

Improved Cre Reporter Transgenic *Xenopus*

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We have produced and characterized improved transgenic reporter lines for detection of Cre recombinase activity during *Xenopus* development. Improvements include choice of fluorophores, which make these Cre reporter lines generally suitable for lineage tracing studies. We also include data for several new parameters affecting survival and transgenesis efficiency using the recently developed meganuclease method of frog transgenesis. These transgenic frogs express cyan fluorescent protein (CFP) under control of the ubiquitous promoter CMV, where CFP is replaced by DsRed2 (a red fluorescent protein) in the presence of Cre. Three independent, high expression, Cre-sensitive lines have been identified that maintain robust fluorophore expression across generations and lack DsRed2 expression in the absence of Cre. A novel use of these lines is to indelibly mark embryonic blastomeres by Cre mRNA injection for permanent fate mapping. Similarly, transgenically expressed Cre under control of tissue-specific promoters will allow detailed analysis of cell lineage relationships throughout embryogenesis, metamorphosis, and adulthood. *Developmental Dynamics* 238:2401–2408, 2009. © 2009 Wiley-Liss, Inc.

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INTRODUCTION

A fundamental component of studies in developmental biology is determination of cell lineage relationships during normal development and after experimental manipulation (Stern and Fraser, 2001). For most adult cell types including stem cells, the precise source populations and developmental pathways are unknown. The model frog *Xenopus laevis* has been at the forefront of embryonic lineage tracing, where a detailed fate map from embryonic blastomeres to early organogenesis was described using cell-impermeant fluorescent dyes (Dale and Slack, 1987; Chalmers and Slack, 2000). However, fluorescence dimin-

ishes with cell division and animal growth.

Two sorts of permanent lineage tracing have been used in frogs, namely grafting and genetic marking using Cre/loxP. Embryonic grafting of green fluorescent protein (GFP)-labeled transgenic tissue into wild-type host was used to follow fate of neural crest (Gross and Hanken, 2005) or tissue type in regenerating tail (Gargioli and Slack, 2004; Lin et al., 2007). Tissue recombination of intestinal tissue layers in vitro between GFP transgenic and wild-type animals was used to identify source tissue of adult intestinal stem cells (Ishizuya-Oka et al., 2009). A special case of lineage tracing

of transgenic cells in wild-type host involved introduction of GFP reporter construct by injection into tail spinal cord (Lin et al., 2007) and muscle (Nakajima and Yaoita, 2003) or by electroporation into tail muscle (Boorse et al., 2006) of premetamorphic tadpoles to follow fate during tail resorption or regeneration. These studies take advantage of *Xenopus* as an excellent system for embryological studies, but inaccessible cells and tissue types, such as internal organs in late stage tadpoles, require different techniques.

The Cre/loxP system used extensively in mice can genetically label any cell type in principle. Cre recombinase activity results in a permanent

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genetic change in a cell, thereby “marking” it and all its progeny to enable cell lineage “tracing” for the study of lineage relationships between cells at different stages of development. The Cre/loxP system labels a cell when Cre recombinase recognizes two loxP sites and deletes or inverts intervening DNA when the loxP sites are oriented in the same or opposite direction, respectively. More than 10 Cre reporter lines have been characterized in mice (Branda and Dymecki, 2004). For example, the Rosa26-Reporter contains a floxed translation stop codon upstream of β -galactosidase (i.e., a stop codon flanked between two loxP sites such that Cre activity removes the stop codon allowing β -galactosidase expression; Soriano, 1999), or Z/EG mice where Cre deletes a floxed alkaline phosphatase upstream of GFP (Novak et al., 2000).

The Cre/loxP system has been shown to work as a proof of principle in frogs, but generally suitable transgenic Cre reporter lines are not currently available. Gargioli and Slack (2004) used a CRE reporter with floxed stop codons between the CMV promoter and GFP, but F0 tadpoles were used and no F1 transgenic lines were characterized (Gargioli and Slack, 2004). Ryffel et al. (2003) characterized a floxed CFP reporter that switched to yellow fluorescent protein (YFP) in the presence of CRE (Ryffel et al., 2003). However, CFP is visible in both CFP and YFP fluorescence filter sets confounding unambiguous analysis. Also, CFP and YFP are too similar for antibodies or in situ hybridization to reliably distinguish them. Waldner et al. (2006) characterized two additional CRE reporter lines, floxed GFP giving rise to DsRed2 or lacZ in the presence of CRE (Waldner et al., 2006). However, the GFP-DsRed2 line showed unexpected activation of both GFP and DsRed2 upon CRE activation. In addition, the lacZ in the GFP-lacZ line exhibited mosaic expression as well as ectopic activation in other cell types when crossed to a muscle-specific CRE inducer line. Also, lacZ may not be appropriate for all cases of lineage tracing because live animal observation is not possible. Furthermore, some tissues (such as the gut) have considerable endogenous β -galactosidase activity, which can cause background problems. Overall, these results and the experi-

ence of the mouse community suggests that to obtain a really useful transgenic model, it is critical to optimize and characterize several independent germ line transmitting transgenic lines—which was a goal of work presented in this brief report.

Our strategy was based on the constructs from Ryffel's lab (Ryffel et al., 2003), which we optimized by using CFP and DsRed2 to avoid problems associated with YFP and lacZ. Importantly, we characterized several independent lines and present them here as a resource to the frog community. To generate these transgenic lines, we used the newly developed meganuclease method of transgenesis in *Xenopus laevis* (Pan et al., 2006) and *X. tropicalis* (Ogino et al., 2006) which is easier to perform, gives higher survival after injection and similar transgenesis efficiency compared with the more laborious REMI technique (restriction enzyme-mediated integration) (Chesneau et al., 2008). Another benefit of the meganuclease method for the current project is the difference in number of insertion sites and copy number per insertion site, which are smaller for the meganuclease method (1–8 copies in 1–2 insertion sites) compared with the REMI method (1–8 copies in 1–6 insertion sites; Kroll and Amaya, 1996; Offield et al., 2000; Sparrow et al., 2000; Chesneau et al., 2008; Ogino and Ochi, 2009). A low copy number and number of insertion sites (ideally one each in a euchromatic genomic region) is important to avoid complications from Cre activity removing or inverting genomic DNA between loxP sites present due to multiple insertions of the transgenesis construct. Few *Xenopus* papers have been published since the original meganuclease descriptions (Seo et al., 2007; Tazumi et al., 2008). Therefore, we also tested several parameters of the meganuclease protocol to optimize the efficiency, the results of which should be generally useful to the *Xenopus* community.

RESULTS AND DISCUSSION

Transgenesis Using Meganuclease Methodology

The *SceI* meganuclease binds to and cleaves DNA sequences containing the 18-base pair site TAGGGATAACA-

GGGTAAT. This sequence is not found in the sequenced vertebrate genomes of frog, zebrafish, human, or mouse. DNA cleavage by *SceI* is asymmetric resulting in a smaller upstream portion and a larger downstream portion to which the enzyme may remain bound as a monomer after cleavage (Perrin et al., 1993; Beylot and Spassky, 2001). The reason why *SceI* endonuclease works in transgenesis and typical restriction enzymes do not is not known, but a possible explanation is that *SceI* may facilitate random integration of a transgene construct by binding to the cleavage products and protecting the foreign DNA from rapid degradation allowing longer time for DNA repair enzymes to work. *SceI* sites are generally placed on either side of the transgene of interest, and in a cell culture system, the orientation of *SceI* sites with respect to the intervening sequence affected the assay results (de Piedoue et al., 2005). To examine the importance of *SceI* site orientation in frog transgenesis, we created a double promoter construct where a ubiquitously expressed CMV:GFP cassette was “outside” of the *SceI* sites (oriented away from each other) and eye-specific CRY:DsRed1 was “inside” (Fig. 1A). Transgenesis with this construct resulted in tadpoles with various combinations of red eyes and green bodies (Fig. 2A; Table 1 row 1), clearly indicating that *SceI* sites facing the transgene is not a requirement for integration. Multiple integrations are common in REMI procedure where co-injection of two plasmids resulted in 80% of transgenic tadpoles containing both plasmids (Marsh-Armstrong et al., 1999) and would not be unexpected with *SceI*. In fact, a full transgenic will occasionally have different intensity fluorescence on the left and right sides suggesting independent integrations at the two cell stage (data not shown). However, *SceI* site orientation with respect to the transgene may have affected integration efficiency of CMV:GFP compared with CRY:DsRed1 because in no case was there a green body lacking red-eyes. In any case, subsequent constructs have the downstream portion of the *SceI* sites oriented toward the transgene to increase integration efficiency.

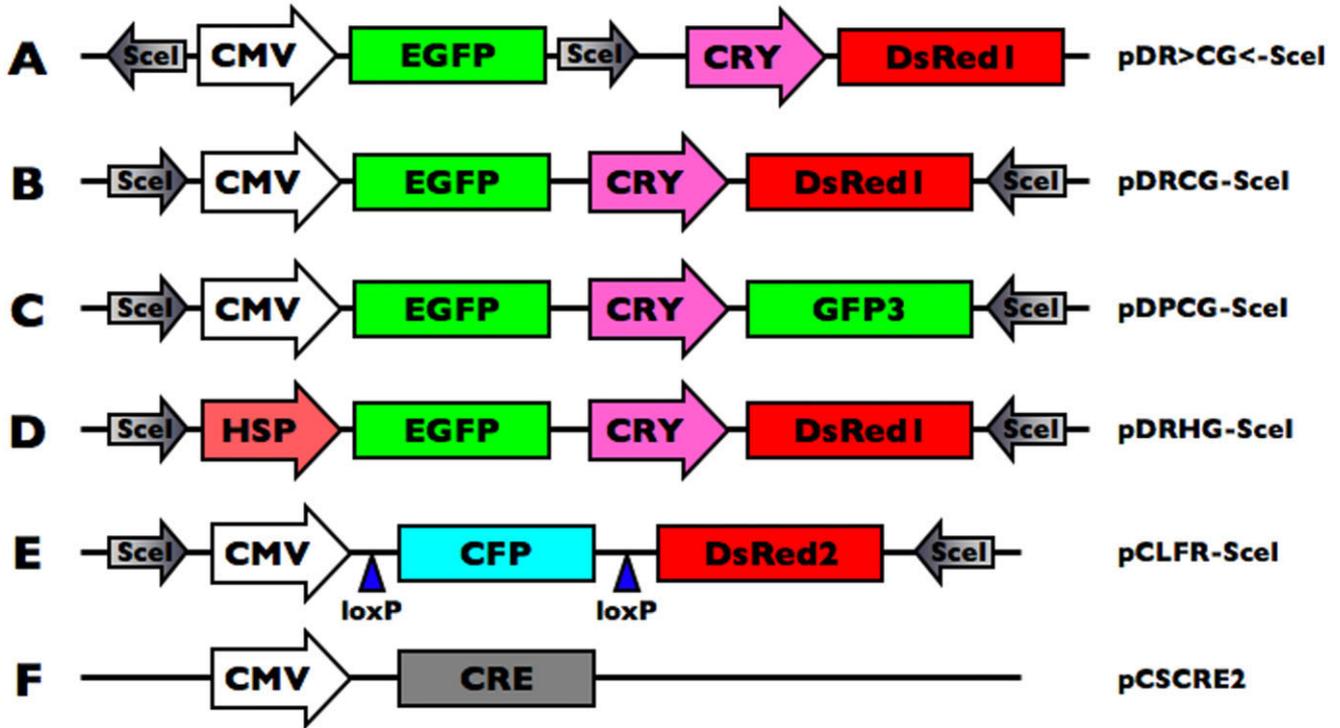


Fig. 1.

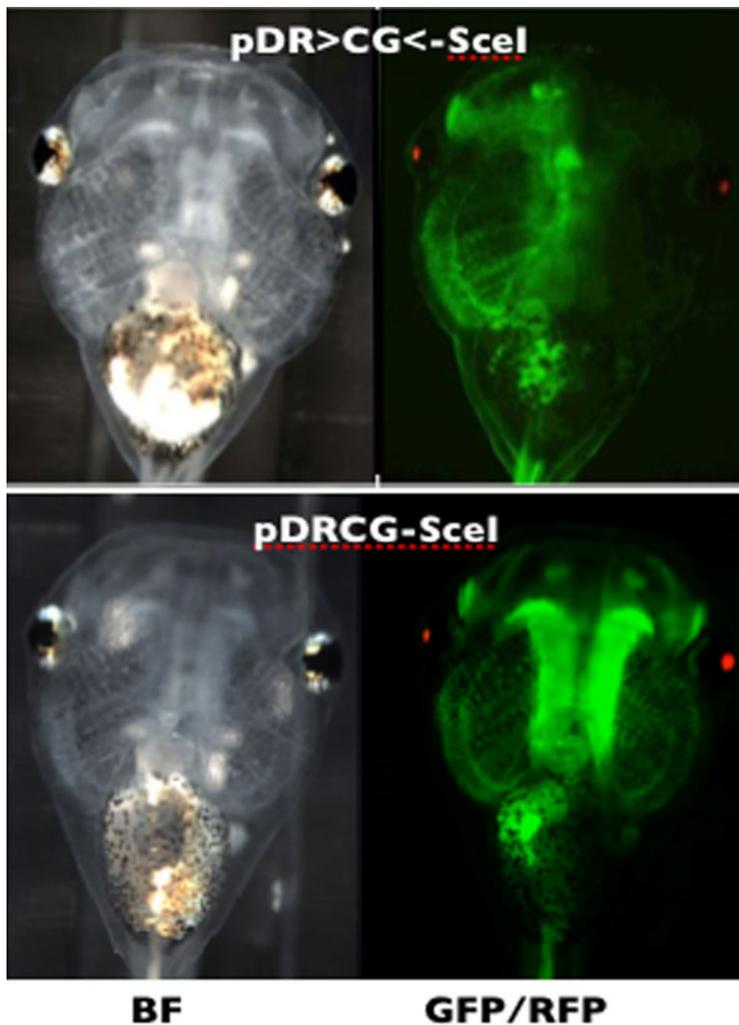


Fig. 2.

Fig. 1. Constructs used for transgenesis. Gray arrows represent 18 base pair *Scel* recognition sequence with arrowhead at larger downstream cleavage product. In pDR>CG<-*Scel*, *Scel* will remain bound to the portion of the plasmid containing CRY:DsRed1 (the “inside” portion) but not CMV:EGFP (the “outside” portion), because downstream ends of *Scel* sites are oriented toward the CRY:DsRed1 cassette. CFP, cyan fluorescent protein; CMV, cytomegalovirus ubiquitous promoter; CRE, Cre recombinase; CRY, gamma crystallin lens-specific promoter; DsRed, *Discosoma sp* red fluorescent protein; EGFP, enhanced green fluorescent protein; GFP3, green fluorescent protein 3; HSP, heat shock-inducible promoter; loxP, Cre recombinase recognition site; YFP, yellow fluorescent protein.

Fig. 2. Exemplar tadpole fluorescence patterns. **Top:** Tadpole injected with pDR>CG<-*Scel* is fully transgenic for CRY:DsRed1 cassette and half transgenic for CMV:GFP cassette. See text for alternate possibilities. **Bottom:** Tadpole fully transgenic for pDRCG-*Scel*. BF, brightfield image; GFP/RFP, merged image from green and red filter sets. CMV, cytomegalovirus ubiquitous promoter; CRY, gamma crystallin lens-specific promoter; DsRed, *Discosoma sp* red fluorescent protein; GFP, green fluorescent protein.

TABLE 1. Transgenesis Conditions and Results

Row	Construct	DNA/embryo	Buffer	Timing	Full Tg	Half Tg	Other Tg	Non-Tg
1	pDR>CG<SceI	40 pg	1.0×	0-60	30% (14)	28% (13)	37% (17) ^a	4% (2)
2	pDRCG-SceI	40 pg	1.0×	0-60	27% (25)	26% (24)	20% (19) ^b	27% (25)
3	pDRCG-SceI	80 pg	1.0×	0-60	20% (10)	22% (11)	47% (24) ^b	12% (6)
4	pDPCG-SceI	40 pg	0.5×	0-60	29% (27)	27% (25)	31% (29) ^c	14% (12)
5	pDPCG-SceI	60 pg	0.5×	0-60	46% (28)	28% (17)	15% (9) ^c	11% (7)
6	pDRCG-SceI	40 pg	0.5×	0-30	26% (5)	47% (9)	5% (1) ^d	21% (4)
7	pDRCG-SceI	40 pg	0.5×	30-60	17% (6)	31% (11)	14% (5) ^d	39% (14)
8	pDRCG-SceI ^e	40 pg	0.5×	0-60	20% (11)	36% (20)	11% (6) ^d	33% (18)
9	pDRCG-SceI	40 pg	1.0×	0-60	6% (5)	30% (23)	30% (23) ^d	34% (26)
10	pDRHG-SceI ^f	40 pg	1.0×	0-60	17% (4)	26% (6)	22% (5) ^g	35% (8)
11	pCLFR-SceI	40 pg	1.0×	0-60	21% (25)	56% (69)	15% (18) ^h	7% (8)
12	pCLFR-SceI + Cre mRNA	40 pg + 200 ng	1.0×	0-60	9% (9)	51% (53)	25% (26) ⁱ	15% (15)
					5 without red 4 with red	34 without red 19 with red		

Embryos were scored between NF40 and NF46. Shading delineates separate experiments.

^aIncludes 3 with 1 red eye only, 1 with 2 red eyes only, 7 with 2 red eyes and half green, 6 with 1 or 2 red eyes, green episomal.

^bIncludes but is not limited to 1 red eye with all green body, no red eye with half or all green body.

^cIncludes 2 green eyes only, 1 green eye, 2 green eyes with half green body.

^dIncludes red or green only on one or both sides.

^eThis row is summation of previous two rows.

^fHeat shocked for 5 min. at 33°C on day 14 and for 1 hr at 33°C on day 15.

^gIncludes 3 with one red eye only, 1 with full green body only, and 1 with half green body only.

^hNon-uniform mosaic CFP expression, most commonly in a handful of tail muscle cells.

ⁱNon-uniform mosaic CFP and DsRed2 expression.

Because DNA quantity has a major effect on tadpole survival and transgenesis efficiency (Ogino et al., 2006), we examined these effects using two transgenesis constructs pDRCG-SceI and pDPCG-SceI, which differ only in the eye color marker for transgenesis (Fig. 1B,C). pDPCG-SceI has two GFP genes, one controlled by the ubiquitous promoter CMV and the other by the lens-specific promoter gamma-crystallin. GFP expression from CMV in the eyes is weaker compared with the gamma-crystallin promoter (unpublished data), but distinguishing them is not a requirement for our purpose here. For pDRCG-SceI, we injected 40 vs. 80 pg per zygote and found reduced tadpole survival at stage NF45 at the higher DNA quantity: 95% survival (n = 122 embryos) for uninjected, 70% (n = 141) for 40 pg, and 46% (n = 128) for 80 pg. For pDPCG-SceI, we observed a similar pattern with the higher DNA amount (60 pg) reducing survival: 89% (n = 109) for uninjected, 87% (n = 107) for 60 pg, and 61% (n = 100) for 40 pg. After 2 weeks of development, we scored transgenesis efficiency in the tadpoles (Table 1, rows 2–5). As previously reported (Ogino et al., 2006;

Pan et al., 2006), we observed a high rate of transgene integration into one-cell of the two-cell stage (“half transgenics”). The higher 80 pg did not increase the percentage of transgenic embryos with consistent transgene expression but, rather resulted in more embryos being categorized as “Other” because of mosaic expression patterns, perhaps due to episomal expression (Table 1, compare rows 2 and 3). The 60-pg injections resulted in higher transgenesis efficiency compared with 40 pg and appears best overall, suggesting that DNA amount per embryo results in a trade-off between mortality and DNA integration efficiency (Table 1, rows 4 and 5). Fluorescence from DsRed1 protein was delayed by at least 2 days relative to when the crystallin promoter is known to be expressed, and often only a streak of red was observed in the eyes. This delay was probably owing to the longer maturation time of this protein, and thus DsRed1 is regarded as inferior to GFP3 as a transgenesis marker.

Because a significant portion of the transgenic animals are half transgenic, we tested whether injecting during the first half of the first cell

cycle improves the efficiency of production of full transgenics. At the same time, we tested whether buffer concentration (1× vs. 0.5×) affects transgenesis efficiency. Injecting early in the cell division increased the frequency of full and half transgenics (26% and 47% in the first 30 min vs. 17% and 31% in the second 30 min; Table 1, rows 6 and 7). The 0.5× buffer concentration improved the transgenesis efficiency (56% in 0.5× vs. 36% in 1×; Table 1, rows 8 and 9), unlike the previous report where buffer concentration made little difference (Ogino et al., 2006). Additional data are required to explain this discrepancy, but the available data attest to the robustness of the method to readily produce large numbers of transgenic tadpoles. These data also suggest that injection within the first 30 min of fertilization improves the chances that the transgene integrates into the genome.

We next verified that the meganuclease method also works well with the heat-shock inducible promoter using the construct, pDRHG-SceI (Fig. 1D; Fu et al., 2002). This construct still had the less reliable CRY:DsRed1 transgenesis marker, so we randomly

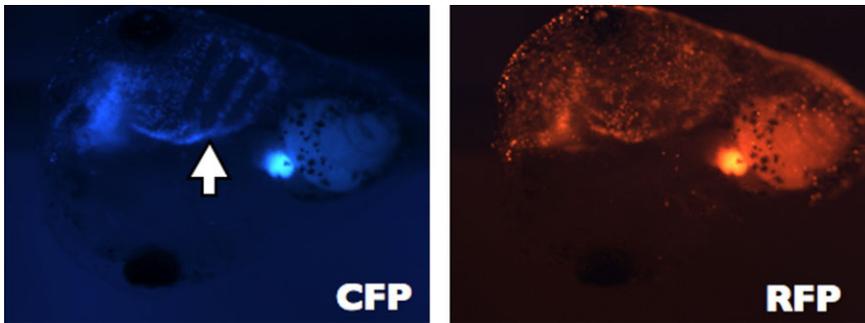


Fig. 3.

Fig. 3. Cre activity in pCLFR-SceI in F0 transgenic tadpole. Tadpole subjected to meganuclease injection followed by Cre mRNA injection at the one-cell stage is half transgenic for pCLFR-SceI as visualized in the CFP filter set. Red fluorescence in the RFP filter set indicates Cre activity removed the CFP to allow DsRed2 expression. The CFP expression is not completely extinguished, especially in the gill cartilages (arrow) for unknown reasons. No embryos lacking Cre mRNA injection showed red fluorescence, indicating that Cre expression is required to allow DsRed2 expression. The strong blue signal in the head region is likely from episomal expression. Autofluorescence can be seen in the intestinal coils and gall bladder. CFP, cyan fluorescent protein; DsRed2, *Discosoma sp* red fluorescent protein; RFP, red fluorescent protein.

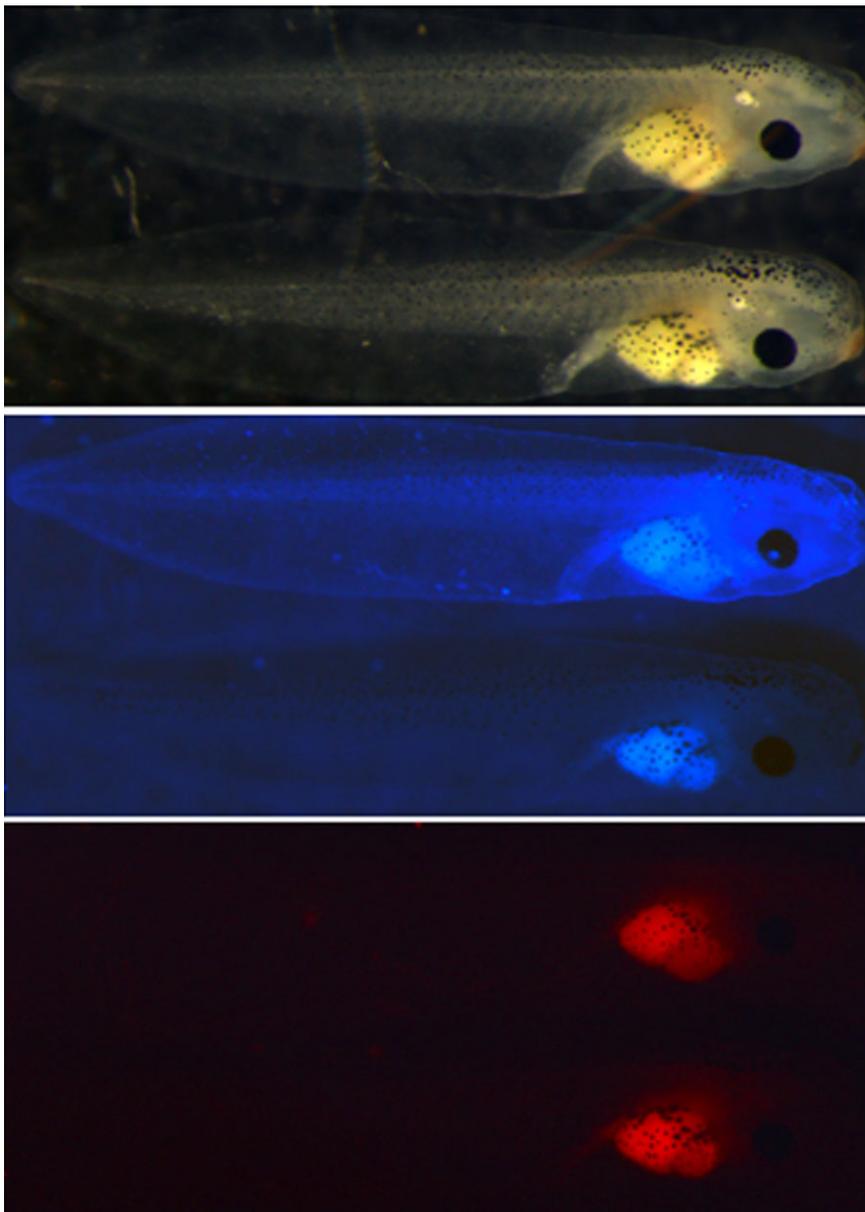


Fig. 4. F1 offspring of pCLFR-SceI founder. Brightfield (top), blue fluorescence (middle), and red fluorescence (bottom) showing transgenic and nontransgenic siblings from Male #4. No red fluorescence was seen in F1 offspring in the absence of Cre activity.

selected 23 of 36 tadpoles that underwent the meganuclease procedure without regard for eye color but lacked green in the body and heat shocked them for two days (Fu et al., 2002). The 13 non-heat-shocked tadpoles still lacked green, but 52% (12 of 23) in the heat shock group had half or full green bodies indicating heat-shock inducibility using this method (Table 1, row 10). Of the heat-shocked tadpoles, a majority gave the expected correlation of red eyes with green body (full and half transgenics, Table 1, Row 10), but three tadpoles had red eyes and no green in the body after heat shock, suggesting integration site variation in transgene expressibility.

Production and Testing of CRE Reporter Lines

We modified the original plasmid pCLFY (Ryffel et al., 2003) by including the *SceI* sites and changing the fluorophores. We changed the fluorophores because CFP is visible in the most common CFP and GFP filter sets, whereas YFP is visible only in the GFP filter set (Ryffel et al., 2003; Waldner et al., 2006). Thus, fluorescence in the CFP filter set would prove presence of CFP, but fluorescence in the GFP filter set could be CFP, YFP, or both. Thus, we replaced YFP with DsRed2 (matures faster than DsRed1 according to the manufacturer) to avoid fluorophore ambiguity. We performed meganuclease with the modified plasmid pCLFR-*SceI* (Fig. 1E) or pCLFR-*SceI* and Cre mRNA. Tadpoles injected with only pCLFR-*SceI* resulted in the expected proportions of

TABLE 2. Germline Transmission and Cre-Sensitivity of pCLFR-*SceI* Founders

pCLFR- <i>SceI</i>	% Blue body ^a (total)	Cre-sensitive?	Strong blue?
Male #1	45% (120), 27% (26)	Yes	No
Male #3	0% (of 110)	Yes ^b	No
Male #4	31% (27)	Yes	Yes
Male #5	42% (53), 44% (28), 53% (42), 49% (38)	Yes	Yes
Female #1	21% (16), 19% (19)	Yes ^c	Yes

Transgenic founders were bred to wild-type partners, and tadpoles were scored between NF40 and NF45. Injections of Cre mRNA occurred at 2- to 32-cell stage. Multiple matings of founders from one to 4 times are listed in the second column.

^aNo red was observed in absence of Cre.

^bTwo of nine survivors after Cre mRNA injection turned bright blue, but not red.

^cPreliminary data using transgenic Cre constructs injected into F1 offspring gave some red cells indicating Cre sensitivity.

full and half transgenics (Table 1, row 11), and importantly had no DsRed2 expression. Surprisingly, only 30% of the full transgenics and 50% of the half transgenics expressed DsRed2 in the animals also receiving Cre mRNA (Fig. 3, Table 1, row 12). This result suggests variability in access to loxP sites on pCLFR-*SceI* likely due to insertion site variation and indicates the need to characterize founders.

To develop a high expression, Cre-sensitive pCLFR-*SceI* reporter line, the strongest blue F0 tadpoles were reared to adulthood and outcrossed to wild type animals to check for germ line transmission. We tested five independent founders for transgene expression levels and Cre-sensitivity in their F1 progeny. Germline transmission frequency was 50% or less, such that a single insertion site and/or epigenetic effects, such as transgene silencing in some of the offspring, could account for the transmission frequencies (Fig. 4; Table 2). Mosaicism in the germ line seems less likely to account for the low transmission frequency because the founders were uniformly blue throughout the body. Cre mRNA was injected to test for recombination ability, and offspring from all but one founder changed from blue to red (Fig. 5A; Table 2). The exception (Male #3), which remains unexplained, had no blue offspring, but when injected with Cre, the offspring became strongly blue with no red. The multiple matings indicated in Table 2 show similar frequency of germline transmission for Male #5 and Female #1. For Male #1, the two different transmission frequencies could be an artifact of the low sample size for the second mating or possible silencing of transgene expres-

sion in the offspring dependent on genetic background of mating partners.

Cre activity on the transgene results in a permanent genetic change in that cell. Because Cre mRNA can be injected into blastomeres or expressed tissue-specifically by developmentally regulated promoters, subsets of cell populations can be followed indefinitely throughout development. We tested the ability to mark a defined cell lineage, A1 blastomeres, which give rise to anterior structures, mostly the brain and epidermis and to a much lesser extent spinal cord and head mesenchyme (Fig. 5B; Dale and Slack, 1987). This feature of permanence is an advantage compared with injecting lineage tracing dyes which get diluted with each cell division during development. Also, transgenic expression of Cre in a pCLFR-*SceI* background was examined by mixing a *SceI* digest of pCLFR-*SceI* with pCSCRE2 (Fig. 1F). This co-injection procedure resulted in animals transgenic for pCLFR-*SceI* and non-integrated mosaic expression of Cre. Only when both pCLFR-*SceI* and pCSCRE2 were co-injected were red cells observed (Fig. 6).

In conclusion, we found the meganuclease method a robust procedure for production of transgenic frogs expressing fluorescent proteins. We used this procedure to make and characterize pCLFR-*SceI* transgenic lines that can serve as an improved reporter for Cre recombinase activity. The use of CFP and DsRed2 is advantageous over CFP and YFP for visual observation because they do not bleed into each other's filter set and for histological analysis because they differ at the mRNA and protein levels so that distinction between them using in situ

hybridization and antibody detection is possible. Cre can be introduced by means of mRNA injection in the embryo to enable lineage tracing from blastomeres beyond early organogenesis to the feeding tadpole through metamorphosis to adulthood. Furthermore, expression of CRE using tissue-specific promoters will enable detailed fate mapping for all cell-types for which transgenic promoters are developed. We have 15–20 F1 adults from each pCLFR-*SceI* transgenic line, and these lines are freely available to the frog community.

EXPERIMENTAL PROCEDURES

Plasmid Construction

The transgenesis plasmid pDR>CG <*SceI* was made by polymerase chain reaction (PCR) amplification using the primers DRB134 5' GGGGGTTCGACATTACCCTGTTATCCCTACATAGCC-AATCAAT-ATGGCGTAT and DRB135 5' GGGGGCGGCCGCATTACCCTGTTATCCCTAGAAT-TAAAAAACCTCCCA-CACCTC on the template pDRCG (gift from L. Fu and Y.-B. Shi) and then cloning the *SalI/NotI*-digested PCR product back into pDRCG digested with the same enzymes. To make pDRHG-*SceI*, the primers I-*SceI*-HSP-F 5' > AATGTCGA-CTAGGGATAACAGGG-TAATGGTATCGATAAAGCTTCGAG-AAAGCTCG and I-*SceI*-CrySV40-R 5' AATACGCGTTAGGGATAACAGGG-TAATTATGACCATGATTACGC-CAAGCGCGCG were used on the template pDRHG (gift from L. Fu and Y.-B. Shi). The PCR product was digested with *SalI* and *MluI* and inserted into pDRHG digested with *SalI*

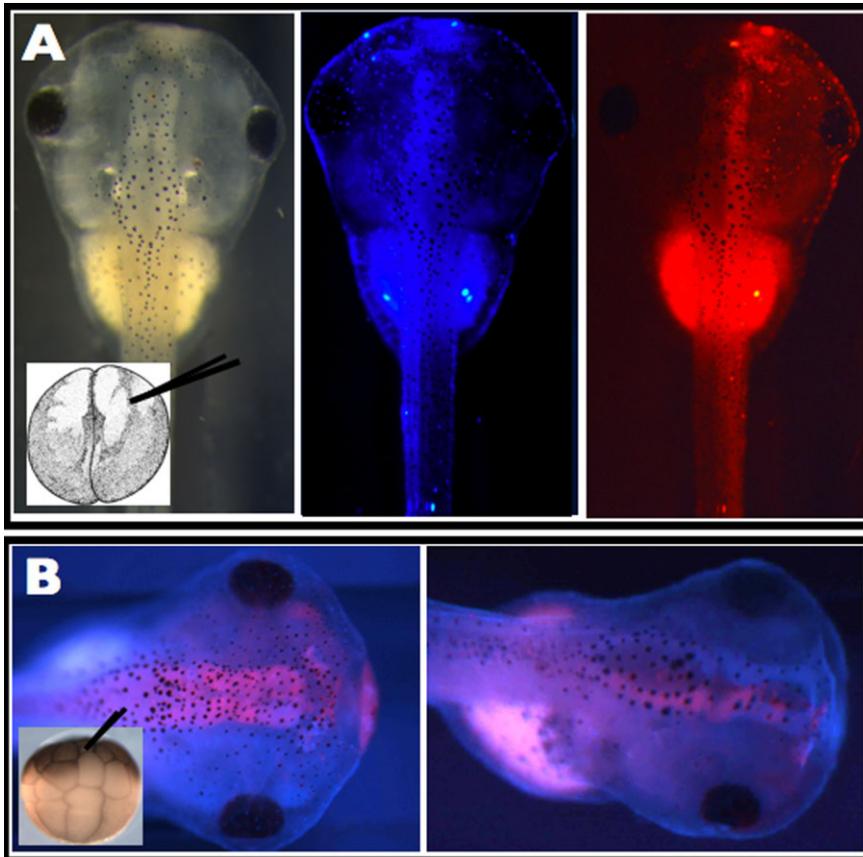


Fig. 5. Lineage tracing by means of Cre mRNA injection into pCLFR-SceI transgenic embryos. A,B: Cre mRNA was injected into one cell of two-cell stage embryos (A) or the A1 blastomeres of the 32-cell stage embryo (B). **A:** Brightfield, CFP, and RFP images show half of the body is exclusively blue, and the other half is mostly red with a few blue cells. The replacement of blue by red fluorescence is particularly evident in brain and tail. **B:** Two examples of tadpoles showing results when both (left) or just one (right) A1 blastomere was injected with Cre mRNA. Intestine contains yolk autofluorescent in both filter sets. Insets show diagrams of injections. CFP, cyan fluorescent protein; RFP, red fluorescent protein.

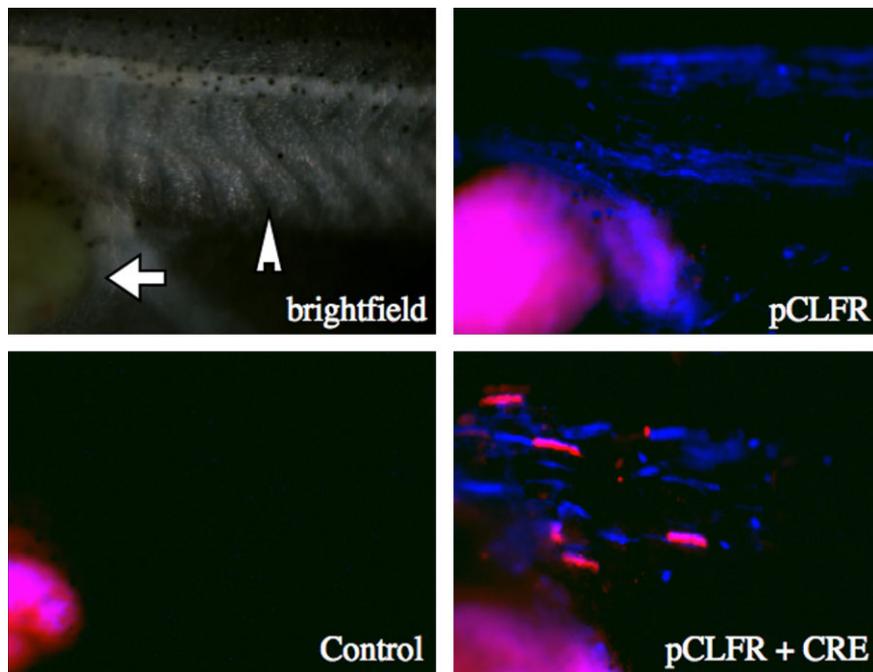


Fig. 6.

and *Bss*HII. To make pDRCG-*SceI*, the primers DRB132 5' GGGGGTCGACTA-GGGTAACAGGGTAATCATAGCCAAT-TCAATATGGCGTAT and I-*SceI*-*CryS*-V40-R were used on the template pDRCG. The PCR product was digested with *SalI* and *MluI* and inserted into pDRCG digested with *SalI* and *Bss*HII. For pDPCG-*SceI*, the *CRY:GFP3* cassette from pDPHG (gift from L. Fu and Y.-B. Shi) was excised using *SphI* and *SpeI* and inserted into pDRCG-*SceI* digested with the same enzymes. The plasmids pCLFY and pCSCRE2 were gifts (C. Walden and G. Ryffel). A *DsRed2*-SV40 polyA fragment amplified by PCR from *DsRed2*-N1 (Clontech) using primers DRB84 5' CATCATCCCG-GGACCG-GTCGCCACCATGGCCTCCTCCGA and DRB85 5' CATCATCCGCGGGCG-GCCGCGCAGTGAAAAAATGCTT-TAT was cloned into *SmaI* and *SacII* sites of pCLFY to make pCLFR. The *Hin*dIII(blunt) - *Bss*HII fragment from pCLFR was cloned into the *AgeI*(blunt) - *Bss*HII sites of pDPCG-*SceI* to make pCLFR-*SceI*. A CRE recombinase fragment amplified by PCR from pCSCRE2 using DRB192 5' CATCATGAATTC-ATGTCCAATTTACTGACCGTACACC and DRB193 5' CATCATGAATTC-CTATAGTTCTAGAGAACCATCGC-CAT was cloned into the *EcoRI* site of pSP64RI (gift from S. Sato) to make pSP64-CRE for mRNA injections. All clones made using PCR were sequence verified.

Transgenesis, mRNA Injections, Transgene Expression

Injections and *SceI* reaction conditions were based on the original de-

Fig. 6. Transgenic expression of Cre in pCLFR-*SceI* induces blue to red conversion. Meganuclease transgenesis procedure was performed with co-injection of pCSCRE2 lacking *SceI* sites to allow mosaic expression of Cre. Brightfield image shows mid-section of tadpole with portions of abdomen (arrow) and tail (arrow head) representing region of tadpole shown in all panels. Control tadpole lacks transgenic fluorescence and only autofluorescent yolk is seen. The pCLFR-*SceI* tadpole shows blue only, whereas mosaic transgenic expression of Cre resulted in several cells showing red fluorescence indicative of Cre-mediated switch from CFP to *DsRed2* expression. CFP, cyan fluorescent protein; *DsRed2*, *Discosoma sp* red fluorescent protein.

scriptions (Ogino et al., 2006; Pan et al., 2006). The *SceI* reaction (200 ng of DNA, 2.3 μ l of *SceI* buffer, 2.3 μ l of 10 \times bovine serum albumin, 2 μ l of *SceI* in 23 μ l of total volume of the reaction mixture) was incubated at 37°C for 40 min then injected into de-jellied zygotes at room temperature, resulting in 2×10^{-3} U of *SceI* and 40 pg of DNA per embryo with a 4- to 10-nl injection using NanoJect injection apparatus (Drummond) or Pico-spritzer III (Parker Hannifin Corp). Reaction conditions, when altered, are noted in Table 1. Embryos were cultured at 18°C and sorted to remove dead or dying embryos 1–2 times per day. Feeding tadpoles were reared at 26–28°C for observation and founders were returned to 18°C for breeding after sexual maturity after 12–18 months. *SceI* was treated like any other restriction enzyme, and no special storage of *SceI* was done. Specifically, we stored *SceI* at –20°C without aliquoting and kept the enzyme on ice during use. In our hands, *SceI* was stable under these conditions at least one year of frequent use. For testing Cre sensitivity, Cre mRNA (Ambion mMessage mMachine) was injected at the one- to two-cell stage (200 pg/cell) in F1 embryos or subsequent to meganuclease injections. For blastomere lineage tracing, Cre mRNA was injected at the 16–32 cell stage (50 pg/cell) in F1 embryos. Transgene expression was observed using a Leica fluorescence dissection microscope with CFP, GFP2, and RFP filter sets.

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