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Site-specific integration of transgene targeting an endogenous *lox*-like site in early mouse embryos

Masanori Ito · Keitaro Yamanouchi · Kunihiko Naito · Michele P. Calos · Hideaki Tojo

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Abstract Functional *lox*-like sequences have been identified within the yeast and mammalian genome. These hetero-specific *lox* sites also allow Cre recombinase to specifically target efficient integration of exogenous DNA into the endogenous pseudo-*lox* (ψ*lox*) sequences that occur naturally in the host genome using a Cre/*loxP* integrative recombination system. We investigated whether the Cre/ψ*lox* system is useful for site-specific integration of transgenes and for improving the production efficiency of transgenic animals. This is the first report on Cre-mediated integrative recombination targeting an endogenous *lox*-like sequence termed pseudo-*loxm5* (ψ*loxm5*) in early mouse embryos. We characterized the Cre/ψ*loxm5* system in embryonic environment. Cre-expressing plasmid and a transgene (CMV/*LacZ* gene) flanked by ψ*loxm5* and ψ*loxcorem5* sites were co-microinjected into the pronucleus of fertilized mouse oocytes. The injected eggs were transferred into foster mothers, and the recombination products were investigated. The results show that the ψ*loxm5* site is an active substrate for Cre-mediated recombination in the mouse embryonic environment. The transgenesis efficiency was up to 27% (6/22). The site-specific integration of the transgene into the endogenous ψ*loxm5* site was found in 50 % of the transgenic pups. Our findings demonstrated that the Cre/ψ*loxm5* integrative recombination system is an efficient and simple strategy for targeting an endogenous *lox*-like site in mammalian embryos.

Keywords Cre · Microinjection · Pseudo-*lox* · Site-specific integration · Transgenic animal

M. Ito (✉) · K. Yamanouchi · K. Naito · H. Tojo
Laboratory of Applied Genetics, Graduate School of Agricultural and Life Sciences, The University of Tokyo,
1-1-1 Yayoi, Bunkyo-ku,
Tokyo 113-8657, Japan
e-mail: itomasanori@med.toho-u.ac.jp

M. P. Calos
Department of Genetics, School of Medicine, Stanford University,
Stanford, CA 9405-5120, USA

H. Tojo
Yamazaki College of Animal Health Technology,
4-7-2 Minamioosawa, Hachioji,
Tokyo 192-0364, Japan

Present Address:
M. Ito
Department of Pharmacology, School of Medicine,
Faculty of Medicine, Toho University,
5-21-16 Omori-Nishi, Ota-ku,
Tokyo 143-8540, Japan

Introduction

Transgenic animals generated by standard DNA microinjection methods show an extreme variability in expression of the introduced gene. This variability is a result of the position effects on gene expression caused by random integration of the transgene into the host genome, and from the highly variable copy numbers of the integrated foreign gene. Further, the efficiency of the transgenesis process has been intolerably low, particularly in farm animals. An appealing strategy to circumvent such constraints would involve site-specific integration of the introduced sequences into an appropriate position in the genome. Homologous recombination can serve as an alternative for site-specific DNA recombination, but its efficiency is low (Vega 1991). The Cre/*loxP* recombination system is highly specific and efficient in eukaryotic cells. Cre has been shown to efficiently carry out intra-molecular and inter-molecular recombination in both prokaryotic and eukaryotic cells

(Sauer 1987; Sauer and Henderson 1988; Sauer and Henderson 1990). Furthermore, Cre has been reported to catalyze recombination at *loxP* sites having a certain degree of homology with the wild-type *loxP* (Lee and Saito 1998; Sauer 1992; Sauer 1996; Thyagarajan et al. 2000). Mutant *lox* sites have been developed to increase the efficiency of Cre-mediated insertion to avoid re-excision (Albert et al. 1995; Araki et al. 1997; Liu et al. 2007; Chatterjee et al. 2010; Kameyama et al. 2010).

Recombinase-mediated integration would be more useful for genetic engineering of animals if the sites that could act as the targets for the Cre recombinase occurred endogenously at appropriate locations in the mammalian genome such as in regions outside of the functional endogenous genes. Such a strategy would likely result in iso transgenic lines having identical expression levels of the transgene. Functional cryptic *lox* sites have been shown to occur naturally in the genome of yeast (Sauer 1996), human, and mouse (Thyagarajan et al. 2000). An illegitimate Cre-dependent chromosomal rearrangement has been reported in the genome of a Cre transgenic mouse in the absence of the *loxP* sequences (Schmidt et al. 2000). Furthermore, it has been reported that chromosome analysis after Cre expression in mammalian cells revealed numerous chromosomal aberrations and an increased number of sister chromatid exchanges (Loonstra et al. 2001). Such genomic rearrangement strongly suggests the presence of functional cryptic *lox* sites in the mouse genome.

We focused on the endogenous ψ*loxm5*, which has been identified in the mouse genome and has been shown to function in Cre-mediated recombination assays performed in bacteria and cultured mammalian cells (Thyagarajan et al. 2000). In this study, we investigated whether the Cre/ψ*loxm5* system is useful for site-specific and stable integration of the trangene in mice. Here, we show an efficient and simple strategy for introducing a foreign gene into the mammalian genome, targeting an endogenous *lox*-like site in mouse embryos using the Cre/*loxP* integrative recombination system.

Materials and methods

Plasmid construction

Three kinds of plasmids were used in the present study. The Cre-expressing plasmid, pCAG/NCre (Sakai and Miyazaki 1997), containing the bacteriophage P1 Cre gene driven by the CAG (cytomegalovirus immediate-early enhancer, chicken β-actin promoter and fusion intron of chicken β-actin and rabbit β-globin) promoter (Niwa et al. 1991), and including the polyadenylation signal (Fig. 1b), was the kind gift of Dr. I. Saito (The University of Tokyo, Japan). The reporter plasmid (pCMV/loxP/LacZ) (Fig. 1a) was constructed by inserting an arbitrary 1.5-kb bacterial sequence, as a stuffer, at the *Swa* I site between the two *loxP* sites of the UL WL plasmid (RGB DNA Bank, Japan). The resulting *loxP* flanked stuffer was excised and ligated at the *Eco*RI/*Sal*I sites of the pCMV-SPORT-βgal (Life Technologies, Tokyo, Japan). Pseudo-loxm5 plasmid (pψloxm5), containing the βgal-expressing gene (*LacZ*) driven by cytomegalovirus (CMV) promoter, was used. The CMV/*LacZ* construct was flanked by two ψ*lox* sequences: ψ*loxm5* and ψ*loxcorem5*

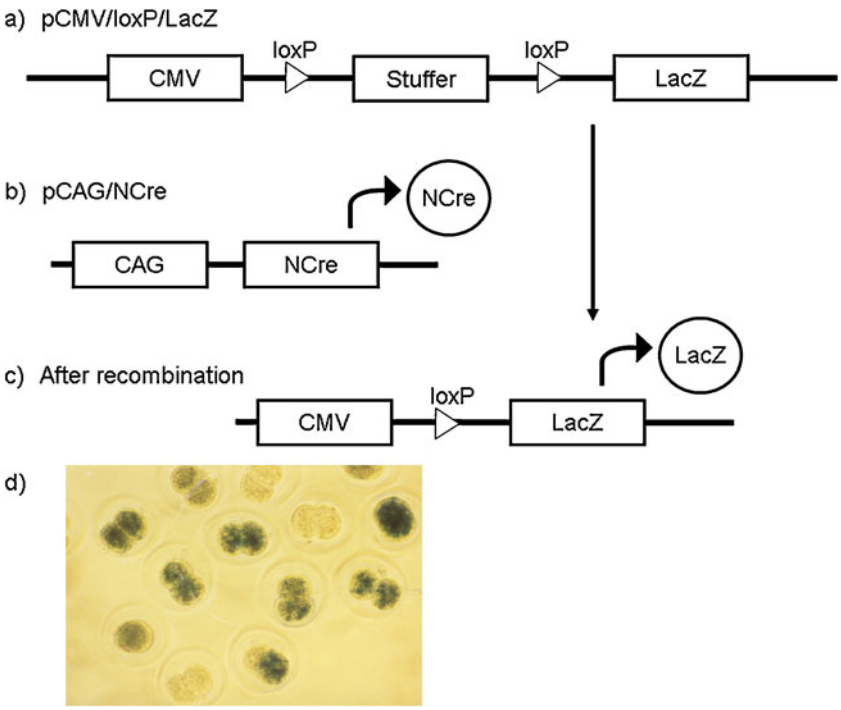

Fig. 1 Cre-transient expression, recombination, and X-gal staining of early-stage embryos. The structure of the target plasmid (pCMV/loxP/LacZ) (**a**) and its recombination form (**c**) are shown. The *lacZ* gene is silent before recombination. After expression of pCAG/NCre (**b**) in fertilized eggs. Cre-mediated recombination induces deletion of a floxed stuffer fragment and results in the expression of *lacZ* gene. pCMV/loxP/LacZ and pCAG/NCre were co-microinjected into the pronucleus of mouse fertilized eggs. The surviving eggs were stained with X-gal after 10 h of culturing (**d**)

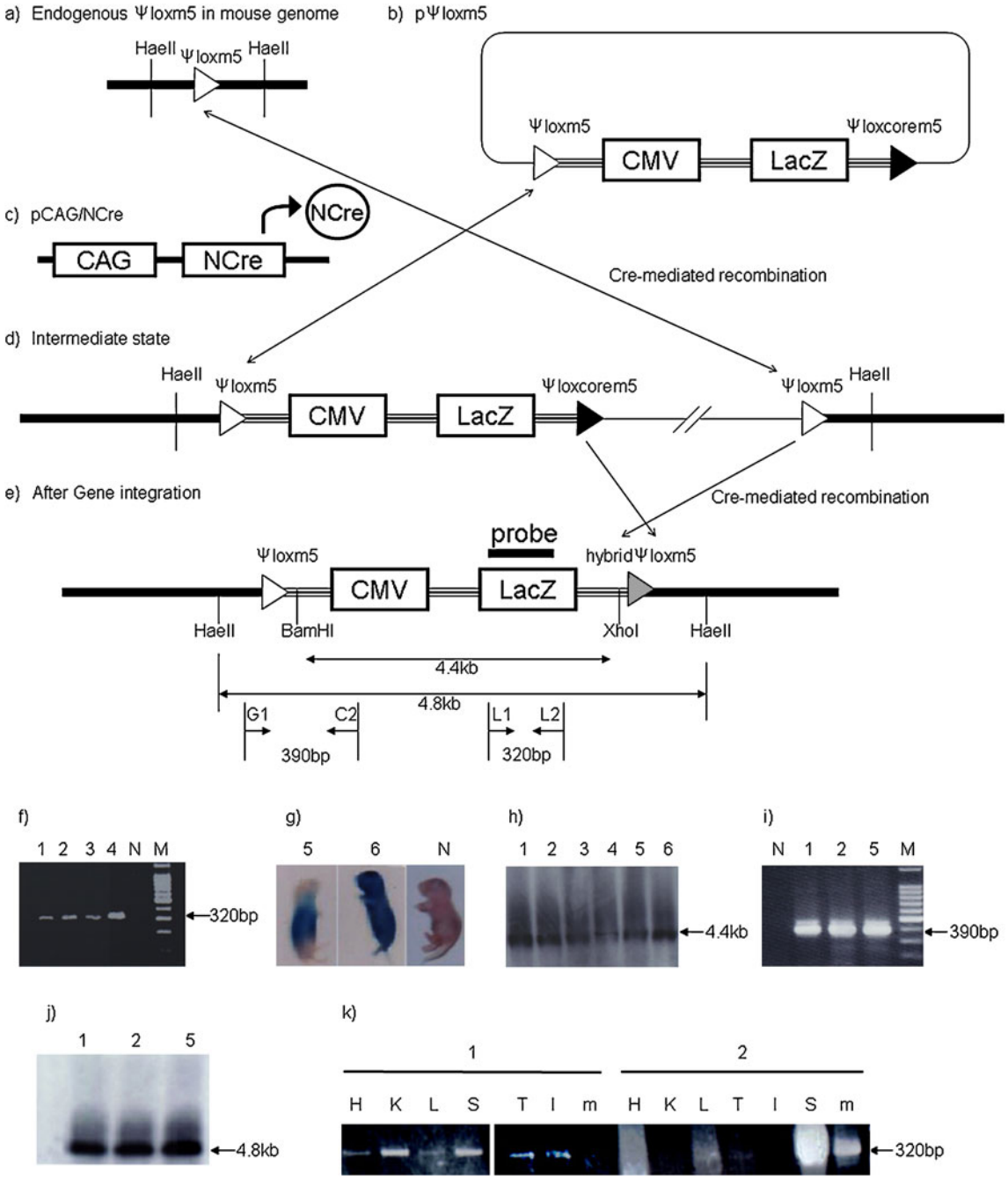

Fig. 2 Strategy and analysis for targeting the endogenous ψ*loxm5* site. The structure of the targeted ψ*loxm5* locus (**a**) and the plasmid pψloxm5 (**b**), and their recombination forms (**d**, **e**) are shown. pψloxm5 and Cre-expressing plasmid (pCAG/NCre) (**c**) were co-microinjected into the pronucleus of mouse fertilized eggs. In the first step, Cre-mediated recombination results in insertion of the transgene, containing its plasmid backbone (**d**). In the second recombination, Cre mediates single-copy integration of the transgene into the endogenous ψ*loxm5* locus of the mouse genome (**e**). PCR analysis of DNAs from mice using L1 and L2 primers from the *LacZ* gene (**f**). X-gal staining of 15 dpc fetuses (**g**). Southern blot analysis of transgene integration using DNA digested with *Bam*HI and *Xho*I (**h**). Analysis of site-specific integration of the transgene by PCR using the indicated primers (G1 and C2) from the endogenous targeted site, and CMV from the transgene (**i**). Analysis of single-copy and site-specific integration of the transgene by Southern blotting using DNA digested with *Hae*II (**j**). DNAs from different tissues of tg1 and 2 mice were extracted and analyzed by PCR for analysis of stable integration of the transgene into various tissues (**k**). N, non-transgenic; M, size marker; H, heart; K, kidney; L, liver; S, skin; T, testis; l, lung; m, muscle

(Fig. 2b). For the ψ*loxcorem5*, a sequence that contains the 13 bp palindromic inverted repeats was derived from *loxP*, and the 8 bp core was derived from the ψ*loxm5*. This arrangement was used in order to maximize the binding of Cre recombinase to the palindoromes (Thyagarajan et al. 2000).

Collection of mouse zygotes and DNA microinjection

Seven-week-old B6C3F1 (C57BL/6J X C3H/He) mice purchased from a commercial breeder (Sankyo Labo-service, Tokyo, Japan) were used for the collection of fertilized eggs. Eight-to-ten-week-old ICR female mice were used as foster mothers. Mice were maintained at 22±2°C and 50±10% humidity on a 14:10-h light-dark cycle, and provided with food and water *ad libitum*. B6C3F1 females were superovulated by injecting 5 IU each of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) at 48-h intervals. Collection of pronuclear stage zygotes and culturing of embryos were carried out according to the standard protocol (Hogan et al. 1994). Pronuclear microinjection of DNA was performed according to the methods used in our previous study (Seo et al. 2000). To test the efficiency of Cre-mediated recombination at an early stage of embryonic development (Fig. 1), 3 ng/μl of pCAG/NCre plasmid and 1 ng/μl of pCMV/loxP/LacZ plasmid were co-injected into the pronucleus of fertilized eggs. To test the endogenous ψ*loxm5* site-specific integration (Fig. 2), 3 ng/μl of pCAG/NCre plasmid and 1 ng/μl of pψloxm5 plasmid were co-injected into the pronucleus of fertilized eggs. The present experiments were conducted according to the guidelines for the use and care of laboratory animals set out by the College of Agriculture, The University of Tokyo.

Analysis of Cre/ψ*loxm5* recombination

Genomic DNA was extracted from tissue or embryo. Samples were collected into tubes containing lysis buffer (10 mM Tris, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween20, 60 μg/ml proteinase K) and incubated at 55°C, followed by phenol/chloroform purification. For the analysis of transgenesis, DNAs were analyzed by polymerase chain reaction (PCR) and Southern blotting. For PCR, L1 primer (5'-GCG-TTA-CCC-AAC-TTA-ATC-G-3') and L2 primer (5'-TGT-GAG-CGA-GTA-ACA-ACC-3') were used for amplification of the sequences within the *LacZ* gene (Ito et al. 2002; Ito et al. 2005). For Southern blotting, DNA was digested with *Bam*HI and *Xho*I, electrophoresed, blotted onto membrane, and hybridized with the ^{32}P-labeled probe from the *lacZ* gene. To investigate the site-specific integration of the transgene into the targeted ψ*loxm5* site, DNAs from transgenic mice were analyzed by PCR and Southern blotting. For PCR, G1 primer (5'-GTT-TAC-TAA-ATG-CCA-GTG-CTT-TG-3') designed from the endogenous *loxm5* site on the genome (accession No. AF033025) and C1 primer (5'-CAT-GTA-CTG-GGC-ATA-ATG-CCA-GG-3') from the transgene were used. For Southern blotting, DNA was digested with *Hae*II located at the 5' and 3' flanking regions of the targeted locus.

Detection of βgalactosidase (βgal) activity

X-gal staining was performed according to the methods used in our previous study (Ito et al. 2002; Ito et al. 2005; Ito et al. 2006). The embryos were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS)(-) for 15 min. After the embryos had been washed three times with PBS(-) containing 0.05% bovine serum albumin (BSA), they were transferred to a solution containing 1.2 mM 5-bromo-4-chloro-3-indolyl β−D-galactopyranoside (X-gal), 2 mM $MgCl_2$, 0.1% Triton X-100, 4 mM $K_4Fe(CN)_6$, and 4 mM $K_3Fe(CN)_6$ in PBS(-), then incubated at 30°C for 24 h. Beta-gal activity in the embryos was identified by a blue color.

Results

Cre transient expression and recombination assay in the mouse embryos

Cre has been reported to carry out an integrative recombination in eukaryotic cells (Sauer and Henderson 1990). Cre-mediated integration would be more useful for generating transgenic animals via pronuclear DNA microinjection if recombination occurred at the one-cell stage of embryonic development; recombination at later stages could result in the production of mosaic animals. To monitor the efficiency of Cre-mediated recombination at the one-cell stage of embryonic development, a transient expression assay was conducted. pCMV/LoxP/LacZ (Fig. 1a) and Cre-expressing plasmid pCAG/NCre (Fig. 1b) were microinjected into the pronucleus of fertilized eggs and allowed to develop in M16 medium for 10 h before X-gal staining. After 10 h of culturing, 49 embryos were still at the one-cell stage, while 81 embryos were cleaved into the two-cell stage. After X-gal staining (Fig. 1d), 23% (11/49) and 65% (53/81) of the embryos were positive at the one-cell and two-cell stages, respectively. The specificity of X-gal staining was confirmed by the fact that embryos injected with pCMV/loxP/LacZ alone were not stained at all. These results suggest that Cre expression and Cre-mediated recombination at *loxP* sites occurred at the one-cell stage of embryonic development.

Genomic ψ*loxm5* site-targeting strategy

As depicted in the experimental outline of Fig. 2, the Cre-mediated integration of the transgene requires two recombination steps. In the first step, the transgene (CMV/*LacZ*) is integrated into with vector backbone. In the second step, Cre is expected to catalyze excision of the vector backbone and to form hybrid ψ*loxm5*. The second recombination should result in a single copy integration of the transgene into the host genome. Out of 16 pups born, four were found to be transgenic by PCR using an L1/L2 primer set (Fig. 2f), a finding that was confirmed by Southern blotting using a probe from the *LacZ* gene (Fig. 2h). To assay the expression and the mosaicism of the integrated transgene, other fetuses that had not been allowed to develop to term were stained with X-gal for βgal activity at 15 days after the embryo transfer. Out of six fetuses, two exhibited βgal activity. One was stained with X-gal over the entire body, and the other showed a mosaic blue deposit in the middle part of the body (Fig. 2g). The transgene integration in X-gal-positive fetuses was confirmed by Southern blotting (Fig. 2h). Next, to investigate whether the transgene was specifically integrated at the endogenous ψ*loxm5* site of the host genome, DNA from the six transgenic mice was analyzed by PCR using specific primer sets, as seen in Fig. 2e. Surprisingly, the 390-bp band was found only in three (tg1, tg2, tg5) of the six transgenic mice (Fig. 2i). Southern blot analysis showed that the correct integration (i.e., site-specific) of the transgene occurred in these three PCR-positive transgenic mice (Fig. 2j). The 4.8-kb bands correspond to a single-copy integrated transgene. These results indicate that the site-specific integration of the transgene occurred in 50% of the produced transgenic mice. To investigate whether the targeting vector was stably integrated into various tissues of the produced transgenic mice, DNAs were extracted from different tissues and analyzed by PCR. The results showed a mosaic integration of the transgene into different tissues (Fig. 2k).

Discussion

We have shown that the naturally occurring ψ*loxm5* site of the mouse genome is a functional sequence for the Cre-mediated DNA recombination event in an embryonic environment. We were able to target the endogenous ψ*loxm5* locus with a high integration frequency for site-specific integration of the transgene. The transgenesis efficiency in the newborn pups and fetuses that had not been to develop to term was 27%. In the present study, the DNA used for microinjection was in a circular form. The integration efficiency of supercoiled DNA following its injection into the pronucleus has been reported to be very low (5.2%) (Brinster et al. 1985). Therefore, this result confirmed that Cre recombinase has the potential to promote insertion of foreign DNA into the defined site of the host genome. One of two X-gal-positive fetuses exhibited the blue deposit throughout its whole body; while the other showed mosaic staining with X-gal only in the middle part of its body (Fig. 2g). Mosaic transgenesis was also observed by PCR analysis of different tissues from tg1 and tg2 (Fig. 2k). This mosaic result would have been caused by the delayed integration of trangene after the one-cell stage of embryonic development or as a result of a second-round recombination of ψ*loxm5*-flanked transgene after its integration. Cre protein may still be produced from the Cre-expressing vector, and the newly inserted DNA surrounded by the ψ*loxm5* sequences is subjected again to excision. It has been reported that after transfecting CHO cells with Cre-expressing plasmid, Cre recombinase is active for 3 days (Koresawa et al. 2000). Fifty percent of our transgenic mice showed the correct integration of the transgene into the targeted ψ*loxm5* locus, which suggests that our trangene was randomly integrated somewhere in the genome of the other three transgenic mice, or that the transgene was specifically integrated into other unknown *lox*-like sequences in the genome. Loonstra et al. (2001) have reported that chromosomal analysis after Cre expression in mammalian cells revealed numerous chromosomal aberrations and an increased number of sister chromatid exchanges, probably due to the occurrence of other functional *lox*-like sequences in the genome. The Cre/*loxP* site-directed recombination system has become an important tool for the genetic manipulation of transgenic animals, and may be important in the future as a tool for human gene therapy. The occurrence of the *lox*-like sequences in the relatively small yeast genome (Sauer 1996) is strongly indicative of the presence of cryptic *lox* sites in most eukaryotic genomes. Interestingly, cryptic *lox* sites were identified by *in silico* analysis in the mammalian genome. Cre-mediated recombination can occur in *E. coli* cells at the *loxP* sites of BAC/PAC (Semprini et al. 2007). The identification of functional *lox*-like sequences in farm animals would be important for exploiting a simple and efficient method for producing transgenic farm animals. Analysis of the *lox* structure will certainly be important for this purpose. Characterization of *lox* sequences has been carried out (Lee and Saito 1998; Missirlis et al. 2006; Sheren et al. 2007).

Conclusions

Our results showed that the Cre/ψ*lox* system is an efficient and simple strategy for targeting an endogenous *lox*-like site of the genome. This system provides an appealing strategy for circumventing the major problems facing

transgenic animal production such as the position effect of transgene expression and variations in copy number of integrated transgenes. Moreover, this strategy can enhance the overall efficiency of transgenic animal production.

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