ELSEVIER

Biomolecular Engineering
17 (2001) 83–85

Biomolecular Engineering

www.elsevier.com/locate/geneanabioeng

Enhanced green fluorescent protein as a useful tag for rapid identification of homozygous transgenic mice

Masahiro Sato [a,*], Toshiteru Watanabe [a], Akiko Oshida [b], Ayako Nagashima [a], Jun-ichi Miyazaki [c], Minoru Kimura [a,b]

[a] *Molecular Medicine Research Center, The Institute of Medical Sciences, Tokai University, Bohseidai, Isehara, Kanagawa, 259-1193, Japan*
[b] *Department of Molecular Life Science, Tokai University, Bohseidai, Isehara, Kanagawa, 259-1193, Japan*
[c] *Department of Nutrition and Physiological Chemistry, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka, 565-0871, Japan*

Received 2 June 2000; received in revised form 26 June 2000; accepted 14 July 2000

Abstract

We developed a technique that simplifies the process of confirming homozygous transgenics at preimplantation stages, which are the earliest stages used in test breeding, using enhanced green fluorescent protein as a tag. All the blastocysts obtained by mating with the combination of Tg/Tg male (homozygous for transgene) × +/+ female exhibited fluorescence.

Keywords: Chicken β-actin promoter; EGFP; Fluorescence; PCR; Transgenic mice

1. Introduction

Homozygous transgenic mice have previously been identified by molecular biological techniques using genomic Southern blots or PCR analyses. However, these DNA analyses often yield incorrect findings concerning homozygosity, and therefore 'test breeding' is always conducted to confirm it. Test breeding can be performed by mating between homozygous mice (for which homozygosity has been previously demonstrated by DNA analysis) and normal mice and subsequent examination by DNA analysis for the presence of a transgene in the resulting progeny. This step is also laborious and time-consuming. We suspected that judgement of whether mice are homozygous or heterozygous could be made at preimplantation stages if early blastocysts are obtained by mating between a transgenic male homozygous for the transgene carrying enhanced green fluorescent protein (EGFP) [1–3] as a noninvasive tag and a normal female. We show here that EGFP can be used for rapid confirmation of homozygous transgenic mice at preimplantation stages, which are the earliest stages used in test breeding.

We constructed a *MNCE* transgene consisting of two expression units, *MBP–NCre* (termed '*MN*') and *CAG–EGFP* (termed '*CE*'). *MN* consists of a myelin basic protein (*MBP*) promoter, which is known to exhibit oligodendrocyte-specific expression of a cDNA (or gene) in vivo [4], and *NCre* gene [*Cre* gene carrying a nuclear localization signal (NLS) sequence at its 5′ end] [5]. *CE* consists of a promoter element, termed *CAG* [6], composed of cytomegalovirus enhancer and a promoter and the 1st intron of chicken β-actin gene and *EGFP* cDNA plus poly(A) signal from *SV40* gene (CLONTECH Laboratories, Palo Alto, CA). The *CAG* promoter system is known to confer ubiquitous and strong expression of a target cDNA both in vitro and in vivo [2,6–8]. *CE* is essentially the same as an *EGFP* expression vector described by Okabe et al. [2], except that an approximately 0.53–kb 3′-nocoding region of rabbit β-globin gene is missing from *CE* (Fig. 1).

The *MNCE* transgene, which had been purified after removal of the pBluescript SK(−) vector (Stratagene, La Jolla, CA) sequence, was microinjected into the pronucleus of a fertilized mouse egg derived from mating between B6C3F1 (CLEA Japan, Inc., Tokyo,

* Corresponding author. Tel.: +81-46-3931121 ext. 2692; fax: +81-463-962892.
E-mail address: masasato@is.icc.u-tokai.ac.jp (M. Sato).

1050-3862/01/$ - see front matter
PII: S1389-0344(00)00070-8

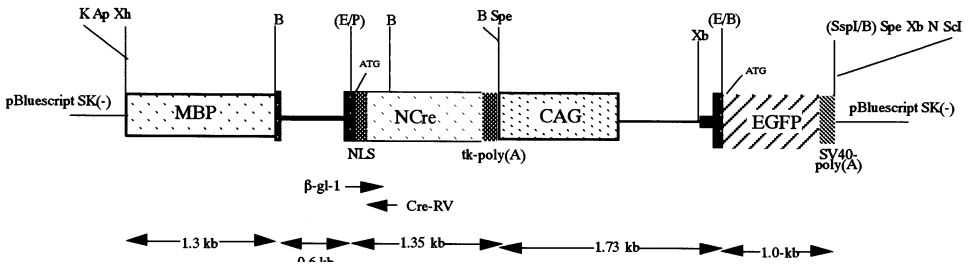

Fig. 1. The *MNCE* transgene construct cloned in pBluescript SK(−) vector. An approximately 2.73-kb EGFP expression unit (termed CE) consists of a promoter element, CAG [6], composed of cytomegalovirus enhancer and promoter and a portion of the 1st intron of chicken β-actin gene, a portion of rabbit β-globin gene (including a portion of the 2nd intron and 3rd exon; shown by solid line), and *EGFP* cDNA plus poly(A) signal from SV40 gene. Upstream of CE, an approximately 3.25-kb NCre expression unit (termed MN), consisting of a myelin basic protein (MBP) promoter [4], a portion of rabbit β-globin gene (including the 2nd intron and 3rd exon; shown by solid line), *NCre* gene [*Cre* gene carrying a nuclear localization signal at its 5′ end] and a poly(A) site of herpes simplex virus thymidine kinase (tk) gene, was placed. Abbreviations are: Ap, *Apa*I; ATG, translation initiation site; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Not*I; P, *Pst*I; ScI, *Sac*I; Spe, *Spe*I; Xb, *Xba*I; Xh, *Xho*I.

Japan; a hybrid between C57BL/6N and C3H/HeN) females and B6C3F1 males (CLEA Japan, Inc.), as previously described [9]. Of 72 F0 mice obtained, at least 15 were found to express EGFP when tails (approximately 5 mm from the tip) isolated at the weaning stage were inspected for EGFP fluorescence under a fluorescence stereomicroscope (SZX12; Olympus, Tokyo, Japan) with DM505 filters (BP460-490 and BA510IF; Olympus). Photographs were taken using a digital camera (FUJIX HC-300/OL; Fuji Film, Tokyo, Japan) attached to the fluorescence microscope and printed out using a digital color printer (CP700DSA; Mitsubishi, Tokyo, Japan). Of the 15 EGFP-expressing F0 mice, one line (male), termed MNCE-53, was used in this study, since it exhibited fluorescence in the tail, body-wall muscle, eye, inner surface of the bottom of the ear and various organs tested, including brain, eye, heart, thymus, lung, pancreas, intestine, kidney, spleen, liver, bladder, skeletal muscle and testis, and transmitted *MNCE* (approximately one copy per diploid cell) to progeny in a Mendelian manner (data not shown).

Genomic DNA from tail biopsies was isolated by the method of Blin and Stafford [10] with slight modification. One set of PCR primers, β-gl-1/Cre-RV (Fig. 1), was used for identification of the introduced *MNCE* DNA in offspring derived from pronuclear injection of eggs. β-gl-1 [11], 5′-CTC CTG GGC AAC GTG CTG GT-3′, corresponded to the 3rd exon of the rabbit β-globin gene sequence [12] from nucleotides 1068 to 1087, and Cre-RV [5], 5′-ATG AAG CAT GTT TAG CTG GCC-3′, corresponded to the 5′ region of the *Cre* gene sequence [13] from nucleotides 761 to 781. This primer set yields 385-bp fragments from the 5′ region of the *NCre* gene, as previously described [5]. The PCR reactions for detection of the 5′ region of the *NCre* gene were carried out as previously described [14]. Forty cycles of PCR were performed with cycle times of 1 min at 94°C, 1 min at 58°C, and 4 min at 72°C. The reaction mixture was then analyzed on 2% agarose gels. The gels were stained with ethidium bromide (EtBr), and the amplified DNA bands were visualized by ultraviolet transillumination. As a positive control, 5 ng of pMNCE DNA was used.

In the case of identification of heterozygous and homozygous F2 mice by PCR, the PCR conditions were the same as used for detecting *MNCE* in tail

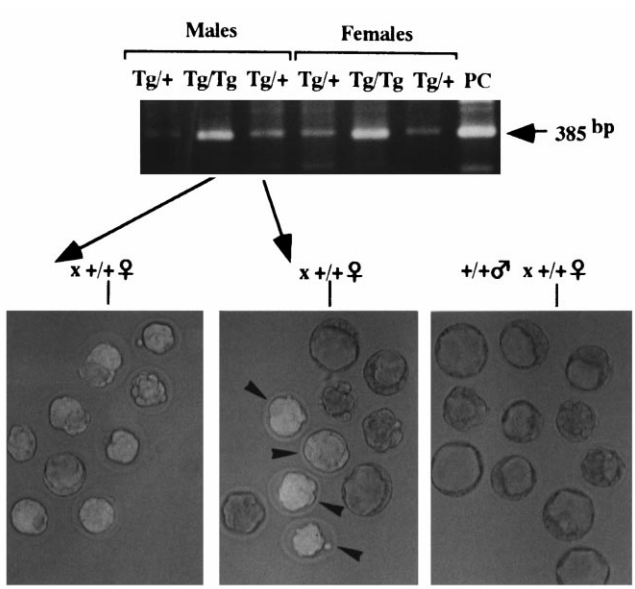

Fig. 2. EGFP fluorescence in early blastocysts derived from mating of the following combinations: Tg/Tg × +/+, Tg/+ × +/+, and +/+ × +/+. The Tg/Tg and Tg/+ mice were obtained by mating between F1 transgenic males and females and subsequent genotyping by PCR, as shown in the upper panel. Lane PC indicates pMNCE plasmid (5 ng) as a positive control. Mating between Tg/Tg male and +/+ female mice resulted in fluorescence in all the embryos tested. Approximately half of embryos (indicated by arrowheads) exhibited fluorescence after mating between Tg/+ male and +/+ female mice, but no embryos exhibited fluorescence after mating between +/+ male and +/+ female mice. Photographs were taken under bright-field illumination to acquire focused images of blastocysts.

DNA, except that the PCR cycle was reduced to 28 cycles, which prevented overproduction of a target fragment and permitted accurate estimation of the amounts of PCR-amplified products. Samples with EtBr-stained 385-bp bands, with approximately twice the intensity of bands in heterozygous samples, were considered those of homozygous mice.

B6C3F1 females, aged 4–5 weeks, were superovulated by injecting 5 IU each of eCG and hCG (Teikoku Zouki, Tokyo, Japan), with injections separated by a 48-h interval. Early blastocysts were collected in M16 medium [15] from the uteri of females that had been mated with adult normal B6C3F1 males or adult F2 transgenic males (heterozygous or homozygous for the *MNCE* transgene) of the MNCE-53 line, at day 3.5. Noon of the day on which vaginal plugs were observed was designated day 0.5 of gestation. These collected blastocysts were directly observed for EGFP fluorescence using a fluorescence microscope (BX60; Olympus) with DM505 filters (Olympus), and photographed as described above.

We first obtained F2 offspring after mating between F1 transgenic males and females, and the genotype of each F2 offspring was determined by PCR (upper panel in Fig. 2). Early blastocysts were obtained by mating with the combinations of Tg/+ male (heterozygous for *MNCE*) × +/+ (normal) female, Tg/Tg (homozygous for *MNCE*) male × +/+ female or +/+ male × +/+ female, and embryos were inspected for fluorescence (lower panel in Fig. 2). As expected, all the blastocysts derived from mating between a Tg/Tg male and +/+ female exhibited fluorescence. Mating of Tg/+ male and +/+ female resulted in expression of fluorescence in approximately 50% of embryos (4/8). No EGFP expression was observed in blastocysts obtained by mating of a +/+ male and +/+ female. These findings indicate that confirmation of homozygous mice at an early stage (at least preimplantation stage) of test breeding is possible.

We also confirmed that approximately 20% of blastocysts derived from mating between a Tg/+ male and Tg/+ female exhibited approximately two-fold stronger fluorescence than the other fluorescent embryos (data not shown), suggesting that homozygous embryos can be identified based on the strength of fluorescence.

Acknowledgements

M. S. and M. K. were supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture, Japan.

References

[1] Cormack BP, Valdivia RH, Falkow S. Gene 1996;173:33–8.
[2] Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. FEBS Lett 1997;407:313–9.
[3] Chiocchetti A, Tolosano E, Hirsch E, Silengo L, Altruda F. Biochem Biophys Acta 1997;1352:193–202.
[4] Kimura M, Sato M, Akatsuka A, Nozawa-Kimura S, Takahashi R, Yokoyama M, Nomura T, Katsuki M. Proc Natl Acad Sci USA 1989;86:5661–5.
[5] Sato M, Watanabe T, Kimura M. Transgenics 1999;2:383–9.
[6] Niwa H, Yamamura K, Miyazaki J. Gene 1991;108:193–200.
[7] Ishii M, Tashiro F, Hagiwara S, Toyonaga T, Hashimoto C, Takei I, Yamamura K, Miyazaki J. Endocr J 1994; 41(Suppl):S9–S16.
[8] Sato M, Kawarabayashi T, Shoji M, Kobayashi T, Hirai S, Tada N. Transgenics 1997;2:153–9.
[9] Hogan B, Costantini F, Lacy E. Manipulating the Mouse Embryo: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1986.
[10] Blin N, Stafford DW. A general method for isolation of high molecular weight DNA from eukaryotes. Nucleic Acids Res 1976;3:2303–8.
[11] Kawarabayashi T, Shoji M, Sato M, Sasaki A, Ho L, Echman CB, Prada C-M, Younkin SG, Kobayashi T, Tada N, Matsubara E, Iizuka T, Harigaya Y, Kasai K, Hirai S. Neurobiol Aging 1996;17:215–22.
[12] Van-Ooyen A, Van der Berg J, Mantei N, Weissmann C. Science 1979;206:337–44.
[13] Sternberg N, Sauer B, Hoess R, Abremski K. J Mol Biol 1986;187:197–212.
[14] Sato M, Kasai K, Tada N. Genetic Anal: Biomol Engin 1995;12:109–11.
[15] Whittingham DG. J Reprod Fert 1971;14(Suppl):7–21.