Note 14). The male or female parent does not matter.
2. F1 double transgenic tadpoles can be identified as above (see Subheading 3.2, step 3) with both red and green fluorescence in the eyes. The parents will be heterozygous for their respective transgenes and thus at most one quarter of the offspring

will be double transgenic. The other offspring can be used as controls, but the best comparison will be between double transgenic animals treated with and without Dox.

3. Treat embryos or tadpoles with 50 μg/mL Dox for 1–3 days, minimum 3–6 h (14). The appropriate stage for Dox treatment will depend on the promoter controlling rtTA expression. Longer Dox treatments will increase the expression level of the Dox-induced transgene.
4. Analyze the experiment for morphological phenotype with or without sectioning and/or examine the effects on cell signaling or gene expression.

Theoretically with the Tet-On system, any transgene can be inducibly overexpressed in any tissue. Three limitations curb the widespread popularity for using this approach. First, few rtTA or TRE lines are currently available, requiring researchers to produce their own, a 2-year commitment at this time. Second, characterization of promoters used to drive rtTA into the tissue of interest is in itself a significant prerequisite for the production of rtTA transgenic lines (see Note 15). However, there are quite a few tissue-specific promoters available that have already been tested in frogs, e.g., expression in lens, neurons, muscle, tadpole skin, intestinal epithelium, exocrine and endocrine pancreas, limbs. Third, transgene expression levels remain a concern requiring careful characterization of transgenic lines to achieve sufficient transgene overexpression to observe a strong phenotype. Nevertheless, the availability of more rtTA and TRE lines will greatly increase the ease with which inducible tissue-specific transgene overexpression can be performed and will provide a significant additional resource to probe the mechanisms of vertebrate development using frogs as a model system.

4. Notes

1. The versions of rtTA and TRE used in frogs are rtTA-2S-M2 (20) and pTREtight (Clontech). Improved rtTA (Tet-On 3G) and TRE (pTRE3G) have been introduced by Clontech but have not been tested in frogs.
2. The TRE:GFP cassette in construct A (Fig. 1) is not necessary for the purposes of this protocol and may be removed from future constructs. The TRE:GFP cassette in construct A was used to characterize the first rtTA line in the absence of existing TRE lines (14).
3. Fluorescent proteins expressed in the lens of the eye are used to identify transgenic animals. If the eye is the organ of interest,

an alternate externally visible marker of transgenesis can be used, e.g., the muscle-specific promoter pCAR (21) can replace CRY to have constitutive GFP expression in the muscle to identify transgenic animals.

4. SceI sites are required for the meganuclease method of transgenesis (see Chapter 12) and are also suitable for the REMI method (see Chapter 11).
5. These insulators reduce the effects of chromosome position effects (14, 22, 23). To avoid enhancer-like carry over effects for uncharacterized promoters, I recommend including an additional HS4 insulator element to flank the promoter:rtTA cassette on the 5′ and 3′ ends. Improved insulator ability has been demonstrated with a 400 base pair sequence (24) but has not been tested in frogs.
6. This protocol uses established *X. laevis* rtTA and TRE transgenic lines (Fig. 1) for ease of characterizing additional rtTA and TRE lines via this protocol. The methods in this protocol are expected to be equivalent for *X. tropicalis* but no rtTA or TRE transgenic lines are thus far available. To begin the process of building a set of Tet-On transgenic lines in *X. tropicalis*, I recommend characterizing transgenic lines using the same transgenesis plasmids as initially used in *X. laevis* (14), which can then be used to characterize the additional lines.
7. The method of choice may depend on the required level of transgene expression. REMI tends to result in higher transgene expression levels compared to the methods using SceI/integrase/transposons because the copy number is much greater, up to 30 copies, compared to 1–8 copies for the other methods. However, the ideal transgene expression level varies with application. Thus, REMI is preferred when using a dominant negative, because expression level of the dominant negative must be high to overcome the activity of the endogenous protein, and even moderate levels of leakiness are unlikely to have an effect. On the other hand, SceI may be better when using cell signaling proteins which typically act at very low concentrations, such that the degree of leakiness may be more of an issue than maximal expression level.
8. A fully green or red fluorescent eye, as opposed to streaks or subsets of fluorescent lens cells, improves but does not guarantee germ-line expression, due to mosaicism particularly in the SceI/integrase/transposon methods. In addition, animals with one solid fluorescent eye should be kept, as it may be indicative of a “half transgenic” where transgene integration occurred at the two-cell stage. Selection of potential founders based on lens-specific GFP expression will eliminate transgenic animals that have germ-line expression but lack fluorescence in the eyes. However, the frequency that this occurs is not known.

9. Due to mosaicism with SceI/integrase/transposon transgenesis methods, the frequency of transgenic offspring is unlikely to be 50%, but more commonly, 5–25% transgenic offspring. Thus, a large number of potential founders will be needed, likely >20 animals, because germ-line transmission based solely on fluorescent protein expression in the eye is around 25%. On the other hand, the REMI method typically results in non-mosaic F0 founders requiring fewer founders to rear for testing because most will be germline.
10. Because females require 2–3 months of recovery time after breeding for re-breeding, it is time efficient to cross female founders directly with pDRTREG-HS4 males and test offspring for germ line expression and a Dox-induced response simultaneously. When females of the pDRTREG-HS4 line are limiting, the rtTA founder males, which can be induced to breed every 1–2 weeks, can first be checked for germline using wild type animals and then soon thereafter crossed with the pDRTREG-HS4 line.
11. Higher Dox-induced GFP expression levels should correlate with the greatest degree of Dox-induced transgene expression when crossed with other TRE lines.
12. Heterozygous transgenic individuals are sufficient for most applications, though homozygous individuals available in the F2 generation from crossing F1 transgenic siblings would enable a twofold higher transgene expression level in cases where increased expression would be useful. However, identifying homozygous individuals will depend on the potentially unreliable ability to detect a twofold difference in lens-specific GFP or DsRed expression.
13. Because females require 2–3 months of recovery time after breeding for re-breeding, it is time efficient to cross female founders directly with pDPCrtTA-TREG-HS4 males and test offspring for germline expression and a Dox-induced response simultaneously. When females of the pDPCrtTA-TREG-HS4 line are limiting, the TRE founder males, which can be induced to breed every 1–2 weeks, can first be checked for germline using wild type animals then soon thereafter crossed with the pDPCrtTA-TREG-HS4 line.
14. Transgenic founders have germline mosaicism (i.e., transgenic or not) explaining the <50% transgenic offspring. Founders also likely have mosaicism among transgenic gametes consisting of different germ cells having different insertion sites and copy numbers. Thus, if transgenic founders are used in this cross, be prepared for low frequencies of F1 double transgenic animals and expect a range of Dox-induced transgene expression levels and corresponding variation in the resulting phenotypes.

Use of F1 transgenic animals as parents in this cross will increase but not guarantee the homogeneity of Dox-induced transgene expression levels and phenotypic results.

15. If the future use of an uncharacterized promoter sequence is for tissue-specific inducible control of transgene expression, I recommend using the protocol outlined in Subheading 3.3 (Characterization of rtTA Founders) rather than first making promoter:GFP transgenic animals. Outside the use of promoter:GFP to mark tissue types, use of promoter:GFP transgenic animals has disadvantages for promoter characterization. Though straightforward, use of promoter:GFP constructs as a way to identify transgenic promoter expression domains limits the resolution of observed promoter activity because the highly stable GFP protein may be visible in cells days or weeks after the promoter has ceased to be active. In addition, transgenic lines from the protocol in Subheading 3.2 but not promoter:GFP are immediately available to carry out tissue-specific inducible control of transgenes by simply crossing to a TRE line.

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