Expression of two transgenes in in vitro matured and fertilized bovine zygotes after DNA microinjection

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Bovine zygotes produced by in vitro maturation—in vitro fertilization (IVM—IVF) were examined for their potential to serve as recipients of transgeness. Pronuclei, which were maximally visible at about 22 h after IVF, were injected with a SV40—LacZ construct (pSVON). Injected zygotes had lower cleavage rates (49.1%, n = 1162, P < 0.01) than did either noninjected controls (87.4%, n = 1420) or noninjected zygotes in which pronuclei were not visible (67.6%, n = 803). Zygotes that were injected into their pronuclei cleaved as well as zygotes injected cytoplasmically. At 48 h after injection, when most embryos had reached the four- and eight-cell stages, more zygotes in the pronuclear group (22.7%, n = 125) stained positively for LacZ than did zygotes in the cytoplasmic group (8.0%, n = 125). A group of zygotes injected into the pronucleus with pSVON was cultured for 9 days. More morulae (10.8%, n = 134) than blastocysts (3.2%, n = 31) expressed the LacZ gene, indicating that silencing of expression occurred as development progressed. Another group of zygotes was injected with a β-actin—LacZ gene construct (pβActinLacZ) and, of the embryos assayed at 48 h, 10.6% (n = 255) stained positively. At 9 days, 36.3% of morulae (n = 91) and 21% of blastocysts (n = 33) expressed the transgene. Almost all putative transgenic embryos injected with either construct showed a mosaic pattern of LacZ expression, with an average of only 2–3 cells staining at the eight-cell stage and the majority of cells in positive blastocysts showing no evidence of expression.

Introduction

Generation of bovine embryos in vitro from oocytes retrieved from the ovaries of slaughtered cows is now a standard procedure. Such embryos remain developmentally competent and can undergo normal development after introduction into recipient females (Leibfried-Rutledge et al., 1989). The potential to produce large numbers of embryos by such in vitro maturation—in vitro fertilization (IVM—IVF) procedures is likely to become increasingly useful for the generation of transgenic livestock (Thomas et al., 1993). At present, gene transfer in farm animals is an inefficient process requiring access to a large pool of one-cell zygotes. It has been demonstrated that bovine zygotes generated in vitro can be used for this, although the overall efficiency of generating transgenic cattle is markedly lower than that routinely reported in mice (Krümpenfort et al., 1991).

Transgenes are often found in their hosts as multiple copies inserted into a single site (Palmiter and Brinster, 1986). It is generally accepted that transgenic animals originate from a single integration event before or concurrent with the first cellular division, although Burdon and Wall (1992) suggested that DNA integration may occur later and in more than one site. Reports from bovine embryos derived in vitro, which were probed for the presence of transgenes with the polymerase chain reaction (PCR), suggest that transgene expression rates can be as high as 38% at the blastocyst stage (Behboodi et al., 1993), although other estimates are more conservative (Thomas et al., 1993). However, there is evidence to suggest that such positives could be false and due to artefacts generated by the PCR system of analysis (Krümpenfort et al., 1994). Furthermore, the detection of a transgene by PCR gives no indication of whether the gene is expressed or even integrated into chromosomal DNA.

To study the expression of microinjected genes in preimplantation embryos, it is necessary to link the reporter gene to a promoter the activity of which is independent of developmental regulation. The SV40 early promoter has been widely used in cell transfection experiments (e.g. Mulligan and Berg, 1980) and is functional in mouse embryos as early as the two- and four-cell stage (Bonnerot et al., 1987; Takeda and Toyoda, 1991). Another useful promoter for such a purpose is that of the cytoplasmic β actin gene, which becomes activated during early embryogenesis (Hayward and Schwartz, 1986). Expression of reporter genes driven by the rat cytoplasmic β actin promoter was reported in microinjected mouse two-cell embryos (Bonnerot et al., 1987) and midgestation embryos (Beddington et al., 1989) and from the chicken promoter at various preimplantation stages (Sands et al., 1993).

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In this study, some of the factors that can affect the success of gene transfer in cattle were examined. The β-galactosidase (LacZ) gene of E. coli was used as a reporter gene, regulated by either the early SV40 (pSVON) or β-actin promoter (pbActin-LacZ) discussed above, and injected into either the pronuclei or the cytoplasm of zygotes to determine the subsequent pattern of transgene expression.

Materials and Methods

Experimental design

In the first experiment, zygotes were examined at various times after insemination to determine when pronuclei were maximally visible. The second experiment was designed to assess the effects of pronuclear injection on subsequent development. In the third experiment, the effects of pronuclear injection or cytoplasmic injection at the one- or two-cell stage on the development of the embryo, and of the expression of the pSVON construct were determined. In the final experiment, the effects of pronuclear injection of an alternative gene (pbActin-LacZ) were examined.

Collection and fertilization of oocytes

Bovine ovaries were collected at an abattoir and transported to the laboratory at 30°C in PBS. Ovarian follicles 2–8 mm in diameter were aspirated with a negative pressure of 2.5 psi. The follicular fluid was pipetted through a 100 μm cell strainer (Becton Dickinson Labware, Franklin Lakes, New York), and the cumulus-oocyte complexes were washed out of the strainer into a Petri dish with TALP-Hepes (Parish et al., 1986). Only oocytes with homogeneous cytoplasm and three or more layers of granulosa cells were selected. Oocytes were matured and fertilized as described by Hernandez-Ledezma et al. (1992). Frozen semen from a single bull was used for all experiments.

Assessment of pronuclear visibility

Pronuclear visibility was assessed by examining different groups of approximately 50 embryos every hour, starting 15 h after introduction of spermatozoa to the fertilization medium. After centrifugation at 13 000 g for 10 min, the presence of pronuclei was visually confirmed under an inverted microscope (Nikon) with Hoffman optics. Subsequently, zygotes with and without pronuclei were cultured separately.

Preparation of constructs for microinjection

The construct, containing 417 bp of the SV40 early promoter (Stacey and Schnieke, 1990) and the LacZ gene from E. coli, was excised from the plasmid pSVON (a gift of R. A. Bowen, Colorado State University) by digestion with BamHI. The fragment was subsequently purified from a 0.8% (w/v) agarose gel by using the Qiaex DNA purification kit (Qiagen Inc., Chatsworth, CA). The pbActinLacZ construct (a gift of J. Cross, University of California, San Francisco), containing a 4.3 kb fragment of the human cytoplasmic β actin promoter, was purified after EcoRI/HindIII digestion of the plasmid. The gene constructs were resuspended in 7.5 mmol Tris–HCl 1–1 (pH 7.4) and 0.15 mmol disodium EDTA 1–2 (Sigma, St Louis, MO) at a concentration of 2.5 μg ml–1.

Microinjection of DNA

After co-culture of gametes, the zygotes were removed, washed in TALP-Hepes and placed in the same buffer containing 300 μg hyaluronidase ml–1 (Sigma) at 37°C for 10 min. Cumulus cells were removed by vortexing for 2 min and the zygotes were transferred to an Eppendorf tube and centrifuged for 10 min at 13 000 g to displace lipids (Loskutoff et al., 1986). Microinjection was performed in a drop of TALP-Hepes under paraffin oil (Sigma). The DNA construct (2–5 pl) was injected into each embryo as described by Hogan et al. (1986). Embryos to be injected cytoplasmically at the one-cell stage were subjected to the same procedures as those injected into the pronucleus. Embryos to be injected cytoplasmically at the two-cell stage were cultured in CZB medium (Chatot et al., 1989) and then treated in the same manner. Centrifugation of embryos (13 000 g for 10 min) to be injected cytoplasmically was performed to facilitate pronuclear visualization and prevent accidental pronuclear injection. In contrast, IVF controls were placed, without further manipulations, into CZB medium directly after fertilization.

Culture of embryos

Embryos were cultured for the first 48 h at 39°C in 5% CO2 in glucose-free CZB medium (Chatot et al., 1989), which had been conditioned by Buffalo rat liver cells (BRL 3A) for 36 h (Hernandez-Ledezma et al., 1993). After 48 h in CZB, embryos were examined to assess the development rate, and eight-cell embryos were selected and placed in TCM 199 containing 10% (v/v) fetal bovine serum and 0.25 mmol sodium pyruvate l–1. This medium had also been conditioned for 36 h by BRL cells.

Staining for LacZ activity

Regardless of developmental stage, embryos were stained at 32 h (two-cell injections), 48 h or 9 days after microinjection. Embryos to be stained were washed in PBS, pH 7.3, and fixed for 5 min in 2% paraformaldehyde/0.2% glutaraldehyde (w/v) in 0.1 mol Na phosphate buffer l–1, pH 7.3 at 4°C. They were washed a further three times in PBS and stained in 0.1 mol sodium phosphate buffer l–1, pH 7.3, containing 1 mg 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside ml–1 (X-Gal; Sigma) at 37°C in air for 24 h (Dannenberg and Suga, 1981).

Statistical analyses

Data were analysed by chi-squared tests and multiple regression coefficient analyses (Systat Inc., 1992).
Results

Pronuclear development

Pronuclear visibility was assessed over time in a total of 770 zygotes, consisting of eight replicates. While pronuclei could be found in only 7% of embryos examined 15 h after the addition of spermatozoa to the oocytes, pronuclear visibility increased to 79% after 22 h, and began to decline to 35% at 26 h. Maximal visibility ranged from 63% to 91%, depending on the day of collection.

Development of injected, nonpronuclear and noninjected zygotes

The development of injected versus noninjected embryos was assessed by injecting a total of 1162 zygotes, representing 17 replicates, with pSVON between 19 and 22 h after IVF, with an additional 1920 zygotes serving as noninjected controls. A further group of 803 zygotes was cultured in which pronuclei had not become visible during the period of microinjection. The cleavage was highest in the control group, in which 88% underwent division (1685 of 1920). In contrast, only 49% of microinjected embryos divided (570 of 1162), while cleavage of embryos in which pronuclei had not been visible was intermediate (67%; 543 of 803). The developmental stage reached at 48 h after microinjection, by those embryos that divided, was significantly ($P < 0.01$) affected by treatment (Fig. 1).

Development of zygotes injected into the pronucleus or the cytoplasm

The comparative rates of development of embryos that had been microinjected with pSVON into either their cytoplasm or their pronuclei are shown (Fig. 2). Of the 365 embryos subjected to pronuclear injection, 77.5% (276 of 356) cleaved, while, of the 125 embryos injected intracytoplasmically, a total of 74.4% cleaved. The development rates between the two groups did not differ significantly ($P > 0.1$). Control embryos in this experiment had cleavage rates of 93.9% (387 of 412) with 73.5%, 13.1%, 7.3% and 6.1% reaching the eight-, four-, two- and one-cell stage, respectively.

Transgene expression in embryos injected into the pronucleus or the cytoplasm with the pSVON construct

Zygotes injected into either the pronucleus or the cytoplasm were fixed and stained for pSVON transgene activity after 48 h in culture. Of the embryos injected into the pronucleus, 22.7% (81 of 356) showed evidence of transgene expression (Table 1). A small number (5.0%) of one-cell embryos expressed $\beta$-galactosidase (LacZ) after 48 h in culture. However, the percentage of positive embryos was highest at the four- and eight-cell stages. A significantly smaller percentage (8.0%, $P < 0.01$) of the embryos that had been injected cytoplasmically at the zygote stage expressed the transgene at 48 h (Table 1). The numbers of positives were too small to assess whether expression changed as development proceeded.

In a parallel study, a group of 133 two-cell embryos was injected cytoplasmically into one blastomere with pSVON to evaluate whether delayed DNA injection influenced transgene expression. These embryos were stained 32 h after microinjection. The total percentage of positives was low (6.5%) and staining was always restricted to a single blastomere, regardless of the developmental stage of the embryo (data not shown).

A group of 438 four- and eight-cell embryos was generated after pronuclear injection to determine whether gene expression changed as development proceeded. This group was maintained in culture for a total of 9 days after microinjection,
Table 1. Number of bovine embryos showing evidence of transgene activity after 48 h (one-cell to eight-cell stage) or 216 h (morula/blastocyst stage) after microinjection of the pSVON construct

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Number of embryos (positive/total)*</th>
<th>Number of embryos (positive/total)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pronuclear injection (%)</td>
<td>Cytoplasmic injection one-cell (%)</td>
</tr>
<tr>
<td>One-cell</td>
<td>4/80 (5)</td>
<td>0/32 (0)</td>
</tr>
<tr>
<td>Two-cell</td>
<td>6/35 (11)</td>
<td>2/18 (11)</td>
</tr>
<tr>
<td>Four-cell</td>
<td>28/82 (34)</td>
<td>4/30 (13)</td>
</tr>
<tr>
<td>Eight-cell</td>
<td>43/139 (31)</td>
<td>4/45 (9)</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>1/31 (3)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d. = not determined.
*Numbers of embryos represent 22, 5 and 1 replicates for the three groups, respectively.
**Two-cell embryos were injected 36 h after insemination and stained 32 h later.

Table 2. Number of embryos showing mosaicism after pronuclear injection with either the pSVON or pbActinLacZ construct

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Two-cell</th>
<th>Four-cell</th>
<th>Eight-cell</th>
<th>Morula</th>
<th>Blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSVON</td>
<td>Number positive</td>
<td>6</td>
<td>28</td>
<td>43</td>
<td>25</td>
</tr>
<tr>
<td>Number of mosaics (%)</td>
<td>4 (67)</td>
<td>26 (93)</td>
<td>41 (96)</td>
<td>24 (96)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Number of positive blastomeres per embryo</td>
<td>1.2</td>
<td>1.9</td>
<td>1.8</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>pbActinLacZ</td>
<td>Number positive</td>
<td>2</td>
<td>2</td>
<td>23</td>
<td>33</td>
</tr>
<tr>
<td>Number of mosaics (%)</td>
<td>2 (100)</td>
<td>2 (100)</td>
<td>20 (87)</td>
<td>28 (85)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Number of positive blastomeres per embryo</td>
<td>1.0</td>
<td>1.0</td>
<td>2.9</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Embryos were stained for LacZ expression at either 48 h (assessment of two-, four- and eight-cell stages) or at 216 h (assessment of morulae and blastocysts) after microinjection.

during which time 232 embryos reached the morula and 31 the blastocyst stage (43.1% and 5.8% of cleaving embryos, respectively). Evidence of transgene activity was displayed in 10.8% of the morulae and 3.2% of the blastocysts (Table 1). The percentage of embryos expressing the pSVON construct declined significantly (P < 0.01) from the eight-cell stage to the morula and blastocyst stages.

The majority of stained embryos proved to be expression mosaics (Table 2). At the four-cell stage and beyond, over 90% of all positive embryos demonstrated mosaicism.

Transgene expression in embryos injected with the pbActinLacZ construct

Because the pSVON construct showed evidence of silencing as embryonic development progressed, 379 embryos, representing replicates, were stained for transgene expression after pronuclear microinjection with the pbActinLacZ construct. After 48 h, no LacZ activity could be detected among 47 one-cell embryos, while only 4.8% of two-cell (2 of 42) and 4.5% of four-cell (2 of 44) embryos showed signs of transgene activity (Fig. 3). The proportion of LacZ-expressing embryos increased to 18.9% (23 of 122) as they reached the eight-cell stage. Of those embryos that progressed to the morula and blastocyst stages after culture for a further 168 h (37.9% and 9.6% of cleaving zygotes, respectively), 36.3% (33 of 91) and 21.2% (7 of 33), respectively, showed signs of LacZ activity. The last two values did not differ significantly.

As in the case of the pSVON construct, the majority of embryos injected with pbActinLacZ proved to be expression mosaics at all stages examined, and positive blastomeres were in a minority (Table 2). No attempts were made to quantify the number of positively staining blastomeres at more advanced cell stages, where the proportion of positive cells seemed to vary considerably and where staining was often localized to patches of cells. In blastocysts, for example, a full range of mosaicism was noted and a blastocyst was only rarely entirely positive.

Discussion

Before the progression of transgene expression in developing bovine embryos was studied, a number of preliminary
experiments were conducted to establish the system in this laboratory. In the course of this work, the reports of Xu and Greve (1988) and Kay et al. (1991), that the best time to view pronuclei in bovine embryos produced by IVM–IVF is between 19 and 22 h after coculturing the gametes, were confirmed. That the microinjection procedure reduces initial cleavage of the zygote and subsequent progression to blastocyst was also verified. Essentially similar observations have been made for mouse (Gordon et al., 1980; Petters et al., 1987), pig (French et al., 1993) and ovine (Hammer et al., 1986; Wright et al., 1991), as well as bovine embryos (Hawk et al., 1989; Krömer et al., 1991). Whether the developmental failure stems from mechanical or genetic damage or a combination of both remains unclear. No differences were noted in the present study between the effects of pronuclear and cytoplasmic injections on development, a result that contrasts with a report of Peura and Jänne (1994), in which they suggested that the reduction in development arises from damage to the pronucleus. In the study reported here, the reason why cleavage rates of injected embryos were higher in the second experiment is unclear, but could relate to seasonal differences in oocyte quality or to improved technical skills of individuals performing the microinjections.

About one-third of embryos at the four- to eight-cell stage expressed the LacZ gene after pronuclear injection of pSVON. Clearly the efficiency of producing cleavage stage embryos that express a microinjected gene is considerably higher than the percentage of transgenic calves that are born (Krimpenfort et al., 1991; Hill et al., 1992; Bowen et al., 1994). Conversely, the number of embryos expressing the two gene constructs in our study was lower than the number of putative transgenics that others have estimated using PCR procedures (Behboodi et al., 1993; Bowen et al., 1994). However, it is likely that PCR analysis leads to an overestimation because, as here, it cannot distinguish between integrated and nonintegrated DNA. In addition, values obtained by PCR will include those embryos not expressing the transgene.

As observed for mouse (Brinster et al., 1985), pig (Hammer et al., 1986) and bovine embryos (Lemme et al., 1994), cytoplasmic injection produced fewer positively staining embryos than did pronuclear injection, possibly because DNA introduced into the cytoplasm was less well placed to encounter the transcriptional machinery of the cell. The bovine embryonic genome is not activated until after the four-cell stage, when newly synthesized RNA and proteins can first be detected (Fusi et al., 1989; Barnes and First, 1991). It was surprising, therefore, that pSVON was expressed in one- and two-cell as well as in four- and eight-cell embryos after only 48 h in culture. This observation implies that the onset of transcriptional activity in early embryos may be determined more by the time after fertilization than by the number of cleavage divisions that have occurred. The observation that embryos (n = 148) failed to stain 12 h after pronuclear injection, when the majority had divided only once (data not shown), is consistent with such a hypothesis.

The majority of bovine embryos injected with either of the two gene constructs proved to be mosaics. Almost 100% mosaicism has been observed after microinjection of an SV40-LacZ gene into mouse embryos, although a far higher proportion of blastomeres at the four- and eight-cell stage stain blue than was observed here in bovine embryos (Takeda and Toyoda, 1991). It is possible that such mosaicism results from regulatory events controlling transcription or translation that can silence expression selectively in some blastomeres and not in others. However, a mosaic distribution of an injected gene construct after PCR analysis of individual mouse blastomeres has been reported (Burdon and Wall, 1992). Moreover, the mosaicism in the present study was noted in embryos injected with both constructs, which had promoters that would not be expected to be under similar regulatory control. The most likely explanation for the observed mosaicism is that expression stems largely from nonintegrated DNA, which is transiently transcribed. Powell et al. (1992) showed that bovine zygotes have a high ability to generate large ligation products from cytoplasmically injected DNA. Such concatamerization might ultimately lead to circularization, making the exogenous DNA more resistant to exonucleolytic degradation and more likely to be asymmetrically distributed at cell division. Moreover, circularization could facilitate and enhance transcription from a microinjected gene (Mertz, 1982; Harland et al., 1983; Bevilacqua et al., 1992).

These experiments do not provide much insight into why production of transgenic offspring is so much less efficient in cattle than in mice. One possibility, which might provide an alternative explanation to the one above for the extreme mosaicism noted in bovine embryos, is that the timing of transgene integration depends upon when the embryonic genome is activated. Recombination rates are markedly higher in transcriptionally active DNA (Blackwell et al., 1986; Rødewohld et al., 1987; Schulz et al., 1987; Thomas and Rothstein, 1989; Nickoloff and Reynolds, 1990). Therefore, integration into the mouse genome might occur earlier than in cattle embryos. Integration at the four- or eight-cell stage, being a relatively rare event, would probably create only a single transgenic blastomere in a multicellular embryo and a patchy distribution of expression at later developmental stages. The presence of only a minority of genetically modified
blastomeres by the late morula stage would seriously reduce the likelihood of such 'positive' cells being partitioned to the inner cell mass. Moreover, any young produced would probably be mosaics, with most tissues showing no evidence of the transgene. It is unlikely that the efficiency of producing transgenic farm animals by pronuclear injection can be improved without a better understanding of the events leading to integration. Therefore, a method is required to distinguish between nonintegrated and integrated DNA in preimplantation embryos. Cousens et al. (1994) have described a PCR procedure to distinguish DNA methylation patterns of integrated from nonintegrated DNA in mouse embryos. However, the reliability of such an approach is uncertain, as there are indications that injected DNA undergoes similar changes in methylation regardless of whether it is integrated (Burdon and Wall. 1992). An alternative procedure may be fluorescent in situ hybridization, which has been used for the location of individual genes on human chromosomes (Lawrence et al., 1990; Johnson et al., 1993) and to determine the status of integration of Epstein Barr virus in B cells (Hurley et al., 1991).

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