Expression of RNA from developmentally important genes in preimplantation bovine embryos produced in TCM supplemented with BSA

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This study investigated the effects of a semi-defined culture system on the temporal pattern of expression of RNA from genes involved in compaction and cavitation: gap junction protein connexin43 (Cx43), desmosomal glycoproteins desmoglein I (Dg I), desmocollins I, II and III (Dc I, Dc II, Dc III), desmosomal protein plakophilin (Plako); metabolism glucosetransporter-1 (Glut-1); RNA processing poly(A)polymerase (PolyA); heat shock protein 70.1 (HSP); and trophoblastic function trophoblast protein (TP) in bovine oocytes and embryos generated in vitro using TCM199 supplemented with BSA as the culture medium. Morulae and blastocysts derived in vivo were collected from superovulated heifers and also used for this study. Poly(A)+ RNA was extracted from pools of 20–50 oocytes or embryos, analysed by reverse transcription–polymerase chain reaction and the amplified fragments were verified by sequencing. Assays were repeated at least three times for each developmental stage and provided consistent results in all replicates. In bovine embryos produced in vitro, mRNA encoding Cx43 was detectable up to the morula stage, whereas blastocysts and hatched blastocysts did not express this gene. No transcripts were found for Dg I and Dc I throughout the tested preimplantation stages. Dc II and Dc III transcripts were found from 2–4-cell embryos up to the hatched blastocyst stage. mRNA encoding Plako was detected in immature and mature oocytes and zygotes, while no transcripts were seen in 2–4-cell and 8–16-cell embryos. The gene was expressed again from the morulae to the hatched blastocyst stage. Oocytes and bovine embryos produced in vitro showed transcripts for Glut-1, PolyA and HSP throughout preimplantation development up to the hatched blastocyst stage. The gene encoding TP was transcribed only in blastocysts and hatched blastocysts. Morulae and blastocysts produced in vivo showed the same expression as their in vitro counterparts, with one exception: the in vivo embryos transcribed Cx43. The results of this study reveal for the first time the transcriptional pattern of a set of 'marker' genes involved in various processes in early bovine embryonic development. Transferable morulae and blastocysts produced in vitro expressed most genes similar to their in vivo counterparts. These data contribute to the molecular characterization of this widely used in vitro culture system for bovine embryos and provide a major advance towards production of 'physiologically normal' embryos.

Introduction

In vitro production (IVP) of bovine embryos is a useful tool for basic embryological studies as well as commercial applications. Blastocyst developmental rates between 30% and 40% of the matured oocytes can be obtained with optimized IVP systems (Brackett and Zuelke, 1993; Trounson et al., 1994). In general, bovine embryos are cultured in media supplemented with serum often in a coculture with somatic cells (Bavister, 1995). Serum was found to have a biphasic influence on development of bovine embryos, inhibiting the first cleavage division but enhancing blastocyst development (Pinyopummintr and Bavister, 1991, 1994; Van Langendonckt et al., 1997). Serum has been replaced by BSA to achieve standardization and to improve repeatability. These semi-defined culture conditions for IVP may overcome the variability and the lack of reproducibility of developmental rates and embryo quality (see Bavister, 1995).

Compared with embryos obtained in vivo, embryos produced in vitro display marked differences, for example, in gross morphology, ultrastructure, colour, density, number of cells, size, developmental rate, temperature sensitivity, freezability, viability and pregnancy rates after transfer (Greve et al., 1994). Wrenzycki et al. (1996) have discovered a qualitative difference
at transcription. Postimplantation events can be affected by culture conditions and are thought to be involved in the delivery of abnormally large fetuses or offspring upon transfer of bovine embryos grown in vitro (Krup and den Daas, 1997). The most prominent correlation is that the high serum content of the media is a major factor related to this phenomenon (Thompson et al., 1995).

Preimplantation embryo development is characterized by distinct morphological steps such as compaction, cavitation, and blastocoele expansion, requiring the well-orchestrated expression of genes derived from the maternal and embryonic genome (Kidder, 1992). The burst of genomic activation of embryos grown in vitro occurs at the 8–16-cell stage (Telford et al., 1990), while some activation is seen at the 2–4-cell stage (Plante et al., 1994; Hyttel et al., 1996; Viuff et al., 1996; Bilodeau-Goeseels and Schultz, 1997a). However, RNA transcription of bovine embryos generated in vitro has already been detected at the 1–2-cell stage and appears to be downregulated at the transition from the 2- to the 4-cell stage or at the 4-cell stage (Hay-Schmidt et al., 1997). Embryos have been cultured in a-amanitin (a potent inhibitor of RNA polymerase II) to determine the relative contribution of embryonic gene transcription to the overall transcripts in the early embryo. If transcription was sensitive to a-amanitin, the transcript must be of embryonic origin. However, bovine embryos did not develop after treatment with a-amanitin (Liu and Foote, 1997). Furthermore, requirements for synthesis of new proteins may also depend upon endogenous stores (Rambhatla and Latham, 1995).

Most of the currently available information on transcription of specific genes in preimplantation development stems from mice. In bovine embryo development, the majority of the information on transcriptional activity is derived from studies in which radiolabelled dUTP uptake is used (Camous et al., 1986; Kopecky et al., 1989; Plante et al., 1994; Hyttel et al., 1996; Viuff et al., 1996). However, with the improvement of IVF systems for bovine embryos (Trounson et al., 1994) and the availability of the highly sensitive reverse transcriptase-polymerase chain reaction (RT-PCR), information on the genetic activity of bovine embryos is growing and it is now feasible to characterize the transcriptional patterns of developmentally important genes (Watson et al., 1992; Harvey et al., 1995; Wrenzycki et al., 1996; Betts et al., 1997; Winger et al., 1997).

The genes analysed in this study were chosen to characterize the effects of a widely used IVF system on physiological processes involved in compaction and cavitation, glucose metabolism, RNA processing, stress and early differentiation. The gap junction protein connexin43 (Cx43) is necessary for the maintenance of compaction and, thus, for subsequent blastocyst formation in mice (Lee et al., 1987; Bevilacqua et al., 1989). Desmosomal junctions are part of the epithelial adhesion system involved in mouse blastocoel cavitation and play a role in stabilizing the trophoderm as the blastocyst expands (Fleming et al., 1991; Collins et al., 1995). Desmosomes are composed of transmembrane glycoproteins, such as desmocollins and desmogleins, that are involved in intercellular adhesion, and several cytoplasmic proteins (desmoplakins, plakoglobin, plakophilin) forming the plaque structure and mediating anchorage of cytokeratin filaments (Schwarz et al., 1991; Buxton et al., 1993; Garrod, 1993). Glucose plays an important role in embryonic metabolism. In the mouse blastocyst, most of the apparent glucose uptake is mediated by facilitative carriers, for example, glucosetransporters (Gardner and Leese, 1988; Gardner and Kaye, 1995). The poly(A) tail found at the 3'-ends of nearly all eukaryotic mRNAs is responsible for the initiation of translation and the regulation of mRNA breakdown (Jackson and Standart, 1990; Bachvarova, 1992). Synthesis of the poly(A) tail is carried out by a poly(A) polymerase (Takahagi et al., 1988; Wahle, 1991). During early development, increased temperatures, free radicals and oxygen stress have deleterious effects on embryonic viability and development (Ealy et al., 1993). Thermotolerance and resistance to protein denaturation in general is induced by a family of proteins referred to as heat shock proteins (HSPs). Maternal recognition of pregnancy is initiated by interferon-γ (IFN-γ). IFN-γ, also named TP-1, is a major secretory protein produced by the trophoblast cells of the bovine blastocyst (Hernández-Ledezma et al., 1992; Watson et al., 1992) and plays an important role in pregnancy establishment via its antiluteolytic effect (Bazer et al., 1991).

Here we report the determination of the transcriptional pattern of ten genes involved in the physiological processes mentioned above in bovine preimplantation embryos generated under semi-defined culture conditions. Morulae and blastocysts collected from superovulated cows were included in this study as in vitro controls for the transferable stages.

Materials and Methods

Production of bovine embryos in vitro

Collection of cumulus-oocyte complexes. Bovine ovaries obtained from a local abattoir were transported in PBS (Sigma Chemical Co., St Louis, MO) at 25–30°C to the laboratory, where they were washed twice in fresh PBS. Cumulus–oocyte complexes (COC) were isolated via slicing (Eckert and Niemann, 1995). Briefly, ovaries were cut with razor blades in PBS containing 0.1% BSA (fraction V, Sigma) and 2 IU heparin (Serva, Heidelberg). The resulting suspension was passed through a filter to isolate the COC. Category I COC (with a homogeneous evenly granulated cytoplasm possessing at least three layers of compact cumulus cells) and category II COC (with fewer than three layers of cumulus cells or partially denuded but also with a homogeneous evenly granulated cytoplasm; Pavlok et al., 1992) were pooled in TCM-air (TCM199 containing 1-glutamine and 25 mmol Hepes I−1 (Sigma) supplemented with 22 µg pyruvate ml−1, 350 µg NaHCO3, ml−1, 50 µg gentamicin ml−1 and 1 mg BSA ml−1 (fraction V)).

Maturation in vitro. TCM199 containing 1-glutamine and 25 mmol Hepes I−1 served as the basis for culture media. One millilitre was supplemented with 22 µg pyruvate, 2.2 µg NaHCO3, and 50 µg gentamicin. For oocyte maturation, this medium was supplemented with 0.1% BSA (fraction V, Sigma), 1 µg oestradiol ml−1 (Serva), 0.5 µg FSH ml−1 (Folltropin, Vetpharm, London, Ontario) and 0.06 IU hCG ml−1 (human chorionic gonadotropin, Ekluton, Vermi, Kempen).
Transcription in early bovine embryos

Fresh COC were washed twice in TCM199 supplemented with 0.1% BSA (fraction V) and then divided in groups of 20–25, transferred into 100 µl maturation drops under silicone oil and cultivated in a humidified atmosphere composed of 5% CO₂ in air at 39°C for 24 h. After fixation and staining with acetic orcein, a representative sample of the oocytes was examined for the presence of metaphase II and the first polar body.

Fertilization in vitro. After in vitro maturation, COC were rinsed in fertilization medium (Fert-TALP supplemented with 6 mg BSA ml⁻¹) and fertilized in Fert-TALP containing 10 µmol hypotaurine l⁻¹ (Sigma), 1 µmol adrenalin l⁻¹ (Sigma), 0.1 iu heparin ml⁻¹ (Serva) (HHF) and 6 mg BSA ml⁻¹. Frozen semen from one bull of proven fertility was used for in vitro fertilization (IVF). For IVF, semen was prepared by a modified ‘swim-up’ procedure (Parrish et al., 1986, 1988). Briefly, semen was thawed in a waterbath at 37°C for 1 min. After swim-up separation in Sperm-TALP containing 6 mg BSA ml⁻¹ for 1 h, the semen was washed twice by centrifugation at 350 g and 36°C for 10 min before being resuspended in Fert-TALP supplemented with HHE and BSA. The final sperm concentration added per fertilization drop was 1 x 10⁸ spermatozoa ml⁻¹.

Fertilization occurred during a 18–20 h coinubcation under the same temperature and gas conditions as described for maturation. Representative groups of fertilized oocytes were fixed and stained to control for the success of sperm–oocyte coinubcation. Oocytes with two pronuclei and one sperm tail were considered as normally fertilized.

Culture in vitro. For in vitro culture, zygotes were trans—ferred into 200 µl drops of TCM199 with 0.1% BSA (fraction V). Culture was maintained for a maximum of 11 days under the same conditions as described above. A total of 3200 COC from 380 ovaries was used to produce a complete developmental series from the immature oocyte to the hatched blastocyst. Upon transfer, these bovine blastocysts produced in vitro yielded pregnancy rates of 50% (Pavlok et al., 1992).

Immature and in vitro matured oocytes were treated with trypsin–EDTA to remove the cumulus cells. All oocytes or embryos were washed extensively and the absence of cumulus cells was verified at ×200 magnification to ensure that the transcripts did not originate from residual cumulus cells.

Pools of immature (IO) and matured oocytes (MO), zygotes (Z), 2–4 cell (2–4), 8–10 cell embryos (8–16), morulae (M), blastocysts (B) and hatched blastocysts (HB) were collected at the appropriate developmental times: MO after 24 h of maturation, Z 18–20 h postinsemination (h.p.i.), 2–4 cell stages 44–46 h.p.i., 8–16 cell stages 92–94 h.p.i., M 140–142 h.p.i., B 188–190 h.p.i., HB 112–114 h.p.i. Oocytes and embryos were included in the experiments only when they were derived from IVP runs in which the following minimum developmental rates had been obtained: 70% IVM, 60% normal fertilization, 60% cleavage rate, 20% blastocysts. Oocytes or embryos that had not reached the expected developmental stage at the respective time points were discarded from this study.

Production of bovine embryos in vivo

Holstein Friesian donor cows were superovulated with a single intramuscular injection of 3000 iu pregnant mares’ serum gonadotrophin (Brumegon®) and 1000 iu pregnant mares’ serum gonadotrophin (Metrogn®), Hydrochemie, Munich) between days 9 and 13 of the oestrous cycle followed 48 h later by Cyproteron (Estrumate®, Pitman–Moore, Burgwedel). After 48 h, the donors were inseminated twice at an interval of 12 h when superovulatory oestrous was detected. On day 7 after insemination, morula and blastocyst stages were recovered by nonsurgical flushing of the uterine horns with 300 ml PBS plus 1% newborn calf serum (NBCS, Number 295957; Boehringer, Mannheim) using established procedures.

Preparation of oocytes/embryos for RT–PCR analysis

Morphologically intact ova or embryos generated in vitro or in vivo were used for the experiments. Briefly, oocytes with an extruded polar body were considered to be mature. Fertilization was verified at ×320 magnification by the presence of two polar bodies and two pronuclei. Two–four cell and 18–16 cell stages were identified by their round, fine granulated blastomeres. Embryos with coalesced blastomeres were considered as compacted morulae; embryos containing a clearly visible blastocoel cavity were considered as blastocysts; and hatched blastocysts were completely hatched from the zona pelludica. After washing three times in PBS containing 0.1% PVA (polyvinyl alcohol, Sigma) they were stored in pools of 20–30 at −80°C in a minimum volume (≤5 µl) of medium until use. RNA equivalents of 1–10 oocytes/embryos were chosen for the PCR analysis in the present study as this strategy had been used successfully in numerous investigations using RT–PCR assays of similar sensitivity (Harvey et al., 1995; Davis et al., 1996; Fleming et al., 1997; Winger et al., 1997; Worrard and Schultz, 1997). In contrast, the less sensitive northern blot technology required pools of up to 360 embryos for RNA detection (Bilodeau-Goesels and Schultz, 1997b). In addition, use of pooled material eliminates potential effects of the cell cycle on amounts of specific mRNAs (Moore et al., 1996).

Isolation of RNA

Poly(A)+ RNA was isolated using a Dynabeads mRNA DIRECT Kit (Dynal®, Oslo) according to the manufacturer's instructions with minor modifications. Briefly, oocytes or embryos were lysed by adding 150 µl of lysis–binding buffer (100 mmol Tris–HCl 1 M, pH 8.0, 500 mmol LiCl 1 M, 10 mmol EDTA 1 M, 1% (w/v) Lithium dodecylsulfate (LiDS), 5 mmol dithiothreitol (1 M), vortexed for 10 s, centrifuged at 12 000 g for 15 s and incubated at room temperature for 10 min. Prewashed Dynabeads® Oligo (dT)₂₅ (10 µl) was pipetted into the fluid. After 5 min incubation at room temperature for binding poly(A)+ RNAs to oligo (dT) Dynabeads, the beads were separated using a Dynal MPC-E-1 magnetic separator, washed once using 100 µl washing buffer 1 (10 mmol Tris–HCl 1 M, pH 8.0, 0.15 mol LiCl 1 M, 1 mmol EDTA 1 M, 0.1% (w/v) LiDS) and three times with 100 µl washing buffer 2 (10 mmol Tris–HCl 1 M, pH 8.0, 0.15 mol LiCl 1 M, 1 mmol EDTA 1 M). Poly(A)+ RNAs were then eluted from the beads by
incubation in 11 μl sterile water at 65°C for 2 min and aliquots were used immediately for RT.

As a positive control, total RNA was also extracted from bovine tongue epithelium by a modification of the method of Chomczynski and Sacchi (1987). The scraped epithelial cells were homogenized in solution D (4 mol guanidine thiocyanate 1−1, 0.5% (w/v) sarcosyl, 100 mmol 2-mercaptoethanol 1−1, 25 mmol sodium citrate 1−1, pH 7.0). Subsequently, 85 μl of 2 mol sodium acetate 1−1, pH 4.0, 850 μl of phenol (acid, Aqua HPLC–Phenol, Roth), and 170 μl chloroform–isoamyl alcohol mixture (49:1) were added to an 850 μl aliquot of the homogenate, with thorough mixing after the addition of each component. The final suspension was shaken vigorously for 10 s and cooled on ice for 15 min. Samples were centrifuged at 10 000 g for 20 min at 4°C. After centrifugation, the aqueous phase was transferred to a fresh tube, mixed with 1 ml isopropanol, and then held at −80°C for 60 min to precipitate RNA. The sample was centrifuged again under the same conditions and the resulting RNA pellet was washed twice using 70% ethanol. After drying at 37°C, the pellet was dissolved in 100–200 μl sterile water and aliquoted in 30 μl portions. Contaminating genomic DNA was digested by incubating the samples with 10 μl DNase (Boehringer, Mannheim) for 30 min at 37°C. DNase was inactivated by heating samples to 80°C for 10 min. Tissue RNA concentration and purity were determined by spectrophotometry. The integrity of the RNA was analysed by ethidium bromide staining after electrophoresis on an agarose (1%) gel.

Reverse transcription

Poly(A)+ RNA isolated from different numbers of oocytes or embryos (20–50) or 200 ng total RNA from tissue (tongue epithelium) was reverse transcribed into cDNA in a total volume of 20 μl. The reaction mixture consisted of 1 × RT buffer (50 mmol KCl 1−1, 10 mmol Tris–HCl 1−1, pH 8.3, Perkin–Elmer, Wursterstetten), 5 mmol MgCl2 1−1, 1 mmol of each dNTP 1−1 (Amersham, Brunswick), 2.5 μmol random hexamers 1−1 (Perkin–Elmer), 20 μl RNase inhibitor (Perkin–Elmer) and 50 μl murine leukaemia virus (MuLV) reverse transcriptase (Perkin–Elmer). The mixture was overlaid with mineral oil to prevent evaporation. The RT reaction was carried out at 25°C for 10 min, and then at 42°C for 1 h followed by a denaturation step at 99°C for 5 min and flash cooling on ice.

Polymerase chain reaction

PCR was performed with cDNA equivalents corresponding to 1–10 oocytes or embryos or 0.5–5.0 ng tissue RNA in a final volume of 100 μl of 1 × PCR buffer (20 mmol Tris–HCl 1−1, pH 8.4, 50 mmol KCl 1−1, Gibco BRL, Eggenstein), 1.5 mmol MgCl2 1−1, 200 μmol of each dNTP 1−1, 1 μmol of each sequence specific primer using a PTC-200 thermocycler (MJ Research, Watertown, MA). During hot start, 1 μl Taq DNA polymerase (Gibco) was added at 72°C. PCR primers (Table 1) were designed from the coding regions of each gene sequence using the OLIGO® program.

The PCR program used an initial step at 99°C for 5 min and 72°C for 2 min (hot start) followed by 35 cycles of 15 s at 95°C for DNA denaturation, 15 s at different temperatures for annealing of primers (Table 1), and 15 s at 72°C for primer extension. The last cycle was followed by a 5 min extension at 72°C and cooling to 4°C. After addition of 10 μl of 10 × loading buffer (0.25% (w/v) xylene cyanol and 25 mmol EDTA 1−1 in 50% glycerin), the RT–PCR products were subjected to electrophoresis on a 2% agarose gel containing 0.5 μg ethidium bromide ml−1 together with a molecular weight marker (pBR322DNA–MspI Digest, New England Biolabs GmbH, Schwalbach) and photographed on an 312 nm UV transilluminator. As negative controls, tubes were always prepared in which RNA or reverse transcriptase was omitted during the RT reaction. The assays were repeated at least three times with different oocyte or embryo batches and provided consistent results.

The identity of the RT–PCR fragments was confirmed by sequencing the products from each primer pair using an automated DNA sequencing device (DNA sequencer, model 4000L, Li-Cor, Lincoln, NE). The original gel was scanned into a computer. The products of each gene transcript were mounted as one picture using Harvard Graphics® for Windows, Version 2.0 (Software Publishing Corp., Santa Clara, CA) after digitalization using the CAM® program (Cybertech CS-1 Image Documentation System, Berlin, Germany) to demonstrate the temporal mRNA expression pattern from the immature oocyte to the hatched blastocyst.

Results

Production of bovine embryos in vitro

Oocytes and embryos from 27 IVP runs were used in this study. The rate (ranges) of development (percentage of embryos relative to the total number of oocytes) were as follows: 73–82% maturation rate. 62–75% normal fertilization, 53–72% 2–4 cell stages, 44–63% 8–16 cell stages, 33–46% morulae, 23–32% blastocysts, 11–22% hatched blastocysts.

Validity of the RT–PCR assay

cDNA samples were prepared from pools of embryos to determine the representative pattern of mRNA expression in preimplantation bovine embryos derived from this semi-defined culture system yielding high developmental rates (see Brackett and Zuelke, 1993; Trounson et al., 1994). Each reverse-transcribed cDNA sample was tested for the absence of contaminating genomic DNA by omitting reverse transcriptase, or for contaminating exogenous RNA or DNA by omitting embryonic RNA during the RT reaction (data not shown). Amplified fragments were never detected in these negative controls. The RT–PCR assay was sensitive enough to detect bovine mRNA from tongue epithelium at levels from 0.5 to 5.0 ng (data not shown). This corresponds well to the total amount of RNA (2.4 ng) found in the bovine oocyte (Bilodeau-Goeseels and Schultz, 1997a). Analyses were repeated with increasing numbers of embryos or using specific priming of the RT reaction, as well as increasing the number of PCR cycles, to verify that the failure to detect a transcript was not attributed to sensitivity problems (data not shown). The
Transcription in early bovine embryos

Table 1. Primers used for polymerase chain reaction

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences and positions</th>
<th>Annealing temperature (°C)</th>
<th>Fragment size (bp)</th>
<th>Sequence references [EMBL accession no.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Connexin 43 (Cx43)</td>
<td>5’primer (736-765) = GGGAAAGGAGCTTATACCACTACTACCAC 3’primer (1222-1251) = CCACTCTAAAGAATAACCCATAGCTTA</td>
<td>60</td>
<td>516</td>
<td>Lash et al. (1990) [J05535]</td>
</tr>
<tr>
<td>Desmoglein I (Dg I)</td>
<td>5’primer (836-850) = GCACAGTGGATGTCACGAGCTTG 3’primer (1590-1620) = GAAGTAGAGGCTATAGCAGGTAG</td>
<td>53.5</td>
<td>785</td>
<td>Koch et al. (1991) [X58466]</td>
</tr>
<tr>
<td>Desmocollin I (Dc I)</td>
<td>5’primer (2412-2436) = GGTGGGTTGTTCTGTTGTTGTA 3’primer (2875-2899) = GTACTCTCCAGCTGTTGCCTCC</td>
<td>55</td>
<td>488/441 (insertion of 46 bp, 2803-2848)</td>
<td>Collins et al. (1991) [X56966]</td>
</tr>
<tr>
<td>Desmocollin II (Dc II)</td>
<td>5’primer (2085-2109) = CTCCTGGAGATGACAAGTGTATTC 3’primer (2503-2527) = GCGGATCTCTTTCTCTGATTTAT</td>
<td>57</td>
<td>443/397 (insertion of 46 bp, 2396-2441)</td>
<td>Koch et al. (1992) [M81190]</td>
</tr>
<tr>
<td>Desmocollin III (Dc III)</td>
<td>5’primer (2088-2117) = GCCATACTTCTGGGATGGAGCAGCATTTC 3’primer (2555-2584) = GTGATGCCAGTGCTCTGCTCAGTAC</td>
<td>57</td>
<td>497/454 (insertion of 43 bp, 2499-2541)</td>
<td>Yue et al. (1995) [L33774]</td>
</tr>
<tr>
<td>Plakophilin (Plako)</td>
<td>5’primer (1337-1361) = CCCGTCGAGCCGGAGCTCTTTCGA 3’primer (1580-1604) = CCGTGTAGGCTCCGCTCCTGGTA</td>
<td>64</td>
<td>268</td>
<td>Heid et al. (1994) [Z37975]</td>
</tr>
<tr>
<td>Glucosetransporter 1 (Glut-1)</td>
<td>5’primer (1609-1638) = AAGGAGCTTGGACCTCGGGAGCTGACT 3’primer (1908-1933) = TGGCGGCTGACAGCTCCTGCTGATAAAA</td>
<td>59</td>
<td>327</td>
<td>Boado and Pardridge (1991) [M80448]</td>
</tr>
<tr>
<td>Poly(A)polymerase (PolyA)</td>
<td>5’primer (886-915) = GTTTCTCTGGTGTTCTGCTGATGC 3’primer (1108-1137) = TGAGCTCTTCCTGCTCCTATTCCA</td>
<td>57</td>
<td>252</td>
<td>Raabe et al. (1991) [X63436]</td>
</tr>
<tr>
<td>Heat shock protein 70.1 (HSP)</td>
<td>5’primer (1861-1890) = AAGGTCGCTGGACAACTGCGAGGTGATT 3’primer (2319-2348) = ACTTTGGAGATTCAACAGAAGCGGTTGAAAAA</td>
<td>59</td>
<td>488</td>
<td>DeLuca-Flaherty and McKay (1990) [X53827]</td>
</tr>
<tr>
<td>Trophoblast protein (TP)</td>
<td>5’primer (420-423) = GCTATCTCTGTGCTCCAGATGATG 3’primer (755-778) = AGTACGATGATGCTCCACCCATC</td>
<td>57</td>
<td>359</td>
<td>Imakawa et al. (1989) [G163764]</td>
</tr>
</tbody>
</table>

Detection of PolyA mRNA from pooled embryos demonstrated that RT had been achieved, indicating the presence of intact RNA. Only good quality embryos were selected for use in this study. Lequarre et al. (1997) have suggested that embryo quality and mRNA contents are correlated.

RNA expression of developmentally important genes in preimplantation bovine embryos

Transcripts for Cx43 were detected up to the morula stage in IVP oocytes or embryos by the fragment of 516 bp. Therefore, the origin could be maternal and embryonic. Morulae and blastocysts produced in vivo showed transcription of the Cx43 gene (data not shown). Transcripts of Dc II and Dc III were detectable from the 2–4 cell stage onwards in IVP embryos, indicated by the predicted sizes of the fragments, 443/397 bp and 497/454 bp, suggesting an embryonic origin of both splice variants. The transcripts were also found in in vivo stages. mRNA encoding Plako was detected in immature and in vitro mature oocytes and zygotes generated in vitro. The transcript could not be amplified from IVP 2–4 and 8–16 cell embryos. Transcription resumed at the morula stage, suggesting the temporal separation of maternal and embryonic expression. Morulae and blastocysts generated in vivo transcribed the gene as shown by the fragment of the expected size of 268 bp. No transcripts were found for Dg I and Dc I at any stages (data not shown). However, fragments with the expected sizes were detected using bovine tongue epithelium total RNA samples (785 bp and 488/441 bp, respectively).
Transcripts encoding Glut-1, PolyA and the HSP were detectable throughout bovine preimplantation development in vitro as indicated by amplicons with the expected sizes of 488 bp, 252 bp and 327 bp, respectively, suggesting that these transcripts are of both maternal and embryonic origin. These products were also identified in bovine morulae and blastocysts produced in vivo. Trophoblast protein was transcribed at the blastocyst stage in IVP embryos, indicating an embryonic origin. The 359 bp fragment was not detectable in morulae produced in vivo, whereas it was found in blastocysts produced in vivo. The transcriptional pattern of the genes with detectable fragments throughout bovine preimplantation development in vitro is shown (Fig. 1).

The results of the transcription pattern in all preimplantation stages of bovine oocytes or embryos generated in vitro (Table 2) and of morulae and blastocysts derived in vivo (Table 3) are summarized. RT-PCR products were sequenced to ensure the specificity of PCR amplification. The obtained sequences possessed identical nucleotide sequences to the published bovine DNA sequence data.

Discussion

In the present study, the effects of a semi-defined culture system on the temporal mRNA expression pattern of ten...
developmentally important genes has been analysed in bovine oocytes and embryos produced \textit{in vitro} using RT–PCR. The transcriptional pattern of the transferable stage morulae and blastocysts produced under semi-defined culture conditions was compared with that of their \textit{in vivo} counterparts. The transcripts determined may be of maternal (oocyte: 2–4 cell...
stage) origin only or may be of both maternal and embryonic (beginning at the 8–16 cell stage) origin. These transcripts are involved in compaction and cavitation (Cx43, Dg I, Dc I, II, III, Plako), metabolism (Glut-1), RNA processing (PolyA), stress (HSP), and maternal recognition of pregnancy (TP).

During murine preimplantation development, functional gap junctions are first observed at compaction at the eight-cell stage (Lo and Gilula, 1979; Goodall and Johnson, 1984) and mRNA encoding Cx43 and Cx43 protein are detected from the four-cell stage onwards (Nishi et al., 1991). The rate limiting step of gap junction coupling is regulated by the insertion of nascent subunits into plasma membranes downstream of transcription and translation (De Sousa et al., 1993). Expression of Cx43 before the morula stage could indicate the presence of precursor pools (McLachlin et al., 1983; Valdimarsson et al., 1991) and, thus, the preparatory step for compaction and blastulation. No gap junction coupling was visible in bovine blastocysts produced in vitro (Prather and First, 1993), and the Cx43 gene was transcribed up to the morula stage in IVF bovine embryos, whereas the transcript was found in blastocysts produced in vivo (Wrenzycki et al., 1996). In the present study, similar culture conditions were used as in the previous study with two exceptions. The semen used for IVF stemmed from a different bull and maturation and culture media were

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### Table 2. Transcription of developmentally important genes in preimplantation bovine embryos generated in vitro

<table>
<thead>
<tr>
<th>Genes</th>
<th>1O</th>
<th>MO</th>
<th>Z</th>
<th>2–4</th>
<th>8–16</th>
<th>M</th>
<th>B</th>
<th>HB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Connexin43 (Cx43)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Desmoglein 1 (Dg 1)</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>Desmocollin I (Dc I)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Desmocollin II (Dc II)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Desmocollin III (Dc III)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Plakophilin (Plako)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Glucosetransporter 1 (Glut-1)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Poly(A)polymerase (PolyA)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Heat shock protein 70.1 (HSP)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trophoblast protein (TP)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

+ , transcripts detectable; −, transcripts not detectable (representing consistent results from three replicates, each representing 1–10 oocytes/embryos). 1O, immature oocytes; MO, matured oocytes; Z, zygotes; 2–4, 2–4 cell embryos; 8–16, 8–16 cell embryos; M, morulae; B, blastocysts; HB, hatched blastocysts.

### Table 3. Transcription of developmentally important genes in in vivo derived bovine morulae and blastocysts

<table>
<thead>
<tr>
<th>Genes</th>
<th>Cx43</th>
<th>Dg 1</th>
<th>Dc 1</th>
<th>Dc II</th>
<th>Dc III</th>
<th>Plako</th>
<th>Glut-1</th>
<th>PolyA</th>
<th>HSP</th>
<th>TP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morulae</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Blastocysts</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

+ , transcripts detectable; −, transcripts not detectable (representing consistent results from three replicates, each representing 2 morulae or 1 blastocyst).

Cx43, connexin-43; Dg 1, desmoglein 1; Dc, desmocollin; Plako, plakophilin; Glut-1, glucosetransporter 1; HSP, heat shock protein 70.1; TP, trophoblast protein.
supplemented with BSA instead of oestrus cow serum (OCS). Serum was substituted by BSA because serum has been attributed with perturbations in embryo morphology, metabolism, ultrastructure and postimplantation development (Dorland et al., 1994; Gardner, 1994; Thompson et al., 1995). Since identical transcription patterns were detected for Cx43 with both BSA and serum, it was concluded that neither serum nor BSA nor the bull affect transcription of this gene. Further studies are needed to investigate the effects of the lack of Cx43 expression in bovine embryos derived in vitro on developmental capacity in vivo. In rat ovaries, it has been shown that gonadotrophins regulate expression of mRNA encoding Cx43 and Cx43 protein (Granot and Dekel, 1997). In addition, a similar expression pattern was found for the GCS (glutamylcysteine synthetase) gene in IVP bovine embryos (Harvey et al., 1995).

Desmosome formation is correlated with the onset of blastocoei formation in mouse embryos (Fleming et al., 1991; Collins et al., 1995). Three types of bovine desmocollins have been described (Legan et al., 1994), each being the product of a different gene and being expressed as a pair of alternatively spliced forms (a and b). The b forms possesses an additional 46 bp in Dc I and II (43 bp in the case of Dc III) exon (Legan et al., 1994). The a and b forms of Dc II are transcribed in mouse oocytes and cleavage stages up to the early eight-cell stage, are never detected in compact eight-cell embryos, and appear again from the 16-cell stage onwards (Collins et al., 1995). In mouse blastocysts, mRNA encoding Dc II is localized within the trophoderm. After isolation and culture of ICMs from early mouse blastocysts, the outer cells start to form a new trophodermal layer indicated by an increase in Dc II transcription (Collins et al., 1995). The present data show for the first time that the transcripts of Dc II and Dc III in bovine embryos are of embryonic origin and both splice variants are detectable. In contrast, Dc I and Dg 1 were not transcribed throughout bovine preimplantation development. The transcriptional pattern of Plako suggests that the transcripts are first of maternal and subsequently of embryonic origin. These results indicate that Dc II, Dc III and Plako are the first components of desmosomes expressed during bovine preimplantation development.

Glucose was found to be detrimental to bovine embryos in vitro up to the morula stage (Takahashi and First, 1992). During in vitro development from the morula onwards, glucose is the preferred energy substrate (Rieger et al., 1992a, b), as it is in embryos in vivo (Javed and Wright, 1991). Glut-1 was found to be distributed in the trophoderm and inner cell mass cells of mouse embryos, while Glut-2 was restricted to the inner trophodermal membranes (Aghayan et al., 1992). The present data demonstrate that Glut-1 is transcribed throughout preimplantation bovine development, suggesting that the transcript is of maternal and embryonic origin. Glut-1 is expressed in a similar pattern in mouse embryos to that in bovine embryos, whereas Glut-2 is expressed from the eight-cell stage onwards in the mouse embryo (Hogan et al., 1991; Aghayan et al., 1992). Glut-1 acts as a regulatable transporter in mouse blastocysts, responsive to IGF-I and insulin, and mediated by the IGF-I receptor (Pantaleon and Kaye, 1996). IGF-I was found to have a beneficial effect on the developmental capacity of bovine embryos (Herrler et al., 1992). Bovine embryos produced in vitro transcribe the IGF-I receptor throughout preimplantation development (Watson et al., 1992). These findings support the hypothesis that the positive effect of IGF-I is attributed to an enhanced glucose uptake by these embryos. Furthermore, Glut-1 transcription and glucose uptake are known to be affected by in vitro culture conditions. Concentrations of mRNA encoding Glut-1 and glucose incorporation were significantly reduced in mouse blastocysts produced in vitro compared with blastocysts grown in vivo (Morita et al., 1994). In addition, the metabolism of IVP embryos was increased compared with that of the embryos in vivo, indicating the involvement of several genes in the regulation of embryo metabolism (Kuruna and Niemann, 1992).

Polyadenylation regulates gene expression via post-transcriptional modulation, translation or stability of mRNA. Extension of a pre-existing short poly(A) tail is associated with mRNA activation, and deadenylation is correlated with cessation of translation (Paynton et al., 1988). Maternal mRNAs gain or lose their poly(A) tail in the cytoplasm at specific timepoints during development (Bachvarova et al., 1985; Paynton and Bachvarova, 1994). The present study demonstrates that the gene encoding this protein is transcribed throughout development in preimplantation bovine embryos. A transient polyadenylation of a maternal mRNA was found after fertilization of mouse oocytes (Temeles and Schultz, 1997). Several mRNAs, for example those encoding tissue plasminogen activator (tPA) and hypoxanthine phosphoribosyltransferase (HPRT), are known to be polyadenylated during oocyte maturation (Huarte et al., 1987; Vassalli et al., 1989; Salles and Strickland, 1995). Brevini-Gandolfi et al. (1997) have demonstrated that the developmental capacity of bovine oocytes is correlated with the length of the poly(A) tail of epidermal growth factor and Oct4 transcripts. The adenylation status of most of the specific bovine mRNAs remains unknown.

Maternal exposure to increased temperatures during ovulation and early embryonic development is correlated with embryonic death (Ealy et al., 1993; Edwards and Hansen, 1996, 1997). The sensitivity of maturing oocytes and early embryos to increased temperature has been attributed to the lack of an appropriate mechanism that confers heat resistance. Molecules of the HSP70 family were found to provide thermoprotection by refolding damaged proteins and protecting RNA (Duncan and Hershey, 1989; Nover and Scharf, 1991). The data from the present study demonstrate that transcripts encoded by the HSP70.1 gene are detectable throughout bovine preimplantation development, suggesting a stage-specific mechanism of bovine oocytes and embryos to heat shock. In oocytes, heat shock altered the development to blastocysts, whereas morulae were not affected (Edwards and Hansen, 1996, 1997). Transcription of the mouse HSP70.1 gene is enhanced at the onset of zygotic genome activation and the amount of transcription was found to be modulated by in vitro culture conditions. The transcriptional activity of HSP70.1 was 15 times higher in mouse embryos generated in vitro than in the embryos in vivo (Christians et al., 1995). Furthermore, timing of induced thermotolerance (a phenomenon whereby exposure to a mild heat shock confers thermotolerance to a subsequent more severe heat shock) differed for mouse embryos that developed in culture as compared with the embryos in vivo (Ealy and Hansen,
There is some evidence that heat shock-induced synthesis of HSP precedes induction of thermotolerance (Edwards et al., 1995), suggesting similar factors are involved in thermotolerance (Edwards and Hansen, 1997). It has also been proposed that HSP70 is switched on by free oxygen radicals that are correlated with embryonic arrest and cell death (Johnson and Nasr-Esfahani, 1994). During heat shock, cellular damage is caused by a variety of factors and a single thermoprotectant is probably unable to protect cells from damaging effects (Arechiga et al., 1995). On the basis of these findings, HSP70.1 could prove a useful tool to indicate stress caused by suboptimal culture conditions.

Trophoblast protein is secreted by the trophoderm of bovine conceptuses between days 15 and 24 of gestation (Bartol et al., 1985). This antiluteolytic signal is primarily responsible for inhibiting uterine production of luteolytic concentrations of PGF2α (Bazer et al., 1991). The present data confirm the report of Watson et al. (1992) that mRNA encoding TP found in bovine blastocysts and hatched blastocysts indicates an embryonic origin.

Among all the transcripts analysed, the only difference between morulae and blastocysts generated in vitro and in vivo was observed for Cx43, confirming our findings for bovine embryos cultured in serum-enriched medium (Wrenzycki et al., 1996). This suggests that expression of the Cx43 gene is not affected by either serum or BSA and that this gene could be a suitably sensitive candidate to be included in a set of appropriate marker genes indicating the quality of an IVF system for bovine embryos. It has been shown that culture conditions after gene expression in mouse preimplantation embryos (Anbari and Schultz, 1993; Vernet et al., 1993; Ho et al., 1994, 1995; Shim et al., 1996). Furthermore, in vitro culture has profound effects on post-implantation development and is thought to be associated with increased duration of gestation and mortality as indicated by abnormally large fetuses and offspring after transfer of IVF embryos (Kruij and denDaas, 1997). Therefore, further investigations are needed to improve in vitro culture conditions of preimplantation embryos. The use of a determined set of genes covering a broad range of physiological systems in the embryos to assess the quality of the in vitro production system will be instrumental in improving the quality of the embryos produced. Morphological criteria are not sufficient to assess embryo quality in an objective manner because IVF bovine embryos are more difficult to evaluate than their in vivo counterparts (Farin et al., 1995). The quantitative pattern of gene transcription in bovine preimplantation embryos generated in vivo and in vitro are currently being investigated using a semi-quantitative RT–PCR assay to gain insight into the regulation of transcription.

In conclusion, for the first time the ontogenetic pattern of the transcription of a broad set of developmentally important genes has been demonstrated in preimplantation bovine embryos generated in a semi-defined in vitro culture system. The difference between in vitro versus in vivo blastocysts found for the Cx43 gene is probably caused by the extended in vitro culture of bovine embryos. Therefore, the composition of an optimized culture system for preimplantation embryos is of critical importance for both commercial applications and basic studies.

The authors are grateful to E. Lemme and K-G. Hadelin for their skilled technical assistance. This research was supported by grant Ni 256/12-1 from the Deutsche Forschungsgemeinschaft (DFG).

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