Journal of Animal and Feed Sciences, 12, 2003, 71–81

The use of green fluorescent protein (GFP) to select bovine embryos

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(Received 5 June 2002; revised version 21 October 2002; accepted 20 December 2002)

ABSTRACT

A major factor limiting the transgenesis in domestic animals is the inefficiency of maintaining large numbers of recipients carrying nontransgenic foetuses. The objectives of this study were: 1. to determine the influence of green fluorescent protein (GFP) construct injection on the development of bovine embryos, 2. to identify and select the GFP positive bovine embryos, and 3. to determine the rate of mosaicism in transgenic embryos. Cattle oocytes were matured and fertilised *in vitro* and zygotes were microinjected with pCX-EGFP construct consisting of CMV-IE enhancer, chicken β-actin promoter, cDNA of GFP (EGFP-732 bp) and rabbit β-globin polyadenylation sequences. Embryos from control (64) and microinjected (198) groups were cultured *in vitro*. After 168 h of culture, morula and blastocysts were observed in 39.06% of control and in 23.23% of injected group. We obtained three GFP positive embryos (1.51% of injected zygotes and 6.52% of morulae/blastocysts). One of them was 100, second 75 and third 25% GFP positive (66.7% of mosaicism). Use of *gfp* gene reporter to select bovine embryos is useful method to increase transgenic offspring, because GFP marker allows to choice only transgenic embryos and transfer them to recipients.

KEY WORDS: *gfp* reporter gene, transgenic bovine embryos

INTRODUCTION

Transgenic animals are very important for scientific, pharmaceutical and agriculture purposes. There are a lot of ways to insert heterologous recombinant DNA into the genome of living organism (Krimpenfort et al., 1991). One of the most important methods is pronuclear microinjection that has been used for successful generation of transgenic mammalian species, such as: mice, rats, rabbits, pigs, sheep, goats and cattle (Wall, 1997).

Microinjection of DNA into the pronuclei of one-cell embryos work very well in mice, but it remains inefficient and expensive in domestic animals. In large domestic animals, this approach generates 0.02 to 2% of transgenic offspring from microinjected zygotes (Cousens et al., 1994) but in cattle less than 1% (Chen et al., 1999). There are several limitations to this approach: 1. a large number of recipients need to be maintain through the gestation period, 2. a low efficiency of transgene incorporation, 3. a requirement for a large number of oocytes for microinjection. These make the production of transgenic animals, especially cows, extremely difficult and expensive (Wall, 2002).

Preselection of transgenic embryos before transfer into the surogate recipient is the way to reduce the cost of transgenic cattle production. This may be achieved by reporter gene technology as their gene products are easy detectable, with low background activities from endogenous gene products. The ectopic levels of reporter gene expression have no adverse effects on either the physiology or development of the individual cell.

At present, many authors have used the reporter gene technique in the preimplantation-stage screening protocol of many species of animals produced *via* pronuclear microinjection (Ikawa et al., 1995; Takada et al., 1997; Kato et al., 1999). This procedure allows the identification of presumptive transgenic embryos that can be transferred into surrogate recipients, thereby increasing the overall efficiency and reducing the cost of transgenic animal production (Keiser et al., 2001).

A number of reporter genes have been used: *Escherichia coli* β-galactosidase gene (*lac*Z) (Connolly et al., 1994); *chloramphenicol acetyltransferase* - (*cat*) (Gorman et al., 1982); *firefly luciferase* (*luc*), (De Wet et al., 1987) and *horseradish peroxidase* (Connolly et al., 1994).

One of the most important reporter genes that can be used in preimplantation-stage screening protocols is the green fluorescent protein (*gfp*) from the jellyfish *(Aequorea Victoria*), which, when expressed in either eukaryotic or prokaryotic cells and illuminated by blue or UV light, yields a bright green fluorescence (Chalfie et al., 1994; Baumann et al., 1998). It has been recently suggested that green fluorescent protein (GFP) may be a good and convenient detection system for transgenesis in intact living cells and organisms. GFP may “open the door” as a fusion tag to monitor protein localization within the living cells (Cubitt et al., 1995).

There are a number of advantages of GFP transgenic embryos. Among these is the production of therapeutic specific proteins in milk or urine (Chalfie et al., 1994; Chiocchetti et al., 1997; Zhou et al., 1997; Baumann et al., 1998) and the labelling of some specific tissues such as the ureteric bud (Srinivas et al., 1999). GFP can also be used as a biomarker for selection of transfected cells (Chan et al., 1999; Arat et al., 2001).

The objectives of the present research were: 1. to determine the influence of *gfp* construct injection on the development of bovine embryos, 2. to identify and select the GFP-positive bovine embryos, and 3. to determine the rate of mosaicism in transgenic embryos.

MATERIAL AND METHODS

In vitro maturation of bovine oocytes

The cumulus-oocyte complexes (COCs) were collected from bovine ovaries which were obtained from a slaughterhouse and matured in TCM199 buffered with Hepes 25 mM supplemented with 10% heat-inactivated FBS, 0.02 IU/ml pFSH (Sigma), 1 μmg/ml 17 β-estradiol (Sigma), 0.2 mM sodium pyruvate (Merck) and 50 μmg/ml gentamicin sulphate. Twenty immature cumulus-oocytes complexes were placed in one well of a 4-well culture dish (Nunc) and cultured in 450 μl TCM 199 supplemented solution for 24 h in 38.5ºC, 5% CO_2 in humidified air (Marguant-Le Guienne et al., 1989).

In vitro fertilization of bovine oocytes

Spermatozoa obtained from a single bull were prepared by swim-up procedure according to the method of Marguant-Le Guienne et al. (1989). Ten matured oocytes after being washed in fertilization medium were placed in 4-well culture dishes in 450 μml Fert-TALP medium supplemented with 6 mg/ml BSA FAF, 0.2 mM sodium pyruvate, 50 μmg/ml gentamicin sulphate, 20 μmM penicillamine (Sigma), 10 μmM hypotaurine (Sigma), 1 μmM epinephrine (Sigma) and 2 μmg/ml heparin (Sigma). The COCs and spermatozoa were adjusted to a final concentration of $1x10^6$/ml and were co-cultured for 24 h at 38.5ºC in 5% CO_2 of humidified air (Parrish et al., 1986).

Microinjection gfp gene construct into bovine zygotes

After 24 h of culture, the presumptive zygotes were washed 3 times in TCM-199 buffer (supplemented with Hepes 25 mM, 50 μg/ml gentamicin sulphate, 100 IU

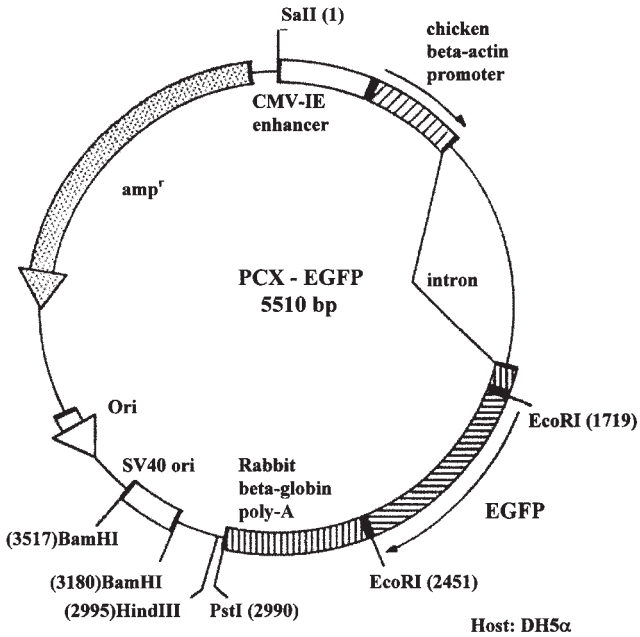

Figure 1. Construction of pCX-EGFP transgene. pCX-EGFP (5510 bp) contains the CMV-IE enhancer, chicken beta-actin promoter, cDNA of EGFP, and rabbit beta-globin poly-A signal

penicillin, 50 μg/ml streptomycin) and then were centrifuged at 12000 x g for 6 min in order to visualize the pronuclei. Zygotes were then transfered to a 3 cm Petri dish into a drop of TCM-199 buffer (containing 25 mM Hepes, 50 μg/ml gentamicin sulphate, 100 IU penicillin, 50 μg/ml streptomycin) and covered with mineral oil (Sigma). The dish with the zygotes was placed under the Nikon Inverted Microscope with Nomarski contrast optics. Injection of DNA solution was conducted manually using a fine glass pipette into the male pronucleus; the time of injection was noted by observing the swelling of the pronucleus. The transgene construct, pCX-EGFP, (gift of Professor M. Okabe, Ossaka University, Japan) used in the present study is shown in Figure 1. It consists of CMV-IE enhancer, chicken β-actin promoter, cDNA of GFP (EGFP) (732 bp) and rabbit β-globin polyadenylation sequences. The pCX-EGFP transgene was diluted to a concentration of 5.0 ng/μl and used for pronuclear microinjection into bovine zygotes at 24 h post insemination.

In vitro culture of bovine embryos

Embryos from control and microinjected groups were co-cultured with Vero/BRL cell monolayers in 40 μl of Menezo B-2 medium (ART of CCD) plus 10% heat

inactivated FBS under mineral oil up to 168 h after insemination at 38.5ºC in 5% CO_2 of incubator. The culture medium was renewed twice during this time. After 48 h of culture, 20 µl of medium was removed and 20 µl of fresh Menezo B-2 medium supplemented with 10% FBS was added but after 144 h of culture, 20 µl of medium was removed and 20 µl of Menezo B 2 was added (Duszewska et al., 2000).

Fluorescence analysis of GFP expression in bovine embryos

Expression of GFP in embryos was detected after 168 h of culture by a standard fluorescein isothiocyanate (FITC) filter for developmental stages and GFP expression using excitation in 488 nm. The transgenic embryos were scored for GFP positive blastomeres within an embryo.

RESULTS AND DISCUSSION

The influence of gfp construct injection on the development of bovine embryos

The development injected and non-injected (control) bovine embryos are presented in Table 1. In this study, in total two hundred and sixty two bovine zygotes were used. One hundred and ninety eight were injected with the *gfp* transgene and sixty four served as a control. After 48 h of culture, 47.98% of the zygotes from the injected group cleaved compared with 81.25% for the control group. After 168 h of culture, a higher percentage of morulae and blastocysts were observed in the control (39.06%) than in injected group (23.23%). Corresponding results for our previous studies were 44 and 32.2% (Duszewska et al., 2000; Rosochacki et al., 2001). The greater negative effect on embryo development in the present study may be due to the increase in microinjected DNA concentration (3 ng in our previous study vs 5 ng in this one), an observation that was also shown by Behboodi et al. (2001). It should be pointed out, that the development of compacted morulae and blastocysts from 48 h cleaved embryos was very similar, being 48% in both groups. The efficiency in producing morulae/blastocysts after a pronuclear injection was lower (23.23%)

TABLE 1

In vitro development of microinjected (GFP-positive) and non-injected (control) bovine embryos

Groups	No of zygotes/ No of injected zygotes	No of cleaved embryos %	No of morulae/blastocysts development %	No of GFP positive embryos after 168 h %
Injected	198/198	95(47.98)	46(23.23)	3(1.52)
Control	64/0	52(81.25)	25(39.06)	0

than in our previous results, but comparable with other authors, who obtained an efficiency in producing blastocysts between 5 and 24% (Krimpenfort et al., 1991; Wall and Seidel, 1992; Bowen et al., 1994; Chan et al., 1999; Ideta et al., 2002). A higher percentage of morulae and blastocysts was obtained by Wang et al. (2001) but they also obtained a higher percentage of viable zygotes after injection (83%). The yield of morulae/blastocysts resulting from 1-cell *in vivo* derived embryos that had survived microinjection could have been even 50% higher than from equivalent *in vitro*-derived embryos (Krimpenfort et al, 1991; Behboodi et al., 1993; Behboodi et al., 2001). In our work we used only *in vitro* derived oocytes.

The identification and selection of the GFP positive bovine embryos

The majority of transgenic animals generated by pronuclear microinjection appear to be mosaic in both somatic cells and germ cells for the pattern of DNA integration (Wall, 1966). Three pronuclear microinjection experiments were performed using the pCX-EGFP gene construct (Figure 1). The coding sequence for enhanced GFP expression (EGFP, Ossaka, Japan) was fused to the chicken β-actin promoter and β-globin polyadenylation sequences, and this construct was used to produce transgenic bovine embryos. Embryos were diagnosed as GFP positive by visual assessment if the level of fluorescent emission was greater than that detected due to autofluorescence from the noninjected group (Figure 2).

The bovine embryos were detected for green fluorescence after 168 h as the expression of transcription events occurs later in bovine embryos than in other species (mouse, rabbits), and also the bovine embryos are much more sensitive to environmental conditions than the others (our unpublished results). In the mouse, the transcription occurs in two-cell embryos as well as expression of microinjected DNA into the pronuclei (Brinster et al., 1982; Stevens et al., 1989). This indicates that promoter function during early embryogenesis as well as transcriptional activity first appears with the formation of the zygotic nucleus (Martinez-Salas et al., 1989). However, not many attempts have been made to determine the precise timing of bovine gene expression after DNA microinjection.

In this experiment after 168 h the green fluorescent appeared in 3 embryos out of 198 injected zygotes, which constitutes 1.51% of transgenesis and 6.52% of morulae/blastocyst transgenic embryos.

The overall efficiency in producing live transgenic cattle is reported to be less than 1% of the total number of microinjected embryos (Brem, 1993; Brem and Muller, 1993; Wall, 1996), but a lot of them could be mosaics, so the efficiency of producing 100% transgenic animals is much lower and ranges from 0.038 to 0.22% (Chen et al., 1999). Takeda et al. (1997) obtained 10% of embryos expressing blastocyst-stage GFP. A high percentage was also obtained by Wang and al. (2001), who used cytomegalovirus promoter (CMV). In our experiment the chicken

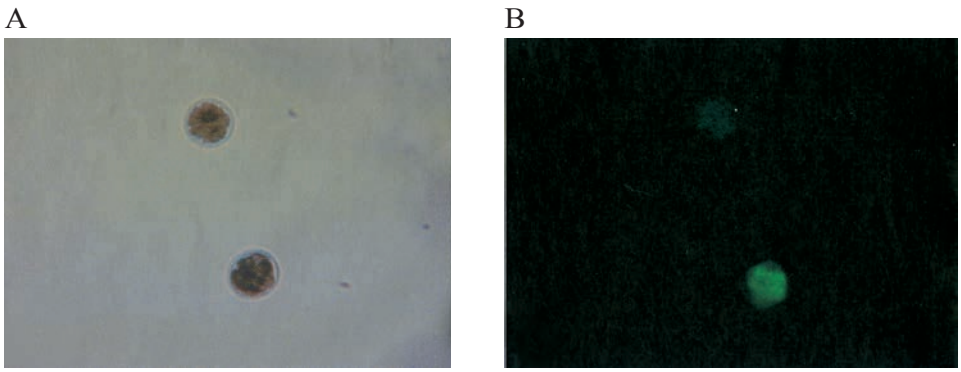

Figure 2. The bovine embryos injected with pCX-CGFP; a-two embryos were viewed under light microscopy, b-two embryos were viewed under UV light microscopy, one of them was GFP positive

β-actin promoter was used. According to Nakamura et al. (1998) this promoter, with its luciferase reporter gene was expressed more intensively in bovine morulae and blastocysts than gene constructs consisting of thymidine kinase with SV40 early promoters. From three transgenic embryos, one embryo was 100% GFP positive. This means that the pCX-EGFP gene construct was integrated during the first cell cycle before DNA replication. In our previous report (Rosochacki et al., 2001) we got no 100% transgenic embryos and only 5 mosaic ones. The category of 100% transgenic embryos was 0.50% of the injected zygotes and 2.17% of the morulae/blastocysts. This low percent of 100% category in the transgenesis may be caused by various factors: nature of the DNA construct or DNA fragment preparation, timing of microinjection and the nature of microinjected embryos (maturation *in vitro*).

In the work of Behboodi et al. (2001) 2578 *in vitro* fertilized 1-cell embryos were microinjected but no transgenic calves were born. However, they did obtained one transgenic bull calf from the microinjection 8193 of two-cell *in vivo* fertilized embryos. They have shown that the new-born bull was transgenic. One possible explanation is that only one blastomere was a founder of the transgenic offspring (the other was destroyed) or that the transgenic calf was mosaic but not for the tissues that were examined. These results emphasize the enormous inefficiency associated with the generation of transgenic cattle by simple microinjection. Thus the alternative strategy based on the use of reporter genes and nuclear transfer of transfected somatic cells could be more appropriate.

The incidence of mosaicism in transgenic embryos

In this experiment we obtained two mosaic embryos after *gfp* gene injection. One of them was 75% GFP positive and one was 25% GFP positive. In our previous

work (Rosochacki et al., 2001), 5 mosaic embryos were obtained. These comprised 25-75% *gfp* incorporation. This could be caused by late integration of transgene during embryonic preimplantation development (Wall and Seidel, 1992).

Mosaicism in transgenic animals produced by pronuclei microinjection was first recognised when offspring derived from transgenic founder mice were found to be nontransgenic (Palmiter et al., 1984). In transgenic mosaic animals, not all the body cells contain the transgene, as DNA integration into the genome occurs randomly and segregation of the transgene into cells is mosaic in most cases. It can be seen in uneven distribution and expression of transgene in different tissues or by the failure to transmit the transgene to offspring (Palmiter et al., 1984; Evans et al., 1994). Also, because of the existence of episomal transgenes, not all of those embryos expressing the reporter gene are transgenic (Muramatsu and Nakamura, 1997). Wall (1997) also points out that because plasmids may remain unincorporated into the genome and can segregate during cell division (producing mosaic embryos), that the ideal *gfp* reporter would somehow signal its incorporation into the genome. Of various strategies that have been used to identify transgenic/mosaic embryos prior to transfer to the recipients (Bowen et al., 1994; Ikawa et al., 1995; Chan et al., 1999) none preclude the identification of false positives due to the persistence of non-integrated microinjected DNA until the late stages of development. The loss of potential transgenic offspring may be due to the false negatives in transgenic mosaicism. This is a very important problem to the application of transgenic technology in livestock (Wall, 1997). Therefore, the current procedure that simplifies the detection of transgenic embryos would be very useful in establishing transgenic lines of animals, particularly cows. GFP would be useful as a marker of gene expression, particularly in embryogenesis, since the observation of live embryo is a direct monitoring of gene expression. Reporter *gfp* together with structural gene of interest and proper promoter could be a promising to get large bioreactors.

CONCLUSIONS

Our results support the idea that the use of *gfp* gene reporter to select transgenic bovine embryos could be a useful method to increase the efficiency of production of transgenic offspring by ensuring that only transgenic embryos are transferred to recipient animals. The other, not 100% transgenic embryos might be served as a donors of transgenic cells after disaggregation, which can also be used through cloning procedure to get more 100% transgenic embryos and transfer them also to the recipients to produce only transgenic animals.

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STRESZCZENIE

Wykorzystanie GFP do selekcji zarodków bydlęcych

Celem badań było: 1. zbadanie wpływu mikroiniekcji genu reporterowego *gfp* na rozwój zarodków bydlęcych, 2. identyfikacja i selekcja GFP pozytywnych zarodków, oraz 3. określenie odsetka zarodków mozaikowych. Oocyty izolowano z jajników od ubitych w rzeźni krów. Dojrzewanie i zapłodnienie prowadzono w warunkach *in vitro*. Do uzyskanych zygot wprowadzano gen pCX-EGFP, złożony z enhancera CMV-IE, promotora beta-aktyny kury, cDNA białka GFP i sygnału poliadenylacji pochodzącego z beta-globiny królika. Zarodki z grupy kontrolnej (64) i grupy doświadczalnej (198) były hodowane *in vitro*. Po 168 godzinnej hodowli, w grupie kontrolnej uzyskano 39,06% zarodków w stadium moruli i blastocysty, natomiast w grupie doświadczalnej 23,23%. Uzyskano 3 zarodki GFP pozytywne (1,51% w stosunku do zygot po mikroiniekcji oraz 6,52% w stosunku do morul i blastocyst). Jeden z zarodków był w 100, jeden w 75 i jeden w 25% GFP pozytywny; mozaicyzm wynosił 66,7%. Wykorzystanie genu reporterowego *gfp* pozwala na wczesną selekcję zarodków bydlęcych i przeniesienie tylko transgenicznych zarodków do biorczyń, co ma ogromne znaczenie w uzyskaniu w pełni transgenicznego potomstwa i znacznego obniżenia kosztów transgenezy.