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 1 (Dg 1), 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 1 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 Langendonck *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

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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* (Kruip and denDaas, 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 blastocoel 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 vivo* 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 α -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 α -amanitin, the transcript must be of embryonic origin. However, bovine embryos did not develop after treatment with α -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; Kopečný *et al.*, 1989; Plante *et al.*, 1994; Hyttel *et al.*, 1996; Viuff *et al.*, 1996). However, with the improvement of IVP 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 IVP 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 trophectoderm as the blastocyst expands (Fleming *et al.*, 1991; Collins *et al.*, 1995). Desmosomes are composed of transmembrane glycoproteins, such as desmocolins 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 (Takagaki *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 τ (IFN τ). IFN τ , also named TP-1, is a major secretory protein produced by the trophoblast cells of the bovine blastocyst (Hernandez-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 vivo* 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 L-glutamine and 25 mmol Hepes l⁻¹ (Sigma) supplemented with 22 μ g pyruvate ml⁻¹, 350 μ g NaHCO₃ ml⁻¹, 50 μ g gentamicin ml⁻¹ and 1 mg BSA ml⁻¹ (fraction V)).

Maturation in vitro. TCM199 containing L-glutamine and 25 mmol Hepes l⁻¹ served as the basis for culture media. One millilitre was supplemented with 22 μ g pyruvate, 2.2 μ g NaHCO₃ and 50 μ g gentamicin. For oocyte maturation, this medium was supplemented with 0.1% BSA (fraction V, Sigma), 1 μ g oestradiol ml⁻¹ (Serva), 0.5 μ g FSH ml⁻¹ (Folltropin[®], Vetrephearm, London, Ontario) and 0.06 iu hCG ml⁻¹ (human chorionic gonadotropin, Ekluton[®], Vemie, Kempen).

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 μ l 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 μ mol hypotaurine l⁻¹ (Sigma), 1 μ mol adrenalin l⁻¹ (Sigma), 0.1 iu heparin ml⁻¹ (Serva) (HHE) 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 1×10^6 spermatozoa ml⁻¹.

Fertilization occurred during a 18–20 h coincubation 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 coincubation. Oocytes with two pronuclei and one sperm tail were considered as normally fertilized.

Culture in vitro. For *in vitro* culture, zygotes were transferred into 200 μ l 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 $\times 200$ 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), 8–16 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[®], Hydrochemie, Munich) between days 9 and 13 of the oestrous cycle followed 48 h later by Cloprostenol (Estrumate[®], Pitman–Moore, Burgwedel). After 48 h, the donors were inseminated twice at an interval of 12 h when superovulatory oestrus 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 matured. Fertilization was verified at $\times 320$ 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 pellucida. 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 μ l) 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; Worrad and Schultz, 1997). In contrast, the less sensitive northern blot technology required pools of up to 360 embryos for RNA detection (Bilodeau-Goeseels 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 (Dyna[®], Oslo) according to the manufacturer’s instructions with minor modifications. Briefly, oocytes or embryos were lysed by adding 150 μ l of lysis-binding buffer (100 mmol Tris–HCl l⁻¹, pH 8.0, 500 mmol LiCl l⁻¹, 10 mmol EDTA l⁻¹, 1% (w/v) Lithium dodecylsulfate (LiDS), 5 mmol dithiothreitol l⁻¹), 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 μ l) 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 μ l washing buffer 1 (10 mmol Tris–HCl l⁻¹, pH 8.0, 0.15 mol LiCl l⁻¹, 1 mmol EDTA l⁻¹, 0.1% (w/v) LiDS) and three times with 100 μ l washing buffer 2 (10 mmol Tris–HCl l⁻¹, pH 8.0, 0.15 mol LiCl l⁻¹, 1 mmol EDTA l⁻¹). 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 l^{-1} , 0.5% (w/v) sarkosyl, 100 mmol 2-mercaptoethanol l^{-1} , 25 mmol sodium citrate l^{-1} , pH 7.0). Subsequently, 85 µl of 2 mol sodium acetate l^{-1} , pH 4.0, 850 µl of phenol (acid, Aqua[®]-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 iu 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 l^{-1} , 10 mmol Tris-HCl l^{-1} , pH 8.3, Perkin-Elmer, Vaterstetten), 5 mmol MgCl₂ l^{-1} , 1 mmol of each dNTP l^{-1} (Amersham, Brunswick), 2.5 µmol random hexamers l^{-1} (Perkin-Elmer), 20 iu RNase inhibitor (Perkin-Elmer) and 50 iu 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 l^{-1} , pH 8.4, 50 mmol KCl l^{-1} , Gibco BRL, Eggenstein), 1.5 mmol MgCl₂ l^{-1} , 200 µmol of each dNTP l^{-1} , 1 µmol of each sequence specific primer using a PTC-200 thermocycler (MJ Research, Watertown, MA). During hot start, 1 iu 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 l^{-1} in 50% glycerol), 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

Table 1. Primers used for polymerase chain reaction

Genes	Primer sequences and positions	Annealing temperature (°C)	Fragment size (bp)	Sequence references [EMBL accession no.]
Connexin43 (Cx43)	5'primer (736–765) = GGGAAAGAGCGATCCTTACCACACTACCAC 3'primer (1222–1251) = CCACCTCCAATGAAACAAAATGAACACCTA	60	516	Lash <i>et al.</i> (1990) [J05535]
Desmoglein 1 (Dg 1)	5'primer (836–850) = GCAGATGGTATGTCAGCAGAGTGTG 3'primer (1596–1620) = GAAGTAAGGCCATATGCAGAGGTAG	53.5	785	Koch <i>et al.</i> (1991) [X58466]
Desmocollin I (Dc I)	5'primer (2412–2436) = GGTGTGGGTTCTGTATTGTTGTTA 3'primer (2875–2899) = GTAGTCTTCGCAGAGCTTGTGTTC	55	488/441 (insertion of 46 bp, 2803–2848)	Collins <i>et al.</i> (1991) [X56966]
Desmocollin II (Dc II)	5'primer (2085–2109) = CTCCTGGCGATGACAAAGTGATTC 3'primer (2503–2527) = GCCGATCCTCTTCCTTCGTAGTTAT	57	443/397 (insertion of 46 bp, 2396–2441)	Koch <i>et al.</i> (1992) [M81190]
Desmocollin III (Dc III)	5'primer (2088–2117) = GCCATACTTCTGGGCATAGCACTACTTTTC 3'primer (2555–2584) = GTGATGGCATGTGCTCTTCGTCCTGATTAC	57	497/454 (insertion of 43 bp; 2499–2541)	Yue <i>et al.</i> (1995) [L33774]
Plakophilin (Plako)	5'primer (1337–1361) = CCCGTGGACCCCGAGGTCTTCTTCA 3'primer (1580–1604) = CGGTGTAGGCGTTGCGGGCGTTGTA	64	268	Heid <i>et al.</i> (1994) [Z37975]
Glucosetransporter 1 (Glut-1)	5'primer (1609–1638) = AGGAGCTGTTCCACCCCTGGGAGCTGACT 3'primer (1906–1935) = TGTGGGTGAAGGAGACTCTGGCTGATAAAA	59	327	Boado and Pardridge (1991) [M60448]
Poly(A)polymerase (PolyA)	5'primer (886–915) = GTTTCCTCGTGTTCTTCTGGGCTATGC 3'primer (1108–1137) = TGGAGTTCTGTTGTGGGTATGCTGGTGTA	57	252	Raabe <i>et al.</i> (1991) [X63436]
Heat shock protein 70.1 (HSP)	5'primer (1861–1890) = AAGGTGCTGGACAAGTGCCAGGAGGTGATT 3'primer (2319–2348) = ACTTGGAAAGTAAACAGAAACGGGTGAAAAA	59	488	DeLuca-Flaherty and McKay (1990) [X53827]
Trophoblast protein (TP)	5'primer (420–423) = GCTATCTCTGTGCTCCATGAGATG 3'primer (755–778) = AGTGAGTTTCAGATCTCCACCCATC	57	359	Imakawa <i>et al.</i> (1989) [G163764]

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* matured 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 1 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).

Fig. 1(a).

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

Fig. 1(b).

Fig. 1. Transcripts (reverse transcriptase-polymerase chain reaction (RT-PCR) products) of developmentally important genes in preimplantation bovine oocytes or embryos generated *in vitro*. Each lane represents the RT-PCR product derived from Poly(A)⁺ RNA from the equivalent of ten (oocytes – 2–4 cell embryos), five (8–16 cell embryos), two (morulae) or one (blastocysts and hatched blastocysts) oocytes or embryos. Lane 1, molecular weight marker (to verify the size of the amplified fragments); lane 2, positive control (bovine tongue); lane 3, negative controls omitting RNA; lane 4, immature oocytes; lane 5, matured oocytes; lane 6, zygotes; lane 7, 2–4 cell embryos; lane 8, 8–16 cell embryos; lane 9, morulae; lane 10, blastocysts; lane 11, hatched blastocysts. Each figure is a montage of individual gels run for each group of oocytes or embryos showing the presence or absence of fragments of the expected size(s) for each gene transcript. In the case of Dc I (data not shown), Dc II and Dc III, both splice variants of the gene transcript were detectable.

developmentally important genes has been analysed in bovine oocytes and embryos produced *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 *in vivo* counterparts. The transcripts determined may be of maternal (oocyte: 2–4 cell

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

Genes	Developmental stages examined							
	Suggested maternal transcription only				Suggested maternal and embryonic transcription			
	IO	MO	Z	2-4	8-16	M	B	HB
Connexin43 (Cx43)	+	+	+	+	+	+	—	—
Desmoglein 1 (Dg 1)	—	—	—	—	—	—	—	—
Desmocollin I (Dc I)	—	—	—	—	—	—	—	—
Desmocollin II (Dc II)	—	—	—	+	+	+	+	+
Desmocollin III (Dc III)	—	—	—	+	+	+	+	+
Plakophilin (Plako)	+	+	+	—	—	+	+	+
Glucosetransporter 1 (Glut-1)	+	+	+	+	+	+	+	+
Poly(A)polymerase (PolyA)	+	+	+	+	+	+	+	+
Heat shock protein 70.1 (HSP)	+	+	+	+	+	+	+	+
Trophoblast protein (TP)	—	—	—	—	—	—	+	+

+, transcripts detectable; —, transcripts not detectable (representing consistent results from three replicates, each representing 1–10 oocytes/embryos).

IO, 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

	Cx43	Dg 1	Dc I	Dc II	Dc III	Plako	Glut-1	PolyA	HSP	TP
Morulae	+	—	—	+	+	+	+	+	+	—
Blastocysts	+	—	—	+	+	+	+	+	+	+

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

Cx43, connexin43; Dg 1, desmoglein 1; Dc, desmocollin; Plako, plakophilin; Glut-1, glucosetransporter 1; HSP, heat shock protein 70.1; TP, trophoblast protein.

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 1, 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 tran-

scription 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 IVP 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

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 (glutamyl-cysteine synthetase) gene in IVP bovine embryos (Harvey *et al.*, 1995).

Desmosome formation is correlated with the onset of blastocoel 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 form 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 trophectoderm. After isolation and culture of ICMs from early mouse blastocysts, the outer cells start to form a new trophectodermal 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 trophectoderm and inner cell mass cells of mouse embryos, while Glut-2 was restricted to the inner trophectodermal 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 pro-

duced *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 (Khurana 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 phosphoribosyl-transferase (HPRT), are known to be polyadenylated during oocyte maturation (Huarte *et al.*, 1987; Vassalli *et al.*, 1989; Sallés 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 rRNA (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,

1994). 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 (Aréchiga *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 trophectoderm 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 PGF_{2α} (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 IVP system for bovine embryos. It has been shown that culture conditions alter 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 IVP embryos (Kruip 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 IVP 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.

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