Journal of Reproduction Engineering
Vol.8, Supplement, 337-345, 2005

CRTEIL, a Cre-*loxP*-based system allowing change of expression of red to green fluorescence upon transfection by a Cre expression vector

Masahiro Sato*

Division of Basic Molecular Science and Molecular Medicine, School of Medicine, Tokai University, Bohseidai, Isehara, Kanagawa 259-1193, Japan

ABSTRACT

We developed a new Cre-*loxP*-based system, termed CRTEIL, using two fluorescent proteins [enhanced green fluorescent protein (EGFP) and HcRed1] and firefly luciferase (luc) cDNAs. As a tester plasmid, we constructed the pCRTEIL-6 plasmid consisting of cytomegalovirus enhancer/chicken β-actin promoter (CAG), a portion of the rabbit β-globin gene, *loxP*-flanked DNA sequence [containing HcRed1 cDNA and chloramphenicol acetyltransferase gene], EGFP cDNA, internal ribosomal entry site (IRES), and luc cDNA. When pCRTEIL-6 and Cre-expression plasmid (pCAG/NCre-5) were co-transfected into NIH3T3 cells, some of these cells exhibited EGFP (green fluorescence) or HcRed1 (red fluorescence) or both. Single transfection with pCRTEIL-6 resulted in predominant expression of HcRed1. Furthermore, luc activity was much higher in co-transfected than in singly transfected cells. *In vivo* electroporation-mediated gene delivery of both pCRTEIL-6 and pCAG/NCre-5 into murine oviductal epithelium yielded the same results as obtained with *in vitro*-transfected NIH3T3 cells. These findings indicate that the CRTEIL system works well both *in vitro* and *in vivo*. This system will be powerful in various fields such as cell lineage analysis and basic research in regenerative medicine, since descendants of cells exhibiting newly activated gene expression can be continuously monitored in non-invasive conditions.

Received for publication: Sep.14, 2005.
Accepted: Sep.28, 2005.
*Correspondence: Dr. M. Sato

Key Words: *Cre*-loxP *system; EGFP; fluorescent protein; HcRed1; lineage analysis; luciferase*

INTRODUCTION

The bacteriophage P1 Cre recombinase, a 38 kDa protein, recognizes specific 34 bp sequences called *loxP* sites, and catalyzes site-specific recombination between two *loxP* sites [1,2]. The Cre-*loxP* system has been considered an important tool for manipulation of genomic sequences and gene expression including tissue-specific activation or inactivation of genes, as well as cell type-specific gene targeting [3].

Araki et al. [4] first utilized this system for manipulation of mouse preimplantation embryos. They constructed a CAG-CAT-Z transgene, in which expression of the chloramphenicol acetyltransferase (CAT) gene flanked by the *loxP* sequences occurs under control of the ubiquitously active cytomegalovirus enhancer/chicken β-actin promoter (CAG) [5]. Expression of the lacZ gene, which is located downstream of the *loxP*-flanked CAT sequence in the CAG-CAT-Z transgene, does not occur in the absence of Cre recombinase protein. When Cre expression vector DNA was injected into the pronuclei of fertilized eggs carrying the CAG-CAT-Z transgene, expression of lacZ protein was induced, indicating that expression of the lacZ gene is induced after removal of the *loxP*-flanked CAT sequence by Cre recombinase. Once such recombination occurs, expression of the lacZ gene in descendent cells will continue throughout embryogenesis.

Using a strategy similar to that of Araki et al. [4], we previously constructed an expression plasmid, pCETZ-17, which consists of CAG, *loxP*-flanked DNA sequence [containing enhanced green fluorescent protein (EGFP) cDNA], and lacZ gene [6]. This Cre-*loxP*-based system was useful for cell lineage analysis in mice. For example, when one blastomere of the 2-cell embryos derived from CETZ-17 transgenic mice was microinjected with pCAG/NCre, its descendant exhibited Cre-mediated recombination in the integrated transgenes and exhibited lacZ activity. However, the offspring of the other blastomere, into which no injection was performed, remained silent for lacZ activity. Histochemical detection of lacZ activity is always associated with fixation of tissues and subsequent staining with X-Gal, a substrate for lacZ. This procedure often hampers serial microscopic observation of cells expressing newly activated gene

products, and some tissues (i.e., adult ovaries, oviducts, and epididymides) are rich in endogenous activity for lacZ.

In this study, we constructed a new plasmid pCRTEIL-6 containing CAG, *loxP*-flanked sequence (HcRed1 cDNA and CAT), EGFP cDNA, internal ribosomal entry site (IRES), and firefly luciferase (luc) cDNA. Cells carrying pCRTEIL-6 in their genome exhibit HcRed1 (red fluorescence), but neither EGFP nor luc. However, upon transfection with a Cre-expression vector, they will exhibit EGFP (green fluorescence), because the *loxP*-flanked sequence in the integrated pCRTEIL-6 transgenes is removed by transiently-expressed Cre protein. This gene switching can be observed in viable cells and the behavior of descendants of the cells exhibiting Cre-mediated gene activation, which would exhibit EGFP continuously, can be monitored under a fluorescence microscope.

MATERIALS AND METHODS

Construction of vectors

The reporter plasmid pCRTEIL-6 (Fig. 1) was constructed through several cloning steps. First, pCRT-17, an intermediate product for pCRTEIL-6, was constructed by inserting HcRed1 cDNA + poly(A) sites of the SV40 gene (Clontech Laboratories, Inc., Palo Alto, CA.) in front of the 5' end of CAT in the pCAG-CAT-lacZ [4] from which the lacZ gene had already been removed. A DNA fragment containing EGFP cDNA, IRES, and luc cDNA was then placed immediately downstream of the *loxP* site located at the 3' end of the CAT gene in pCRT-17 to obtain pCRTEIL-6. The resulting pCRTEIL-6 has a backbone of pBluescript SK(-) (Stratagene, La Jola, CA). For expression of Cre, we used pCAG/NCre-5 plasmid [7]. pCE-29 plasmid [8] was also used as a positive vector for expression of EGFP in murine cells.

Cells and transfection

NIH3T3 cells were first seeded onto gelatin-coated 6-well dishes (#4810-020; Iwaki Glass Co., Tokyo, Japan) at a density of 10^6 cells/well one day before transfection and grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Co., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen Co.) at 37°C in an atmosphere of 5% CO2 in air at 37°C. For transfection of a single plasmid, four μg of plasmid DNA was mixed with 8 μl of LF2000 (#11668-027; Invitrogen Co.) in Dulbecco's modified phosphate-buffered saline without Ca^{2+} and Mg^{2+}, pH 7.4 [PBS(-)] and a total of 100 μl solution was prepared according to the manufacturer's protocol. For co-transfection, two plasmids (3 μg for each) were mixed with 12 μl of LF2000 in PBS(-). These DNA/liposome complex

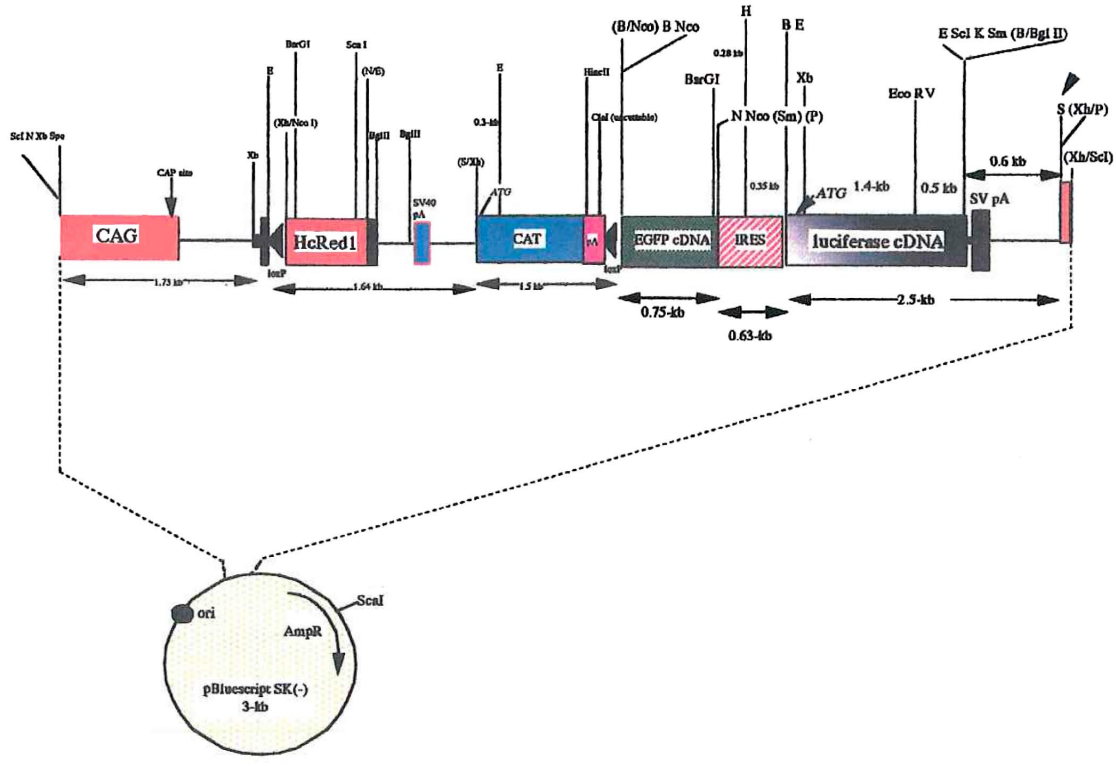

Fig. 1. Structure of the target plasmid (pCRTEIL-6). Before recombination, the *loxP*-flanked HcRed1/CAT hybrid sequence is expressed under control of the CAG promoter in cells carrying pCRTEIL-6, while the EGFP and luc cDNAs are silent. Cre-mediated recombination results in deletion of the HcRed1/CAT sequence and expression of the EGFP and luc cDNAs. Parentheses indicate enzymatic sites destroyed. Amp^R, ampicillin resistance gene; ATG, translation initiation site; B, *Bam* HI; CAG, cytomegalovirus enhancer + chicken β-actin promoter; CAP site, transcription start site; CAT, chloramphenicol acetyltransferase gene; E, *Eco* RI; EGFP, enhanced green fluorescent protein cDNA; IRES, internal ribosomal entry site; H, *Hin* dIII; K, *Kpn* I; N, *Not* I; Nco, *Nco* I; ori, replication origin; P, *Pst* I; pA, poly(A) sites; ScI, *Sac* I; Sm, *Sma* I; Spe, *Spe* I; SV40 pA, poly(A) sites of SV40 gene; Xb, *Xba* I; Xh, *Xho* I.

were added to the cell culture and incubated for 1 day at 37°C. After transfection, cells were observed for fluorescence, as described below.

Observation for fluorescence

Cells were observed using an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan) with DM505 filters (BP460-490 and BA510IF) and DM600 filter(BP545-580 and BA6101F),

which were used for EGFP and HcRed1 monitoring, respectively. For detection of fluorescence in dissected oviducts, an Olympus BX40 dissecting microscope was used. Microphotographs were taken using a digital camera (FUJIX HC-300/OL; Fuji Film, Tokyo, Japan) attached to the fluorescence microscope and printed out using a Mitsubishi digital color printer (CP700DSA; Mitsubishi, Tokyo, Japan).

Gene transfer to oviductal epithelium by* in vivo *electroporation (EP)

Intraoviductal injection was performed as described previously [9]. One μl of solution containing plasmid DNA and trypan blue (TB; 0.05% at final concentration) was slowly injected with a glass micropipette, which had been attached to a mouth-piece, into the ampulla of an oviduct. The DNA introduced per oviduct was pCE-29 (0.2 μg), pCRTEIL-6 (0.2 μg), pCAG/NCre-5 (0.2 μg) or pCRTEIL-6 (0.2 μg) + pCAG/NCre-5 (0.2 μg). After finishing injection, the micropipette was rapidly removed. The oviductal regions were then subjected to *in vivo* EP. Eight square-wave pulses with a pulse duration of 50 msec and electric field intensity of 50 V were administered from a square-wave pulse generator (T820; BTX Genetronics, Inc., San Diego, CA). One day after *in vivo* EP, the oviducts were subjected to observation for fluorescence and then to lysis for measurement of luc activity.

Luciferase assay

Luc assay was performed using a kit [Dual-Luciferase Reporter Assay System (No. E1910); Promega Co., Madison, WI]. The oviducts isolated 1 day after *in vivo* EP were homogenized in 1 ml of 1 X reporter lysis buffer (Promega Co.). After centrifugation at 15,000 rpm for 10 min at 4°C, the supernatant (200 μl) was transferred to a fresh Eppendorf tube. Cells 1 day after transfection were collected with a cell scraper and precipitated after brief centrifugation. Cell pellets were then lysed with 1 ml of 1 X reporter lysis buffer. Relative light units (RLU) obtained with luc were measured for 5 sec following a 2-sec delay after the addition of the lysate (10 ml) to 50 ml of luc assay substrate (Promega Co.) using a luminometer (#TD-20/20; Turner Designs Instrument, Sunnyvale, CA). RLU were normalized to micrograms of tissue protein added in the luc assay. Tissue protein determinations were performed using Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA).

RESULTS AND DISCUSSION

Cre-mediated recombination in vitro

To test whether Cre-mediated excision of the *loxP*-flanked HcRed1/CAT sequence in the pCRTEIL-6 construct results in generation of EGFP fluorescence, NIH3T3 cells were co-transfected with pCRTEIL-6 and pCAG/NCre-5 DNA by the lipoplex method. One day after transfection, some of these cells exhibited both red and green fluorescence (indicated by arrowheads in Fig. 2e,f), suggesting incomplete Cre-mediated recombination in the introduced pCRTEIL-6 construct. There were a few cells preferentially expressing green fluorescence (indicated by arrows in Fig. 2e,f), suggesting the occurrence of complete Cre-mediated recombination in the transfected cells. Interestingly, cells expressing only red fluorescence, suggesting incorporation of two constructs at the same time into a single cell, were extremely rare in this system. In the control experiment in which cells were transfected by pCRTEIL-6 alone, some cells exhibited HcRed1-derived red fluorescence but not EGFP-derived green fluorescence (Fig. 2b *vs.* 2c). These findings indicate that the Cre-*loxP* system using pCRTEIL-6 as a reporter plasmid works well with cultured murine cells.

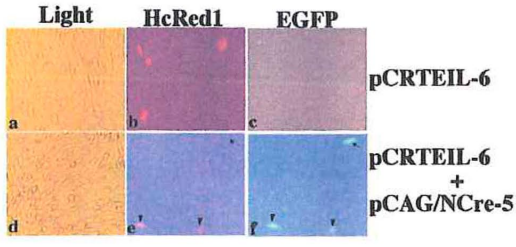

Fig. 2. Cre-mediated excision *in vitro*. (a-c) NIH3T3 cells transfected with pCRTEIL-6 alone. When cells were inspected for fluorescence 1 day after transfection, HcRed1-derived red fluorescence (but not EGFP-derived green fluorescence) was predominant. Microphotographs **(a-c)** indicate the same cells. **(d-f)** NIH3T3 cells co-transfected with pCRTEIL-6 and pCAG/NCre-5. Some cells exhibited green fluorescence predominantly [indicated by arrows in **(e)** and **(f)**], while other cells exhibited both types of fluorescence [indicated by arrowheads in **(e)** and **(f)**]. Microphotographs **(d-f)** indicate the same cells. **(a,b)** Photographs taken under light (Light); **(b,e)** photographs taken under UV illumination using filters for detection of red fluorescence (HcRed1); **(c,f)** photographs taken under UV illumination using filters for detection of green fluorescence (EGFP).

Cre-mediated recombination in vivo

We next examined whether this Cre-*loxP* system using pCRTEIL-6 as a reporter plasmid can also be used *in vivo*. For this purpose, gene delivery into oviductal epithelium was performed by DNA injection into the lumen of oviducts and subsequent EP [9]. Instillation of pCE-29 plasmid yielded bright green

fluorescence throughout the ampulla portion (Fig. 3b), but not red fluorescence (Fig. 3c). When pCRTEIL-6 plasmid DNA was singly introduced, no fluorescence for EGFP was observed (Fig. 3e). Instead, red fluorescence was observed in some oviductal epithelial cells (Fig. 3f). Co-injection with pCRTEIL-6 and pCAG/NCre-5 plasmids resulted in generation of both red and green fluorescence, although green fluorescence appeared to be weaker than

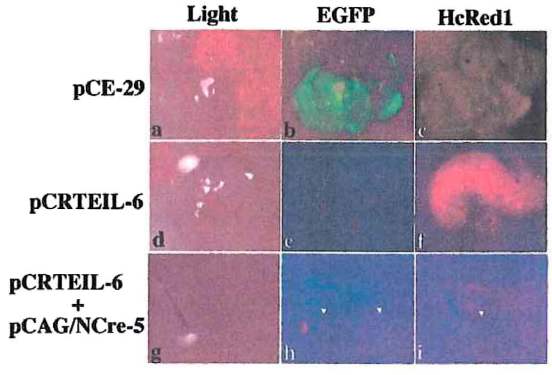

Fig. 3. Cre-mediated excision *in vivo*. (a-c) Oviducts transfected with pCE-29 alone. EGFP-derived green fluorescence (but not HcRed1-derived red fluorescence) was observed. **(d-f)** Oviducts transfected with pCRTEIL-6 alone. HcRed1-derived red fluorescence (but not EGFP-derived green fluorescence) was observed. **(g-i)** Oviducts co-transfected with pCRTEIL-6 and pCAG/NCre-5. Both types of fluorescence [indicated by arrows in **(h)** and **(i)**] were seen, although green fluorescence appeared to be stronger than red fluorescence. **(a,d,g)** Photographs taken under light (Light); **(b,e,h)** photographs taken under UV illumination using filters for detection of green fluorescence (EGFP); **(c,f,i)** photographs taken under UV illumination using filters for detection of red fluorescence (HcRed1).

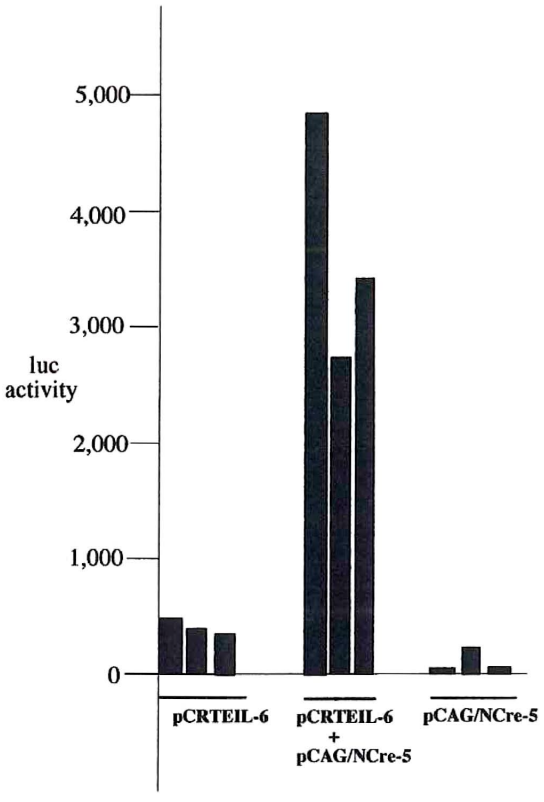

Fig. 4. Luciferase reporter gene activity in oviduct sampled 1 day after *in vivo* gene delivery to oviducts. Oviducts were *in vivo*-transfected with pCRTEIL-6 alone (negative control), pCRTEIL-6, and pCAG/NCre-5 (experiment) or pCAG/Ncre-5 alone (negative control). Oviducts were isolated from each mouse at the indicated day to measure luc activity. Of a total of 5 females injected with DNA, 3 oviducts were subjected to measurement of luc activity for each transfection group.

red fluorescence (indicated by arrows in Fig. 3h, i). These findings indicate that the Cre-*loxP* system using pCRTEIL-6 works well even *in vivo*.

Induction of luciferase activity by co-transfection with pCRTEIL-6 and pCAG/NCre-5

The IRES sequence is present between EGFP and luc cDNAs in the pCRTEIL-6 plasmid (Fig. 1). Therefore, both proteins (EGFP and luc) should be produced at the same time in a cell into which pCRTEIL-6 and pCAG/NCre-5 have been co-transfected. In fact, we observed increase in luc activity repeatedly when oviductal epithelial cells were co-transfected with pCRTEIL-6 and pCAG/NCre-5 (Fig. 4).

In this study, we have described switching of gene expression from red to green fluorescence by the Cre-*loxP* system when pCRTEIL-6 plasmid is used as a tester plasmid. This Cre-*loxP* system using pCRTEIL-6 has great advantages over the previous CETZ-17 system [6], because the process of change of gene expression can be easily monitored in non-invasive conditions. Furthermore, it is possible to trace the fate of the descendants that express EGFP under physiological conditions. For finer analysis of the fate of cells transfected with exogenous genes using the pCRTEIL-6-based Cre-*loxP* system, it will be convenient to use a transgenic mouse line carrying the CRTEIL-6 transgene. This experiment is now underway.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Kimi Araki (Kumamoto University) for allowing us to use pCAG-CAT-Z plasmid. This study was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture, Japan.

REFERENCES

1) Sternberg N, Hamilton D. Bacteriophage P1 site-specific recombination. I. Recombination between *loxP* sites. *J. Mol. Biol.* 150, 467-486, 1981.
2) Hoess RH, Ziese M, Sternberg N. P1 site-specific recombination: nucleotide sequence of the recombining sites. *Proc. Natl. Acad. Sci. U.S.A.* 79, 3398-3402, 1982.
3) Porter A. Controlling your losses: Conditional gene silencing in mammals. *Trends Genet.* 14, 73-79, 1998.
4) Araki K, Araki M, Miyazaki J, Vassalli P. Site-specific recombination of a transgene

in fertilized eggs by transient expression of Cre recombinase. *Proc. Natl. Acad. Sci. U.S.A.* 92, 160-164, 1995.

5) Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transformants with a novel eukaryotic vector. *Gene* 108, 193-200, 1991.
6) Sato M, Yasuoka Y, Kodama H, Watanabe T, Miyazaki J, Kimura M. New approach to cell lineage analysis in mammals using the Cre-*loxP* system. *Mol. Reprod. Dev.* 56, 34-44, 2000.
7) Sato M, Watanabe T, Kimura M. Embryo transfer *via* oviductal wall: An alternative method for efficient production of transgenic mice. *Transgenics* 2, 383-389, 1999.
8) Sato M, Ishikawa A, Kimura M. Direct injection of foreign DNA into mouse testis as a possible *in vivo* gene transfer system *via* epididymal spermatozoa. *Mol. Reprod. Dev.* 61, 49-56, 2002.
9) Sato M: Intraoviductal introduction of plasmid DNA and subsequent electroporation for efficient in vivo gene transfer to murine oviductal epithelium. *Mol. Reprod. Dev.* 71, 321-330, 2005.