FLP and Cre recombinase function in *Xenopus* embryos

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ABSTRACT

The use of the site-specific DNA recombinases FLP and Cre is well-established in a broad range of organisms. Here we investigate the applicability of both recombinases to the *Xenopus* system where they have not been analyzed yet. We show that injection of FLP mRNA triggers the excision of an FLP recombination target (FRT)-flanked green fluorescent protein (GFP) sequence in a coinjected reporter construct inducing the expression of a downstream β-galactosidase gene (*lacZ*). The FLP-mediated gene activation can be controlled in *Xenopus* embryos by injecting a mRNA encoding a fusion of FLP to the mutant ligand binding domain of the human estrogen receptor whose activity is dependent on 4-hydroxytamoxifen. We also demonstrate that a Cre reporter injected into fertilized eggs is fully recombined by Cre recombinase before zygotic gene transcription initiates. Our results indicate that in *Xenopus* embryos Cre is more effective than FLP in recombining a given quantity of reporter molecules. Finally, we present FLP-inducible double reporter systems encoding two fluorescence proteins (EYFP, ECFP, DsRed or GFP). These novel gene expression systems enable the continuous analysis of two reporter activities within living embryos and are expected to allow cell-lineage studies based on recombinase-mediated DNA rearrangement in transgenic *Xenopus* lines.

INTRODUCTION

A complex network of interactions between distinct gene activities determines the pattern of gene expression in a developing organism. The basic principles involved in these developmental processes have been conserved to a large degree during evolution. To analyze the mechanisms of early development in vertebrates, the frog *Xenopus* is a most attractive model, as in this amphibian species the development occurs outside of the female and thus can easily be observed and manipulated. Furthermore, *Xenopus* embryos are available in large amounts and require a relatively low infrastructure for breeding. Recently, the attractiveness of *Xenopus* has been further increased by the ability to generate transgenic frogs (1–3), allowing gene manipulation to analyze the molecular basis of organogenesis in vertebrates in detail.

In mice and mammalian cell cultures, the use of site-specific DNA recombinases, such as FLP from *Saccharomyces cerevisiae* and Cre from bacteriophage P1, to manipulate genomic DNA by DNA rearrangement is a well-established approach (4). Both FLP and Cre recombinases can be applied to alter gene structures to either ablate or turn on gene activities. An *in vitro* comparative analysis of FLP and Cre has shown that FLP is more thermolabile than Cre (5). Furthermore, the lower DNA binding capacity of FLP requires more FLP than Cre to excise a given amount of recombination substrate (6). The significance of site-specific DNA recombinases has been further increased by the finding that the time point of a desired recombination event can be regulated within the living organism by expressing DNA recombinases whose activity is dependent on synthetic steroid hormones (7–9). Thus, the ligand-regulated DNA recombinases FLPER(T) and CreER(T), which have recently been obtained by fusing the recombinase open reading frame to a mutated form of the human estrogen receptor (ER) ligand binding domain (LBD), are inducible by the synthetic anti-estrogen 4-hydroxytamoxifen, but do not respond to the natural ligand estrogen (9,10).

In the present communication we demonstrate that both constitutive FLP and Cre recombinase as well as tamoxifen-regulated FLPER(T) recombinase function effectively in *Xenopus*, providing powerful tools to induce gene expression in *Xenopus* embryos. In addition, we present FLP inducible double reporter systems allowing the simultaneous analysis of two fluorescent proteins within living *Xenopus* embryos.

MATERIALS AND METHODS

Plasmids

The *pac* gene in pSVpaZ11 (5) was replaced by a green fluorescent protein (GFP) gene derived as a *Hin*dIII–*Xba*I fragment from plasmid pCSGFP-2 (3). Subsequently, the FRT-GFP-EpA-FRT-lacZ-LpA cassette was excised with *Avr*II/*Not*I and inserted between the *Xba*I and *Not*I sites of pCS2+ (11) yielding plasmid pCMV:GFP(FRT)lacZ. In analogy, pCMV:GFP(loxP)lacZ was constructed based on pSVpaX1 (5). The FLP reporters encoding different fluorescent proteins [pCMV:EYFP(FRT)ECFP in Fig. 3A; pCMV:ECFP(FRT)EYFP in Fig. 3B; pCMV:DsRed(FRT)GFP in Fig 3C; pCMV:GFP(FRT)DsRed in Fig. 3D] are based on the pCMV:GFP(FRT)lacZ reporter. The GFP cassette was replaced

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by a *Hin*dIII–*Xba*I fragment encoding either the EYFP gene from pEYFP-N3 (Clontech), the ECFP gene from pECFP-N3 (Clontech) or the DsRed gene from pDsRed1-N1 (Clontech), whereas the *lacZ* sequence was replaced by a *Bam*HI–*Xba*I fragment encoding ECFP, EYFP, GFP or DsRed. These *Bam*HI–*Xba*I fragments were generated by subcloning the fluorescent proteins into pCS2+ (11). pCSFLPe was constructed by cloning a 1.5 kb fragment of pOGFlpe6 (12), containing the FLPe coding region, into *Bam*HI/*Xho*I excised pCS2+ (11). pCSFLPER(T) was constructed by cloning a 2.4 kb fragment of p22LFE1 (10), containing the FLPER(T) coding region, into the *Bam*HI and *Eco*RI sites of pCS2+ (11). pCSCre was constructed by cloning an *Eco*RI–*Not*I fragment of Cre-pBSSKII (a kind gift of T.Schweer, Essen) containing the Cre/pA gene into pCS2+ (11). The vector pCSCreER(T) encoding the 4-hydroxytamoxifen regulated Cre was generated by inserting the *Eco*RI fragment of pCre-ER(T) (9) into pCS2+ (11).

Preparation of synthetic mRNA

pCSCreER(T), pCSFLPe and pCSFLPER(T) were linearized with *Pvu*II, pCSCre was linearized with *Not*I. Capped synthetic mRNA was transcribed using SP6 polymerase (13).

Embryos and microinjections

Xenopus laevis embryos were obtained by standard procedures and staged according to Nieuwkoop and Faber (14). Synthetic mRNA (3.5 ng) was microinjected either exclusively or in combination with reporter DNA (200 pg) into one blastomere of two cell stage embryos derived from *in vitro* fertilized eggs. Embryos were cultured in 0.1× MBS(15) at 20.5°C. For 4-hydroxytamoxifen treatment embryos were cultured in 0.1× MBS containing 1 µM 4-hydroxytamoxifen (Sigma).

X-gal staining

For X-gal staining, larvae were fixed for 15 min at room temperature in 50 mM Na-cacodylate (pH 7.3) containing 1% glutaraldehyde. Embryos were washed for 5 min in Fe/Na-phosphate solution [7.2 mM Na_2HPO_4, 2.8 mM NaH_2PO_4, 150 mM NaCl, 1 mM $MgCl_2$, 3 mM $K_3(Fe(CN)_6)$ 3 mM $K_4(Fe(CN)_6)$, pH 7.2]. Subsequently, embryos were stained for 2 h at 37°C by using fresh Fe/Na-phosphate solution containing 0.1% Triton X-100 and 0.027% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), refixed for 1 h in MEMFA (0.1 M MOPS, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde, pH 7.4) and stored in 100% methanol.

Microscopy

Living and fixed larvae were analyzed with the Leica MZ/FLIII stereomicroscope and photographed with the KAPPA camera PS 30 C (KAPPA opto-electronics GmbH, Gleichen) except for the embryos shown in Figure 2 which were photographed with the KAPPA camera CF 15/4 MC(C) (KAPPA opto-electronics GmbH). Fluorescence was monitored using a 425/475 nm filter set to detect ECFP, a 480/510 nm filter set to detect either GFP or EYFP and a 546/590 nm filter set to detect DsRed. Embryos analyzed with distinct filter sets were photographed with identical exposure times. Embryos analyzed at normal light and embryos analyzed upon X-gal staining were photographed without any filter.

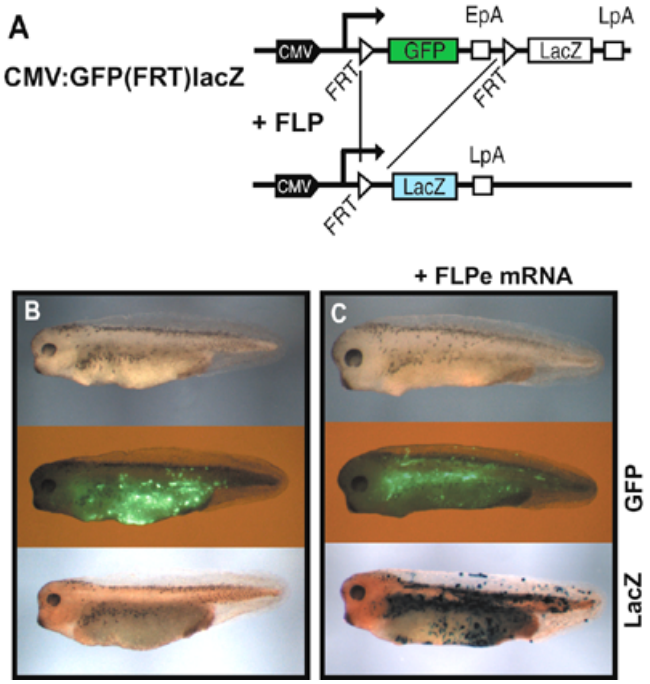

Figure 1. Constitutive FLP activity in *Xenopus* embryos. (**A**) The FLP reporter construct CMV:GFP(FRT)lacZ is schematically drawn with the CMV promoter/enhancer driving the expression of the GFP and *lacZ* genes. The position of the SV40 early (EpA) and late (LpA) polyadenylation signals as well as the FRT target sites of the FLP are given. (**B** and **C**) Stage 36 *Xenopus* larvae were derived from eggs injected with CMV:GFP(FRT)lacZ DNA (B) or coinjected with CMV:GFP(FRT)lacZ DNA and FLPe mRNA (C). The living animals were photographed in normal light (top) or in fluorescence light (middle). X-gal staining was obtained after fixation of the larvae (bottom).

RESULTS AND DISCUSSION

To test the ability of FLP to function in *Xenopus* embryos, we designed the FLP inducible gene expression system shown in Figure 1A. In the construct CMV:GFP(FRT)lacZ the human cytomegalovirus (CMV) early enhancer/promoter drives the expression of a marker gene encoding GFP which can readily be monitored in living embryos by fluorescence microscopy (16). The SV40 early poly(A) region (EpA) terminates the transcription, thus blocking the expression of the downstream inducible marker gene β-galactosidase (*lacZ*). The GFP coding sequence and the EpA that are placed between two FLP recombination targets (FRTs) can be excised by FLP-mediated DNA recombination, inducing CMV driven expression of *lacZ*. The enzymatic activity of LacZ is detectable by X-gal staining on fixed *Xenopus* larvae (16). To introduce FLP recombinase into *Xenopus* embryos we injected synthetic FLP mRNA into fertilized eggs, a most efficient way to get early expression of a exogenous protein, as translation occurs immediately after fertilization, whereas transcription starts several hours later at mid blastula transition when zygotic gene activation initiates (17). As wild-type FLP is thermosensitive (5) we used the variant FLPe which shows enhanced thermostability *in vitro* as well as *in vivo* (12).

Figure 1B illustrates an example of stage 36 larvae (14) derived from a fertilized *Xenopus* egg injected with the FLP

Table 1. Efficiency of FLP and Cre mediated DNA recombination in *Xenopus* embryos

Recombinase reporter	Recombinase mRNA	4-OH-tamoxifen	GFP		LacZ	
			Positive	Negative	Positive	Negative
CMV:GFP(FRT)lacZ	–	–	35	0	0	35
	FLPe	–	21	9	21	9
CMV:GFP(FRT)lacZ	FLPER(T)	–	11	0	0	11
	FLPER(T)	+	28	0	17	11
CMV:GFP(loxP)lacZ	–	–	20	0	0	20
	Cre	–	0	86	53	33

The number of *Xenopus* larvae showing green fluorescence and LacZ staining upon injection of reporter DNA and recombinase mRNA is given.

reporter DNA CMV:GFP(FRT)lacZ. The intense green fluorescence detectable under the fluorescence microscope implies a strong expression of GFP. The mosaic expression pattern reflects the distribution of the reporter DNA within the injected embryo (18). X-gal staining on the fixed larvae revealed no β-galactosidase activity. Thus, the polyadenylation signal in front of the *lacZ* cassette proved to be efficient in precluding any activity from the downstream *lacZ* gene. In contrast, Figure 1C shows an example of larvae derived from an egg coinjected with FLP reporter DNA and FLPe mRNA. Within this embryo nearly all GFP-positive cells revealed β-galactosidase activity upon X-gal staining, indicating FLPe-mediated DNA recombination. The coexpression of GFP and *lacZ* reflects that the reporter molecules available in a given cell are not fully excised by FLPe action. This may indicate that FLPe-mediated DNA recombination is a relatively slow event, possibly affected by FLPe inactivation due to protein degradation. In conclusion, among 30 embryos coinjected with FLP reporter DNA and FLPe mRNA, we observed 21 larvae expressing GFP. All these GFP-positive embryos revealed β-galactosidase activity upon X-gal staining (Table 1). The residual nine embryos lacking GFP expression revealed no β-galactosidase activity upon X-gal staining, indicating that these larvae are derived from eggs not properly injected (Table 1). Finally, 35 control embryos injected exclusively with FLP reporter DNA showed GFP expression, but no blue cells could be observed upon X-gal staining (Table 1). Thus, our data give strong evidence that FLPe functions effectively and reproducibly in developing *Xenopus* embryos.

To investigate the conditional function of FLP, we used the steroid-regulated DNA recombinase FLPER(T). In FLPER(T), the FLP gene is fused to the G521R mutant LBD of the human ER (10). This mutant LBD has a reduced affinity for estrogen, whereas its affinity for the anti-estrogen 4-hydroxytamoxifen (T) is unchanged. Figure 2A illustrates an example of stage 30 larvae derived from a fertilized *Xenopus* egg coinjected with CMV:GFP(FRT)lacZ reporter DNA and FLPER(T) mRNA. The intense green fluorescence detectable under the fluorescence microscope indicates a strong expression of GFP. On the fixed embryo no β-galactosidase activity could be monitored upon X-gal staining with the exception of a single cell (marked by the arrow in Fig. 2A). In comparison, Figure 2B shows an example of larvae derived from an egg incubated with 4-hydroxytamoxifen upon coinjection of FLP reporter DNA

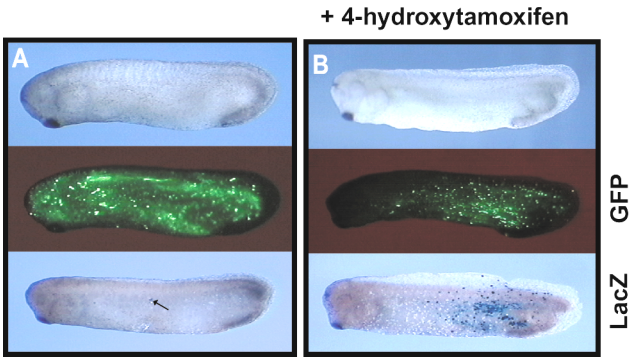

Figure 2. 4-Hydroxytamoxifen-regulated FLP activity in *Xenopus* embryos. (**A** and **B**) Stage 30 *Xenopus* larvae were derived from fertilized eggs coinjected with CMV:GFP(FRT)lacZ DNA and FLPER(T) mRNA. Embryos were cultured either in the absence (A) or in the presence of 4-hydroxytamoxifen (B). The living embryos were photographed in normal light (top) or in fluorescence light (middle). X-gal staining was obtained after fixation of the larvae (bottom).

and FLPER(T) mRNA. In this embryo, the majority of GFP-positive cells revealed β-galactosidase activity upon X-gal staining. By analyzing 28 GFP-positive embryos derived from eggs coinjected with FLP reporter DNA and FLPER(T) mRNA that were cultured in the presence of 4-hydroxytamoxifen, we detected β-galactosidase activity within 17 larvae, whereas no blue cells could be monitored within 11 larvae (Table 1). Moreover, we analyzed 11 GFP-positive control embryos coinjected with FLP reporter DNA and FLPER(T) mRNA which were cultured in the absence of 4-hydroxytamoxifen. X-gal staining on these embryos revealed no β-galactosidase activity in six larvae, whereas in five larvae, single cells (one to five cells) were stained blue, indicating a low background activity of FLPER(T) in the non-induced state.

Our data demonstrate that FLPER(T) successfully induces conditional gene expression in *Xenopus* embryos treated with 4-hydroxytamoxifen. The low background activity of FLPER(T) that is detectable in the absence of the ligand does not affect the identification of 4-hydroxytamoxifen-mediated FLP action. Larvae cultured in the anti-hormone solution up to the feeding larvae stage 45 (14) show normal morphology, indicating that the concentration of the anti-estrogen (10^{-6} M) has no adverse effect on the embryonic development. Never-

theless, only 60% of the injected larvae revealed FLPER(T)-mediated expression of *lacZ* upon the administration of 4-hydroxytamoxifen. This is in contrast to the high efficiency of FLPe-mediated DNA excision shown above, but possibly reflects that the FLP used in FLPER(T) contains the mutation F70L that has recently been shown to decrease FLP activity in comparison with both wild-type FLP and the mutant FLPe (5,12).

When using the FLP inducible reporter system CMV:GFP(FRT)lacZ, embryos have to be fixed to analyze the expression of the *lacZ* gene. To allow the continuous observation of FLP-mediated changes in gene expression, we constructed alternative FLP-inducible reporters encoding two distinct fluorescent proteins. As shown in Figure 3, we combined genes encoding either yellow (EYFP) and cyan (ECFP) fluorescent protein or red (DsRed) and green (GFP) fluorescent protein. The expression of the reporter genes can readily be monitored by fluorescence microscopy on living embryos using different wavelengths to excite the fluorophores, and distinct barrier filters to minimize background fluorescence due to partially overlapping emission spectra. Embryos injected with CMV:EYFP(FRT)ECFP reporter DNA showed a strong expression of EYFP (Fig. 3A). Notably, the yellow fluorescence of EYFP looks very similar to the green fluorescence of GFP, although both proteins are characterized by distinct excitation and emission spectra. Characteristically, the high intensity of the yellow emission causes some bleed-through using the filter set for detecting ECFP (Fig. 3A,

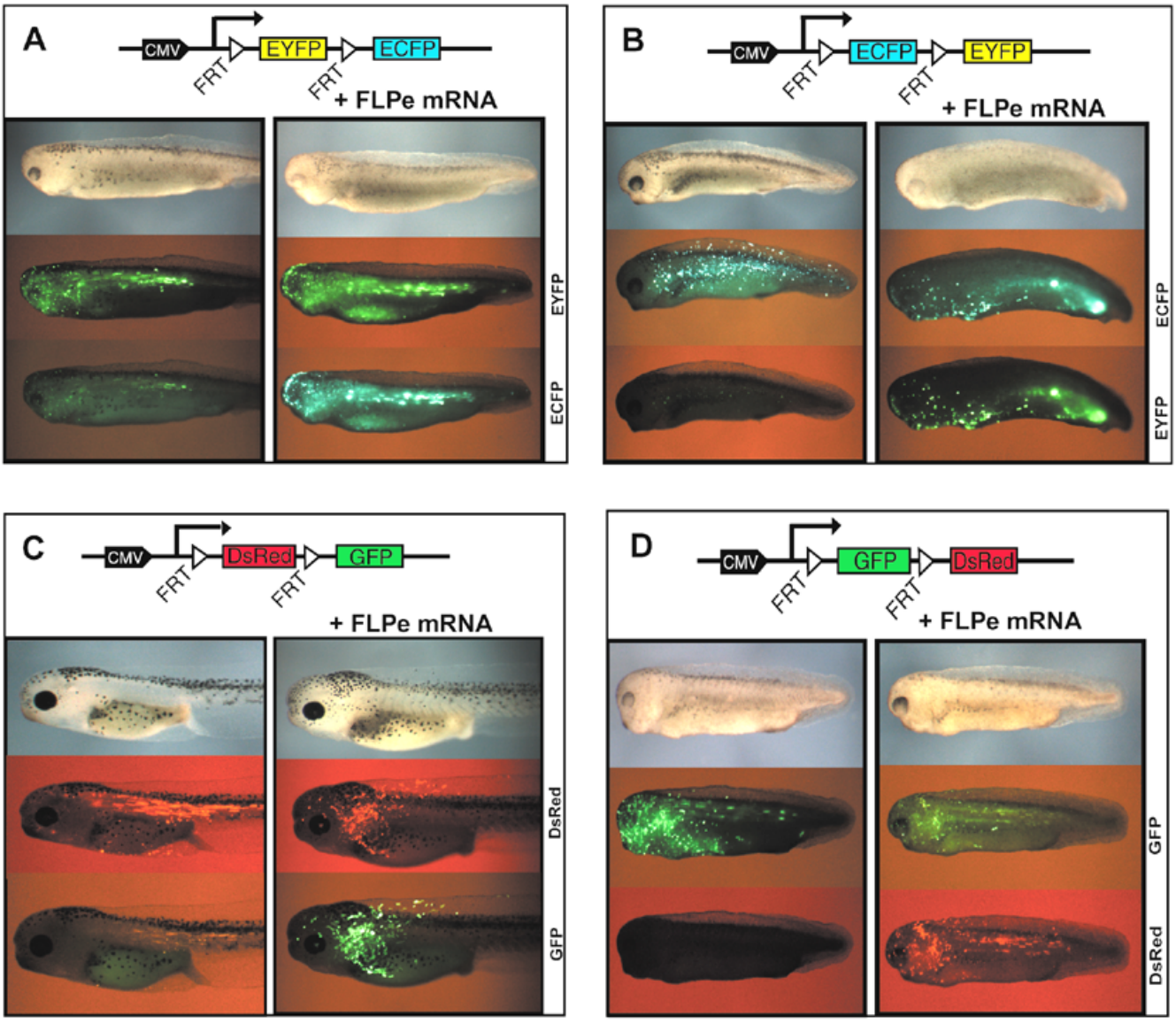

Figure 3. FLP reporters encoding different fluorescent proteins. The FLP inducible reporters CMV:EYFP(FRT)ECFP (**A**), CMV:ECFP(FRT)EYFP (**B**), CMV:DsRed(FRT)GFP (**C**) and CMV:GFP(FRT)DsRed (**D**) are schematically drawn in each panel. *Xenopus* larvae were derived from eggs injected with reporter DNA (left panels) or coinjected with reporter DNA and FLPe mRNA (right panels). The living larvae were photographed in normal light (top picture in each panel) or in fluorescence light using defined filter sets (middle and bottom picture in each panel). The excitation/barrier filters were 480/510 nm for EYFP and GFP, 425/475 nm for ECFP and 546/590 nm for DsRed.

bottom of left panel). In embryos coinjected with CMV:EYFP(FRT)ECFP DNA and FLPe mRNA, most of the EYFP-positive cells revealed in parallel FLPe induced expression of ECFP (Fig. 3A, bottom of right panel). Expression of both EYFP and ECFP could be monitored starting already in the late gastrula (data not shown). In FLPe-induced embryos with high expression levels of the reporter DNA, the cyan fluorescence appeared green due to the background fluorescence of EYFP (data not shown). Commonly, the signal intensity of ECFP is low compared to EYFP. Therefore, we also tested the reporter construct CMV:ECFP(FRT)EYFP combining the two fluorescence markers in reverse order. In embryos coinjected with this reporter DNA and FLPe mRNA, the ECFP-positive cells revealed in parallel FLPe induced expression of EYFP (Fig. 3B, right panel) which was not affected by the background fluorescence of ECFP (Fig. 3B, bottom of left panel). Alternatively, we investigated the combination of a red (DsRed) and green (GFP) fluorescent protein. Embryos injected with CMV:DsRed(FRT)GFP reporter DNA showed a bright red fluorescence indicating strong expression of DsRed. The high intensity of the red emission causes an orange background fluorescence apparent with the filter set for detecting GFP (Fig. 3C, bottom of left panel). In embryos coinjected with reporter DNA and FLPe mRNA, most of the DsRed-positive cells revealed in parallel FLPe induced expression of GFP (Fig. 3C, right panel). FLPe-induced embryos with high expression levels of the reporter DNA showed green fluorescence that appeared yellowish due to the background fluorescence of DsRed (data not shown). To avoid this cross contamination we used the reporter construct CMV:GFP(FRT)DsRed combining the two fluorescence markers in reverse order. In embryos coinjected with this reporter DNA and FLPe mRNA, the GFP-positive cells revealed FLPe-induced expression of DsRed which was never affected by background fluorescence of GFP (Fig. 3D, bottom of left panel). Expression of GFP could be monitored first in the late gastrula (stage 13), whereas red fluorescence could not be detected prior to stage 35 (data not shown). This possibly reflects that the rate of fluorophor formation in DsRed is slow in comparison with the rate of fluorophor formation in GFP, ECFP and EYFP (19). Furthermore, we observed that the yellow–green background fluorescence that is apparent in the gall bladder and the gut of *Xenopus* larvae using filter sets for detecting ECFP, GFP or EYFP is completely absent using the filter set for detecting DsRed.

In conclusion, the FLP inducible double reporter systems presented here allow the detection of two distinct fluorescent signals within a single cell of a living *Xenopus* embryo and additionally offer the possibility of analyzing two gene activities simultaneously. By using the constructs CMV:ECFP(FRT)EYFP and CMV:GFP(FRT)DsRed, the expression of the inducible genes (EYFP and DsRed) is not affected by background fluorescence of the selectable reporters (ECFP and GFP). Thus, these constructs provide powerful tools to generate transgenic reporter lines which can be applied to cell-lineage studies based on FLP-mediated DNA recombination.

To compare the function of FLP and Cre recombinases in *Xenopus* embryos, we designed the Cre inducible reporter CMV:GFP(loxP)lacZ (Fig. 4A) where the two FRTs of the corresponding FLP reporter (Fig. 1) are substituted by two Cre

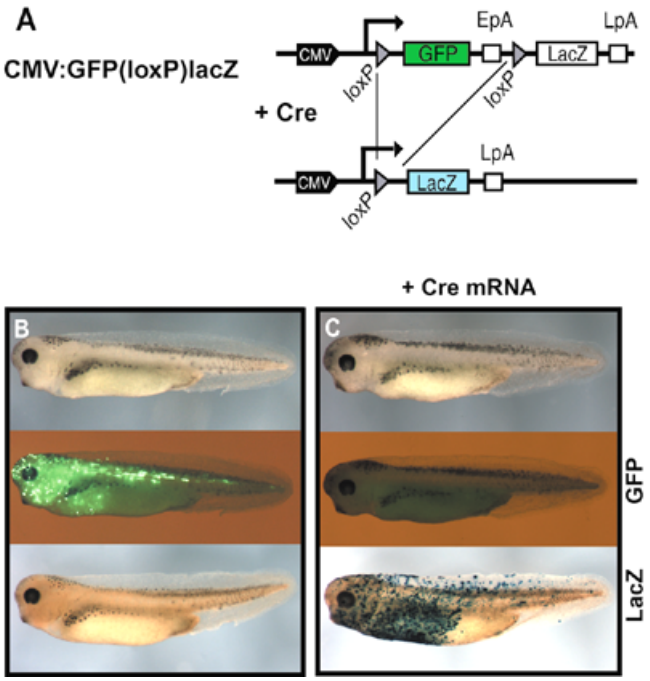

Figure 4. Constitutive Cre activity in *Xenopus* embryos. (**A**) The Cre reporter construct CMV:GFP(loxP)lacZ is schematically drawn with the CMV promoter/enhancer driving the expression of the GFP and *lacZ* genes. The position of the SV40 EpA and LpA signals as well as the target sites of the Cre recombinase (loxP) are given. (**B** and **C**) Stage 38 *Xenopus* larvae were derived from eggs injected with CMV:GFP(loxP)lacZ DNA (B) or coinjected with CMV:GFP(loxP)lacZ DNA and Cre mRNA (C). The living animals were photographed in normal light (top) or in fluorescence light (middle). X-gal staining was obtained after fixation of the larvae (bottom).

recognition targets (loxPs). Figure 4B illustrates an example of stage 38 larvae derived from a fertilized egg injected with Cre reporter DNA. The intense green fluorescence detectable under the fluorescence microscope implies a strong expression of GFP, whereas X-gal staining on the fixed embryo revealed no β-galactosidase activity. Thus, in analogy with the FLP reporter, the early poly(A) region in front of the *lacZ* cassette proved to be efficient in occluding any transcription from the downstream *lacZ* gene. Figure 4C shows an example of larvae derived from an egg coinjected with Cre reporter DNA and Cre mRNA. Characteristically, we failed to detect any GFP expression, but observed a strong β-galactosidase activity upon X-gal staining. As summarized in Table 1, among 86 embryos coinjected with Cre reporter DNA and Cre mRNA, no GFP expression could be monitored at all developmental stages. While 53 of these larvae revealed β-galactosidase activity upon X-gal staining, the residual 33 embryos which lacked both GFP and β-galactosidase expression, were obviously derived from eggs not properly injected. Finally, in 20 embryos injected exclusively with Cre reporter DNA, the expression was restricted to GFP and no blue cells could be monitored upon X-gal staining (Table1).

To test the conditional function of Cre in *Xenopus* embryos, we used the tamoxifen-regulated fusion construct CreER(T) (9) which was designed in analogy with FLPER(T) described above. Upon coinjection of CMV:GFP(loxP)lacZ reporter DNA and CreER(T)mRNA into fertilized eggs, we observed a

strong expression of *lacZ* already in the absence of 4-hydroxytamoxifen (data not shown). This finding implies a high activity of Cre in the non-induced state and renders the applicability of CreER(T) to control Cre action in *Xenopus* embryos by the administration of tamoxifen questionable.

In summary, we have shown that embryos coinjected with CMV:GFP(FRT)lacZ and FLPe express *lacZ* in parallel with GFP at all developmental stages starting at mid blastula transition, whereas embryos coinjected with CMV:GFP(loxP)lacZ and Cre express *lacZ* exclusively, indicating that the substrate molecules have been recombined entirely and irreversibly before mid blastula transition when zygotic transcription initiates. Thus, our data demonstrate that both FLP and Cre can be applied to induce gene expression in *Xenopus* embryos, although Cre is much more effective than FLP in recombining a given quantity of excision substrate. This finding is in accordance with a kinetic analysis showing that Cre has a higher affinity for its target sites than FLP, indicating that lower quantities of Cre are sufficient to bind and excise the substrate molecules (6). The generation of *Xenopus* lines carrying either the DNA recombinases or the reporters as a transgene will give us more insight into the applicabilities of FLP and Cre to the *Xenopus* system. We assume that transgenic lines will provide powerful tools to analyze the *Xenopus* development at the molecular level by novel aspects: transgenic reporter lines can be crossed with lines expressing DNA recombinases in a cell-type-restricted manner to trace the activity of a given promoter during embryogenesis. Thereby, the use of *Xenopus* instead of mammals allows a continous observation of the developing embryo outside of the female and, as the embryo is transparent, it is predestined for fluorescent protein visualization. Moreover, we suggest that DNA recombinases can be used to induce the expression of specific regulatory molecules at defined developmental stages to assess their functional role in embryogenesis.

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