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ARTICLE

Conditional Activation of a Single-Copy L1 Transgene in Mice by Cre

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Received 2 April 2008; Accepted 26 May 2008

Summary: The synthetic L1 retrotransposon, *ORFeus*, is useful for probing mechanisms of L1 retrotransposition in vivo and for genome-wide mouse mutagenesis because of its high level of activity. To achieve controlled activation of *ORFeus* in mice, we constructed *ORFeus*^{LSL}, in which *ORFeus* coding sequences were separated from the promoter by a loxP-β-*geo*-stop-loxP (LSL) cassette, and derived transgenic mouse lines containing single-copy *ORFeus*^{LSL}. We observed tissue-specific *ORFeus* activation by crossing *ORFeus*^{LSL} to various Cre-expressing lines, specifically in the germ line or the pancreas, providing definite evidence that all host factors and machinery required posttranscriptionally for L1 retrotransposition are available in somatic tissues in living animals. Notably, the single-copy *ORFeus* transgene is about threefold more active per copy than a previously described multicopy *ORFeus* transgene in the germ line and even more active somatically. This conditional transgenic *ORFeus* mouse model should allow further exploration of posttranscriptional cellular requirements for L1 retrotransposition and facilitate the development of *ORFeus* mouse lines suitable for in vivo mutagenesis. genesis 46:373–383, 2008. © 2008 Wiley-Liss, Inc.

Key words: LINE-1; retrotransposon; transgenic mouse; Cre/lox system; conditional; copy number

INTRODUCTION

Long interspersed elements type 1 (LINE-1 or L1) are the most abundant transposable elements in both human and mouse genomes. For example, the mouse genome contains ~600,000 copies of L1, with ~3,000 full-length, potentially active copies (Goodier *et al.*, 2001). Full-length L1s are 6–7 kb, encompassing an internal promoter in the 5′ untranslated region (5′UTR), two nonoverlapping open reading frames (ORF1 and ORF2), and a weak polyadenylation signal in the 3′ UTR (Ostertag and Kazazian, 2001). The expression of full-length L1 transcripts and proteins are mostly confined to gonadal tissues (Branciforte and Martin, 1994; Ergun *et al.*, 2004; Trelogan and Martin, 1995); this pattern of expression is consistent with the evolutionary pressure for L1 to successfully increase its copy number. L1 replicates in the genome by retrotransposition, a copy-and-paste mechanism involving reverse transcription of an RNA intermediate. The retrotransposition activity of a cloned L1 element can be tested ex vivo in cell culture by incorporating a retrotransposition indicator cassette in its 3′UTR (Moran *et al.*, 1996). Native human L1 isolates are more active in such assays as compared to native mouse L1s (Goodier *et al.*, 2001; Naas *et al.*, 1998), but a synonymously recoded, synthetic mouse L1 (termed *ORFeus*) is the most active L1 so far reported (Han and Boeke, 2004). By way of retrotransposition, L1 affects the host genome in various ways and constitutes a major force in driving mammalian genome evolution (Kazazian, 2004). Thus, attempts have been made in establishing mouse models of L1 to further our understanding on L1 biology in vivo, and to develop suitable tools for genome-wide mouse mutagenesis. Several native human L1 elements have been introduced into the mouse genome by transgenesis with modest activity (Babushok *et al.*, 2006; Muotri *et al.*, 2005; Ostertag *et al.*, 2002; Prak *et al.*, 2003). In contrast, the synthetic retrotransposon *ORFeus* is highly active in both somatic tissues and the

Abbreviations: gDNA, genomic DNA; iPCR, inverse PCR; L1, long interspersed element type 1; LSL, loxP-β-geo-stop-loxP.

This article contains supplementary material available via the Internet at http://www.interscience.wiley.com/jpages/1526-954X/suppmat.

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Contract grant sponsor: Life Sciences Research Foundation (Affymetrix Postdoctoral Fellowship); National Institutes of Health.

Published online 8 July 2008 in
Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/dvg.20407

mouse germ line when expressed from a constitutive heterologous promoter, lending great promise for mouse mutagenesis and in vivo L1 studies (An *et al.*, 2006).

A conditional L1 system is highly desirable for these applications. For example, to be used as an efficient insertional mutagen in mice, a transposon is usually equipped with a gene trapping cassette so that, after transposition, the endogenous transcriptional unit where the transposed copy is inserted will be rendered inactive and the site of integration can be easily identified by the transposon tag (Carlson and Largaespada, 2005). A challenge for developing an L1-based mutagenesis system is that having an unregulated potent gene-trapping cassette will invariably affect the fitness of the host animal once the retrotransposition frequency exceeds some threshold. In fact, we have observed an unusually high rate of embryonic lethality and failure to produce by surrogate mothers during microinjection of *ORFeus* transgenes equipped with gene-trapping cassettes (W. An, E. Davis, K. O'Donnell, M. Davisson, M. Wiles, J. Kulik, J. Boeke, unpublished data). Even if an active line is established after extensive screening, it will be genetically unstable, and difficult to maintain due to ongoing retrotransposition. Retroviral and DNA transposon-based systems overcome such problems by separating the cis and trans functions of the transposable element into a binary system (Carlson and Largaespada, 2005; Miller, 1997). However, it is challenging to apply this approach to L1 as it retrotransposes preferentially in cis; L1 proteins expressed from one vector cannot efficiently mobilize a passenger RNA expressed in trans (Esnault *et al.*, 2000; Wei *et al.*, 2001). The underlying mechanism for cis preference is not fully understood but L1 proteins are found to colocalize with the encoding L1 RNA in cytoplasmic ribonucleoprotein particles, which are proposed retrotransposition intermediates (Boeke, 1997; Hohjoh and Singer, 1996; Kulpa and Moran, 2005, 2006; Martin, 1991). Obviously, the cis preference of L1 retrotransposition exerts a restriction on *ORFeus* vector design and requires use of a single vector encoding both L1 proteins and containing any desired utility element such as a retrotransposition indicator cassette and/or gene-trapping cassette.

To explore the possibility of regulating an *ORFeus* transgene in vivo, we exploited Cre/loxP. Cre is a site-specific recombinase from bacteriophage P1 that mediates recombination at a pair of conserved recognition sequences (loxP) (Sauer, 1998). Cre is often used in conditional transgenesis (Nagy, 2000), in which the promoter and the coding region of the transgene is separated by a floxed transcriptional "stop" sequence (usually consisting of strong tandem polyadenylation signals), which blocks the formation of transcripts for the downstream transgene unless the stop sequence is removed by Cre-mediated excision. In one variation on this scheme (Lobe *et al.*, 1999) the triple-polyadenylation stop sequence is preceded by a β-*geo* coding sequence (Friedrich and Soriano, 1991), enabling efficient screening for overexpression in ES cells prior to "investing" in a given line. Here, we adopted the Z/AP strategy in new *ORFeus*^{LSL} transgenic mouse lines, and established single-copy *ORFeus*^{LSL} mouse lines that are tissue specifically activatable via Cre-mediated excision.

RESULTS

Construction of Transgenic *ORFeus*^{LSL} Mouse Lines

Our objectives for regulated *ORFeus* transgenic mouse lines are twofold: First, we would like to suppress *ORFeus* activity in founder animals and subsequently activate it in a spatiotemporally controlled manner in the progeny; Second, we prefer a system that enables us to screen for integration loci compatible with overexpression before committing to a specific ES cell line as the basis of a new line of mice. To this end, we incorporated the Z/AP design (Lobe *et al.*, 1999) by grafting the *l*oxP-β-*geo*-*s*top-*l*oxP (LSL) cassette described for that transgene between the CAG promoter (Niwa *et al.*, 1991) and *ORFeus* coding sequences (Han and Boeke, 2004); this construct is termed *ORFeus*^{LSL} (Fig. 1a). In principle, before introducing Cre, *ORFeus* will not be expressed; once the LSL cassette is excised, *ORFeus*^{LSL} becomes an active *ORFeus*^{L} transgene (Fig. 1b), and fully retrotransposition-competent (Fig. 1c). We transfected *ORFeus*^{LSL} into C57BL/6J ES cells (Koentgen *et al.*, 1993), and derived founder mice from three ES cell clones (1B9, 1E2 and 2G6) with high-level *lacZ* expression and one copy of *ORFeus*^{LSL} as determined by Southern blotting (Fig. S1). Quantitative RT-PCR suggested insignificant β-*geo* transcript variation among the three cell lines. Therefore, we focused our subsequent studies on line 2G6 except as noted.

We used PCR to genotype founders and backcrossed progeny. To monitor Cre-mediated excision of the β-*geo*/stop cassette in *ORFeus*^{LSL} transgene, we devised two PCR assays using three primers (Fig. 1a). A positive signal for a PCR reaction named "floxed" (primers 1 and 2) indicates presence of *ORFeus*^{LSL}; a positive signal for the PCR reaction "excised" (using primers 1 and 3) indicates a recombined allele (*ORFeus*^{L}). To monitor retrotransposition activity, we employed a third PCR reaction "intron" using intron-flanking primers in the retrotransposition indicator cassette (Fig. 1a, primers 4 and 6). Donor transgene amplification of *ORFeus*^{LSL} (Fig. 1a) or *ORFeus*^{L} (Fig. 1b) should generate a 1,370-bp band, whereas *ORFeus* retrotranspositions (Fig. 1c) lack the intronic sequence and present a 470-bp band. A fourth PCR reaction "3′ end" (primers 7 and 8) amplifies the 3′ end of *ORFeus*, and confirms the presence of donor transgene and/or retrotransposition events. Finally, the PCR reaction "cre" amplifies the Cre transgene and the PCR reaction "Hprt" amplifies mouse hypoxanthine guanine phosphoribosyl transferase, an endogenous control for genomic DNA (gDNA). As expected, gDNA from mice heterozygous for *ORFeus*^{LSL} tested negative for the intronless signal in the PCR reaction intron, showing

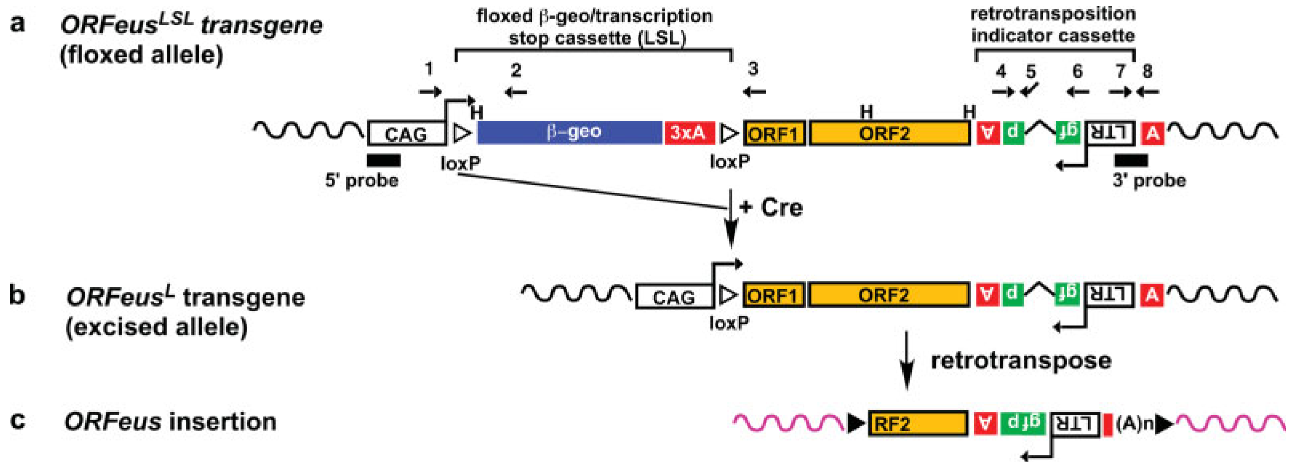


FIG. 1. Construction of *ORFeus*^{LSL} mouse lines. (**a**) Schematic representation of *ORFeus*^{LSL} transgene. The *ORFeus*^{LSL} transgene consists of the following sequence elements from 5′ to 3′: a composite CMV IE enhancer/modified chicken β-actin promoter (CAG; Niwa *et al*., 1991); a floxed β-*geo*/stop cassette comprising a β-galactosidase/neomycin phosphotransferase fusion gene (β-*geo*; Friedrich and Soriano, 1991) and triple tandem copies of SV40 late polyadenylation signal (3xpA; Lobe *et al*., 1999); *ORFeus* ORF1 and ORF2 (Han and Boeke, 2004); a *gfp*-based retrotransposition indicator cassette with its own promoter (inverted LTR) and polyadenylation signal (boxed inverted letter A); and β-globin polyadenylation signal (boxed upright letter A). Note the *gfp* ORF is antisense relative to *ORFeus* transcription direction and is interrupted by an intron. The splicing of this intron from the *ORFeus* transcript restores an intact *gfp* coding sequence, a feature that is diagnostic for retrotransposition events. Approximate locations of genotyping PCR primers (numbered arrowheads) and Southern blotting probes are indicated H, HindIII sites. (**b**) The predicted structure of the *ORFeus* transgene following Cre-mediated excision of the floxed β-*geo*/stop cassette. (**c**) A schematic of an insertion by *ORFeus* retrotransposition.

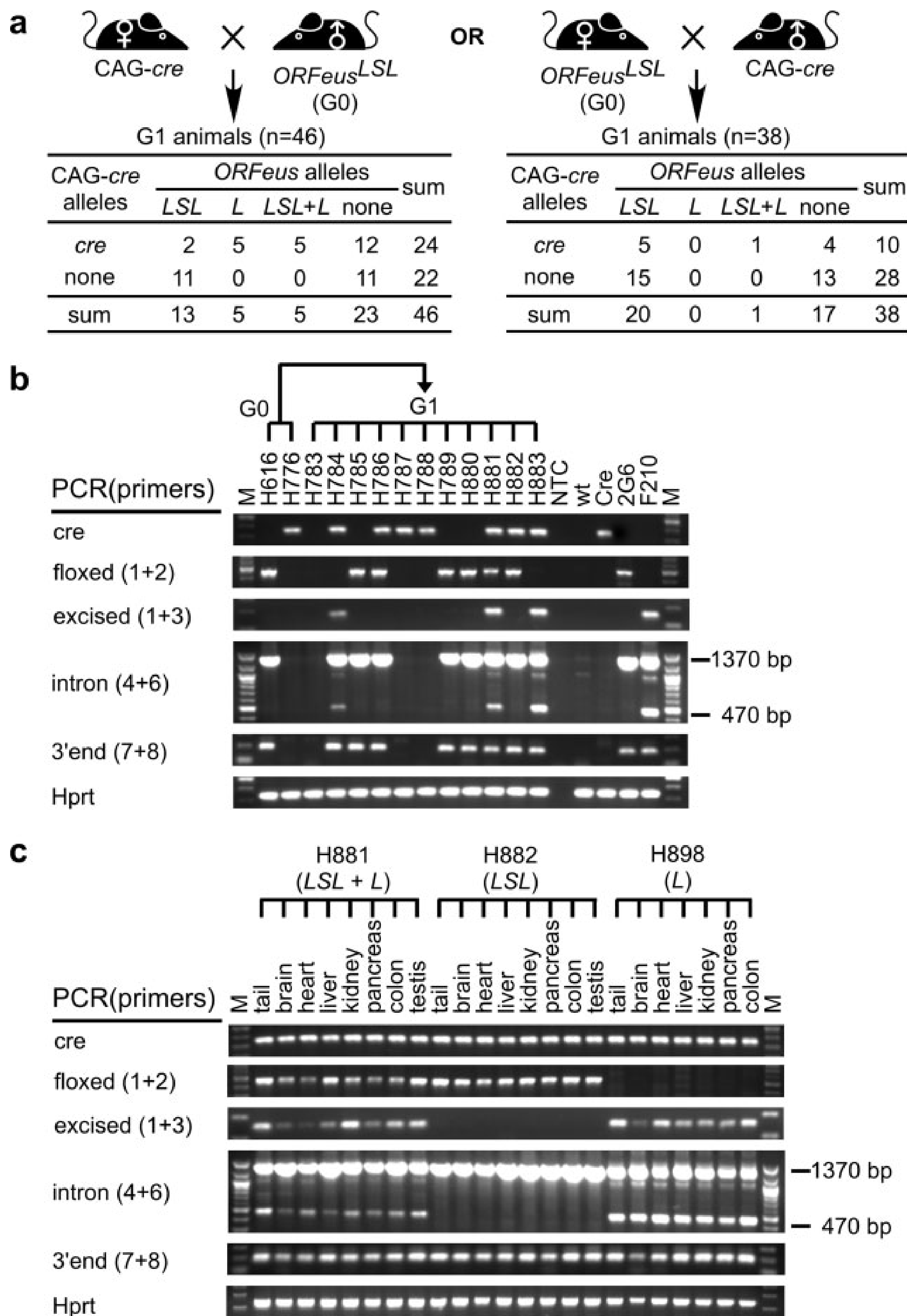
that the β-*geo*/stop cassette effectively blocks the transcription of *ORFeus* and renders it inactive (for example, see parental animal H616 in Fig. 2b).

Ubiquitous Activation of Donor *ORFeus*^{LSL} Transgene by CAG-*cre*

To demonstrate whether *ORFeus* is activated by Cre-mediated excision in *ORFeus*^{LSL} mouse lines, we turned to a ubiquitously expressed Cre mouse line, CAG-*cre* (Sakai and Miyazaki, 1997). The Cre in this mouse line like *ORFeus*^{LSL} itself is regulated by a CAG promoter. Thus, CAG-*cre* should permit *ORFeus* activation in the broadest spectrum of tissue types as the promoters for both Cre and target transgene have the maximal overlap of tissue specificity (Nagy, 2000). This CAG-*cre* mouse line is reported to possess a maternal effect: target transgenes in progeny mice from heterozygous female CAG-*cre* parents undergo complete excision of the floxed sequence even in the absence of inheritance of CAG-*cre*, presumably reflecting the presence of Cre transcripts in oocytes prior to completion of the first meiotic division and subsequent partitioning of Cre RNA/protein to resulting oocytes. In contrast, excision is only seen in Cre-transgene containing progeny mice from male CAG-*cre* parents (Sakai and Miyazaki, 1997). We therefore set up reciprocal breedings between our heterozygous *ORFeus*^{LSL} mice (designated G0) and heterozygous CAG-*cre* animals (Fig. 2a). G1 progeny were born from female (N = 46) and male (N = 38) CAG-*cre* parents, and subsequently genotyped for Cre-mediated excision events and retrotransposition (Fig. 2b). To our surprise, no maternal effect was observed as excision was detected exclusively in the G1 progeny carrying the CAG-*cre* transgene from either CAG-*cre* positive dam or sire. For doubly heterozygous G1 progeny of CAG-*cre* females, 5/12 displayed complete excision in the tail tissue examined (Fig. 2b, animals H784 and H883), 5/12 displayed a mosaic excision pattern, indicated by the presence of both floxed and excised alleles in the same tissue (Fig. 2b, animal H881), whereas the remaining 2/12 showed no sign of excision (Fig. 2b, animal H786). Among the progeny of CAG-*cre* males, Cre-mediated excision was detected in only 1/6 of doubly heterozygous G1 progeny (summarized in Fig. 2a); this individual was also mosaic for *ORFeus*^{LSL} and *ORFeus*^{L} alleles. Hence, the observed excision rate in doubly heterozygous G1 progeny was also much lower than that in the initial study which reported excision in 100% of such animals without mosaicism (Sakai and Miyazaki, 1997). Similar results were obtained from reciprocal crosses between CAG-*cre* and a different *ORFeus*^{LSL} mouse line (line 1E2) (Fig. S2).

We next examined whether the excised *ORFeus*^{L} transgene was active in those G1 progeny that suffered Cre-mediated LSL excision. With intron PCR, we detected intronless signal in the tail tissue of all animals that have undergone either complete (Fig. 2b, animals H784 and H883) or mosaic LSL excision (Fig. 2b, H881), confirming that retrotransposition occurs in cells with an activated copy of *ORFeus*.

We initially examined Cre-mediated excision of the *ORFeus*^{LSL} transgene and subsequent activation of the *ORFeus* transgene in tail biopsies only. To further explore the pattern of Cre-mediated excision in our



CAG-*cre* alleles	*ORFeus* alleles				sum
	LSL	*L*	*LSL+L*	none	
cre	2	5	5	12	24
none	11	0	0	11	22
sum	13	5	5	23	46

CAG-*cre* alleles	*ORFeus* alleles				sum
	LSL	*L*	*LSL+L*	none	
cre	5	0	1	4	10
none	15	0	0	13	28
sum	20	0	1	17	38

FIG. 2. Activation of *ORFeus*^{LSL} by ubiquitous CAG-*cre*. (**a**) Reciprocal breeding scheme and summary of G1 progeny genotypes. G1 mice are tabulated according to the genotyping PCR results for the CAG-*cre* transgene (*cre* or none) and the *ORFeus*^{LSL} transgene. The status of the latter is defined as floxed *ORFeus*^{LSL} allele (LSL), excised *ORFeus* allele (L), mosaic (LSL+L), or none. (**b**) PCR genotyping G1 animals by tail biopsy. Results for G1 progeny from breeding between a female CAG-*cre* mouse (H776) and a male *ORFeus*^{LSL} mouse (H616) are shown along with both parents. Detailed description on individual PCR can be found in the text. Relevant *ORFeus*^{LSL} primers are shown in parentheses. NTC, no template control; wt, wild-type C57BL/6 sample; Cre, *cre* transgene control; 2G6, a sample containing floxed *ORFeus*^{LSL} allele; F210, a sample from F210 mouse line (An *et al*., 2006), which has the excised form of *ORFeus* (*ORFeus*^{L}) in a multi-copy concatemer; M, 100-bp DNA molecular weight ladder (New England Biolabs). (**c**) Genotyping tissues of three animals with distinct *ORFeus* alleles. The animals were selected according to the initial genotyping results with tail biopsy, and were dissected for indicated tissues. All animals were positive for the CAG-*cre* transgene and their *ORFeus* allelic statuses are indicated. Tail samples were included for comparison.

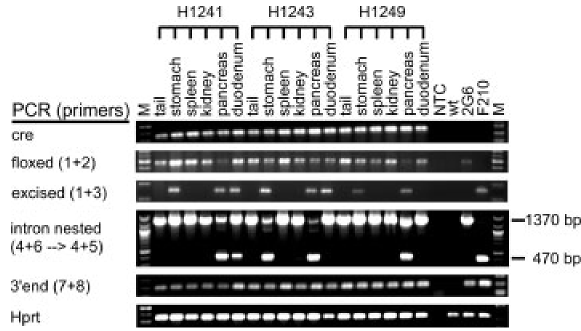


FIG. 3. Tissue-specific *ORFeus*^{LSL} activation by Pdx1-*cre*. Selected tissues from three doubly heterozygous *ORFeus*^{LSL}; Pdx1-*cre* mice were genotyped by PCR for *cre* transgene (*cre*), Cre-mediated excision (floxed and excised), retrotransposition (intron nested), 3′ end of *ORFeus*^{LSL} transgene (3′ end) and endogenous Hprt gene (Hprt). All PCR reactions were performed as in Figure 2b with the exception for detecting retrotransposition activity where a seminested PCR strategy was used.

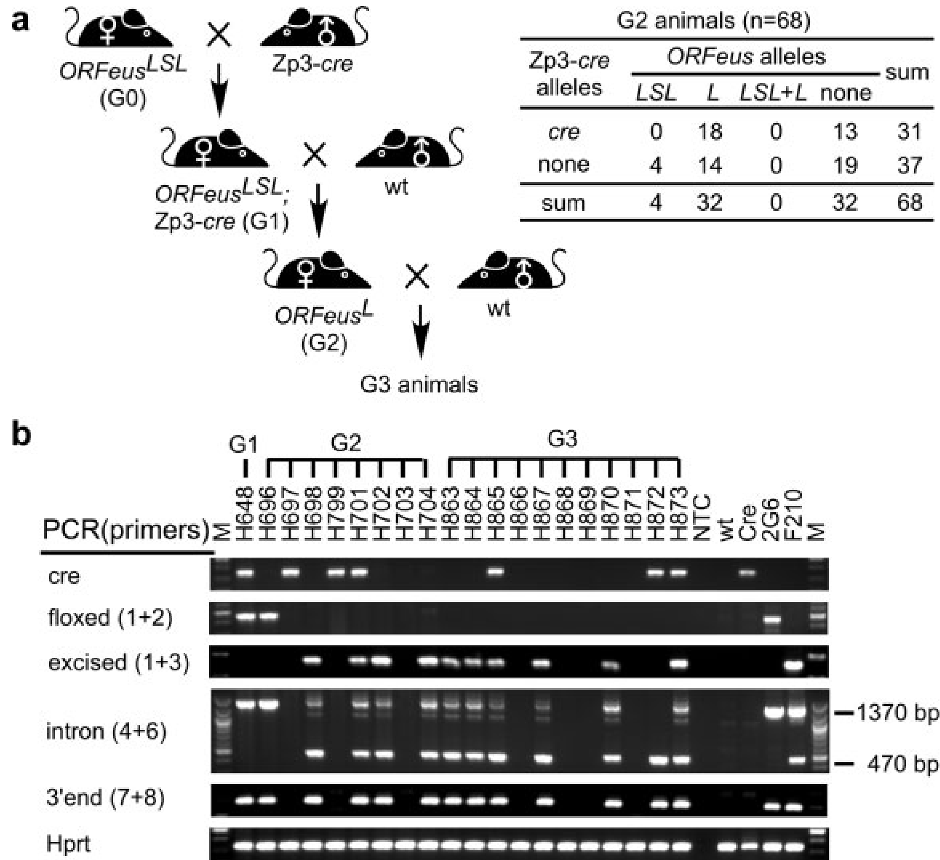
ORFeus^{LSL} transgene, we selected three doubly heterozygous G1 animals representing different states of *ORFeus* alleles ("LSL" only, "L" only, and mixed) deduced from genotyping results on tail biopsies, and inspected a panel of tissues from each of these mice. H881 was mosaic for *ORFeus*^{LSL} and *ORFeus*^{L} alleles, H882 was positive only for *ORFeus*^{LSL}, and H898 was positive only for *ORFeus*^{L}; all carried the CAG-*cre* transgene (Fig. 2c). Interestingly, all other tissues examined had identical results for the status of the *ORFeus* transgene and retrotransposition activity as in the tail for each animal, despite the fact that all three animals carried the CAG-*cre* transgene (Fig. 2c), suggesting that the tail genotype predicts the status of other tissues in the same animal. This consistency of excision in all tissues of the animal suggests that Cre expression levels are set at the whole animal level by the incoming gametic copy of CAG-*cre*. The implication is that the transgene comes in one of at least two states (off or switching on and off) and those states are maintained throughout the development of the animal. The status of the three classes of animals can be simply determined by tail genotyping.

Tissue-Specific Activation of *ORFeus*^{LSL} in Somatic Tissues by Pdx1-*cre*

Having demonstrated that *ORFeus*^{LSL} transgene can be activated in a broad spectrum of tissues by CAG-*cre*, next we sought to test whether *ORFeus*^{LSL} could be activated in a more restrictive, tissue-specific manner by crossing to Pdx1-*cre* (Hingorani *et al*., 2003). The *cre* transgene in this line is regulated by the promoter for the mouse pancreatic and duodenal homeobox 1 gene (*Pdx1*), which directs Cre expression specifically in the pancreas, and to a lesser extent, in the stomach and duodenum (Hingorani *et al*., 2003). We bred heterozygous *ORFeus*^{LSL} mice to heterozygous Pdx1-*cre* animals, and examined a panel of six tissues from three doubly heterozygous *ORFeus*^{LSL};Pdx1-*cre* mice (see Fig. 3). As expected, Cre-mediated excision could be detected in 3/3 pancreatic samples, 3/3 stomach samples, 2/3 duodenal samples, but none of several other tissues examined (Fig. 3, PCR excised), confirming the previously reported tissue specificity. To detect tissue-specific insertion events, we employed the PCR intron assay to detect retrotransposed copies of the activated *ORFeus*^{L} as in Figure 2b. Only the 1,370-bp intron-containing signal was detected in all tissues using the standard assay (not shown). We hypothesized that the inability of detecting the 470-bp intronless signal by the intron-flanking PCR might reflect a low abundance of insertions relative to the donor element in this tissue. The Pdx1-*cre* line we used reportedly displays mosaic expression in the pancreas (Hingorani *et al*., 2003). As such, we expected only a fraction of the pancreatic cells to undergo Cre-mediated excision and activation of the *ORFeus*^{LSL} transgene. To enhance detection sensitivity for retrotransposition events, we devised a seminested PCR strategy in which the intron-flanking PCR product was diluted and amplified in a subsequent PCR (with primers 4 and 5 as in Fig. 1a) using a splicing junction-spanning primer (primer 5), which preferentially amplifies the spliced product. Retrotransposition signals were detected in 3/3 pancreatic samples, 1/3 stomach and 1/3 duodenal samples but not in any other tissues examined (Fig. 3, PCR "intron nested"). Consistent with the prevalence of excised allele of *ORFeus*, all positive samples for the 470-bp band were also positive for Cre-mediated excision events (Fig. 3, PCR excised). *ORFeus* retrotransposition in these tissues was independently confirmed by the recovery of several insertions using inverse PCR (iPCR) (Table S1). Thus L1 machinery is intact in somatic cells in which L1 is not normally expressed.

Germ Cell Specific Activation of *ORFeus*^{LSL} by Oocyte and Spermatid-Specific Cre

The CAG-*cre* mouse line has been used to produce animals with complete excision of the floxed target sequence throughout an animal's body (Sakai and Miyazaki, 1997), but our results indicate that the efficiency is relatively low: 5/12 for doubly heterozygous *ORFeus*^{LSL}; CAG-*cre* G1 animals from female CAG-*cre* parents and 0/6 for those from male CAG-*cre* parents. An alternative strategy for achieving uniform excision is to use Cre-expressing mouse lines under the regulation of germ line specific promoters. To this end, we first tested an oocyte-specific Cre-expressing mouse line, Zp3-*cre*, in which Cre expression is controlled by the mouse *zona pellucida* glycoprotein 3 (Zp3) promoter; expression is restricted to growing oocytes (de Vries *et al*., 2000). We bred *ORFeus*^{LSL} females to Zp3-*cre* males, backcrossed doubly heterozygous *ORFeus*^{LSL};Zp3-*cre* G1 females to C57BL/6 males, and then genotyped the G2 progeny for Cre-mediated excision and retrotransposition (Fig. 4a). Genotyping results for a litter of eight G2 animals are shown along with their G1 maternal parent (Fig. 4b). As expected, no Cre-mediated excision was observed in the



Zp3-*cre* alleles	*ORFeus* alleles				sum
	LSL	*L*	*LSL+L*	none	
cre	0	18	0	13	31
none	4	14	0	19	37
sum	4	32	0	32	68

FIG. 4. Activation of *ORFeus*^{LSL} in female germ line by oocyte-specific Zp3-*cre*. (**a**) Breeding scheme and summary of progeny genotypes. (**b**) Representative genotyping PCR results on tail biopsy for G1, G2, and G3 animals. Symbols used are identical to Figure 2b.

tail biopsy of the doubly heterozygous female G1 parent (Fig. 4b, animal H648) as Zp3-*cre* expression is restricted to oocytes of such females; excised *ORFeus*^{L} transgene was detected in four G2 animals derived from this female, all had undergone complete LSL excision. Notably, three of these four animals showed complete excision even though they themselves do not carry Zp3-*cre* (Fig. 4b, animals H698, H702, and H704). Similar analysis was extended to 68 G2 mice from such breedings (summarized in Fig. 4a). Among 36 mice that inherited *ORFeus*^{LSL}, 18 were positive for Zp3-*cre* and had undergone complete LSL excision; the remaining 18 were negative for Zp3-*cre*. Among these, 14/18 had complete LSL excision, but 4/18 lacked excision. Complete excision in the absence of Zp3-*cre* transgene is not surprising, as data from an independently derived Zp3-*cre* mouse line suggest that Zp3-*cre* is expressed early in oocyte maturation, prior to the completion of meiosis I, and Cre produced in the growing oocyte is sufficient to mediate recombination of the target gene after fertilization (Lewandoski *et al.*, 1997). Overall, our data indicate that Zp3-*cre* is fairly efficient, as 89% (32/36) of target-containing G2 progeny of doubly heterozygous females underwent complete excision. Further analysis on selected tissues from G2 mice that carried either floxed or excised alleles of the *ORFeus*^{LSL} transgene confirms the absence of mosaic excision, and indicates that the genotyping result of the tail tissue is diagnostic of other tissues from the same animal (Fig. S3). Similar results were obtained with a male germ-line specific transgene Prm1-*cre* (O'Gorman *et al.*, 1997) (Fig. S4).

Comparison of Retrotransposition Activity Between Single-Copy and Multiple-Copy *ORFeus* Transgenes

We have demonstrated that Cre/loxP system can be used to control *ORFeus* activation in a tissue-specific manner by using a readily available repository of Cre animals. Such floxed *ORFeus*^{LSL} mouse lines can be easily propagated, as no *ORFeus* expression or activity will interfere with the well being of the animals before Cre-mediated excision. However, an immediate concern for this Cre/loxP-based conditional *ORFeus*^{LSL} transgenic system is the relative level of retrotransposition activity from these single-copy transgenes as opposed to an *ORFeus* mouse line carrying a multicopy *ORFeus* transgene (An *et al.*, 2006), the unit copy of which is structurally identical to the activated single-copy *ORFeus*^{L} in this study, including the single loxP site. To address this

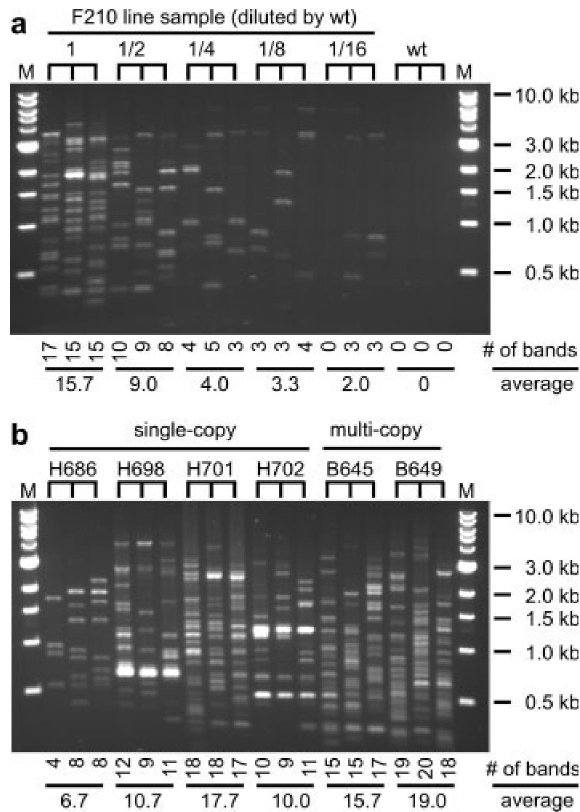


FIG. 5. Comparison of *ORFeus* activity between single-copy transgene and multiple-copy transgene. (**a**) iPCR-based insertion profiling on a dilution series of a multi-copy *ORFeus*-containing sample. A donor-containing sample from line F210 (An *et al.*, 2006) was diluted at indicated dilution factor with wild-type (wt) mouse gDNA samples, and subjected to iPCR amplification. An undiluted F210-derived sample and a wt sample were used as controls. (**b**) Four G2 samples parented by doubly heterozygous $ORFeus^{LSL}$; Zp3-*cre* female G1 mouse were analyzed by iPCR and compared to two independent samples from line F210. The number of discernable bands in each lane is counted and the average among triplicate PCRs for each sample is indicated at the bottom of the gel.

question, we compared the retrotransposition activity of single-copy and multicopy *ORFeus* transgenes by iPCR profiling.

We have previously established that when independent replicative iPCR reactions are performed on the same gDNA sample after restriction and ligation, the number and migration positions of DNA bands vary as the result of stochastic amplification of a complex pool of insertions contained in the sample (An *et al.*, 2006). However, it is not known whether the average number of bands per iPCR reaction for each sample represents the relative abundance of insertion events from sample to sample. To test this directly, we created a dilution series of donor-containing samples from our previous multicopy *ORFeus* mouse line F210 and carried out iPCR reactions on each dilution (Fig. 5a). The results from this experiment indicate that the mean number of bands from independent PCRs mirrors the abundance of insertions in the gDNA preparation, and thus can be used to assess the relative level of retrotransposition activity between different mouse lines. Using the same iPCR technique, we surveyed several $ORFeus^{L}$-containing G2 progeny of $ORFeus^{LSL}$;Zp3-*cre* animals (Fig. 5b, animals H686, H698, H701, H702) and N2 animals from previously described multicopy *ORFeus* line F210 (Fig. 5b, animals B645 and B649). Among G2 individuals carrying activated single-copy $ORFeus^{L}$, we observed relatively broader variation on the average number of iPCR bands per animal as compared to N2 animals from line F210. Overall, the level of retrotransposition activity in somatic tissues for animals carrying single-copy $ORFeus^{L}$ (11 bands/lane) is roughly 65% that of animals from multicopy line F210 (17 bands/lane) if the number of iPCR bands is used as a proxy for retrotransposition activity. In fact, $ORFeus^{L}$ insertions can be readily identified from these G2 animals (Table S1).

To explore the utility of this Cre/loxP-based single-copy $ORFeus^{LSL}$ system for germ line applications, we further evaluated the germ line insertion frequency of this single-copy *ORFeus* transgene. Germ line insertions are defined as retrotransposition events that occur in germ cells of an animal containing an activated allele of the $ORFeus^{LSL}$ transgene. Accordingly, a germ line insertion can be present in $ORFeus^{LSL}$-containing animals if the *ORFeus* transgene has been activated in their transgenic parent's germ cells; alternatively, it can be detected in donorless progeny animals if the retrotransposition event occurs prior to meiosis II in the corresponding germ cell and subsequently segregate away from the donor transgene. To calculate germ line insertion frequency, we considered germ line insertions only in donorless animals so that somatic insertions that occurred in donor-containing animals would not confound the analysis. We bred G2 mice containing an activated $ORFeus^{L}$ allele to wild-type C57BL/6 mice (Fig. 4a), and genotyped 161 G3 animals. Among these mice, 78 were donor-negative (Fig. 4b, animals H866, H868, H869, H871, and H872), and 9% (7/78) had insertion signals by intron PCR (Fig. 4b, animal H872). This is equivalent to an average germ line insertion frequency of 9.4% per animal. In contrast, the germ line insertion frequency is 33% for line F210 which had ~10 copies of donor transgene (An *et al.*, 2006), averaging 3.3% per unit copy. Therefore, our single-copy $ORFeus^{L}$ mouse line is ~threefold more active per copy in the germ line than mouse line F210.

DISCUSSION

We have established a conditional L1 system in which the *ORFeus* transgene is kept totally inactive in mice but can be activated in specific tissues using different Cre mouse lines. The tissue specificity of *ORFeus* activation is jointly determined by the promoter specificity of the target $ORFeus^{LSL}$ transgene and that of the Cre transgene. The use of a ubiquitous promoter to drive the expression of *ORFeus* transgene offers a significant advantage over an alternative design with a more restric-

tive promoter, as the activation of our *ORFeus* transgene will be determined solely by the expression pattern of the Cre transgene (Nagy, 2000). Thus, once a single line is made it has the potential to be utilized in a broader range of studies by exploiting the ever-expanding repository of Cre mouse lines (http://nagy.mshri.on.ca/cre/). This compares favorably with reinvesting time and resources in constructing individual *ORFeus* mouse lines controlled by distinct tissue-specific promoters. Indeed, we have demonstrated the activation of $ORFeus^{LSL}$ transgene globally by ubiquitous CAG-*cre* (Sakai and Miyazaki, 1997), in defined tissue types by pancreas-specific Pdx1-*cre* (Hingorani *et al.*, 2003), and in female and male germ lines by oocyte-specific Zp3-*cre* (de Vries *et al.*, 2000) and spermatid-specific Prm1-*cre* (O'Gorman *et al.*, 1997), respectively. Overall, the tissue specificity is consistent with the known promoter specificity of the Cre transgene. In contrast, prior to crossing to Cre-expressing mice, we detected no excision of the LSL cassette even with a highly sensitive PCR based assay. Thus the Cre/loxP switch allows one to maintain the transgene free of *ORFeus* expression and retrotransposition, i.e. in a genetically stable form. As part of the Z/AP-like design of Cre/loxP configuration, the transgene also incorporates a selection cassette, conferring yet an additional advantage in that active mouse ES cell lines can be selected/evaluated before committing to make a mouse line.

A remarkable feature of our conditional $ORFeus^{LSL}$ system is that it employs a single-copy L1 transgene. All previous efforts on making L1 transgenics used standard transgenesis (An *et al.*, 2006; Babushok *et al.*, 2006; Muotri *et al.*, 2005; Ostertag *et al.*, 2002; Prak *et al.*, 2003), which normally results in a multicopy concatemer of donor transgenes (Palmiter and Brinster, 1986). Such multicopy donor concatemers are not compatible with the Cre/loxP approach as sequences between two distal loxP sites are subjected to Cre-mediated excision, leaving behind transgenes at reduced copy numbers (Garrick *et al.*, 1998; Lakso *et al.*, 1996). Although L1 possesses a multiplicative copy-and-paste mode of replication and a single copy of L1 can theoretically spawn an unlimited number of insertions, our initial concern was whether the reduced copy number in the $ORFeus^{LSL}$ transgene would adversely affect its retrotransposition frequency. To this end, we compared both somatic and germ line retrotransposition frequencies between the activated single-copy $ORFeus^{L}$ and our previously reported ~10 copy *ORFeus* concatemer donor (An *et al.*, 2006), the unit copy of which is structurally identical to the activated single-copy $ORFeus^{L}$ in this study. The single copy line is 65% as active as the multicopy line in somatic tissues and 28% that of the multicopy line in the germ line. However, on a per-copy basis, the single-copy line is about threefold more active than the multicopy line in the germ line and about 6.5-fold more active somatically. A plausible explanation for the high activity of the single-copy line is that the $ORFeus^{LSL}$ transgene was integrated at a highly active genomic locus as the result of *lacZ* screening in ES cells. An alternative yet complementary explanation is that the high level of activity might reflect a potential intrinsic advantage of a single-copy transgene over multicopy transgene arrays as the latter tend to be the target for repeat-induced gene silencing (Garrick *et al.*, 1998). In this regard, a direct comparison of single-copy and multicopy *ORFeus* transgenes at identical genomic loci would be highly desirable. Regardless the underlying mechanisms, our results demonstrate that *ORFeus* can be very effective in retrotransposition when present in even a single copy in the mouse genome.

The efficiency of Cre-mediated recombination is affected by several factors other than the promoter driving Cre expression (Sauer, 1998; Schmidt-Supprian and Rajewsky, 2007). Over the course of this study, we encountered lower-than-expected excision efficiency for several Cre mouse lines tested. For example, the CAG-*cre* mouse line was reported to result in complete excision of the target transgene in all G1 progeny of female CAG-*cre* parents regardless of their CAG-*cre* status (Sakai and Miyazaki, 1997); but we observed mosaic excision in a significant proportion of doubly heterozygous G1 animals and no excision in G1 animals derived from female CAG-cre heterozygotes that did not inherit CAG-*cre*. We also observed mosaic excision of $ORFeus^{LSL}$ transgene using a separate Cre-expressing mouse line under the regulation of human CMV minimal promoter (CMVmini-*cre* (Schwenk *et al.*, 1995); not shown). In addition, we found that two germ line specific Cre mouse lines Zp3-*cre* (de Vries *et al.*, 2000) and Prm1-*cre* (O'Gorman *et al.*, 1997), although resulting in complete excision, were not 100% efficient in the context of our construct. We initially hypothesized that the unexpected excision patterns observed here could reflect chromatin-related differential accessibility of the target loxP locus (Baubonis and Sauer, 1993; Vooijs *et al.*, 2001). Therefore, we set out to test CAG-*cre* and CMVmini-*cre* mouse lines on two independent $ORFeus^{LSL}$ target lines (2G6 and 1E2) that had identical transgene sequence at different genomic loci (Fig. S2 and data not shown). The seemingly identical results obtained from such experiments favor an alternative explanation, i.e. some target sequence-specific effects. The excision efficiency by a different site-specific recombinase, FLP, is known to be adversely affected by an increasing distance between its target sites (Ringrose *et al.*, 1999). The distance between loxP sites in $ORFeus^{LSL}$ is 4.9 kb, at least 2.5-fold longer than that in target transgenes tested by the original reports for these Cre mouse lines (de Vries *et al.*, 2000; O'Gorman *et al.*, 1997; Sakai and Miyazaki, 1997; Schwenk *et al.*, 1995). Thus, our data are consistent with inefficient excision by Cre recombinase on more distantly positioned loxP sites. Overall, among the Cre lines tested, Zp3-*cre* was the most efficient in achieving complete excision of floxed target sequence in all tissues; CAG-*cre*, although less efficient, has the advantage of generating complete excision in a single breeding cycle.

On a different note, our study presents potential evidence that adds to the increasing literature for Cre-induced toxicity (Schmidt-Supprian and Rajewsky, 2007). Such toxicity may explain the apparently low prevalence of CAG-*cre* transgene among G1 progeny of heterozygous CAG-*cre* male parents derived from two independent *ORFeus*^{LSL} lines:10/38 for line 2G6 (Fig. 2a; $P < 0.01$) and 14/47 for line 1E2 (Fig. S2; $P < 0.01$). It appears that this Cre-mediated effect is independent of the presence of exogenous loxP sites as approximately half of CAG-*cre* positive G1 progeny carried the target *ORFeus* transgene. In addition, the segregation of the CAG-*cre* transgene in G1 progeny from heterozygous CAG-*cre* female parents conformed to a Mendelian ratio, suggesting this is an effect on the male germ line. The underlying mechanisms of this effect are a subject for future investigation. Nevertheless, it is noteworthy that the paternal genome may be more susceptible to Cre-mediated actions than the maternal counterpart as paternal DNA is uniquely remodeled from a nucleosome-based to a protamine-based chromatin during spermatogenesis (Kimmins and Sassone-Corsi, 2005). It has been documented that chronic expression of Cre recombinase in postmeiotic spermatids from the Prm1 promoter results in pronounced chromosome rearrangements and complete male sterility (Schmidt *et al.*, 2000).

Our Cre/loxP-based conditional *ORFeus*^{LSL} transgene presents great opportunities for probing L1 biology in vivo. Endogenous human and mouse L1 protein products are predominantly detected in germ cells and rarely in somatic tissues except for certain types of gonadal somatic cells (Branciforte and Martin, 1994; Ergun *et al.*, 2004; Trelogan and Martin, 1995). Scores of disease-causing L1 retrotransposition events have been reported (Ostertag and Kazazian, 2001) but the developmental timing of L1 retrotransposition can be deduced only for two such events: in one case, it apparently originated during maternal meiosis I (Brouha *et al.*, 2002), and in another during early embryogenesis resulting in somatic and germ line mosaicism (van den Hurk *et al.*, 2007). Additional in vivo evidence for L1 retrotransposition in somatic tissues is scarce. There is a single case report on a somatic retrotransposition event from a colorectal cancer patient (Miki *et al.*, 1992). Recent transgenic studies using human L1s under the regulation of endogenous L1 promoters suggest they can retrotranspose not only in mouse germ cells (Muotri *et al.*, 2005; Ostertag *et al.*, 2002) but also in the brain (Muotri *et al.*, 2005), presumably as the result of transcriptional activation of the L1 transgene in these cellular compartments. Driven by a constitutive CAG promoter, our conditional *ORFeus*^{LSL} system is not designed to address the developmental timing of L1 expression, but it is perfectly suited for dissecting cellular requirements for retrotransposition. In fact, using Pdx1-*cre*, our study demonstrates that all of the host factors and machinery required for L1 retrotransposition are intact and available in several somatic tissues (pancreas, duodenum and stomach) in which transposition normally does not occur in the context of a living animal, a question that could not be asked previously. We anticipate further experiments could provide more insights into mechanisms controlling L1 activity in vivo, especially when coupled with inducible Cre-expressing mouse lines. For example, it has been debated whether L1 retrotransposition can occur in nondividing cells. Previous attempts were performed in cell culture with both immortalized cancer cells and primary cells by either transfection or by adenoviral infection (Kubo *et al.*, 2006; Shi *et al.*, 2007), reaching conflicting conclusions. Findings from these studies can be validated by using *ORFeus*^{LSL} mice as an in vivo model, which naturally provide a wide range of both actively dividing and terminally differentiated cell types. Particularly, the effect of the degree of differentiation on retrotransposition may be studied by examining the epidermis, which is composed of multiple stratified layers of increasingly differentiated keratinocytes. An excellent example of using inducible Cre recombinase in studying dynamic Cre-mediated excision and expression of a reporter gene in epidermis has been previously reported (Brocard *et al.*, 1997).

Taken together, by coupling Cre/loxP with *ORFeus* retrotransposon, we have demonstrated a conditional L1 transgenic system that can be regulated in a tissue-specific fashion by crossing to a wide variety of readily available Cre mouse lines. The tight control of L1 activity by Cre-mediated excision should prove instrumental not only in deriving potent gene trap-equipped *ORFeus* lines for use in genome-wide mutagenesis studies but also for probing L1 functions in vivo, especially in conjunction with inducible Cre recombinase.

METHODS

Plasmids

The *ORFeus*^{LSL} transgene contains the following sequence elements: CAG promoter and loxP-β-*geo*-stop-loxP sequences from pQX107 (a gift from Jeremy Nathans), *ORFeus* coding sequences from pBSsmL1 (Han and Boeke, 2004), a modified *gfp*-based retrotransposition indicator cassette from pRSVGFPuvINT, and β-globin polyadenylation signal from pQX107. To make pRSVGFPuvINT, the Rous Sarcoma Virus promoter was PCR amplified from pREP10 (Invitrogen) and cloned into the EcoRV site of pBluescriptSK(−) (Stratagene) to make pBSRSV; a GFPuvINT fragement was converted from the plasmid pBSKS-EGFP-INT (Ostertag *et al.*, 2000) by a series of mutagenesis/fusion PCRs, digested with BamHI/EcoRV and cloned into the corresponding sites of pBluescriptKS(−) to generate pBSGFPuvINT; the RSV promoter from pBSRSV was removed with NheI/AvrII and cloned into the corresponding sites of pBSGFPuvINT to make pRSVGFPuvINT. To make pBSsmL1glob, the β-globin polyadenylation signal was PCR amplified from the plasmid pQX107, digested with BamHI/EcoRI, and cloned into the corresponding sites of pBSsmL1 to produce pBSsmL1glob. The RSVGFPu-

vINT cassette from pRSVGFPuvINT was removed with BamHI/EcoRV and cloned into the BamHI/HpaI sites of pBSsmL1glob to make pBSsmL1GFPuv. The CAG promoter and LSL cassette from pQX107 was removed with NotI/XmnI, blunted with Klenow fragment, and cloned into the BstZ17I sites of pBSsmL1GFPuv to make $ORFeus^{LSL}$, which was sequencingly verified in its entirety.

Transgenic Mice

C57BL/6J Bruce4 embryonic stem cells (Koentgen *et al.*, 1993) were electroporated with NotI-linearized $ORFeus^{LSL}$ transgenic construct and selected with 200 μg/ml G418. G418-resistant ES clones were screened for β-galactosidase (*lacZ*) expression by X-gal staining and the transgene copy number was determined by Southern blotting of HindIII-digested gDNA with 5′ and 3′ probes. Primers for generating these probes are listed in Table S1. Three independent ES clones with single copy transgenes and high levels of *lacZ* expression were selected from ~500 ES cell clones and injected into BALB/c blastocysts, and the resulting chimeric males were mated to C57BL/6J females. Founders were identified by Southern blot and maintained as heterozygotes. All Cre mouse lines used in this study have been reported previously by various groups: CAG-*cre* mice (Sakai and Miyazaki, 1997) were provided by Charles Hawkins; CMV-*Cre* (Schwenk *et al.*, 1995) and Pdx1-*cre* mice (Hingorani *et al.*, 2003) were maintained by Frank Koentgen, and Anirban Maitra, respectively; Zp3-*cre* (de Vries *et al.*, 2000) and Prm1-*cre* mice (O'Gorman *et al.*, 1997) were acquired from Jackson Laboratories (Maine, USA). Protocols for the use of mice were approved by Institutional Animal Care and Use Committee.

Genotyping PCR and iPCR

All primers used in this study are listed in Table S2. Genotyping PCR reactions were performed as previously described (An *et al.*, 2006) except for PCR intron nested, which entails a second round PCR with primers 4 and 5 for 25 cycles by using 1/25 of the amplification product from PCR intron as DNA template. The 3′ genomic junctions of *ORFeus* insertions were recovered from doubly heterozygous $ORFeus^{LSL}$; Pdx1-*cre* G1 animals by a nested iPCR protocol, which includes a second round amplification for 25 cycles by using 1/25 of the first round iPCR reaction of 35 cycles as previously described (An *et al.*, 2006).

ACKNOWLEDGMENTS

We thank Jeremy Nathans for helpful discussions and Charles Hawkins for providing the CAG-*cre* mice.

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