韓國家畜繁殖學會誌 24(4) : 343~346 (2000)
Korean J. Animal Reprod.

Approaches to Improving Production Efficiencies of Transgenic Animals

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Ⅰ. INTRODUCTION

Transgenic animals are very useful for scientific, pharmaceutical, and agricultural purposes. In livestock, transgenic technology has been used forth genetic alteration of farm animals, the production of human proteins inlarge quantities in the milk of transgenic farm animals (Clark et al., 1989; Ebert et al., 1991; Kimpenfort et al., 1991; Wall et al., 1991; Hill et al., 1992; Velander et al., 1994 and etc.), and the generation of animals with organs suitable for xenotransplantation (Pinkert, 1994; Chen et al., 1999). To date, the transfer of foreign genes into farm animals has been performed mainly by microinjection of DNA into the pronuclei of fertilized eggs. However, the overall success rate of transgenic animals in livestock so far has been disappointingly low, eg., the efficiency is 0~5% in swine, and less than 1% in sheep (Pursel et al., 1989) and cattle (Krimpenfort et al., 1991; Hill et al., 1992), compared with the rate in mice where 5 % microinjected develop into transgenic animals.

Recently, McGreath et al. (2000) have succeeded in producing the gene targeted sheep by the use of nuclear transfer from cultured somatic cells transfected with a foreign gene *in vitro*. However, we may need plenty of time until currently employ this method for gene transfer to farm animals. We have been studying to exploit the method for improving production efficiencies of transgenic animals with emphasis of its application to farm animals. The present paper describes three approaches that we have made in our laboratory to improve production efficiencies of transgenic animals, based on the DNA microinjection method.

Ⅱ. CO-INJECTION OF RESTRICTION ENZYME WITH FOREIGN DNA INTO THE PRONUCLEUS FOR ELEVATING PRODUCTION EFFICIENCIES OF TRANSGENIC ANIMALS

Brenneman et al. (1996) reported that the treatment of human cell lines with the restriction endonuclease Xba I, which has a recognition site within the treated region of HPRT homology, increased the frequency of homologous recombination by more than tenfold. We investigated whether or not co-injection of foreign DNA constructs with restriction endonuclease into the pronucleus of mouse zygotes would improve the integration frequencies of foreign DNA into the host genome. Two kinds of DNA constructs that have no Eco RI site in their sequences were used for co-microinjection. With reference to the results of experiments in which Eco RI alone was injected at various amounts varying from $10-9$ to $10-5$ U/nucleus, the amount of $5\times10-8$ U/nucleus which showed survival rate of 60.6% was used for the co-

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injection with DNA. Successful transgenesis of co-injected embryos was identified by the Dpn I-Bal 31 digestion method for single embryos and by PCR method for pups born, respectively. The overall efficiency for the integration of foreign DNA in single embryos and live-born pups obtained by the co-injection procedures were 17.9% compared with 9.1% obtained by the injection of DNA alone. In our laboratory, the mean efficiency of transgenesis in the pups born was less than 11 % (Katsube et al., 1993; Tojo et al., 1993; Ikeda et al., 1994; Hase et al., 1996; Kano et al., 1998; Inuzuka et al., 1999; Kato et al., 1999; Seo et al., 2000). Therefore, the present results suggest that co-injection of foreign genes with restriction enzyme possibly elevates the integration rate of foreign genes into host genomes. The results suggest that co-injection of foreign genes with restriction enzyme may elevate the integration rate of foreign genes into host genomes.

Ⅲ. EFFICIENT SELECTION OF TRANSGENIC MOUSE EMBRYOS USING EGFP AS A MARKER GENE

We have established a reliable method that uses the EGFP (Enhanced Green Fluorescent Protein) gene as a marker for selecting transgenic embryos from preimplantation embryos. Embryos that were subjected to the pronuclear microinjection of the CMV/beta-actin (CAG)/EGFP fusion gene were cultured *in vitro* until they developed into the morulae- or blastocyst-stage. The expression of EGFP was easily observed by a fluorescent microscopy. There appeared to be no damage to the *in vivo* developmental ability of the embryos in response to the EGFP excitation light, which utilized an IB filter for a period of 30 min. Modified PCR analysis using Dpn I and Bal 31 digestion of the embryonic DNA (Seo et al., 1997) showed that all of the embryos expressing EGFP in all their cells were transgenic, while more than half with mosaic expression of EGFP were not transgenic. Approximately 77% of pups born from the embryos that uniformly expressed the EGFP gene were transgenic, while 21.4% of pups from the embryos with mosaic expression were transgenics. The results showed that the use of EGFP as a marker is very useful and reliable for selecting transgenic embryos, and that it is important to transfer the embryos expressing EGFP in all their cells to obtain truly transgenic animals.

Ⅳ. PHENOTYPES OF TRANSGENIC MICE EXPRESSING WAP/hGH-CAG/EGFP FUSION GENE PRODUCED BY SELECTING TRANSGENIC EMBRYOS

Based on our previous results, we constructed the WAP/hGH-CAG/EGFP fusion gene, introduced it into mice, and analyzed the phenotypes of transgenic mice. In WAP/hGH gene, the promoter region of mouse whey acidic protein gene (WAP) was linked to the coding region of human growth hormone gene (hGH). Fifteen of 95 pups born (15.8 %) from the transfer of embryos without selection for transgenic embryos were transgenic. Approximately 85.7 % (6/7) of pups born from the embryos that uniformly expressed the EGFP gene were transgenic, while 44.4% (4/9) of pups from the embryos with mosaic expression were transgenic, and no transgenic pups (0/9) were generated from the EGFP-negative embryos. WAP/hGH was expressed in the trasnsgenic mice as well as EGFP, and hGH was secreted into the milk of nursing transgenic females. The results confirmed that the use of EGFP as a marker is very reliable for selecting transgenic embryos, and that the target gene coupled to a marker gene was expressed in transgenic mice in tissue specific fashion as well as a marker

gene.

V. EFFICIENT SITE-SPECIFIC INTEGRATION OF THE TRANSGENE TARGETING AN ENDOGENOUS LOX LIKE SITE IN EARLY MOUSE EMBRYOS

Transgenic mice or cells lines made by standard DNA transfer methods show an extreme variability in expression of the introduced gene. This occurs from the highly variable copies numbers of the integrated transgene and from position effects on gene expression due to random integration of the transgene into the host genome. Moreover, this phenomena may inactivate an endogenous gene. An appealing strategy to circumvent such constraints would involve site specific integration of introduced sequences into safe position in the genome. Homologous recombination could be an alternative but its efficiency still low (Vega et al., 1991). Cre/loxP recombination system is both highly specific and efficient. Recombinase mediated integration would be more powerful for genetic engineering if sites that could act as targets for the Cre recombinase occurred endogenously in the mammalian genome at safe locations, such as regions outside of the fonctional genes; Cre was shown to carry out efficiently an intra-molecular (excessive) and an inter-molecular (integrative) recombination both in prokaryotic and eukaryotic cells. Furthermore, Cre was shown to catalyses recombination at cryptic or pseudo loxP site which has a remarkable degree of homology to the wild type loxP. A functional lox-like sequences were identified in the genome of bacteria (Sterberget al., 1981) , in yeast (Sauer et al., 1996) and recently in mousse and human genome (Thygarajan et al. 2000). We focused on the endogenous lox like sequence called pseudo loxM5 that occurs naturally on the chromosome 5 in the mouse genome. The Cre/LoxM5 recombination frequency derived from the bacterial excision assay was reported to be 98.7% (Thygarajan et al., 2000). This is the first report on Cre mediated integrative recombination targeting an endogenous lox-like sequence in early mouse embryos. A Cre expressing plasmid and a linear transgene flanked by two loxM5 were co-microinjected into the pronucleus of fertilized oocytes and recombination products were investigated. Approximately 66.7% of pups born were found to be transgenic by PCR, one was mosaic and one was negative. Here, we demonstrate an efficient and simple strategy to target an endogenous lox-like site in the early mouse embryos using Cre/LoxP integrative recombination system.

Finally, our results showed to provide useful procedures for elevating production efficiencies of transgenic farm animals.

VI. REFERENCES

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(접수일자 : 2000. 10. 10. / 채택일자 : 2000. 10. 20.)