A novel reporter mouse strain that expresses enhanced green fluorescent protein upon Cre-mediated recombination

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Abstract The success of Cre-mediated conditional gene targeting depends on the specificity of Cre recombinase expression in Cre-transgenic mouse lines. As a tool to evaluate the specificity of Cre expression, we developed a reporter transgenic mouse strain that expresses enhanced green fluorescent protein (EGFP) upon Cre-mediated recombination. We demonstrate that the progeny resulting from a cross between this reporter strain and a transgenic strain expressing Cre in zygotes show ubiquitous EGFP fluorescence. This reporter strain should be useful to monitor the Cre expression directed by various promoters in transgenic mice, including mice in which Cre is expressed transiently during embryogenesis under a developmentally regulated promoter.

Key words: Cre/*loxP* recombination; Transgenic mouse; Green fluorescent protein; β-Actin promoter; Keratin 5 promoter

1. Introduction

The Cre/*loxP* site-specific recombination system derived from bacteriophage P1 provides a convenient tool for tissue-specific or developmental stage-specific modulation of gene expression in knockout mice and transgenic mice [1]. For these studies, various lines of transgenic mice that express Cre recombinase with different specificity have been generated. The success of Cre-mediated conditional gene targeting depends on the specificity of the Cre recombinase expression in Cre-transgenic mouse lines [2]. We reported previously that a transgenic mouse strain that expresses *Escherichia coli* β-galactosidase after Cre-mediated excision of the *loxP*-flanked chloramphenicol acetyltransferase (CAT) gene serves as a useful tool to monitor the specificity of Cre expression [3,4]. Other reporter strains have also been reported, in which the β-galactosidase–neomycin phosphotransferase fusion gene (β-*geo*), inserted in the ROSA26 locus, is expressed only after Cre-mediated excision of the *loxP*-flanked sequence [5,6].

Green fluorescent protein (GFP), obtained from the jellyfish *Aequorea victoria*, yields green fluorescence when excited with UV light [7–9]. Light-stimulated GFP fluorescence does not require any co-factors, substrates, or additional gene products from *A. victoria*, and can be detected in living cells. Enhanced GFP (EGFP) is a red-shifted GFP variant with humanized codon usage, and the green fluorescence of EGFP is 4–35-fold brighter than that of the wild-type GFP, when excited with blue light [10]. EGFP is suitable for fluorescence microscopy and flow cytometry. Therefore, EGFP has been used as a novel genetic reporter system in many studies both in vitro and in vivo.

To create a simple and efficient method for evaluating the specificity of Cre expression, we have developed transgenic mouse lines carrying a reporter gene construct, CAG-CAT-EGFP, which directs the expression of EGFP upon the Cre-mediated excision of the *loxP*-flanked CAT gene located between the modified chicken β-actin promoter with the CMV-IE enhancer (CAG promoter) [11] and the EGFP gene. Thus, in these reporter mice, the CAT gene is driven by the CAG promoter before recombination, while the EGFP gene is driven by the same CAG promoter only after Cre-mediated recombination. These reporter mice are expected to produce green fluorescence in the tissues where Cre-mediated recombination has occurred, since the CAG promoter is highly active in a wide range of cell types, even in embryos. In fact, the 'green mice' reported by Okabe et al. are transgenic mouse lines expressing EGFP ubiquitously under the CAG promoter [12].

Here we crossed one of these reporter lines to a CAG-Cre transgenic mouse line that expresses Cre under the control of the CAG promoter. We demonstrate that all tissues examined, except for red blood cells and hair, of the double transgenic F1 progeny express EGFP. We also demonstrate that an epidermis-specific expression of EGFP can be obtained in the F1 progeny resulting from a cross between this reporter line and K5-Cre transgenic mice, in which Cre is driven by a keratinocyte-specific promoter [13]. These results indicate that this reporter strain should be a useful tool for monitoring the Cre expression driven by various promoters in transgenic mouse lines.

2. Materials and methods

2.1. Mice

C57BL/6J mice and BDF1 mice were purchased from Clea Japan Inc. (Tokyo, Japan). CAG-Cre transgenic mice expressing Cre recombinase under the control of the CAG promoter were described pre-

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Abbreviations: GFP, green fluorescent protein; EGFP, enhanced GFP; CAT, chloramphenicol acetyltransferase; CAG promoter, modified chicken β-actin promoter with CMV-IE enhancer; FACS, fluorescence-activated cell sorter

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viously [4]. K5-Cre transgenic mice expressing Cre recombinase under the control of the keratin 5 (K5) promoter were described previously [13]. These mice were kept under specific pathogen-free conditions in our animal facility at Osaka University Medical School. All transgenic mice used in this study were heterozygous for the transgene.

2.2. Construction of the transgene and generation of transgenic mice

The reporter transgene plasmid was constructed by inserting a *Bam*HI–*Eco*RI fragment containing EGFP cDNA from pEGFP (Clontech, Palo Alto, CA, USA) and an *Eco*RI–*Sac*I fragment containing the rabbit β-globin polyadenylation signal into the *Bam*HI–*Sac*I site of the pCAG-CAT-Z reporter plasmid in place of the *lacZ* gene [3]. The resulting pCAG-CAT-EGFP reporter plasmid contained the CAG promoter, the CAT gene flanked by directly repeated *loxP* sequences, and the EGFP gene (Fig. 1A). The CAG-CAT-EGFP region was excised from the pCAG-CAT-EGFP plasmid and microinjected into the pronuclei of one-cell embryos of BDF1×BDF1 mice to produce transgenic mice. Founder transgenic mice were identified by PCR and Southern blot analyses of tail DNA, and bred with C57BL/6J mice. PCR primers for the transgene detection were as follows: EGFP forward primer, 5′-AGCAAGGGCGAGGAGCTGTT-3′; EGFP backward primer, 5′-GTAGGTCAGGGTGGTCACGA-3′; Cre forward primer, 5′-AGGTTCGTTCACTCATGGA-3′; Cre backward primer, 5′-TCGACCAGTTTAGTTACCC-3′; CAT forward primer, 5′-CAGTCAGTTGCTCAATGTACC-3′; CAT backward primer, 5′-ATATCACCAGCTCACCGTCTT-3′.

2.3. Southern blot analysis

Tail tips were lysed in 1% sodium dodecyl sulfate (SDS), 0.2 mg/ml proteinase K, 10 mM Tris–HCl (pH 7.5), and 1 mM EDTA at 50°C for 3 h. DNA was extracted with phenol–chloroform and precipitated with ethanol. DNA samples (5 μg) were digested with *Eco*RI and subjected to electrophoresis on a 1.2% agarose gel. After denaturation with 0.5 M NaOH and 1.5 M NaCl, separated DNA fragments were transferred from the agarose gel onto a nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Uppsala, Sweden). The *Bam*HI–*Eco*RI fragment containing EGFP cDNA from pEGFP was fluorescein-labeled by random priming (Random Prime Labeling Module, Amersham Pharmacia) and used as a probe. The blot was hybridized with the probe at 62°C overnight, and processed for autoradiography using chemiluminescence techniques (CPD-Star Detection Module, Amersham Pharmacia), according to the manufacturer's instructions.

2.4. Fluorescence-activated cell sorter (FACS) analysis

Peripheral lymphocytes were prepared by density separation in Lympholyte-M (Cedar Lane Lab., Ontario, Canada), according to the manufacturer's instructions. Thymocytes and splenocytes were prepared by squeezing whole thymuses or spleens between a pair of glass slides in phosphate-buffered saline, followed by hemolysis with ammonium chloride buffer. For anti-CD3 staining, cells were incubated with a biotinylated anti-murine CD3 mAb (clone KT3, Beckman Coulter, Fullerton, CA, USA), followed by avidin–PE conjugate. For anti-CD4 staining, cells were incubated with an R-PE-conjugated anti-murine CD4 mAb (clone L3T4, Pharmingen, San Diego, CA, USA). Cell suspensions were filtered through 70-μm nylon mesh, and subjected to analysis on a FACScan cytometer (Becton Dickinson, San Jose, CA, USA). EGFP was excited at 488 nm and detected using a 510–520-nm band-pass filter.

2.5. Observation of EGFP

Various organs excised from transgenic mice were embedded in OCT compound (Tissue-TEK, Miles, Elkhart, IN, USA) and frozen in liquid nitrogen. Sections (10 μm thick) were cut with a cryostat, and placed on 3-amino-propyltriethoxysilane-coated slides. Specimens were examined without fixation by fluorescence microscopy (Olympus, Tokyo, Japan) with a 460–490-nm band-pass excitation filter and a 510-nm long-pass emission filter.

3. Results

3.1. Establishment of CAG-CAT-EGFP transgenic mice

Eleven founder transgenic mice carrying the CAG-CAT-EGFP transgene (Fig. 1A) were generated, nine of which transmitted the transgene to the next generation. Four of these nine transgenic mouse lines (lines 16, 20, 39, and 42) produced offspring expressing EGFP fluorescence detected by fluorescent microscopy when mated with CAG-Cre transgenic mice (Fig. 3A). This EGFP fluorescence could also be recognized with the naked eye under UV light. Three of the lines (20, 39, and 42) were propagated and used in the following study.

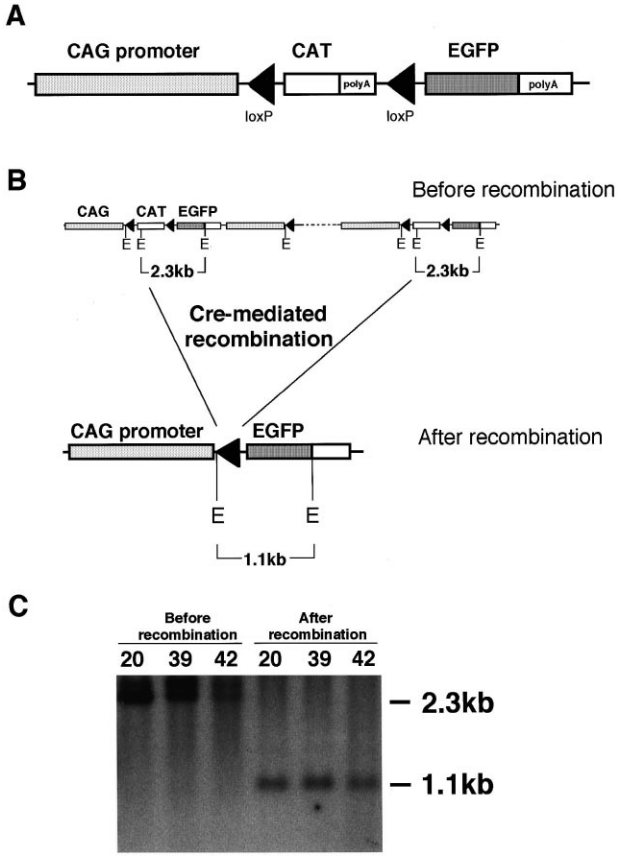

Fig. 1. Structure of the transgene and Cre-mediated recombination. A: Structure of the CAG-CAT-EGFP transgene. The transgene contains the CAG promoter, the *loxP*-flanked CAT gene with the SV40 polyadenylation signal, and the EGFP cDNA with the rabbit β-globin polyadenylation signal. B: Cre-mediated recombination in the transgene. Before recombination, multiple copies of the transgenes connected in a head-to-tail manner are present in a chromosome, and the CAG promoter drives the CAT gene, but not the EGFP gene (upper). Cre-mediated recombination results in the removal of all of the *loxP*-flanked DNA sequences and leaves a single copy of the transgene in which the EGFP gene is driven by the CAG promoter (lower). E, *Eco*RI site. C: Southern blot analysis of *Eco*RI-digested tail DNA of CAG-CAT-EGFP transgenic mice of lines 20, 39, and 42 before and after Cre-mediated recombination. The 2.3-kb band corresponds to the *Eco*RI fragment of the unrecombined transgene. The 1.1-kb band corresponds to the *Eco*RI fragment of the recombined transgene. The EGFP cDNA fragment was used as a probe.

3.2. Cre-mediated recombination in CAG-CAT-EGFP transgenic mice

CAG-CAT-EGFP transgenic lines were mated with heterozygous CAG-Cre transgenic mice and the F1 progeny were examined for EGFP expression by fluorescence microscopy and for genotype by PCR using primer sets specific for the EGFP and Cre genes. Eleven F1 progeny containing both the

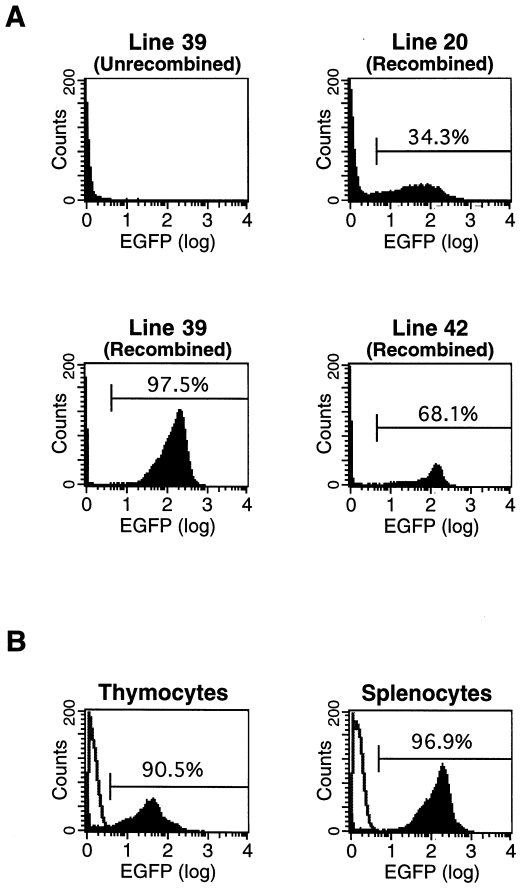

Fig. 2. FACS analysis of the white blood cells of mice with the recombined allele of the CAG-CAT-EGFP transgene. A: Peripheral lymphocytes of the F1 progeny from the reporter mice (lines 20, 39, and 42)×the CAG-Cre transgenic mice expressed EGFP, while those of the parental reporter mice of line 39 with the unrecombined transgene (CAG-CAT-EGFP) showed background fluorescence. B: Thymocytes and splenocytes of the F1 progeny from the reporter mice of line 39×CAG-Cre transgenic mice expressed EGFP uniformly. Gray lines indicate the fluorescence intensity of the cells from the reporter mice of line 39 with the unrecombined transgene.

EGFP and CAG-Cre transgenes were obtained, all of which were fluorescence-positive. Four F1 progeny from CAG-CAT-EGFP transgenic males×CAG-Cre transgenic females were fluorescence-positive and had the EGFP transgene, but not the Cre transgene. We have previously reported that the mature oocytes of heterozygous CAG-Cre transgenic females contain Cre activity sufficient to mediate the deletion of paternally contributed *loxP*-flanked DNA sequences upon fertilization irrespective of the transmission of the Cre gene [4]. Our present results suggest that the *loxP*-flanked CAT gene was readily removed by the maternally derived Cre activity retained in transgene-negative oocytes of CAG-Cre transgenic females.

Southern blot analysis of *Eco*RI-digested tail DNA confirmed that the expected recombination had occurred in the fluorescence-positive F1 progeny from CAG-CAT-EGFP transgenic mice (lines 20, 39, and 42)×CAG-Cre transgenic mice. As shown in Fig. 1C, the tail DNA of parental CAG-CAT-EGFP transgenic mice produced a 2.3-kb band corresponding to the *Eco*RI fragment from the head-to-tail connected transgenes. According to the densitometric analysis, the transgene copy number was 22, 21, and 13 in CAG-CAT-EGFP transgenic mice of lines 20, 39, and 42, respectively. Tail DNA of the fluorescence-positive F1 progeny produced a 1.1-kb band corresponding to the *Eco*RI fragment from a single copy of the recombined transgene, which had lost the whole *loxP*-flanked DNA sequence by Cre-mediated recombination (Fig. 1B).

To examine whether the CAT gene was completely excised by Cre-mediated recombination in the fluorescence-positive F1 progeny from CAG-CAT-EGFP transgenic mice×CAG-Cre transgenic mice, we extracted DNA from the whole embryos at 9.5 days post coitum, and performed PCR analysis for both the CAT gene and the EGFP gene. We detected the PCR product from the EGFP gene, but not from the CAT gene in the fluorescence-positive embryo (not shown). This result indicated that the *loxP*-flanked CAT gene was completely excised by Cre-mediated recombination in the fluorescence-positive F1 progeny from CAG-CAT-EGFP transgenic mice×CAG-Cre transgenic mice, regardless of the multiple transgene copies in CAG-CAT-EGFP transgenic mice.

3.3. FACS analysis of the lymphocytes of mice with the recombined allele of the CAG-CAT-EGFP reporter transgene

We analyzed the expression of EGFP fluorescence in the peripheral lymphocytes of the F1 mice from CAG-CAT-EGFP transgenic mice (lines 20, 39, and 42)×CAG-Cre transgenic mice, by FACS. The peripheral lymphocytes of the F1 mice of line 39 with the recombined allele of the CAG-CAT-EGFP transgene showed a single bright peak of fluorescence. The peripheral lymphocytes of lines 20 and 42 with the recombined allele also showed a single peak, although at lower levels (Fig. 2A). Thymocytes and splenocytes of the F1 mice of line 39 also showed a single bright peak (Fig. 2B). The peripheral lymphocytes of the F1 mice with the unrecombined allele of the transgene did not express fluorescence, and their background fluorescence levels did not differ from those of non-transgenic littermates (not shown).

3.4. Histological analysis of mice with the recombined allele of the CAG-CAT-EGFP reporter transgene

To examine the expression of EGFP fluorescence in various organs of the F1 mice from CAG-CAT-EGFP transgenic mice ×CAG-Cre transgenic mice, we performed histological analysis by fluorescence microscopy. We examined the brain, spinal cord, eye, lung, heart, thymus, liver, kidney, spleen, pancreas, stomach, ileum, colon, and skeletal muscle of the F1 mice from line 39, which had the recombined allele of the CAG-CAT-EGFP transgene. All of these tissues expressed EGFP (Fig. 3). However, red blood cells and hair were negative for EGFP fluorescence (not shown). Skeletal muscle, heart, liver, the epithelium of the gut and pancreatic exocrine tissue expressed strong EGFP fluorescence. Other tissues, including brain, kidney and pancreatic islets, expressed considerably lower levels of fluorescence. As shown in Fig. 3K, pancreatic islets showed very weak fluorescence compared with the exocrine part of the pancreas. However, fluorescence of isolated islets was clearly detectable by fluorescence microscopy (Fig. 3L). On the other hand, we could not detect EGFP fluorescence in any tissue of the F1 mice with the unrecombined allele of the CAG-CAT-EGFP transgene (not shown).

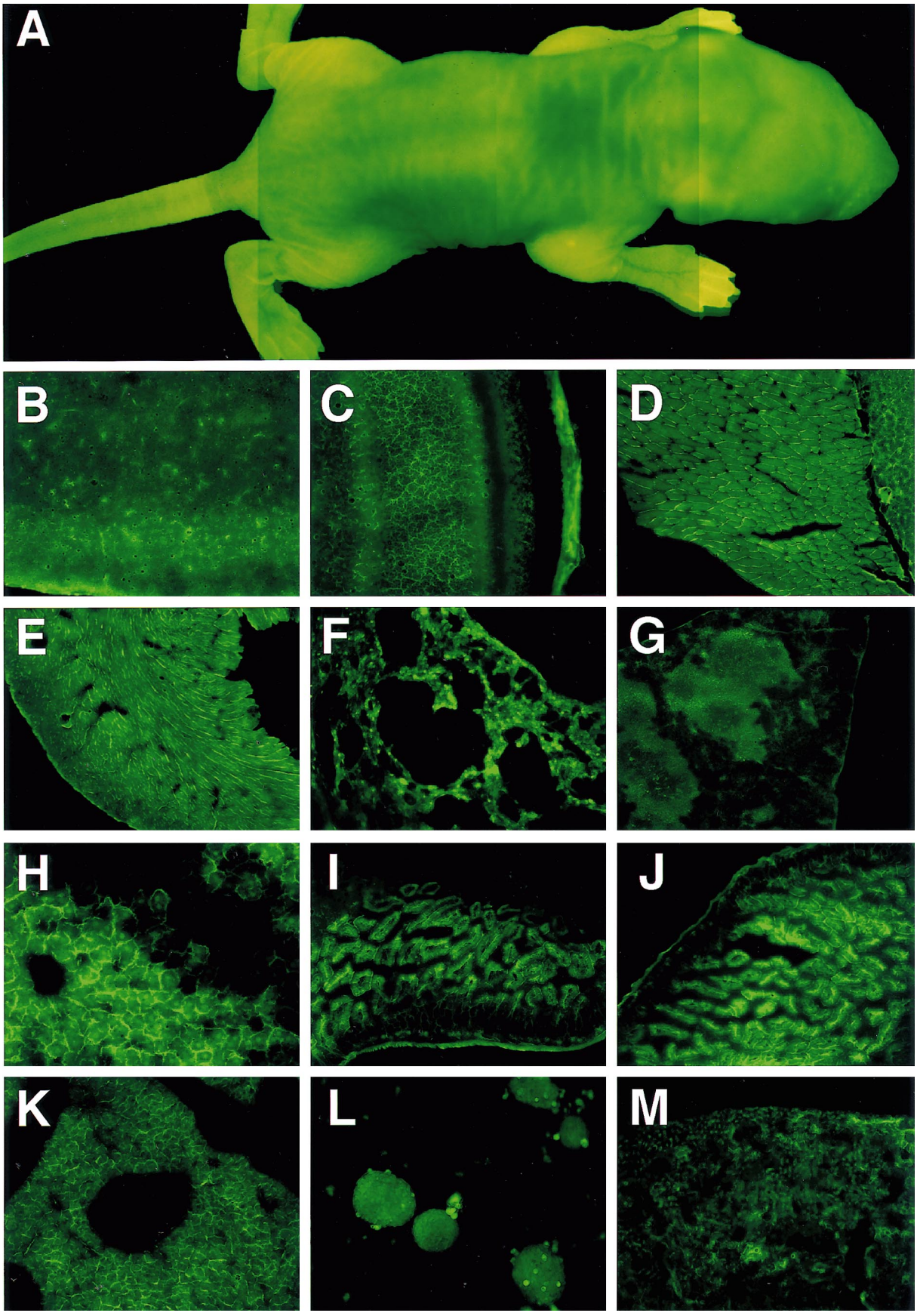
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Fig. 3. Appearance and histological analysis of the F1 progeny from CAG-CAT-EGFP reporter mice of line 39×the CAG-Cre transgenic mice. A: Expression of EGFP on the body surface of a neonatal F1 mouse was examined by fluorescence microscopy. B–M: Histological analysis. Organs of the F1 progeny with the recombined transgene were frozen in liquid nitrogen, and 10 mm thick sections were cut with a cryostat. Pancreatic islets were isolated by collagenase digestion followed by Ficoll density gradient purification. Unfixed specimens were examined by fluorescence microscopy. B, brain; C, eye; D, skeletal muscle; E, heart; F, lung; G, spleen; H, liver; I, ileum; J, colon; K, pancreas; L, isolated pancreatic islets; M, kidney.
←

3.5. *Keratinocyte-specific expression of EGFP in the F1 progeny from CAG-CAT-EGFP reporter mice×K5-Cre transgenic mice*

To examine tissue-specific recombination in this reporter strain, we mated CAG-CAT-EGFP reporter mice of line 39 with K5-Cre transgenic mice, which express Cre exclusively in keratinocytes. As shown in Fig. 4D, the body surface of the double transgenic F1 mouse from CAG-CAT-EGFP reporter mice×K5-Cre transgenic mice expressed fluorescence. A transverse section of its tail showed that the expression of EGFP was limited to the skin (Fig. 4E), while the tail of the F1 mouse from CAG-CAT-EGFP reporter mice×CAG-Cre transgenic mice expressed EGFP not only in the skin, but also in other inner tissues (Fig. 4F). Detailed examination of the back skin of the F1 progeny from CAG-CAT-EGFP reporter mice×K5-Cre transgenic mice revealed that EGFP fluorescence was expressed exclusively in the epidermis and hair follicles as expected from the specificity of the K5 promoter (Fig. 4H). EGFP fluorescence was also observed in the epithelium of the tongue, the esophagus, and forestomach, in which the K5 promoter has been reported to be active (not shown).

4. Discussion

We have generated CAG-CAT-EGFP reporter transgenic mouse lines that express EGFP fluorescence after Cre-mediated excision of the *loxP*-flanked CAT gene sequence within the transgene. The features of this reporter strain are as follows: (1) EGFP is used as the reporter; (2) the ubiquitously strong CAG promoter is used to drive the reporter gene; (3) the reporter gene construct includes the *loxP*-flanked CAT gene sequence between the CAG promoter and the EGFP gene; (4) this reporter gene is switched on by Cre-mediated recombination. In the present study, we demonstrated that the F1 progeny obtained by mating one of these reporter lines (line 39) with CAG-Cre transgenic mice, which produce Cre in zygotes, expressed EGFP fluorescence in all tissues examined. We also demonstrated that tissue-specific expression of EGFP was induced in the double transgenic F1 progeny obtained by mating this reporter line with transgenic mice expressing Cre in a tissue-specific manner. Although strong fluorescence was detected in most of the tissues examined in mice with the recombined allele of the CAG-CAT-EGFP transgene, some tissues such as kidney and spinal cord showed

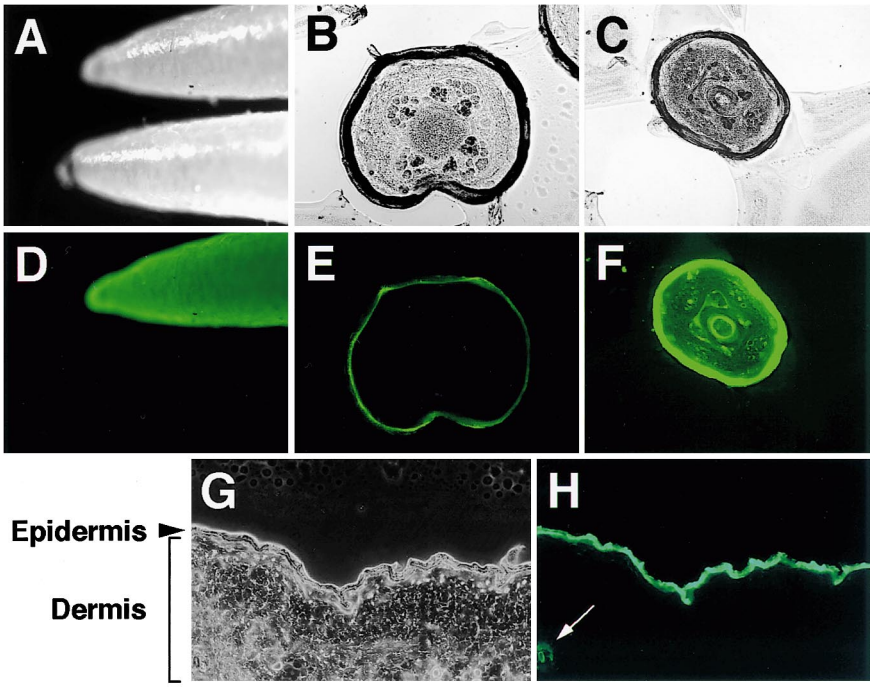

Fig. 4. Epidermis-specific expression of EGFP in the F1 progeny from CAG-CAT-EGFP reporter mice of line 39×K5-Cre transgenic mice. A: Appearance of the tail tips of a double transgenic newborn mouse (upper) and a non-transgenic littermate (lower). B: Transverse section of the tail of a double transgenic F1 mouse from the reporter mice of line 39×K5-Cre transgenic mice. C: Transverse section of the tail of an F1 mouse with the recombined transgene from the reporter mice of line 39×CAG-Cre transgenic mice. G: Detailed examination of the back skin of a double transgenic F1 mouse from the reporter mice of line 39×K5-Cre transgenic mice. D, E, F, and H: The same samples as in A, B, C, and G, respectively, were examined for EGFP expression by fluorescence microscopy. In H, the arrow indicates a hair follicle.

only weak fluorescence especially after paraformaldehyde fixation. Therefore, our reporter mice may not be suitable for the detection of Cre expression specific to these tissues.

EGFP would be a good reporter protein to monitor the expression of Cre, because it is less cytotoxic and simpler to detect than β-galactosidase. Since the fluorescence can be detected in living cells, this reporter strain would be useful in various applications, such as collecting Cre-expressing cells by FACS. However, it is essential to use a strong promoter to drive the EGFP gene in these studies. Previously we found that it was difficult to detect the fluorescence in embryonic stem cells when the EGFP gene was driven by the phosphoglycerate kinase-1 promoter. In this study, we utilized the CAG promoter to drive the reporter gene and could easily detect the fluorescence of EGFP in various tissues by fluorescence microscopy and FACS. This observation is consistent with the description of the previously reported 'green mice', which express EGFP under the control of the CAG promoter [12]. It is known that the ROSA26 locus identified by the gene trapping method has a ubiquitously active promoter. However, this promoter activity does not seem to be as strong as the CAG promoter and would not be appropriate to drive the EGFP-based reporter gene. On the other hand, Cre recombinase can efficiently excise the *loxP*-flanked DNA sequences in mammalian chromosomes and even a residual amount of Cre recombinase is thought to be able to switch on this reporter system, leading to EGFP expression. Thus, our reporter system is expected to be used to detect even weakly expressed Cre, because the EGFP fluorescence driven by the CAG promoter results in such a strong signal.

The CAG-CAT-EGFP reporter mice have multiple copies of the transgene. Although our results indicated that the *loxP*-flanked DNA sequences was completely excised by Cre-mediated recombination in the fluorescence-positive F1 progeny from CAG-CAT-EGFP transgenic mice × CAG-Cre transgenic mice, it is possible that weak Cre expression may result in partial deletion of the *loxP*-flanked DNA sequences and that each cell may express CAT and/or EGFP, depending on the status of the deletion. Thus, it would be necessary to examine the EGFP expression in the F1 progeny from our reporter mice × transgenic mice expressing Cre recombinase at low levels.

The present study indicates that the novel reporter strain of CAG-CAT-EGFP transgenic mice could serve as a simple and efficient tool, both to monitor the tissue specificity of Cre expression in transgenic mice in which the Cre gene is driven by various promoters, and to analyze the fate of cells deriving from progenitors that transiently express Cre during embryogenesis under some developmentally regulated promoter.

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