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Spermatocyte-Specific Gene Excision by Targeted Expression of Cre Recombinase

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Transgenic mice carrying the coding sequence of the Cre recombinase, whose expression was driven by the spermatocyte-specific *Pgk-2* promoter, were generated. These mice were crossed with a reporter transgenic line, which produces β-galactosidase depending on the occurrence of *loxP*-mediated DNA recombination. When DNA of the offspring was analyzed by PCR and Southern blotting, signals that appear after the recombination were detectable only in the testis. Histochemical analyses revealed that β-galactosidase was present in spermatocytes and spermatogenic cells at later differentiation stages. However, the distribution of the protein was not uniform in all spermatocytes. Analyses of genomic DNA of the next generation indicated that recombination took place in about 70% of spermatogenic cells. From these results, we concluded that this transgenic line possessing *Pgk-2*-driven expression of the Cre recombinase should be useful for identifying spermatogenic genes that function at or after the spermatocyte stage. © 2000 Academic Press

Key Words: **Cre/*loxP* recombination system; mouse *Pgk-2;* transgenic mice.**

Mammalian spermatogenesis is a complex process that involves the mitotic proliferation of spermatogonia, the meiotic division of spermatocytes, chromosomal condensation, production of sperm-specific proteins, and the morphogenic differentiation of spermatids to spermatozoa (1, 2). Although numerous detailed cytological studies have been described, the molecular basis for many of the steps of the pathway has yet to be determined. This might be due to the absence of suitable spermatogenic cell lines and methods of introducing genes into spermatogenic cells, which could be used to identify genes and factors involved in mammalian spermatogenesis. Gene disruption technology using embryonic stem cells has been successfully employed to identify genes required for spermatogenesis (3–5). However, this strategy can only be applied to genes whose functions are not required during early development; genes indispensable for either development or spermatogenesis cannot be identified due to death of the affected animals. The development of a conditional gene disruption method in which candidate genes are inactivated only in the testis or at particular stages during spermatogenic differentiation is thus needed.

The Cre recombinase of bacteriophage P1 recognizes a 34-bp sequence called *loxP*, and catalyzes recombination between two *loxP* sequences, resulting in loss of the sequences in between. This Cre/*loxP* recombination system has been successfully used for tissue- or cell type-specific gene inactivation or deletion (6–10), and for excision, integration or translocation of DNA segments (11–13). In this study, we adopted this technique in order to develop a spermatocyte-specific gene excision method. To do so, the Cre recombinase was specifically expressed in spermatogenic cells using a 1.4-kbp DNA region including the mouse *Pgk-2* promoter, which has been shown to induce transcription of the downstream sequences in pachytene spermatocytes (14).

MATERIALS AND METHODS

Generation of transgenic mice. The entire Cre-coding sequence was inserted into the *Xho*I/*Not*I site of P2ASVβ (14) so that the Cre sequence was located just downstream of the 1.4-kbp mouse *Pgk-2* promoter. The DNA was purified using QIAEX (Qiagen, Catsworth, CA) and microinjected into the pronuclei of fertilized eggs of BCF_1 (C57BL/6 × C3H) mice as previously described (15). The resulting transgenic mouse line was named Pgk2-Cre Tg.

Reverse transcription-mediated PCR. Total RNA (2 μg) extracted from various mouse organs (16) was used to synthesize cDNA with random hexamers as primers, and the cDNA was subjected to PCR. Amplified DNA products were separated on a 2% agarose gel and visualized by staining the gel with ethidium bromide.

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PCR and Southern blotting of genomic DNA. DNA was prepared from tails of transgenic mice using a QIAamp Tissue Kit (Qiagen) and subjected to PCR with primers Cre1 (5′-AGGTTCGTTCACTCATGGA) and Cre2 (5′-TCGACCAGTTTAGTTACCC) to amplify the Cre-coding sequence, or with primers L1 (5′-GCGTTACCCAACTTAATCG) and L2 (5′-TGTGAGCGAGTAACAACC) to amplify the reporter *CAG-CAT-Z* sequence, or with primers CA1 (5′-CTGCTAACCATGTTCATGCC) and L3 (5′-GGCCTCTTCGCTATTACG) to amplify the recombined *CAG-CAT-Z* sequence (17) (see Fig. 1). PCR products were separated on a 2 or 1.4% agarose gel and visualized with ethidium bromide. For Southern blotting, DNA was prepared from various organs of transgenic mice by treatment with SDS and proteinase K followed by phenol extraction. The DNA (40 μg) was digested with *Eco*RI, separated on a 1% agarose gel, and transferred onto a nitrocellulose membrane. The membrane was hybridized with a ^{32}P-labeled 1.4-kbp DNA fragment containing β-galactosidase-coding sequence. Hybridization signals were visualized using an image analyzer (Molecular Imager; Bio-Rad, Hercules, CA).

Histochemical analysis. Testes were fixed with phosphate-buffered saline containing 1% formaldehyde, 0.2% glutaraldehyde, and 0.02% Nonidet P-40 at 4°C for 2 h. The fixed testes were embedded with OCT compound, frozen at −80°C, and cut into sections of 14–20-μm thickness. The sections were washed with phosphate-buffered saline, incubated in a solution containing 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 1 mg/ml of *5*-bromo-*4*-chloro-*3*-indolyl-β-D-galactoside (X-gal) in phosphate-buffered saline for 5–12 h at 37°C, and examined under a microscope after counterstaining with Nuclear Fast Red. Spermatogenic cell-types were determined based on the size and morphology of spermatogenic cells and their nuclei, as described previously (1, 18, 19).

RESULTS

Transgenic mice in which the Cre recombinase was expressed under control of the spermatocyte-specific *Pgk-2* promoter were generated (see Fig. 1). A total of 15 founder animals containing the transgene were analyzed, and two lines showed testis-specific expression of Cre mRNA (Fig. 2A). The two transgenic lines possessed one and 35 copies of the transgene, respectively, and stably passed the transgene on to their progeny (data not shown). These mice were then mated with mice carrying the reporter *CAG-CAT-Z* sequence (17) to examine the occurrence of Cre-mediated recombination. This reporter sequence was constructed to result in β-galactosidase expression when the *CAT* sequence flanked by *loxP* is eliminated through Cre-mediated recombination (see Fig. 1). When genomic DNA of the progeny male mice (Pgk2-Cre/CAG-CAT-Z Tg) was analyzed by Southern blotting, testis-specific excision of the *CAT* sequence was observed (Fig. 2B).

To determine the timing of the onset of recombination during spermatogenic differentiation, we examined the presence of β-galactosidase in testicular cells. When testis sections prepared from Pgk2-Cre/CAG-CAT-Z Tg were treated with X-gal, signals were observed with spermatocytes and spermatids, but not with pre-meiotic spermatogenic cells, Sertoli cells or cells present in the testicular interstitium (Figs. 3A, 3B, and 3D). Mice carrying the *CAG-CAT-Z* sequence but not crossed with Pgk2-Cre Tg did not give any

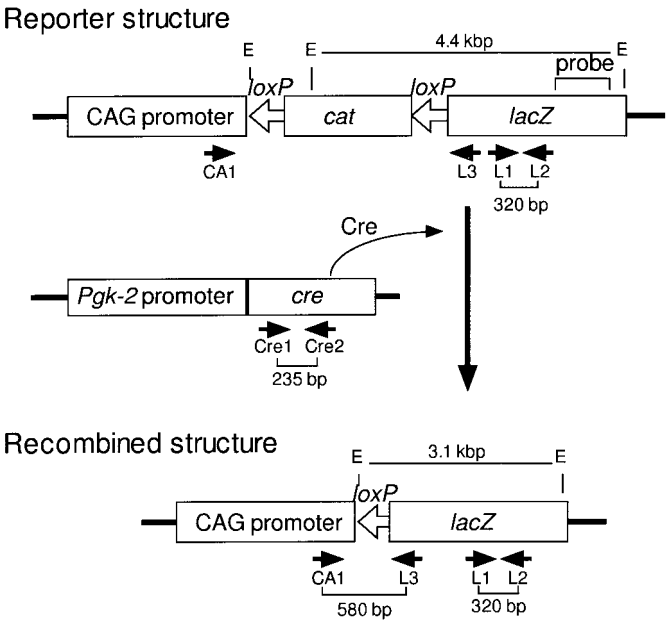

FIG. 1. Schematic representation of transgene constructs and Cre-mediated recombination. The structures of the transgenes used in this study are shown; the *CAG-CAT-Z* sequence as a reporter for *loxP*-mediated recombination and the Cre-expressing DNA with the mouse *Pgk-2* promoter. Shown at the bottom is the structure of the reporter sequence after recombination. Horizontal closed arrows indicate the position and direction of primers for PCR together with the expected sizes of amplified DNA. In Southern blotting, recombined and nonrecombined sequences were detected as 4.4- and 3.1-kbp DNA fragments, respectively, after digestion with *Eco*RI (E).

signal (Fig. 3C), and the cell types with positive signals corresponded to those in which the *Pgk-2* promoter is active (14), indicating that β-galactosidase expression was dependent on the presence of the Cre recombinase. However, some cross sections of the seminiferous tubules gave no signal, and positivity was not uniform among spermatocytes and spermatids within the same sections. This mosaic pattern of β-galactosidase distribution is likely to reflect the presence of spermatogenic cells in which recombination did not occur. To more directly examine whether spermatogenic cells with the original *CAG-CAT-Z* sequence exist, Pgk2-Cre/CAG-CAT-Z Tg mice were mated with wild-type BCF_1 females, and DNA from the progeny was analyzed (Fig. 4). Of 17 mice that carried the *lacZ* sequence, 12 gave the 580-bp DNA derived from the recombined sequence (lanes 4 and 6) while five did not (lanes 3 and 5). This suggested that Cre-mediated recombination took place in about 70% (12/17) of spermatogenic cells. All these data collectively indicated that Cre-mediated recombination was induced in an organ and spermatogenic stage-specific manner, but that this event did not take place uniformly among the corresponding spermatogenic cells and that some of these cells retained the unmodified reporter gene.

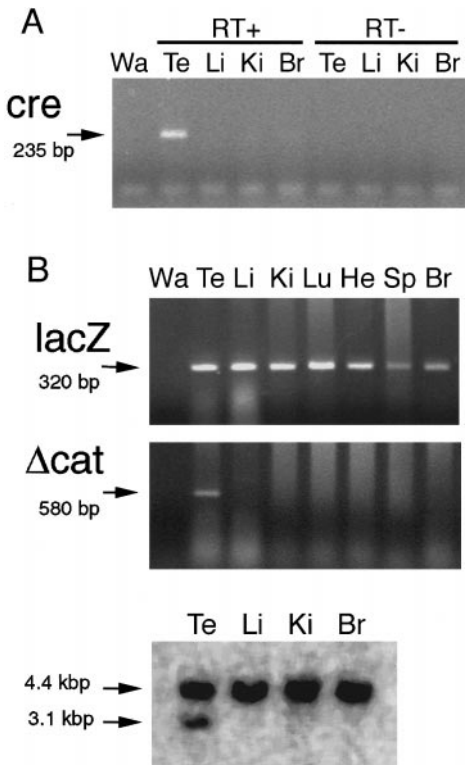

FIG. 2. Testis-specific expression of Cre mRNA and Cre-mediated recombination of the *CAG-CAT-Z* sequence. RNA and genomic DNA were prepared from various organs of Pgk2-Cre/CAG-CAT-Z Tg: Te, testis; Li, liver; Ki, kidney; Lu, lung; He, heart; Sp, spleen; Br, brain. (A) Reverse transcription-mediated PCR for detection of Cre mRNA. Control reactions with no reverse transcriptase (RT-) and with water alone (Wa) were included. (B) PCR (top two panels) and Southern blotting (bottom panel) for detection of the recombined sequence. Δ*cat* (indicated with an arrow) denotes a PCR product detectable only with recombined genomic DNA (see Fig. 1). Note that the longer PCR product derived from the unmodified *CAG-CAT-Z* sequence is not shown in the panel.

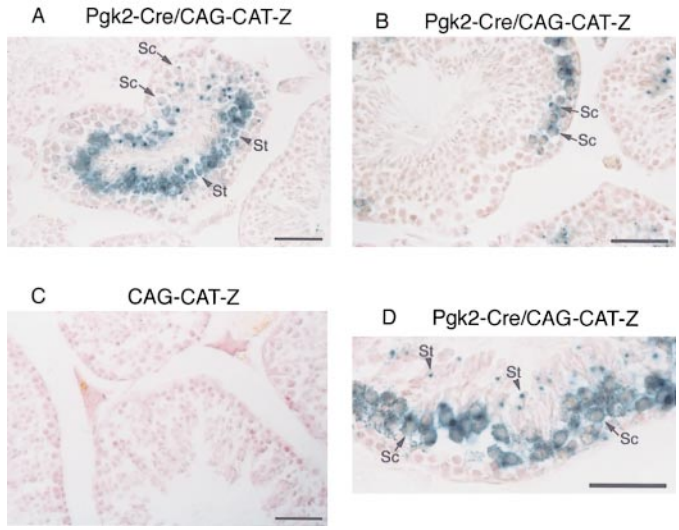

FIG. 3. Distribution of β-galactosidase in the testis of Pgk2-Cre/CAG-CAT-Z Tg mice. Testis sections from Pgk2-Cre/CAG-CAT-Z Tg mice (A, B, and D) and mice carrying only the *CAG-CAT-Z* sequence (C) were histochemically analyzed for the presence of β-galactosidase by staining with X-gal. The sections were counterstained with Nuclear Fast Red. Arrows and arrowheads point to spermatocytes (Sc) and spermatids (St), respectively. Bar = 50 μm.

DISCUSSION

In this study, we aimed to established a method by which DNA segments are excised in a manner specific to testis and spermatocytes. To do so, the Cre recombinase was expressed in spermatocytes using the mouse *Pgk-2* promoter. Transgenic mice that specifically express Cre in the testis were obtained, and a cross with these mice induced spermatocyte-specific DNA recombination in female mice carrying a reporter for *loxP*-dependent DNA excision. However, for unknown reasons, the occurrence of Cre/*loxP*-mediated recombination seemed not to be uniform among spermatocytes: the reporter gene in about 30% of the spermatogenic cells was left unmodified. We believe that this system has the advantage that the fate of spermatogenic cells with disrupted genes can be analyzed in comparison with those with intact genes within the same testis sections.

Transgenic mice in which the Cre recombinase is expressed in a spermatogenic cell-specific manner have been generated. O'Gorman *et al.* used the *Prm-1* promoter to restrict the expression to haploid spermatids (20). Vidal and colleagues generated transgenic mice in which the *Sycp1* promoter caused Cre to be expressed in primary spermatocytes (21). Our Pgk2-Cre Tg mice are, to our knowledge, the third example of spermatogenic cell-restricted expression of the Cre recombinase. All of these three systems should be useful for disrup-

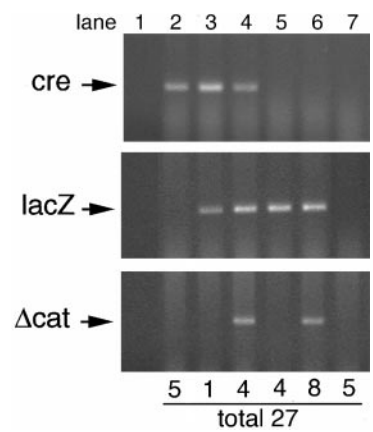

FIG. 4. PCR analysis of genomic DNA from progeny after a cross between Pgk2-Cre/CAG-CAT-Z Tg males and wild-type BCF_1 females. Positions of the *cre*, *lacZ*, and recombined *CAG-CAT-Z* (Δcat) sequences are indicated by arrows. At the bottom are the numbers of mice showing the indicated pattern of PCR products. Lane 1 contained a control reaction with no DNA.

tion of candidate spermatogenic genes at various particular stages during spermatogenic differentiation.

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REFERENCES

1. Russell, L. D., Ettlin, R. D., Sinha Hikim, A. P., and Clegg, E. D. (1990) Histological and Histopathological Evaluation of the Testis, Cache River Press, Clearwater, FL.
2. Kierszenbaum, A. L. (1994) Mammalian spermatogenesis in vivo and in vitro: A partnership of spermatogenic and somatic cell lineages. *Endocr. Rev.* **15,** 116–134.
3. Knudson, C. M., Tung, K. S., Tourtellotte, W. G., Brown, G. A., and Korsmeyer, S. J. (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science* **270,** 96–99.
4. Sassone-Corsi, P. (1997) Transcriptional checkpoints determining the fate of male germ cells. *Cell* **88,** 163–166.
5. Yuan, L., Liu, J. G., Zhao, J., Brundell, E., Daneholt, B., and Hoog, C. (2000) The murine SCP3 gene is required for synaptonemal complex assembly, chromosome synapsis, and male fertility. *Mol. Cell* **5,** 73–83.
6. Gu, H., Marth, J. D., Orban, P. C., Mossmann, H., and Rajewsky, K. (1994) Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science* **265,** 103–106.
7. Tsien, J. Z., Huerta, P. T., and Tonegawa, S. (1996) The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. *Cell* **87,** 1327–1338.
8. Rohlmann, A., Gotthardt, M., Hammer, R. E., and Herz, J. (1998) Inducible inactivation of hepatic LRP gene by cre-mediated recombination confirms role of LRP in clearance of chylomicron remnants. *J. Clin. Invest.* **101,** 689–695.
9. Shibata, H., Toyama, K., Shioya, H., Ito, M., Hirota, M., Hasegawa, S., Matsumoto, H., Takano, H., Akiyama, T., Toyoshima, K., Kanamaru, R., Kanegae, Y., Saito, I., Nakamura, Y., Shiba, K., and Noda, T. (1997) Rapid colorectal adenoma formation initiated by conditional targeting of the Apc gene. *Science* **278,** 120–123.
10. Stec, D. E., Davisson, R. L., Haskell, R. E., Davidson, B. L., and Sigmund, C. D. (1999) Efficient liver-specific deletion of a floxed human angiotensinogen transgene by adenoviral delivery of Cre recombinase in vivo. *J. Biol. Chem.* **274,** 21285–21290.
11. Sauer, B., and Henderson, N. (1988) Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc. Natl. Acad. Sci. USA* **85,** 5166–5170.
12. Smith, A. J., De Sousa, M. A., Kwabi-Addo, B., Heppell-Parton, A., Impey, H., and Rabbitts, P. (1995) A site-directed chromosomal translocation induced in embryonic stem cells by Cre-loxP recombination. *Nature Genet.* **9,** 376–385.
13. Sakai, K., Mitani, K., and Miyazaki, J. (1995) Efficient regulation of gene expression by adenovirus vector-mediated delivery of the CRE recombinase. *Biochem. Biophys. Res. Commun.* **217,** 393–401.
14. Ando, H., Haruna, Y., Suzuki, M., Yamada, S., Okabe, M., and Nakanishi, Y. (2000) Ectopic activation of the transcription promoter for the testis-specific mouse Pgk-2 gene upon elimination of a cis-acting upstream DNA region. *Develop. Growth Differ.* in press.
15. Toyonaga, T., Hino, O., Sugai, S., Wakasugi, S., Abe, K., Shichiri, M., and Yamamura, K. (1994) Chronic active hepatitis in transgenic mice expressing interferon-gamma in the liver. *Proc. Natl. Acad. Sci. USA* **91,** 614–618.
16. Chomczynski, P., and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156–159.
17. Sakai, K., and Miyazaki, J. (1997) A transgenic mouse line that retains cre recombinase activity in mature oocytes irrespective of the cre transgene transmission. *Biochem. Biophys. Res. Commun.* **237,** 318–324.
18. O'Gorman, S., Dagenais, N. A., Qian, M., and Marchuk, Y. (1997) Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **94,** 14602–14607.
19. Oakberg, E. F. (1956) A description of spermatogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. *Am. J. Anat.* **99,** 391–409.
20. Numata, M., Ono, T., and Iseki, S. (1994) Expression and localization of the mRNA for DNA (cytosine-5)-methyltransferase in mouse seminiferous tubules. *J. Histochem. Cytochem.* **42,** 1271–1276.
21. Vidal, F., Sage, J., Cuzin, F., and Rassoulzadegan, M. (1998) Cre expression in primary spermatocytes: A tool for genetic engineering of the germ line. *Mol. Reprod. Dev.* **51,** 274–280.