Estimation of the Efficiency of Transgenic Rabbit Production Following GFP Gene Microinjection into Rabbit Zygotes

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ABSTRACT : The present study was conducted to evaluate the efficiency of transgenic rabbit production by DNA microinjection using EGFP (Enhanced Green Fluorescent Protein) gene. In this experiment EGFP coding sequences fused to CMV promoter were microinjected into rabbit one-cell embryos, and then GFP expression and gene integration were evaluated in preimplantation embryos and fetuses recovered on day 15 of pregnancy to determine efficiency of transgenic rabbit production. Effect of DNA concentration was also tested on development *in vitro* following microinjection and transgene integration in fetuses. Development of embryos in vitro was decreased by DNA microinjection, but the rates of pregnancy and implantation were not significantly affected by microinjection. As development progressed *in vitro* percentage of GFP expression in rabbit embryos was decreased, resulting GFP expression detected in 37.5% of blastocysts. The efficiencies for production of transgenic fetuses were 4.0% and 7.6%, respectively, when 10 ng/μl and 20 ng/μl of DNA concentration were microinjected. Transgenic fetuses were confirmed by GFP expression and PCR analysis of fetus genomic DNA. These results indicated that DNA microinjection itself damaged embryo development and DNA concentration affected the efficiency of transgenic rabbit production. (*Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 10 : 1367-1372*)

Key Words : GFP Gene, Microinjection, Transgenic Rabbit, DNA Concentration

INTRODUCTION

Microinjection of DNA into pronucleus of one-cell embryo has successfully been used for generating transgenic animals. This method is still the most reliable one for the production of transgenic livestock animals that have obstacles in somatic cell nuclear transfer. However, the efficiency of transgenic animal production by microinjection is low in other animals compared to mouse. Preselection of DNA-microinjected embryos before embryo transfer into recipients has been reported to increase the efficiency of transgenesis (Ikawa et al., 1995; Takada et al., 1997). Recently, we have developed embryo transfer technique for *in vitro* cultured rabbit blastocysts and successfully produced kits following transfer of those blastocysts into oviducts of recipients (manuscript in preparation).

The green fluorescent protein (GFP) gene isolated from jellyfish Aequera Victoria or its mutant EGFP (Enhanced GFP) which amplifies its excitation peak by 50 times compared to wild type of GFP has been established as a useful marker for monitoring gene expression in situ (Cormack et al., 1996; Crameri et al., 1996; Roger et al., 1995). Previous reports in which the GFP gene as a selectable marker was successfully transfected into embryos of mouse, bovine or non mammalian species such as Zebrafish and Drosophilia confirmed that GFP DNA can be used for monotoring gene expression in embryos (Chalfie et al., 1994; Cui et al., 1994; Amsterdam et al., 1995; Ikawa et al., 1995; Yeh et al., 1995; Chan et al., 1997; Takada et al., 1997; Zernicka et al., 1997; Chan et al., 1999). There have also been some studies in which expression of GFP in cytoplasm had no harmful effect on embryo development (Ikawa et al., 1995; Zernicka et al., 1997). No study has been reported for the usefulness of GFP as genetic marker for the production of transgenic rabbits.

The purpose of the present study was to determine the overall efficiency of transgenic rabbit production as well as the assessment of potential use of GFP as a genetic marker for transgenic rabbit production by detecting the expression of GFP gene in both embryos and fetuses following microinjection of GFP DNA into pronucleus of rabbit embryos. And the effect of DNA concentrations on the efficiency of transgenic rabbit production was tested.

MATERIALS AND METHODS

Gene and microinjection

EGFP gene fused to CMV promoter was linearized by digestion with restriction enzyme and vector plasmid sequences were removed. Linearized DNA was electrophorased and DNA band was purified with gene clean Kit (Gene clean II, Bio 101) and resuspended with TE buffer (10 mM Tris, 0.1 mM EDTA). Final DNA concentration was adjusted to either 10 ng/μl or

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20 ng/μl. Microinjection was performed under micromanipulator with a DIC Optic (Nikon, Japan). DNA solution was injected into the pronucleus of the one-cell embryo with confirming swelling of pronuclear membrane.

Superovulation, *in vitro* culture and embryo transfer

Sexually matured New Zealand White rabbits were superovulated by subcutaneous injection of 0.4, 0.4, 0.5, 0.5, 0.5 and 0.5 mg FSH at 12 hr intervals (Kennelly et al., 1965). And they were injected with 30 mg HCG intervenously 12 hr after the last FSH injection. Twenty hours after mating with males twice, one-cell zygotes were collected by flushing oviducts with DPBS containing 0.3% BSA solution. One-cell embryos were washed three times with RDH medium (a 1:1:1 mixture of RPMI, DMEM and Hams F-10). Embryo culture was performed in 50 μl drop of RDH medium containing 0.3% BSA and 25 mM Taurine under the condition of 5% CO_2 and 39℃ for 72 hr. Cell number of expanded blastocysts was counted by the method reported previously (Pursel et al., 1985). Embryos were transferred shortly after microinjection into oviduct of synchronized recipient. About 8 to 12 embryos were transferred to each oviduct of recipient.

Detection of GFP expression in embryos and fetuses

Detection of transgenic embryo was performed by direct visualization of green fluorescence with using Meridian Ulima Z CSLM Argon Laser confocal microscope (excitation WL: 488, filter: UV + VIS 460 / 40BP Filter) as embryo development was advanced to 4~8-cell, morular and blastocyst stage. For detection of GFP expression in fetuses, fetuses were collected from the uterus at 15 day after embryo transfer by hysterectomy and whole fetus was directly exposed in confocal microscopy.

Detection of GFP gene in fetuses with PCR

In order to prepare genomic DNA of fetuses for PCR, crude tissues were incubated at 55℃ overnight in lysis buffer with proteinase K and DNA was purified by extraction with phenol:chloroform (1:1) solution, and precipitated with 2 volumes of ethanol. Precipitated DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA). Primers for GFP detection are as follows:

GFP 5 : 5′ — CAA GGA CGA CGG CAA CTA CAA GAC — 3′

GFP 3 : 5′ — GTC GAT TAT GAT CAG TTA TCT AGA TCC — 3′

The PCR was performed under condition of denaturation at 94℃ for 1.5 min, annealing at 55℃ for 2 min and extension at 72℃ for 2 min with total of 35 cycles in PCR machine (Perkin Elmer). PCR amplification was confirmed by 2% agarose gel electrophoresis following PCR reaction.

Data analysis

The rates of embryo survival, pregnancy and transgenesis were subjected to 2×2 Chi-square test. Differences in average cell number and average fetuse number between experimental groups were analyzed by Student-t test (Snedecor and Cochran, 1967).

RESULTS

***In vitro* embryo development after microinjection**

DNA microinjection into rabbit embryos resulted in the decrease in viability and development of embryos in this experiment. After 72 hr culture, 75.0% and 68.4% of embryos microinjected with 10 ng/μl and 20 ng/μl of DNA, respectively, developed to blastocysts while 87.5% of uninjected embryos developed to blastocysts (table 1). The difference in the percentage embryos that developed to blastocysts between uninjected and 20 ng/μl of DNA-injected groups were significant. Table 1 also showed that DNA microinjection caused embryos to degenerate (0.0% vs 7.0% and 10.5%, for uninjected vs 10 ng/μl and 20 ng/μl of DNA-injected groups, respectively). DNA microinjection and DNA concentration did not affect significantly the cell number of expanded blastocysts (151 vs 145 and 148, for uninjected vs 10 ng/μl and 20 ng/μl of DNA-injected groups, respectively).

Table 1. The viability and development of rabbit embryos microinjected with EGFP gene following culture for 72 hr

Experimental group[c]	No. of embryos injected	No. of embryos degenerated (%)	No. of morula (%)	No. of blastocyst (%)	Average cell number of expanded BLd
Control	48	0 (0.0)	6 (12.5)	42 (87.5)[a]	151
10 μg/μl	100	7 (7.0)	17 (17.0)	75 (75.0)[ab]	145
20 μg/μl	76	8 (10.5)	12 (15.8)	52 (68.4)[b]	148

[a,b] Values with different superscripts within each column differ ($p<0.05$).

[c] Control : intact embryos, 10 μg/μl and 20 μg/μl : DNA concentrations microinjected.

[d] Blastocysts and values do not significantly differ ($p>0.05$).

Pregnancy rate and average fetus number after DNA microinjection

At 15 days after EGFP gene microinjection, rabbit fetuses were recovered from the uterus of recipients by hysterectomy to evaluate pregnancy and implantation rates as well as number of fetuses per pregnant recipient. There was no difference between injected group and uninjected group in the pregnancy and implantation rates. The pregnancy rate of recipients following embryo transfer in this experiment was 60% in uninjected group and 57.1% or 66.7% in injected groups with the concentration of 10 ng/μl or 20 ng/μl, respectively (table 2). Table 2 also showed that implantation rate of embryos following transfer into recipients was 23.0% in uninjected and 17.3% and 23.6% in injected groups with the concentration of 10 ng/μl or 20 ng/μl, respectively. In this experiment, the number of fetuses per pregnant recipient decreased in higher DNA concentration (7.0 and 5.8 in the groups injected with 10 ng/μl and 20 ng/μl of DNA, respectively, compared to 7.8 in uninjected group).

GFP expression of microinjected rabbit embryos during *in vitro* development

When GFP expression in embryos following microinjection with 10 ng/μl GFP DNA was observed at 4~8-cell, morular and blastocyst stages during *in vitro* development, GFP was expressed in 62.5%, 45.0% and 37.5% of embryos, respectively (table 3), suggesting that the proportion of embryos expressing GFP decreased as development was in progress. As shown in figure 1 (A and B), embryos expressing GFP following DNA microinjection could be clearly identified and green fluorescence in some embryos appeared in a mosaic manner in which green fluorescence was not emitted uniformly in all blastomeres of embryos.

GFP expression and transgenic rate in fetuses after DNA microinjection

At day 15 after EGFP DNA microinjection, fetuses were recovered from the uterus and all fetuses were examined for green fluorescence *in situ*. As shown in table 4, 2 out of 50 fetuses (4.0%) examined under fluorescent microscopy emitted green fluorescence in 10 ng/μl group, while 7.6% (4/52) of fetuses in 20 ng/μl group appeared to be expressing GFP. Therefore, transgenic efficiency was higher in embryos microinjected with 20 ng/μl than with 10 ng/μl of DNA concentration. As shown in figure 1 (C and D), intense fluorescence appeared in brain, vertebra and muscle of fetuses probably due to the tissue-specificity of the CMV promoter.

To confirm the existence of transgene in fetuses, PCR using genomic DNA of fetuses was performed with GFP-specific primers. Intense band with the size identical to GFP plasmid DNA control following PCR appeared in all GFP-positive fetuses (figure 2). That PCR band was not amplified in DNA of GFP-negative fetuses. Therefore, it appeared that PCR result was consistent with fluorescent observation in fetuses in this experiment.

DISCUSSION

This study was performed to investigate the effect of DNA concentrations on the efficiency of transgenic rabbit production by DNA microinjection in addition to examine the potential use of GFP as a genetic marker for transgenesis in rabbits. To attain these aims, we microinjected EGFP gene into rabbit zygotes and monitored green fluorescence *in situ* under a confocal microscope after 72 hr culture *in vitro*. We

Table 2. Efficiency of pregnancy and implantation after EGFP gene microinjection into rabbit zygotes

Experimental group[a]	No. of embryos transferred	No. of recipient	No. of pregnant recipient (%)	No. of fetuses (%)	Average no. of fetuses
Control	200	10	6 (60.0)	47 (23.0)	7.8[b]
10 ng/μl	162	7	4 (57.1)	28 (17.3)	7.0[bc]
20 ng/μl	148	9	6 (66.7)	35 (23.6)	5.8[c]

[a] Control : intact embryos, 10 ng/μl and 20 ng/μl : DNA concentrations microinjected.
[b,c] Values with different superscripts within each column differ ($p<0.05$).

Table 3. GFP-expressing efficiency of rabbit embryos after microinjection of EGFP gene during pre-implantation period[a]

No. of embryos observed at each stage	No. of fluorescent embryos at 4~8-cell stages (%)	No. of fluorescent embryos at morular stage (%)	No. of fluorescent embryos at blastocyst stage (%)
80	50 (62.5)	36 (45.0)	30 (37.5)

[a] One cell-embryos were microinjected with DNA concentration of 10 μg/μl.

also transferred the microinjected one-cell embryos to the oviducts of the pseudopregnant recipients and collected fetuses at day 15 of pregnancy in order to detect green fluorescence from the whole fetuses and analyze genomic DNA of fetuses for identification of transgenic fetuses.

In the present experiment, development of rabbit embryos into blastocysts was affected by DNA microinjection (table 1). And cell numbers of expanded blastocysts derived from microinjected zygotes were not significantly lower than uninjection group. More embryos with DNA microinjection were degenerated than uninjected embryos and the degenerated embryos appeared at the early stages. This result was similar with the previous results that microinjection DNA into porcine or rabbit embryos increased the portion of embryos that were degenerated or arrested at 1-cell stage (French et al., 1993; Chrenek et al., 1998). William et al. (1992) suggested that this reduced development of microinjected embryo may be caused by the introduction of DNA (and/or its contaminant) but not by the mechanical damage to the nuclear or cytoplasmic membrane. Although we did not completely understand what caused retardation of embryo development following DNA microinjection in this experiment, it is considered that DNA micro-

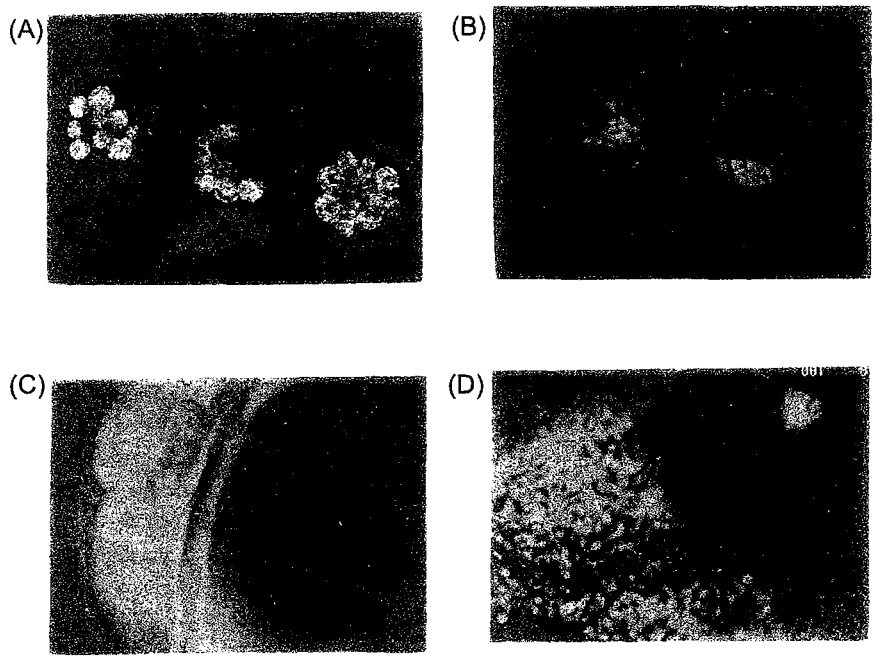

Figure 1. GFP expression in rabbit embryos microinjected with GFP gene fused to CMV promoter and rabbit fetuses derived from embryos microinjected with GFP DNA. (A) Morular embryos, (B) Blastocysts, (C) Dorsal area of fetus, (D) Abdominal area of fetus

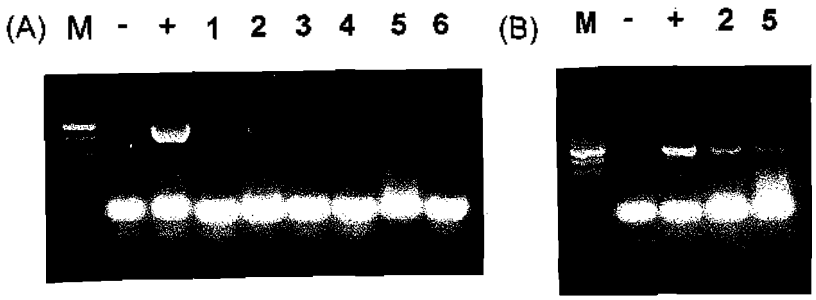

Figure 2. PCR analysis of genomic DNA of rabbit fetuses derived from embryos microinjected with GFP gene fused to CMV promoter. (A) Two GFP-positives out of 6 feuses following GFP amplification. (B) Reamplification of genomic DNA of two fetuses. M, 100bp DNA marker; -, negative control DNA; +, positive plasmid DNA; 1-6, genomic DNAs of rabbit fetuses

Table 4. GFP expression and transgenic efficiency in the rabbit fetuses derived from embryos microinjected with GFP gene

Gene concentration	No. of fetuses examined	No. of fluorescent fetuses	No. of transgenic fetuses (%)
10 ng/μl	50	2	2 (4.0)
20 ng/μl	52	4	4 (7.6)

injection itself had the effect on embryos development *in vitro* since there was no difference in embryo development between two different DNA concentration groups. There was no difference in pregnancy and implantation efficiency between uninjected and two microinjected groups in this experiment, which was contrast to in vitro development data, probably due to exclusion of early retarded embryos before transfer to oviducts of recipients.

Transgenic efficiency in the group of DNA concentration of 20 ng/μl was higher than in the group of DNA concentration of 10 ng/μl whereas the development of zygotes microinjected with high DNA concentration into blastocysts was lower than that with low DNA concentration (table 1 and 4). This tendancy is in agreement with other reports for mouse (Brinster et al., 1985) and bovine embryos (Menck et al., 1998). Overall transgenic efficiency in this experiment was a little higher than that in other transgenic rabbit studies (Hammer et al., 1985; Wang et al., 1996).

The number of embryos expressing GFP following microinjection was decreased as development was advanced in the present experiment (table 3), in aggreement with Chauhan et al. (1999) who studied bovine embryos microinjected with GFP construct. Mosaic pattern of GFP expression in the rabbit embryos observed in this experiment (figure 1. A) has also been reported in other species (Chan et al., 1999; Kato et al., 1999). The decrease of the number of GFP-positive embryos in the later development stages may be attributed to the fact that in the early development of embryos unintegrated DNA segments following microinjection were existed with its transcriptional action and those unintegrated DNA segments were excluded as embryo development advanced. From confocal microscopy with whole body of fetuses, total six fetuses were positive in GFP and the same fetuses were confirmed to be transgenic in the analysis of genomic DNA by PCR. This result suggested that GFP can be used as a fusion gene for detection of transgene expression in transgenic rabbits.

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