J. Biochem. 122, 977-982 (1997)

Efficiency of Recombination by Cre Transient Expression in Embryonic Stem Cells: Comparison of Various Promoters[1]

Kimi Araki, Takashi Imaizumi, Keiji Okuyama,[2] Yuichi Oike, and Ken-ichi Yamamura[3]

Department of Developmental Genetics, Institute of Molecular Embryology and Genetics, Kumamoto University School of Medicine, Kuhonji 4-24-1, Kumamoto 862

Received for publication, June 18, 1997

The Cre-*lox*P recombination system of bacteriophage P1 is frequently utilized in genetic manipulation in embryonic stem (ES) cells. The level of Cre expression is critical to induce *lox*P site-specific recombination in ES cells. To compare the efficiency of recombination, we constructed four Cre expression vectors driven by different promoters: cytomegarovirus/chicken β-actin (CAG) promoter, human polypeptide chain elongation factor 1α (hEF-1α) promoter, mouse phosphoglycerate kinase-1 (mPGK) promoter, and polyoma enhancer/herpes simplex virus thymidine kinase (MC1) promoter. We introduced these Cre expression vectors by electroporation into three ES cell lines carrying a single copy of CAG-*lox*P-chloramphenicol acetyltransferase (CAT) gene-*lox*P-β-galactosidase (β-gal) gene construct. Since the Cre-mediated recombination leads to excision of the CAT gene, the efficiency of recombination can be monitored as β-gal expression. No selection system was used in the experiments. The maximum recombination frequency was obtained when the CAG promoter was used, followed by the hEF-1α promoter, the mPGK promoter and the MC1 promoter in order. These results indicate that the efficiency of recombination in transient expression system correlates with the promoter activity of Cre expression vector. Thus, it is important to choose the promoter for effective recombination by Cre.

Key words: embryonic stem cells, expression vectors, Cre recombinase, *lox*P site-specific recombination, transient expression.

Genetic manipulation of embryonic stem (ES) cells, such as gene disruption through homologous recombination, is a powerful tool for the study of mammalian developmental genetics. Recently, the Cre-*lox*P recombination system of bacteriophage P1 has gained prominence as a new and effective tool for many kinds of genome engineering (see below) in ES cells (*1*). Cre recombinase catalyzes reciprocal site-specific recombination between two specific 34-bp sites called *lox*P (*2*). The intramolecular recombination of two *lox*P sites positioned head-to-tail or head-to-head results in deletion or inversion of the intervening DNA sequences, and an intermolecular recombination causes integration or reciprocal translocation at the *lox*P site. Since the recombination does not require additional cofactors, this system has been successfully used in yeast (*3, 4*), plants (*5*), mammalian culture cells (*4, 6*), and mice (*7-9*).

The combination of the Cre-*lox*P system and homologous recombination in ES cells has provided new approaches, such as tissue-specific knock-out (*10, 11*), inducible knock-out (*12*), site-directed mutagenesis (*13-15*), site-directed gene insertion (*16, 17*), multiple gene targeting (*18*), site-directed chromosomal translocation (*19, 20*), and long targeted deletion (*21, 22*), inversion and duplication (*21*). In these systems, while recombination *in vivo* is induced by mating with a Cre-expressing transgenic line or microinjection of Cre expression vector into pronuclei of fertilized eggs, recombination in ES cells is achieved by the electroporation of Cre expression vector in circular form. These approaches can be classified into two groups depending on the distance of *lox*P sites, *i.e.*, the size of DNA manipulated.

In the first group, which includes the first five approaches, Cre-*lox*P system is utilized for the removal of exogenous selectable marker genes, such as neomycin resistance (*neo*^{R}) gene, from targeted clones. Therefore, the size of DNA segment excised by Cre is a few kbp and its recombination efficiency is relatively high. Thus, it is possible without using a selection system to obtain an ES clone that has undergone Cre-mediated excision in a frequency of 4-16% (*18, 23*). Nevertheless, in many cases, herpes simplex virus thymidine kinase (*tk*) gene is used as negative selection marker to select excised ES clones. This is because *tk*-negative selection system works so effectively

[1] This work was supported by grants from the Ministry of Education, Science, Sports and Culture, a grant from the Yamanouchi Foundation for Research on Metabolic Disorders, a grant from the Osaka Foundation for Promotion of Clinical Immunology, and a grant from the Science and Technology Agency. K. Araki and T. Imaizumi contributed equally to this work.

[2] Present address: OTSUKA GEN Research Institute, 463-10 Kagasuno, Kawauchi-cho, Tokushima 771-01.

[3] To whom correspondence should be addressed. Tel: +81-96-373-5316, Fax: +81-96-373-5321

Abbreviations: CAG, modified chicken β-actin; CAT, chloramphenicol acetyltransferase; ES, embryonic stem; hEF-1α, human polypeptide chain elongation factor 1α; MC1, polyoma enhancer/herpes simplex virus thymidine kinase; mPGK, mouse phosphoglycerate kinase-1; *neo*^{R}, neomycin resistance; NLS, nuclear localization signal; *tk*, thymidine kinase; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

that almost all surviving ES clones carry a Cre-*lox*P mediated deletion (*24*). However, in recent years, *tk*-negative selection has been shown to have an adverse effect, that is, the expression of *tk* gene prevents germ-line transmission (*25, 26*). Therefore, it is desirable to develop an effective Cre expression vector for efficient Cre-mediated excision instead of the *tk*-negative selection system.

The second group includes site-directed chromosomal translocation and long targeted deletion, inversion, and duplication. In this group, Cre-*lox*P system was utilized as a tool for chromosome engineering through the recombination between two *lox*P sites locate 2-3 cM apart or on different chromosomes. Consequently, such chromosomal recombination frequency is very low. To achieve such chromosomal recombination by Cre, positive selection marker genes, such as a hypoxanthine phosphoribosyltransferase gene (*19, 21*), have been used. Only upon Cre-mediated recombination one part of a selection marker gene fuse with the remaining part of the maker gene, resulting in the generation of the functional selection marker gene. In this case, the number of recombinants itself is very small. Therefore, it is also important to develop an effective Cre expression vector in this category.

The expression level of the *cre* gene is expected to affect directly the efficiency of recombination. In this study, we have compared four promoters that are frequently used for expression of marker genes. These include modified chicken β-actin (CAG) promoter (*27*), human polypeptide chain elongation factor 1α (hEF-1α) promoter (*28*), mouse phosphoglycerate kinase-1 (mPGK) promoter (*29*), and polyoma enhancer/herpes simplex virus thymidine kinase (MC1) promoter (*30*). We report here that the CAG promoter gave the strongest Cre activity, with a maximum recombination frequency of 26% without use of a selection system.

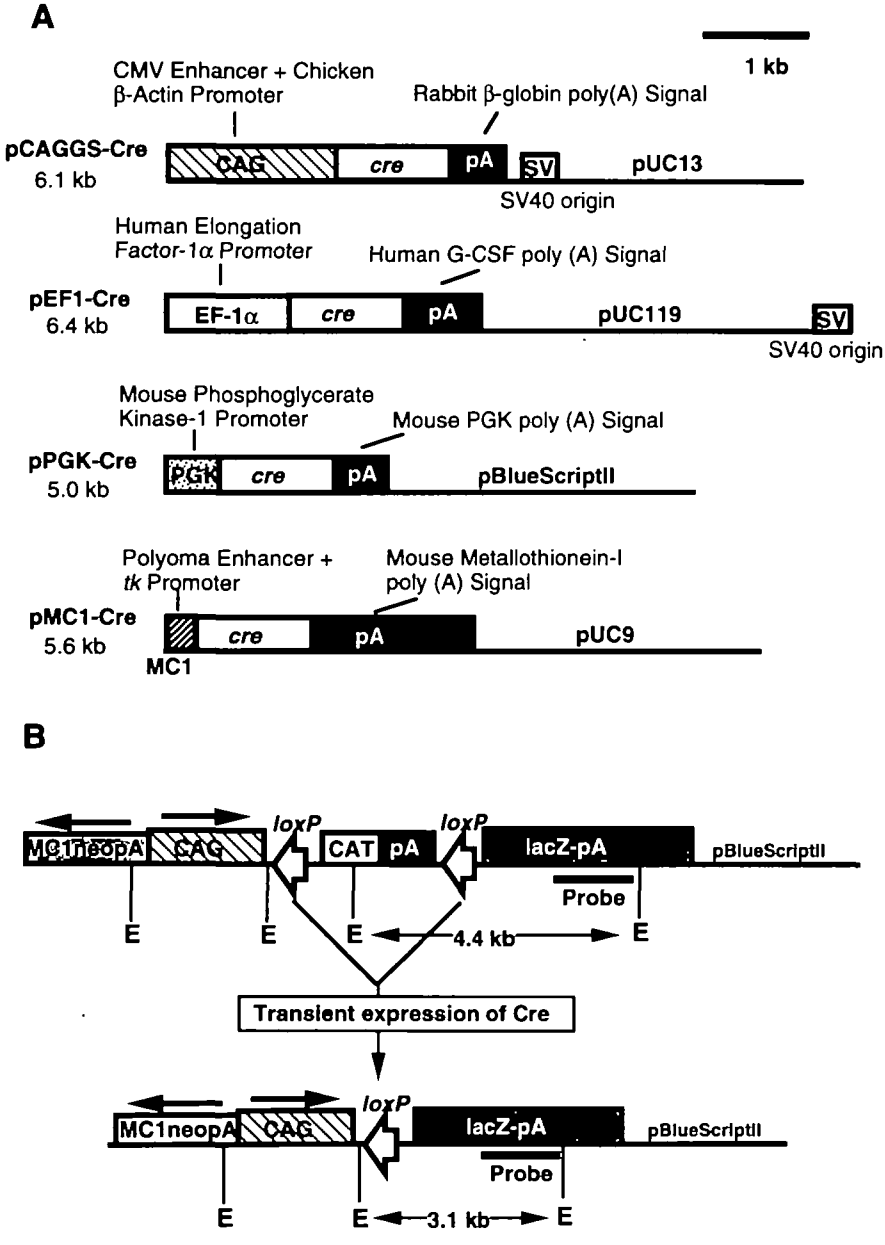

Fig. 1. **(A) Structure of the Cre expression vectors.** Plasmid maps are shown in linearized form, whereas the plasmids were used in their circular forms. **(B) Experimental design for the detection of Cre-mediated recombination.** Horizontal open arrows represent *lox*P sites. Vertical arrows indicate the direction of transcription. CAG, CAG promoter; pA, polyadenylylation signals; E, *Eco*RI; Probe, the probe used for Southern blotting in Fig. 4. The structure of the pCATZ-neo transgene construct is shown in the upper part. Before recombination, the CAT gene is expressed, and *Eco*RI digestion produces a 4.4-kbp fragment. In the lower part, the structure after recombination is shown. Recombination results in excision of the CAT gene flanked by two *lox*P sites and in expression of *lacZ*. *Eco*RI digestion produces a 3.1-kbp fragment.

MATERIALS AND METHODS

Plasmids and DNA Construction—We constructed four expression vectors, pCAGGS-Cre, pEF1-Cre, pPGK-Cre, and pMC1-Cre (Fig. 1A). Plasmid pCAGGS-Cre[4] has been described (*9*). Plasmids pEF1-Cre and pPGK-Cre were constructed by inserting the *cre* fragment from pBS185 (*4*) (Life Technologies) into the *Xba*I site of pEF-BOS (*28*, gift from S. Nagata) and the *Hin*dIII site of pBlKS(−)mPGKp/pA (gift from H. Niwa). Plasmid pMC1-Cre was constructed by ligating the fragment containing the *cre* gene and poly(A) signal from pBS185 (Life Technologies) into the *Mlu*I and *Hin*dIII sites of pMC1NeopolyA (Stratagene). The structures of these Cre expression vectors are shown in Fig. 1A. Plasmid pCAGGS-lacZ used as a control has been also described. Plasmid pCATZ-neo used for isolation of cell lines (see below) was constructed from pCAG-CAT-Z (*9*) by inserting a *neo*^{R} gene derived from pMC1NeopolyA (Stratagene) into a *Sal*I site. The map of linearized pCATZ-neo is shown in Fig. 1B.

Cell Culture and Electroporation—The ES cell line, TT2 (*31*), was grown as described (*32*) except for the use of G418-resistant primary mouse embryo fibroblasts as feeder layers.

In the case of electroporation with the pCATZ-neo plasmid, 40 μg of *Sal*I-digested DNA and 1×10^7 TT2 cells were used. The cells were electroporated with a Bio-Rad Gene Pulser set at 200 V and 950 μF, and after 24 h they were fed with medium supplemented with G418 (200 μg/ml). Selection was maintained for 7 days, then colonies were picked into 24-well plates and expanded for freezing. The clones were examined by Southern blotting and chloramphenicol acetyltransferase (CAT) assay to select cell lines showing a single-copy integration pattern and CAT gene expression, as described below.

The same conditions were used for electroporation of the circular forms of Cre expression vectors (30 μg each). Half of the electroporated cells from a cuvette were first plated onto a single 60-mm plate and grown for 48 h. They were then replated onto two 60-mm plates at 3×10^2 cells per plate to form colonies. After 1 week, colonies were stained

[4]Requests for plasmid pCAGGS or pCAGGS-Cre should be addressed to: Dr. Jun-ichi Miyazaki, Department of Nutrition, Osaka University School of Medicine, Yamadaoka 2-2, Suita, Osaka 565

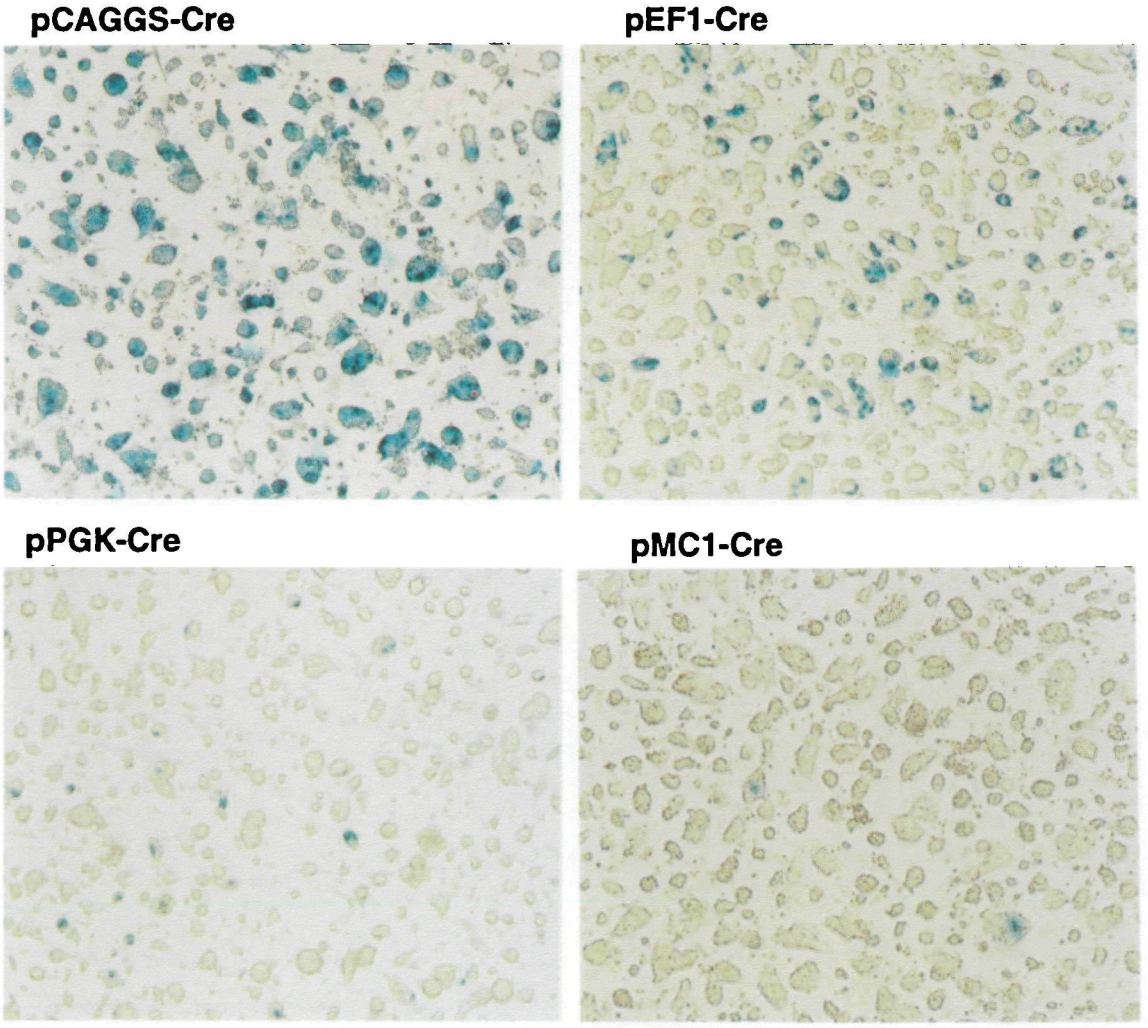

Fig. 2. **X-gal staining of CATZ29 24 h after electroporation with the Cre expression vectors.** Half of the electroporated cells were plated on a 60-mm plate and incubated for 24 h. The Cre expression vectors used are indicated at the top of each photo. Magnification, ×16.

with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) as described (*33*) or picked up and expanded for DNA analyses.

Detection of CAT Activity—The CAT activity of ES cell clones were determined with a CAT-ELISA kit from Boehringer Mannheim. Cells from each well of a 6-well plate were lysed in 0.5 ml of lysis buffer from the kit, then spun for 10 min at 4°C to remove cellular debris. Two hundred microliters of cell extract was used for the assay. The protein concentration of the supernatant was determined with a kit from Bio-Rad (based on the Bradford method). CAT activities were normalized against the amount of protein used for the assay.

Southern Hybridization—Cells were lysed with SDS/proteinase K, then treated with phenol/chloroform, 1 : 1 (v/v) twice, precipitated with ethanol, and dissolved in 10 mM Tris-HCl, pH 7.5/1 mM EDTA. Six micrograms of genomic DNA was digested with *Eco*RI, electrophoresed on a 0.9% agarose gel, then blotted onto a nylon membrane (Boehringer Mannheim). Hybridization was performed using a DIG DNA Labeling and Detection Kit (Boehringer Mannheim).

RESULTS

Construction of Cell Lines Carrying a Single CAG-loxP-CAT-loxP-LacZ Plasmid—Plasmid pCATZ-neo was designed to assess the efficiency of Cre-mediated recombination by *LacZ* expression (Fig. 1B). In this vector, a CAT gene with poly (A) signal flanked by two *lox*P sites is located between the CAG promoter and *LacZ* gene. Consequently, Cre-*lox*P-mediated recombination leads to deletion of the CAT gene, thereby allowing *LacZ* expression. Since this vector does not contain a negative selection marker gene for selecting cells in which Cre-*lox*P-mediated deletion has occurred, the percentage of *LacZ*-expressing cells represents the efficiency of recombination induced by the Cre expression.

We introduced the linearized vector pCATZ-neo into ES cells by electroporation, and established three ES cell lines carrying a complete single copy of pCATZ-neo vector. These cell lines, CATZ25, CATZ29, and CATZ31, were examined for CAT activity. As shown in Fig. 3, cell line CATZ25 showed the highest CAT activity, and the others showed almost the same, lower level. We confirmed that no *LacZ* expression was observed in these cell lines on X-gal staining before recombination.

Comparison of the Frequencies of Cre-Mediated Recombination among the Cre Expression Vectors—Figure 1A shows the Cre expression vectors used in this experiment. The CAG promoter and the hEF-1α promoter have been reported to have strong activity in mammalian cells (*27, 28*). The mPGK promoter and the MC1 promoter are often used to drive drug-resistant genes in ES cells (*33*). The MC1 promoter is also used to express Cre or nuclear localization signal (NLS)-Cre (*10, 13, 14, 16, 19, 20, 23, 24, 34*). The *cre* gene used in our vectors is derived from plasmid pBS185 (*4*), in which the DNA sequences surrounding the initiation ATG codon of *cre* were changed according to Kozak. Though plasmids pCAGGS-Cre and pEF1-Cre contain the replication origin of simian virus 40, this should not affect the expression in ES cells.

We introduced the Cre expression vector plasmids into the cell line CATZ29 by electroporation and observed *LacZ* expression 24 h later by X-gal staining to estimate the recombination efficiencies. As shown in Fig. 2, the frequencies of stained cells were clearly different among the vectors, being highest with pCAGGS-Cre and lowest with pMC1-Cre. Similar staining results were obtained when the other cell lines used (data not shown).

To evaluate the recombination frequency, we replated electroporated cells to form colonies derived from a single cell, stained the colonies with X-gal, then scored them. As shown in Fig. 3, the recombination frequencies with pCAGGS-Cre, pEF1-Cre, pPGK-Cre, and pMC1-Cre were 15-26%, 9-13%, 4-9%, and $<0.3\%$, respectively. These results demonstrate that pCAGGS-Cre is the most efficient Cre expression vector in ES cells. Although the recombination frequency varied among the cell lines used, the cell line CATZ25 always showed the highest frequency regardless of the vector. Interestingly, cell line CATZ25 showed the highest CAT activity among the three lines.

We also examined the percentage of blue cells when ES cells were electroporated with pCAGGS-lacZ. Only a small percentage of ES cells were stained with X-gal. As described, this is much lower than that (15-26%) induced by pCAGGS-Cre, suggesting that Cre-mediated recombination is induced at a lower amount of Cre than the detectable level of β-galactosidase on X-gal staining.

Analysis of Recombination by Southern Blotting—To confirm whether deletion of the CAT gene is due to the Cre-*lox*P mediated recombination, genomic DNAs were

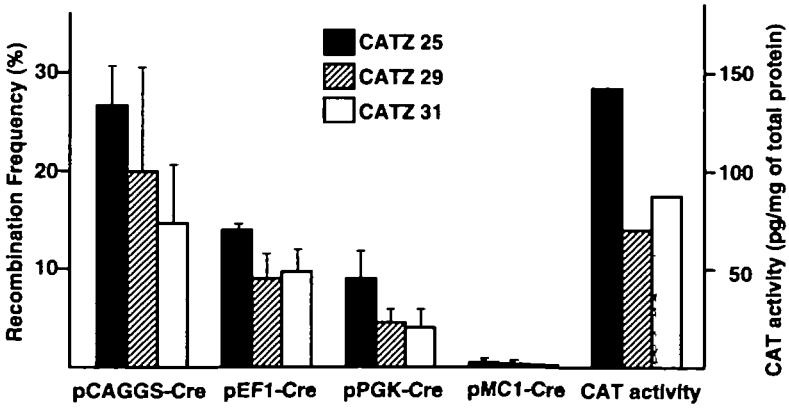

Fig. 3. **Recombination frequencies and CAT activity in each cell line.** The electroporated cells with each Cre expression vector were plated at a low density to form colonies. After culture for 7 days, colonies were stained with X-gal and scored. Percent recombination was calculated as the number of blue colonies divided by total number of colonies. Values represent means ± SD for five experiments. CAT activity was measured by use of a CAT-ELISA kit (Boehringer Mannheim).

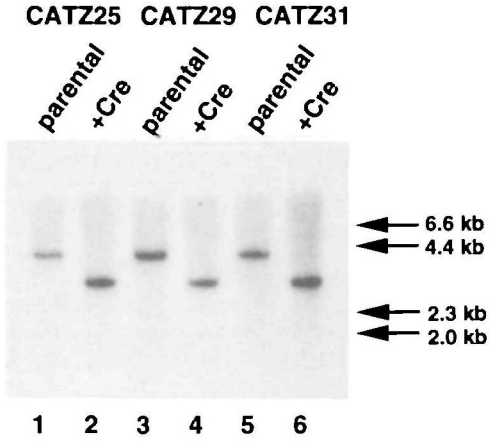

Fig. 4. **Southern blot analysis of genomic DNAs prepared from parental cell lines (parental) and subclones after Cre-mediated recombination (+Cre).** After electroporation with pCAGGS-Cre, cells were plated at a low density to form colonies, then picked up and expanded for X-gal staining and DNA analysis. An X-gal staining positive clone from each parental cell line was chosen for DNA analysis. The probe used for this Southern analysis is shown in Fig. 1B. *Eco*RI-digested DNA (6 μg) was loaded in each lane.

prepared from clones showing positive X-gal staining. As shown in Fig. 1B, DNAs from these clones should give a 3.1-kbp band, instead of the 4.4-kbp band in the parental cell lines, when analyzed by Southern blotting using a part of *lacZ* sequence as a probe. In agreement with this, all the X-gal positive clones gave the 3.1-kbp band (Fig. 4, lanes 2, 4, and 6), in contrast to the 4.4-kbp band in the parental cell lines (lanes 1, 3, and 5). These results demonstrate that the expected recombination occurred between *lox*P sites in the X-gal positive clones.

DISCUSSION

We have shown here that the recombination efficiency by Cre transient expression significantly varied depending on the promoter activity of the Cre expression vector, and that pCAGGS-Cre is the most efficient to induce recombination in ES cells. We obtained clones that had undergone the Cre-mediated recombination in high frequency (15–25%) in the absence of negative selection after electroporation of pCAGGS-Cre. On the contrary, the recombination frequency obtained by use of pMC1-Cre was very low (<0.3%), suggesting that a negative selection system is necessary when this vector is used.

Our results suggest that the recombination frequencies are proportional to their promoter activities. Soriano *et al.* reported that PGK-neo construct showed two times higher transformation efficiency than MC1-neo construct (*29*). Contrary to this, our results showed a 30-fold difference between PGK and MC1 promoter (9% *versus* 0.3% in CATZ25 line). DiSanto *et al.* (*23*) reported that the frequency of Cre-mediated deletion was 4% without any selection system using Cre expression vector pIC-Cre, in which the *cre* gene is driven by MC1 promoter. This frequency is higher than our result with pMC1-Cre. Although the reason for these differences is not known, they might be due to differences in experimental conditions, such as vector structures, cell lines, electroporation conditions, or chromosomal positions of vectors integrated.

We have not examined the optimum conditions for electroporation in this study. Thus, it may be possible to increase the recombination frequency by optimizing these conditions. A preliminary experiment showed that the frequency increased up to 50% using electroporation conditions of 3 μF, 800 V, 3×10^7 cells, and 100 μg of pCAGGS-Cre. Furthermore, Kellendonk *et al.* (*35*) reported that NLS-Cre was almost two times more efficient than Cre without NLS in green monkey CV-1 cells. Thus, use of NLS to localize Cre in the nucleus may be another approach to increase the frequency.

The most popular vector used to drive Cre expression in ES cells is pMC-Cre (*13, 16, 19, 20, 23, 33*), which contains MC1 promoter and NLS-Cre. Although NLS was reported to increase the recombination efficiency two times, the recombination efficiency of the plasmid pMC-Cre is at most 8%. Another plasmid used to express Cre is pBS185, carrying the human cytomegarovirus major immediate early promoter (CMV promoter) (*19, 22*). Smith *et al.* (*19*) reported that these two plasmids, pMC-Cre, and pBS185, showed similar recombination frequency. Although Abuin and Bradley (*18*) reported a relatively high recombination frequency of 16% using pOG231 as Cre expression vector, they did not describe the promoter of pOG231. In any case, we think pCAGGS-Cre has at least the same or higher activity as to Cre expression.

We observed that recombination efficiencies varied among the cell lines used, and that the highest CAT gene-expressing cell line gave the highest recombination efficiency. We obtained a similar result for Cre-mediated site-directed integration (*36*), in which the highest lacZ-expressing cell line gave the highest site-directed integration efficiency. Since all the cell lines used in both experiments carried a single copy of the transgene, the chromosomal position effect is considered to be the cause of the differences in the level of expression and recombination frequency. In other words, recombination efficiency might be proportional to the level of expression.

As described, plasmid pCAGGS-Cre can induce recombination in high efficiency in ES cells. Thus, pCAGGS-Cre will be a particularly useful tool for inducing large chromosomal alterations such as translocation, inversion and deletion, because the recombination efficiency of large-scale genome alteration is very low. In addition, pCAGGS-Cre will be useful for excising selectable marker genes after homologous recombination in ES cells.

We wish to thank Y. Kiyonaga for the technical assistance. We thank Drs. S. Nagata and H. Niwa for the gift of pEF1-BOS and pBlKS(−) mPGKp/pA, respectively.

REFERENCES

1. Rossant, J. and Nagy, A. (1995) Genome engineering: the new mouse genetics. *Nature Med.* 1, 592-594
2. Hoess, R.H. and Abremski, K. (1990) The Cre-*lox* recombination system in *Nucleic Acids and Molecular Biology* (Eckstein, F. and Lilley, D.M.J., eds.) Vol. 4, pp. 99-109, Springer-Verlag, Berlin/Heidelberg
3. Sauer, B. (1987) Functional expression of the cre-*lox* site-specific recombination system in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 7, 2087-2096
4. Sauer, B. and Henderson, N. (1990) Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recom-

binase. *New Biologist* **2**, 441-449
5. Dale, E.C. and Ow, D.W. (1991) Gene transfer with subsequent removal of the selection gene from the host genome. *Proc. Natl. Acad. Sci. USA* **88**, 10558-10562
6. 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
7. Lakso, M., Sauer, B., Mosinger, B., Jr., Lee, E.J., Manning, R.W., Yu, S.H., Mulder, K.L., and Westphal, H. (1992) Targeted oncogene activation by site-specific recombination in transgenic mice. *Proc. Natl. Acad. Sci. USA* **89**, 6232-6236
8. Orban, P.C., Chui, D., and Marth, J.D. (1992) Tissue- and site-specific DNA recombination in transgenic mice. *Proc. Natl. Acad. Sci. USA* **89**, 6861-6865
9. Araki, K., Araki, M., Miyazaki, J., and Vassalli, P. (1995) Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. *Proc. Natl. Acad. Sci. USA* **92**, 160-164
10. Gu, H., Marth, J.D., Orban, P.C., Mossmann, H., and Rajewsky, K. (1994) Deletion of a DNA polymerase β gene segment in T cells using cell type-specific gene targeting. *Science* **265**, 103-106
11. Hennet, T., Hagen, F.K., Tabak, L.A., and Marth, J.D. (1995) T-cell-specific deletion of a polypeptide *N*-acetylgalactosaminyl-transferase gene by site-directed recombination. *Proc. Natl. Acad. Sci. USA* **92**, 12070-12074
12. Kühn, R., Schwenk, F., Aguet, M., and Rajewsky, K. (1995) Inducible gene targeting in mice. *Science* **269**, 1427-1429
13. Brusa, R., Zimmermann, F., Koh, D.-S., Feldmeyer, D., Gass, P., Seeburg, P.H., and Sprengel, R. (1995) Early-onset epilepsy and postnatal lethality associated with an editing-deficient *GluR-B* allele in mice. *Science* **270**, 1677-1680
14. Ferradini, L., Gu, H., De Smet, A., Rajewsky, K., Reynaud, C.-A., and Weil, J.-C. (1996) Rearrengement-enhancing element upstream of the mouse immunoglobulin kappa chain J cluster. *Science* **271**, 1416-1420
15. Torres, R.M., Flaswinkel, H., Reth, M., and Rajewsky, K. (1996) Aberrant B cell development and immune response in mice with a compromised BCR complex. *Science* **272**, 1804-1808
16. Wang, Y., Schnegelsberg, P.N.J., Dausman, J., and Jaenisch, R. (1996) Functional redundancy of the muscle-specific transcription factors Myf5 and myogenin. *Nature* **379**, 823-825
17. Kitamoto, T., Nakamura, K., Nakao, K., Shibuya, S., Shin, R.W., Gondo, Y., Katsuki, M., and Tateishi, J. (1996) Humanized prion protein knock-in by Cre-induced site-specific recombination in the mouse. *Biochem. Biophys. Res. Commun.* **222**, 742-747
18. Abuin, A. and Bradley, A. (1996) Recycling selectable markers in mouse embryonic stem cells. *Mol. Cell. Biol.* **16**, 1851-1856
19. Smith, A.J.H., 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
20. Van Deursen, J., Fornerod, M., Van Rees, B., and Grosveld, G. (1995) Cre-mediated site-specific translocation between non-homologous mouse chromosomes. *Proc. Natl. Acad. Sci. USA* **92**, 7376-7380
21. Ramírez-Solis, R., Liu, P., and Bradley, A. (1995) Chromosome engineering in mice. *Nature* **378**, 720-724
22. Li, Z.-W., Stark, G., Gotz, J., Rulicke, T., Muller, U., and Weissmann, C. (1996) Generation of mice with a 200-kb amyloid precursor protein gene deletion by Cre recombinase-mediated site-specific recombination in embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **93**, 6158-6162
23. DiSanto, J.P., Muller, W., Guy-Grand, D., Fischer, A., and Rajewsky, K. (1995) Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor γ chain. *Proc. Natl. Acad. Sci. USA* **92**, 377-381
24. Gu, H., Zou, Y.-R., and Rajewsky, K. (1993) Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-*lox*P-mediated gene targeting. *Cell* **73**, 1155-1164
25. Huttner, K.M., Pudney, J., Milstone, D.S., Ladd, D., and Seidman, J.G. (1993) Flagellar and acrosomal abnormalities associated with testicular HSV-*tk* expression in the mouse. *Biol. Reprod.* **49**, 251-261
26. Braun, R.E., Lo, D., Pinkert, C.A., Widera, G., Flavell, R.A., Palmiter, R.D., and Brinster, R.L. (1990) Infertility in male transgenic mice: disruption of sperm development by HSV-*tk* expression in postmeiotic germ cells. *Biol. Reprod.* **43**, 684-693
27. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193-199
28. Mizushima, S. and Nagata, S. (1990) pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* **18**, 5322
29. Soriano, P., Montgomery, C., Geske, R., and Bradley, A. (1991) Targeted disruption of the *c-src* proto-oncogene leads to osteopetrosis in mice. *Cell* **64**, 693-702
30. Thomas, K.R. and Capecchi, M.R. (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51**, 503-512
31. Yagi, T., Tokunaga, T., Furuta, Y., Nada, S., Yoshida, M., Tsukada, T., Saga, Y., Takeda, N., Ikawa, Y., and Aizawa, S. (1993) A novel ES cell line, TT2, with high germline-differentiating potency. *Anal. Biochem.* **214**, 70-76
32. Niwa, H., Araki, K., Kimura, S., Taniguchi, S., Wakasugi, S., and Yamamura, K. (1993) An efficient gene-trap method using poly A trap vectors and characterization of gene-trap events. *J. Biochem.* **113**, 343-349
33. Joyner, A.L. (1993) *Gene Targeting: A Practical Approach*, Oxford University Press, New York
34. van der Hoeven, F., Zákány, J., and Duboule, D. (1996) Gene transpositions in the *HoxD* complex reveal a hierarchy of regulatory controls. *Cell* **85**, 1025-1035
35. Kellendonk, C., Tronche, F., Monaghan, A.P., Angrand, P.O., Stewart, F., and Schutz, G. (1996) Regulation of Cre recombinase activity by the synthetic steroid RU 486. *Nucleic Acids Res.* **24**, 1404-1411
36. Araki, K., Araki, M., and Yamamura, K. (1997) Targeted integration of DNA using mutant *lox* sites in embryonic stem cells. *Nucleic Acids Res.* **25**, 868-872