EARLY SCREENING OF GENE EXPRESSION OF SV40 DRIVEN LACZ INTRODUCED INTO BOVINE EMBRYOS

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Summary

The present study was conducted to assess gene expression of bacterial lacZ driven by the SV40 promoter at early developmental stages of bovine embryos. The lacZ gene was linearized with BamHI digestion, and introduced into the pronucleus by microinjection at 20 hrs after the commencement of *in vitro* fertilization. Intact bovine blastocysts were not stained with X-Gal, suggesting that there is no endogenous beta-galactosidase activity in these blastocysts. In contrast, the bovine blastocyst cells microinjected with the lac*Z* gene exerted a characteristic greenish-blue color originating from the bacterial beta-galactosidase activity, albeit at a low rate, i.e. 2.1% of the total fertilized oocytes injected. It was concluded, therefore, that the lac*Z* gene driven by the SV40 promoter could be used for an indirect screening method in which the presence of transgene is evaluated from the product of transgene expression.

(**Key Words** : Gene Expression, SV40 Promoter, Lac*Z*, Bovine Embryo, Microinjection)

Introduction

Recent advancement of the nonsurgical techniques associated with *in vitro* fertilization (IVF) and culture of bovine oocytes allows the supply of practically unlimited number of embryos available for embryo transfer (Goto et al., 1988; Eyestone et al., 1991). Based on this technology, transgenic cattle have been produced by microinjecting a foreign gene into the pronucleus of zygotes, followed by transferring the injected embryos to recipient cattle (Roschlau et al., 1989; Krimpenfort et al., 1991; Hyttinen et al., 1994). Generally speaking, however, the production of transgenic farm animals is prohibitively expensive due to the long gestation period, small number of offspring per gestation and high maintenance costs including the need for a large number of recipient animals.

Besides these, a very low transgenesis rate is one of the major obstacles to be overcome. The reported efficiency of producing transgenic cattle was impracticably low. Krimpenfort et al. (1991) reported, for example, that the production rate of cattle expressing transgenes was only at 0.2% of microinjected oocytes. This problem could be solved by developing a reliable screening method for identifying embryos that carry integrated transgenes prior to the embryo transfer. In this connection, several attempts have been made, and reported in the literature.

One of the possible methods is to detect directly injected transgenes. The use of direct PCR with transgene-targeted primers for embryo selection has shown to yield a high number of false positives (Bowen et al., 1993; Horvat et al., 1993). A better selection method has been reported by using methylated gene constructs for microinjections with adequate restriction enzyme digestion prior to the PCR analysis (Jänne et al., 1992). However, this method has been questioned by the observation that changes in DNA methylation patterns are independent of transgene integration in mouse embryos (Burdon and Wall, 1992).

Another possible approach might be the detection of marker proteins originated from a reporter gene microinjected together with transgenes of interest. This does not require a biopsy procedure which might decrease the survival rate of embryos transferable to recipient cattle. However, the indirect protein detection method may also confer false positive signals unless enough time is allowed to minimize transient gene expression originating from episomally existing microinjected genes.

In the present study, an attempt was made to establish basic conditions of the indirect screening method whether

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or not the product of transgene expresion i.e. proteins, could be detected at early developmental stages of bovine oocytes.

Materials and Methods

In vitro maturation and fertilization of ovarian oocytes

In vitro maturation and IVF was done basically according to the method of Goto et al. (1994) with slight modifications. Cumulus-oocyte complexes were aspirated from small (1-7 mm in diameter) antral follicles in bovine ovaries obtained from a local slaughterhouse. They were washed twice with phosphate-buffered saline (Whittingham, 1971) supplemented with 0.3% (w/v) bovine serum albumin (Gibco, New York), followed by washing three times with maturation medium. The maturation medium was Hepes buffered TCM-199 (25 mM) (Gibco) supplemented with 10% (v/v) heat-treated calf serum, streptomycin (100 μg/ml) (Cosmobio, Tokyo), and penicillin (100 units/ml) (Cosmobio, Tokyo). The oocytes with an intact, non-expanded cumulus mass and evenly granulated cytoplasm were then cultured for 24 hrs.

IVF was performed using frozen-thawed semen which had been diluted with BO medium (Brackett and Oliphant, 1975) without bovine serum albumin but with caffeine (10 mM) and heparin (200 μg/ml) (Sigma, St. Louis). The spermatozoa were washed with this medium and centrifuged at 500 × g for 5 min. This washing procedure was repeated twice. The spermatozoa were then resuspended to a concentration of 12.5×10^6 cells/ml, and were preincubated at 39℃ for 2 hrs in the BO medium supplemented with 5 mM caffeine, 100 μg/ml heparin, and 10 mg/ml bovine serum albumin (Sigma, St. Louis). IVF was conducted by co-incubating spermatozoa (12.5×10^6 cells/ml) and cumulus-oocyte complexes (15-20 per 100 μl drop) at 39℃ for 6 hrs.

In vitro culture of one-cell embryos and microinjection

After the 6-hr IVF, the cumulus-oocyte complexes were washed three times with the maturation medium. Then, the development culture of the cumulus-oocyte complexes was done by using the fresh maturation medium. At 16 hrs after the commencement of IVF, one-cell embryos were suspended in the TCM-199 medium supplemented with 0.1% (w/v) hyaluronidase for 10 min, and were stripped of cumulus cells by pipetting. The embryos were then washed three times with the maturation medium to remove the remaining hyaluronidase.

Denuded fertilized oocytes were centrifuged at 11,000 × g at 30℃ for 10 min to visualize pronuclei, and microinjected with a transgene as reported previously (Gordon et al., 1980; Brinster et al., 1981, 1985). The transgene used was the bacterial lacZ reporter gene fused to the simian virus 40 early promoter and enhancer (pSVGal) (Promega, Madison, U.S.A.), which had been linearized by BamHI digestion, purified, diluted with the TE buffer (1 mM Tris and 25 μM EDTA, pH 7.5) to give the final concentration of 3.6 ng/μl, and filtered through a 0.20 μm filter. Zygotes were microinjected with 1 to 3 pl of the DNA solution (500 to 1,500 copies of the transgene) at approximately 18-22 hrs after the commencement of IVF.

After microinjection, the embryos were co-cultured to the blastocyst stage with the cumulus cells that had been prepared prior to *in vitro* maturation. The medium for embryo development was the same as that for *in vitro* maturation medium. Expression of the lacZ gene was detected by histochemical X-Gal staining for bacterial beta-galactosidase (Sambrook et al., 1989) after removing zona pellucida and the cumulus cells by digesting with 0.2% (w/v) pronase at 39℃ for 5 min.

Statistical analysis

The data were treated statistically by the χ^2 test using a 2 × 6 contingency table to assess the effect of centrifugation (Snedecor and Cochran, 1980).

Results

The number of bovine embryos reaching different developmental stages (from the 2-cell to blastocyst) with or without the centrifugation treatment is given in table 1. The results indicated that the embryos developed to blastocysts at about 10 to 11% of the total irrespective of the centrifugation treatment. The development of bovine embryos was not significantly affected by the centrifugation treatment according to the χ^2 test.

Intact bovine blastocysts were not stained with X-Gal, except for a few cumulus cells (figure 1), suggesting that these blastocysts did not have endogenous beta-galactosidase activity. The bovine oocytes expressing bacterial beta-galactosidase by X-Gal staining are presented in figure 2. A characteristic greenish-blue color due to beta-galactosidase activity was seen in some embryos microinjected. The number of embryos expressing beta-galactosidase activity at each developmental stage is given in table 2. Five 1-cell stage oocytes, two 2-cell stage oocytes and one blastocyst out of 48 fertilized oocytes were stained greenish-blue with X-Gal, showing the

expression of the transgene. The overall efficiency for obtaining the beta-galactosidase-positive blastocysts was at 2.1% (1/48).

TABLE 1. EFFECT OF CENTRIFUGATION OF THE DEVELOPMENT OF BOVINE ZYGOTES

Developmental stage	Centrifugation treatment[1]			
	−		+	
	No.	%[2]	No.	%[2]
2-Cell	262	−	38	−
4-Cell	203	77.5	28	73.7
8-Cell	169	64.5	28	73.7
16-Cell	138	52.7	24	63.2
Compact morula	105	40.1	18	47.4
Blastocyst	30	11.5	4	10.5

[1] Centriluged at 11,000 x g at room temperature for 10 min.
[2] Proportion of embryos that have successfully completed each stage to the initial number of fertilized oocytes.

There was no significant effect of the centrifugation treatment on the proportion of embryos according to the χ^2 test ($\chi^2 = 1.10$, df = 5, $P > 0.1$).

TABLE 2. EXPRESSION OF BACTERIAL LacZ GENE DURING THE DEVELOPMENT OF OOCYTES FERTILIZED *IN VITRO* AFTER MICROINJECTION

Developmental stage	Total No.	%[1]	No. of expression[2]
Injected oocytes	48	−	−
1-Cell	48	100	5
2-Cell	11	22.9	2
4-Cell	10	20.8	0
8-Cell	5	10.4	0
16-Cell	3	6.3	0
Compact morula	2	4.2	0
Blastocyst	2	4.2	1

[1] Proportion of embryos that have successfully completed each stage to the initial number of fertilized oocytes.
[2] The number of bovine fertilized oocytes stained greenish-blue with X-Gal.

Discussion

The development of a good screening method for the presence of the transgene and its integration into the embryonic genome is of the uppermost importance to reduce the production cost of transgenic farm animals. In the literature, there have been several reported methods mainly based on the amplification of transgenes by means of PCR with some modifications. So far, however, all of these have suffered from the shortcoming that they tend to confer a large number of false positives (Burdon and Wall, 1992; Janne et al., 1992; Bowen et al., 1993; Horvat et al., 1993). Alternative quick and reliable methodology is definitely needed.

In the present study, an attempt was made to establish basic conditions of the indirect screeening method whether or not the product of transgene expression, i.e. proteins, could be detected at early developmental stages of bovine oocytes. Bacterial beta-galactosidase was chosen in the present study as a marker protein to be dected. To activate

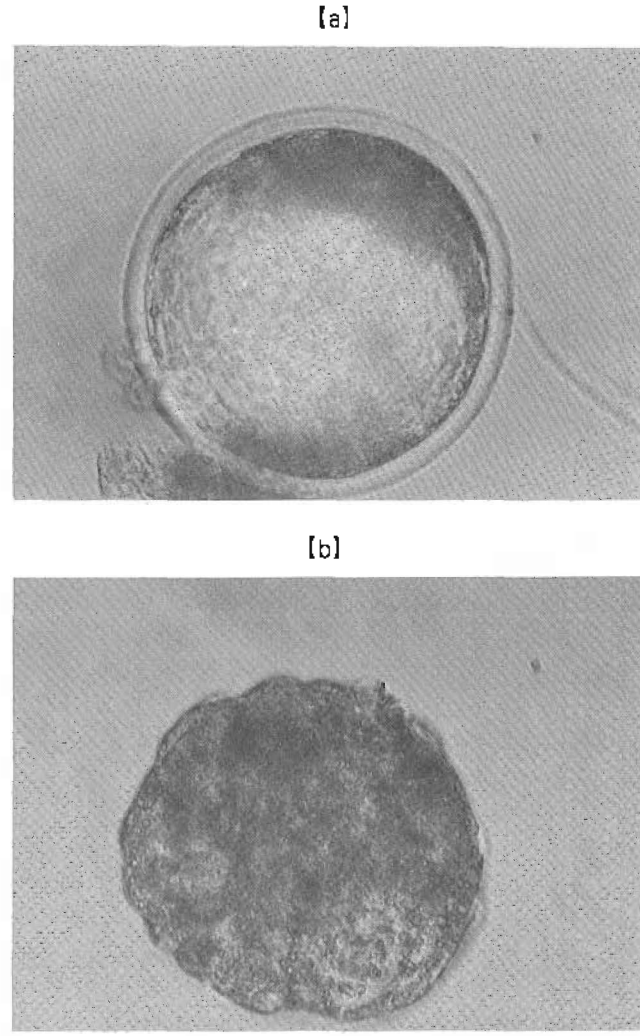

Figure 1. X-Gal staining of intact bovine blastocysts with (a) or without (b) cumulus cells and zona pellucida which was removed by digesting with 0.2% pronase (x 400). No greenish-blue color characteristic to bacterial beta-galactosidase was detected in the control blastocysts except for the cumulus cells.

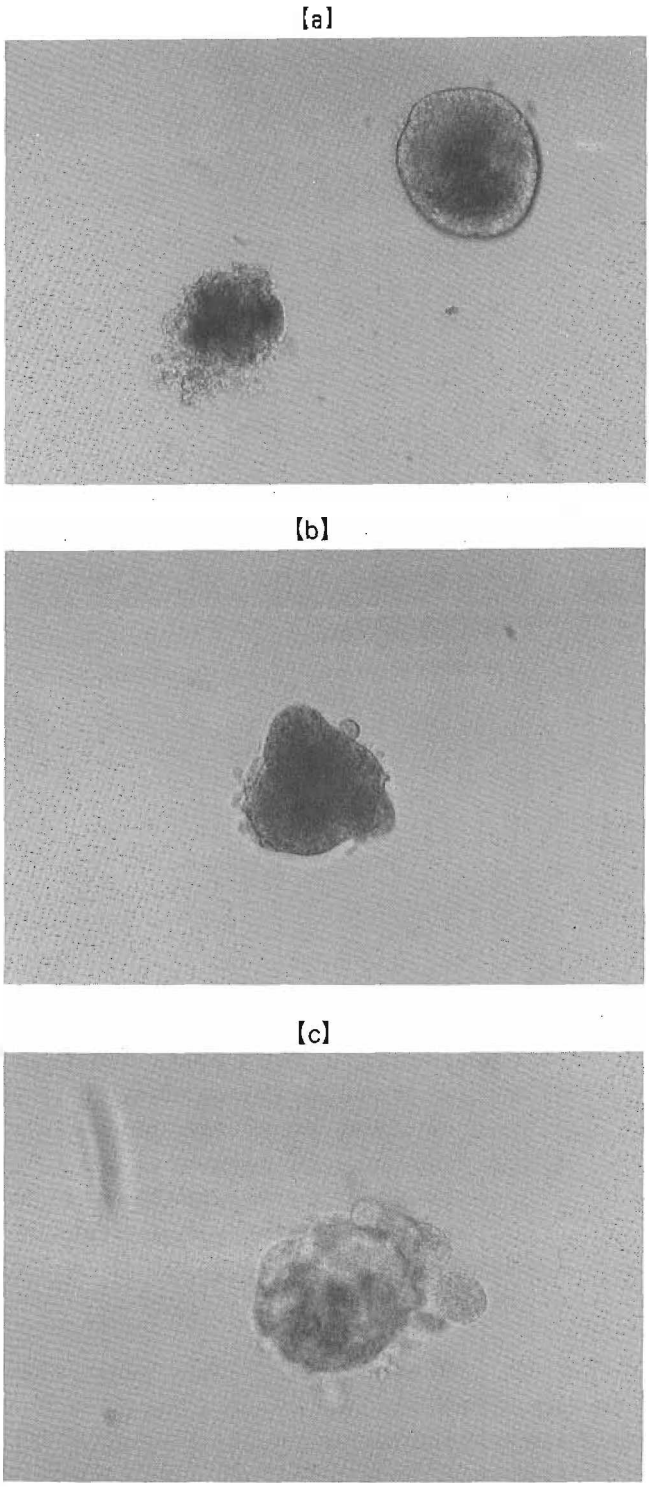

Figure 2. X-Gal staining of bovine embryos at 1-cell (a, X 200), 2-cell (b, X 200) and blastocyst (c, X 300) stages after microinjecting pSVGal into the pronucleus of *in vitro* fertilized bovine oocytes at the 1-cell stage. A 1-cell embryo which did not express beta-galactosidase is also shown on the right in (a).

the galactosidase gene expression in bovine embryos, the SV40 early promoter and enhancer were used, because it was found that in mouse preimplantation embryos the gene expression of bacterial beta-galactosidase was driven by the SV40 promoter (Takeda and Toyoda, 1991). As demonstrated clearly, albeit at a low gene expression rate, some embryos exhibited the greenish-blue color characteristic to bacterial beta-galactosidase by X-Gal staining in the present study. In contrast, no characteristic greenish-blue color was detected in intact control bovine oocytes. Accordingly, the lacZ gene fused to the SV40 promoter could be used for identifying the presence of the product of reporter gene expression, hence the reporter gene itself, in bovine zygotes at 2-cell to blastocyst stages. Thus, the method described here in the present study may at least be useful to optimize conditions of DNA microinjection at the early developmental stages to raise transgenic cattle with good yield.

Obviously, however, the indirect protein detection method may also confer false positive signals if a significant portion of the gene expression is transient, resulting from episomally existing microinjected genes. It was implied from the PCR study that cattle may not degrade DNA as readily as mice (Krisher et al., 1994). If this were true, a double check would have to be made to eliminate the false positive signals as much as possible by using the combination of direct PCR and indirect protein detection methods, for example.

It was found previously that the centrifugation treatment had no detectable adverse influence on survival rates of the cattle zygote (Wall and Hawk, 1988), which is in good agreement with the present result. In the present study, however, the efficiency of embryos successfully differentiated to blastocysts was low compared with those reported in the literature (Eyestone et al., 1991; Hamano and Kuwayama, 1992) in spite of the fact that the overall production efficiency of transgene expression, 2.1% (1/48) was comparable to that reported in the literature (Gagne et al., 1993). Therefore, the method for culturing IVF embryos should be improved. For this purpose, inclusion of insulin in the maturation and development medium might elevate the overall efficiency of bovine embryo development because the hormone stimulates the proliferation of cultured cumulus cells (Hoshi et al., 1991). In addition, the time of microinjection could also be an important factor affecting the embryo development efficiency. Krisher et al. (1994) demonstrated that microinjection at 11 hrs after the commencement of IVF resulted in highe survival rates of bovine embryos than did the microinjection at 19 hrs, which was close to the present condition, i.e. at about 18-22 hrs.

Because the detection of beta-galactosidase expression by X-Gal staining requires fixation of cells, the microinjected bovine embryo is no longer usable for embryo transfer to recipient cattle even if the presence and expression of the reporter gene has been confirmed. Therefore, the development of live detection of transgenic embryos is necessary. Lin et al. (1994) have demonstrated that this is possible in embryos of transgenic zebrafish expressing the bacterial lac Z gene. Further study is now in progress to establish a better method in which the detection of transgenes could be done in living bovine embryos.

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