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Glucose Metabolism During Bovine Preimplantation Development: Analysis of Gene Expression in Single Oocytes and Embryos

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ABSTRACT Glucose metabolism of the bovine embryo is low during the first cleavages and increases sharply after the major resumption of the genome (8–16 cells). The mRNA level for genes involved in glucose metabolism was tested by RT-PCR on individual oocytes and embryos at different stages of development. These genes were: glucose transport GLUT-1, hexokinase (HK), glucose-6-phosphate-dehydrogenase (G6PDH), and glucose-phosphate-isomerase (GPI); actin was used as a reference transcript. RT-PCR results revealed three types of oocytes or embryos: *positive* with a PCR signal for each transcript considered, *nul* with no signal for any transcript, and *heterogeneous* with a PCR signal for some transcripts and none for others. The number of nul and heterogeneous samples was higher for slow than for fast-cleaving embryos (81% vs. 36%), and the proportion of positive embryos increased significantly at the 16-cell and morula stages ($P < 0.002$), suggesting a correlation between mRNA content and developmental capacity. In positive embryos, GLUT-1 mRNA level was reduced by half during maturation and fertilization. Actin and hexokinase mRNA levels decreased during the first cleavages, but significantly increased at the 16-cell and morula stages, respectively. GPI transcript remained stable throughout development, whereas there was a significant rise for G6PDH at the 4-cell stage, perhaps due to a polyadenylation process. Finally, the absence or decrease in intensity of several transcripts at the blastocyst stage suggests suboptimal culture conditions. *Mol. Reprod. Dev. 48:216–226, 1997.* © 1997 Wiley-Liss, Inc.

Key Words: bovine oocyte; bovine embryo; RT-PCR; gene expression; glucose switch

INTRODUCTION:

During early preimplantation stages, mammalian embryos do not metabolize much, if any, glucose. Moreover, in most species, in vitro early development is hampered in the presence of glucose, whereas later it may be required to support normal morula-blastocyst transition (reviewed by Leese, 1995; Barnett and Bavister, 1996).

Glucose uptake by in vitro produced bovine embryos increases steadily from the two-cell stage up to hatching blastocyst, whereas the increase in metabolism occurs mainly in two steps: one corresponding to the major onset of embryonic transcription (8–16 cell stage), the other at the time of compaction (Rieger et al., 1992). Although there is always some activity of the pentose phosphate pathway, the main metabolic pathway for glucose after the 8–16 cell stage and up to the blastocyst stage is glycolysis (Thompson et al., 1996).

Enzymatic studies in mouse and human embryos revealed that the switch to glucose utilization involves mainly the enzyme 6-phosphofructokinase (PFK) (Barbehenn et al., 1974) with a possible role for hexokinase and glucose transport (Gardner and Leese, 1988). Enzymatic reactions may be regulated at various levels. The amount of enzyme depends on the level of transcription, the stability of the message, and its rate of translation, but the activity of the protein also may depend on posttranscriptional regulation.

The purpose of this work was to study the regulation of transcription for several enzymes involved in glucose metabolism in individual in vitro produced bovine embryos according to their developmental stage and kinetics of cleavage, which is considered to be a criterion of developmental capacity (Bolton et al., 1989; Van Soom et al., 1992; Grisart et al., 1994; McKiernan and Bavister, 1994).

The bovine embryo relies on maternal transcripts and proteins up to the eight-cell stage when the major onset of zygotic transcription occurs (Frei et al., 1989). Metabolic changes during this period could depend on variable patterns of transcription for some of the enzymes we measured: hexokinase (HK) and the glucose transporter GLUT-1, which, as reported above, have been involved in the glucose switch, glucose-6-phosphate dehydrogenase (G6PDH) as a potential indicator

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of the pentose phosphate pathway (PPP) activity, and glucose-phosphate-isomerase (GPI) being involved in glycolysis. β-actin was chosen as a reference transcript.

MATERIALS AND METHODS

Embryo Production

Oocytes were collected by puncturing ovarian follicles from slaughtered cows. Intact cumulus-oocyte complexes (COCs) were matured for 20 hr in TCM 199 supplemented with 10% fetal calf serum (FCS), then fertilized with percoll separated spermatozoa at a concentration of 2.10^6 per ml in TALP medium. At 24 hr postinsemination (HPI), zygotes were stripped of cumulus cells by vortexing and cultured for 7 days in 30 µl droplets of modified SOF medium with 3 mg/ml of BSA under 5% CO_2 and 5% O_2 (Van Langendonckt et al., 1997), which contained 25–30 embryos.

In a first experiment, two-cell embryos were selected at 30 HPI, pooled, and cultured in drops already used for the first 10 hr of culture. One control drop was left intact. Each drop was assigned to one particular developmental stage. Embryos were selected at 45 HPI for the four-cell stage, 70 HPI for the 5–8 cell stage, 118 HPI for the 9–16 cell stage, 140 HPI for the morula stage, and 165 HPI for the blastocyst stage. This timing was first established in conditioned medium (Grisart et al., 1994), but was quite similar in mSOF + BSA (Van Langendonckt et al., 1997).

In a second experiment, some immature, mature, and fertilized oocytes (20 HPI) were harvested. The remaining zygotes were cultured and collected 30 HPI for the two-cell, 45 HPI for the four-cell, and 70 HPI for the eight-cell stages. In this case, uncleaved oocytes and two-cell embryos at 45 HPI, as well as four-cell embryos at 70 HPI, were also collected as samples of slow-cleaving or blocking embryos.

Once selected according to this scheme, oocytes and embryos were treated with 0.25% trypsin for 5 min at 37°C, vortexed for 5 min to remove all cumulus cells, washed five times in PBS without Mg^{++} and Ca^{++}, then stored individually in a minimal volume of deionised water (1–3 µl) at −70°C. Complete removal of cumulus cells by this method was confirmed by examining under a fluorescent microscope 20 immature oocytes treated the same way, stained with Hoechst 33342, and examined for contaminating nuclei.

RNA Extraction and Reverse Transcription

After the addition of 20 µg of glycogen as a carrier, the total RNA of each individual oocyte or embryo was extracted with 100 µl of ultraspec (Biotecx) and 20 µl of chloroform, vortexed 15 sec, then centrifuged to separate organic and aqueous phases. RNA was precipitated by adding two volumes of ethanol to the aqueous phase. The RNA pellet obtained after centrifugation was washed with 75% ethanol, then briefly dried before being dissolved in 5 µl of deionised DEPC-treated water. In the second experiment, a fixed amount of a reporter RNA was added to each sample in order to check the reproducibility of the method. This reporter RNA was produced from a mutated piece of the bovine hexokinase cDNA under a SP6 RNA polymerase promoter and followed by a polyA tail.

To unravel RNA secondary structure, samples were heated at 90°C for 5 min, then quick-chilled on ice, and immediately reverse-transcribed. Reverse transcription was performed at 37°C for 1 hr in a 10 µl volume containing 50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 200 ng oligod$T_{(12–18)}$ (Pharmacia); 1 mM of each dNTP (Pharmacia), 15 U. RNAGuard (Pharmacia), and 100 U. MMLV reverse transcriptase (Gibco-BRL). After reverse transcription, the volume of each sample was increased to 30 µl.

PCR Amplification

Primers. Primer pairs were obtained from Eurogentech (Allandale, NJ) or from Gibco-BRL (Gaithersburg, MD). These 20-base-long primers were selected according to the bovine sequence for hexokinase (Griffin et al., 1989), glucose transport GLUT-1 (Boado and Pardridge, 1991), and for β-actin (Degen et al., 1983). When the bovine sequence was not available, primers were chosen from interspecies conserved regions of the cDNA according to the mouse sequence for glucose-6-phosphate-dehydrogenase (G6PDH) (Zollo et al., 1993) and the human sequence for glucose-phosphate-isomerase (GPI) (Walker et al., 1990). All these primer pairs spanned at least an intron in order to differentiate RNA from DNA amplification (Table 1). The location and size of the introns were predicted according to rat or human genomic data. The selected introns were rather small, so DNA and RNA amplified bands were in the same size range, except for GLUT-1. Amplicons of the expected size were subsequently digested by one or two restriction enzymes in order to confirm their identity.

Amplification. Each amplification was done separately on 1/6th of the reverse transcription reaction in a 50 µl final volume containing 1.5 mM $MgCl_2$; 50 mM KCl; 10 mM Tris-HCl, pH 8.3 with 0.5 µM of primers; 0.05 mM of each dNTPs (Pharmacia, Gaithersburg, MD), and 1 unit of Taq polymerase (Dynazym). After a hot start of 6 min at 95°C, 35 cycles of amplification were performed as follows: denaturation at 95°C for 40 sec, annealing at 55–59°C (according to the primer pairs used) for 50 sec, extension at 72°C for 1 min. A final extension step of 5 min at 72°C ended the reaction.

Detection and quantitation. One-tenth of the amplification product was separated on 4% agarose gel and stained with ethidium bromide or 6% acrylamide gel coloured with silver nitrate. The image of each gel was digitized and the signal intensity for each PCR product quantitated by densitometric scanning with a computer-assisted image analysis system (NIH image v 1.56). The conditions of staining and imaging were normalized from one gel to another according to the intensity of the 267 bp band of molecular weight pBR322/Hae III.

Semiquantitative RT-PCR Assay

For each pair of primers some preliminary amplification experiments were carried out on a fixed amount of

TABLE 1. Primers Used for PCR

Gene	Primer sequence	Reference	Size		RNA restriction	
			DNA bp	RNA bp	Enzyme	Fragments size
B-ACTIN						
Sens	5′ GCGTGACATCAAGGAGAAGC 3′	bovine (Degen)	643	432	Dde I	216, 140, 54
Antisens	5′ TGGAAGGTGGACAGGGAGGC 3′	rat (Nudel)				
HK						
Sens	5′ TGCGGCTCTCTGATGAAACT 3′	bovine (Griffin)	355	166	Dde I	137, 29
Antisens	5′ TCCAGGGCGATGAAATCTCC 3′	rat (Kogure)			Sma I	94, 72
G6PDH						
Sens	5′ CAAGATGATGACCAAGAAGC 3′	mouse (Zollo)	407	200	Ava I	163, 37
Antisens	5′ AGCAGTGGTGTGAAGATACG 3′	human (Chen)				
GLUT-1						
Sens	5′ CGCTATTTGTGGTGGAACGA 3′	bovine (Boado)	?	345	Dde I	216, 129
Antisens	5′ CGGTGAAGATGATGAAGACG 3′	rat (Williams)			Eag I	322, 23
GPI						
Sens	5′ CGCCCAACCAACTCTATTGT 3′	human (Walker)	188	101	Bgl II	77, 24
Antisens	5′ CAGATGATGCCCTGAACGAA 3′					

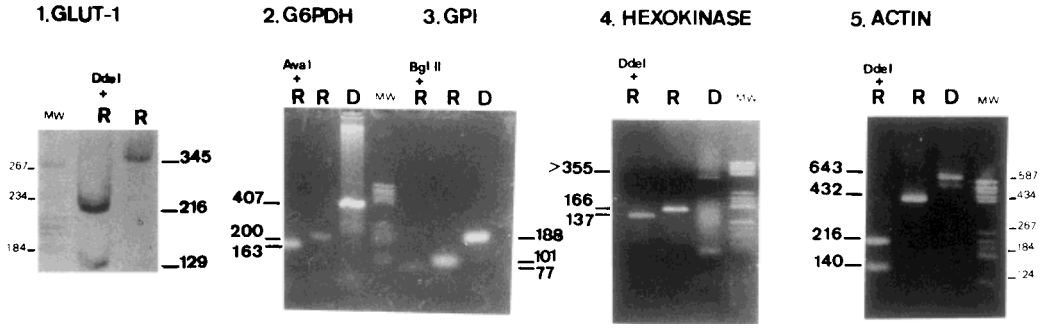

Fig. 1. PCR and RT-PCR products obtained on DNA (D) or RNA (R) extracted from bovine oviduct cells and amplified using five different pairs of primers. The validation of the PCR product on RNA is done by restriction diagnosis (Ava I, Bgl II, or Dde I + R). All sizes are in base pairs and bands <60 bp are not visualized on these gels. pBR322/Hae III fragments are used as molecular weight (MW).

RNA derived from bovine oviduct cells. These cells were scraped from a fresh oviduct and cultured for 24 hr under TCM 199 with 10% calf serum before being harvested. RNA was extracted with ultraspec as for oocytes and embryos, then quantified by spectrophotometry. As the bovine oocyte contains ~1 ng of total RNA (Olszanska and Borgul, 1993), 1 ng of RNA from oviduct cells was reverse transcribed and amplified as described previously. At this RNA concentration, the amplification reaction was stopped at every cycle from cycles 26–40, and the range of cycle numbers over which the amplification reaction was clearly exponential was established. This cycle number subsequently served to check that amounts of added RNA, ranging from 5 ng to 0.1 ng, gave a proportional output of amplicons.

The efficiency of RNA extraction and reverse transcription was checked by the PCR product that was obtained for the reference RNA added to each sample in the second experiment.

Statistical Analysis

The significance of differences in proportions was investigated by using logit linear models (Grizzle et al., 1969; Freeman, 1987). Levels of different mRNAs were analyzed by log-normal linear ANOVA models (McCullagh and Nelder, 1983). Different features of the observed proportions and mRNA level trends were assessed by using local polynomial contrasts; the a posteriori character of these contrasts is accounted for by the Scheffe method (Arnold, 1971). The statistical significance of the minima and maxima was assessed by testing the nullity of local quadratic contrasts. Null hypotheses were rejected at the conventional $P = 0.05$ level of significance.

RESULTS

Validity of the RT-PCR Assay

Primer pairs were chosen to give a different signal for RNA or DNA amplification, and the identification of all RT-PCR products was confirmed by restriction digestion. Examples of PCR results are presented in Figure 1. Several nonreverse transcribed embryos and other negative controls at different levels of the manipulation were included (data not shown).

Preliminary studies on amplification reactions showed that, for a fixed amount of initial RNA (1 ng), our system detected a linear increase in PCR products after 30 or 35 cycles of amplification according to the primer pairs (Fig. 2A). With 35 cycles, the intensity of ampli-

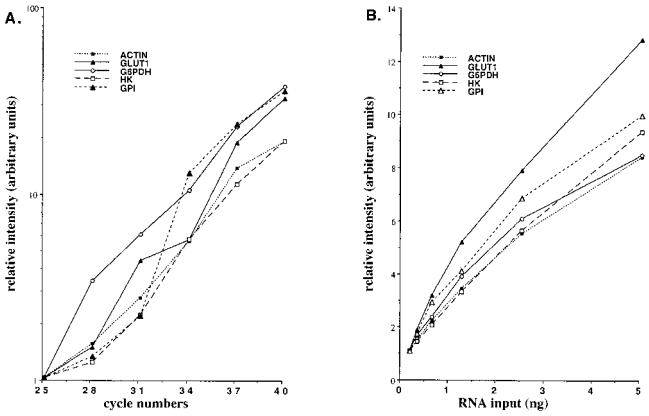

Fig. 2. Validation of the semiquantitative RT-PCR procedure regarding the number of cycles (**A**) or regarding the amounts of RNA input (**B**). mRNA was isolated from oviduct cells, reverse transcribed then amplified separately with the five different pairs of primers. After gel separation, RT-PCR products were quantitated by densitometric scanning of the digitized image. Densities were expressed in A relatively to the signal obtained with 25 cycles, RNA input was fixed at 1 ng. In B, it was expressed relatively to the signal obtained with 0.15 ng, PCR cycles number was fixed to 35.

TABLE 2. Developmental Distribution of Oocytes and Embryos According to Their mRNA Content

Stage	n	Positive n (%)*	Nul n (%)	Hetero-geneous n (%)*	G type n (%)[a]	A type n (%)[a]
Oocytes	85	54 (64)	12 (14)	19 (22)	11 (58)	6 (32)
2-cell	25	20 (80)	2 (8)	3 (12)	2 (67)	1 (33)
4-cell	38	23 (60)	3 (8)	12 (32)	7 (58)	2 (17)
8-cell	47	22 (47)	6 (13)	19 (40)	14 (74)	2 (11)
16-cell	24	16 (67)	2 (8)	6 (25)	4 (67)	0 (0)
Morula	18	16 (89)	0 (0)	2 (11)	2 (100)	0 (0)
Blasto	29	18 (62)	5 (17)	6 (21)	5 (83)	1 (17)
Slow cleaving	32	6 (19)	9 (28)	17 (53)	17 (100)	0 (0)
Total samples	298	175 (59)	39 (13)	84 (28)	62 (74)	12 (14)
Total fast cleaving[b]	181	115 (64)	18 (10)	48 (27)	34 (71)	6 (13)

*Overall significant differences in developmental distribution ($P < 0.05$).
[a]Percentage expressed in function of the number of heterogeneous samples only.
[b]Sum of the 2-, 4-, 8-, 16-cell, morula, and blastocyst.

cons was linearly correlated with variable amounts of initial RNA, ranging from 0.1 ng up to 5 ng, which is in the range of bovine oocyte total RNA (Fig. 2B). Consequently, the intensity of each PCR product obtained on a single oocyte or embryo depends on the initial quantity of mRNA in the sample.

The PCR products obtained on the exogenous polyA-RNA, which was added in the second experiment, were quite similar from sample to sample and confirmed the reproducibility of the method (see Fig. 6). The signal was completely lost for one embryo out of the 160 treated. That sample was excluded from our results.

General Transcriptional Data

The yield of blastocysts in the control drop was 30% vs. 62% for two-cell embryos selected at 30 HPI. In the first experiment, 10 mature oocytes and 129 fast-cleaving embryos could be individually analyzed by RT-PCR. In the second experiment, 160 samples were treated, 32 of them were delayed oocytes or embryos. As the results obtained from the two experiments were not significantly different, they were gathered in Table 2. The data on one- and two-cell embryos at 45 HPI, as well as four-cell embryos at 70 HPI, were pooled in one category called "slow-cleaving embryos." The total of the 2-, 4-, 8-, 16-cell, morula, and blastocyst embryos collected under the time scheme described above were named "fast-cleaving embryos."

Out of the 298 samples treated, 175 (59%) displayed a signal for each PCR performed and were called positive; 39 (13%) did not give PCR products with any primer pairs except for the exogenous added RNA. For these oocytes or embryos, the RNA was degraded before the manipulation, so they were called nul. Finally, 84 (28%) oocytes or embryos showed a strong signal for some primer pairs and none for other pairs. They were called heterogeneous.

Positive samples represented 64% of the oocytes and fast-cleaving embryos against only 19% for the slow-cleaving ones. The relationship between the percentage of positive samples and their developmental stage was tested with logit-linear modeling. Significant ($P < 0.05$) differences between developmental stages were found (Fig. 3). The quadratic behavior between the two-cell and morula stages shows a highly significant minimum ($P = 0.002$) at the eight-cell stage. The two maxima of the curve at the two-cell and morula stages are not significant ($P = 0.09$ and $P = 0.06$, respectively), although it is approaching significance for the morula peak. The same analysis with heterogeneous samples gave the opposite result as the distribution of nul oocytes or embryos did not show statistically significant variation according to developmental stage.

Different heterogeneous types could be observed when one or more mRNAs were undetectable. Two cases were frequent (88%): either a nul actin and a strong signal for G6PDH, called G type (I in Fig. 4.), or the opposite with no signal for G6PDH and a strong actin signal, called A type (II in Fig. 4). The other signals were variable, hexokinase, and GPI, being mostly positive, and GLUT-1, mostly negative (GPI and GLUT-1 signals are not shown in Fig. 4). The A type is present mainly in the early stages, and none is described in slow-cleaving embryos, whereas the "G type" is the only one described for this category and the most frequent one for all stages (Table 2).

Expression Pattern for Different Genes Analyzed

In order to design temporal patterns of expression, only positive samples of the two experiments were considered. In the first experiment, the patterns went from the mature oocyte up to the blastocyst stage; in the

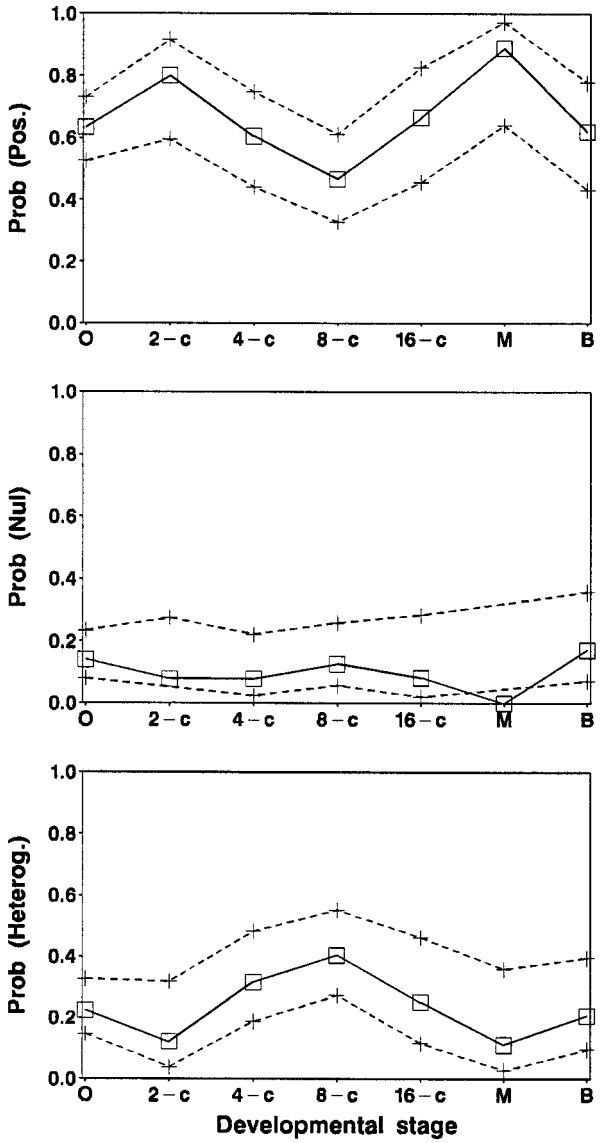

Fig. 3. Probability of occurence (solid line) for different "RNA categories" of oocytes or embryos as a function of the developmental stage (probabilities estimated by the observed proportions, Table 2). The confidence interval ($P = 0.95$) is given by the dotted line. Statistical analysis of these categorical data was done by linear models. Results mostly showed a significant minimum of positive embryos at the eight-cell stage ($P = 0.002$), as well as a correlated maximum of heterogeneous embryos at the same stage. The proportion of nul embryos showed no significant variation throughout development. Note that the approximation used to compute the linear categorical model does not provide an accurate confidence interval when the observed proportion is zero, which is the case at the morula stage. O: immature, mature, and fertilize oocytes; 2-c: 2-cell; 4-c: 4-cell; 8-c: 8-cell; 16-c: 16-cell; M: morula; B: blastocyst. Pos.: positive sample with a PCR product for each transcript analyzed, Nul: samples with no PCR product for any transcript analyzed; Heterog.: heterogeneous samples with a PCR product for some transcripts, whereas none for others.

second experiment, they went from the immature oocyte to the 8-cell stage, the data being excluded from delayed embryos. In that second experiment, GLUT-1 results were available, but there was no more information on endogenous hexokinase as it competed for amplification with our reporter RNA. The results obtained for actin, G6PDH, and GPI in the two experiments were not statistically different, so they were pooled in order to design temporal patterns of expression (Fig. 5). For these three genes, the data are expressed as the mean of 15–24 individual values for each developmental stage.

Actin. There was a small decrease from the immature oocyte up to the eight-cell stage, the actin mRNA level being finally reduced by half ($P = 0.01$). Actin mRNA then showed a sharp, highly significant rise starting at the 16-cell stage and lasting up to the morula stage ($P = 0.001$). The drop at the blastocyst stage was also significant ($P = 0.01$).

Glucose-6-phosphate-dehydrogenase. The striking feature here was a maximum in PCR products at the four-cell stage. This increase can be visualised in Figure 6. The quadratic component of this peak was highly significant ($P = 0.003$).

Glucose-phosphate-isomerase. This transcript was very stable during maturation, fertilization, and preimplantation development. Individual values within or between stages were quite similar.

Results on hexokinase and GLUT-1 were derived from one experiment only, experiment 1 for hexokinase and experiment 2 for GLUT-1. As a consequence, there were no data available for hexokinase about immature and fertilized oocytes, whereas for GLUT-1, there were no data available for development after the ZGA. Between 7–18 individual values were pooled for each developmental stage (Fig. 5).

Hexokinase. There was a highly significant drop in the mRNA level starting at the four-cell stage and reaching a minimal value at the 8- and 16-cell stages ($P = 0.001$), followed by a sharp rise at the morula stage ($P = 0.001$). As for actin, a significant drop in the amount of PCR products was observed as the embryo proceeded to the blastocyst stage ($P = 0.05$).

GLUT-1. There was a strong decrease occurring during maturation and fertilization. The level reached at the two-cell stage was significantly different from the value observed in the immature oocyte ($P = 0.01$). From the two-cell stage up to the eight-cell stage, the amount in PCR products did not change.

DISCUSSION

About 30–35% of the bovine oocytes originated from abattoir ovaries, matured and fertilized in vitro, reach the blastocyst stage in culture (Brackett and Zuelke, 1993). Not only are the oocytes collected in that way an extremely heterogeneous form of material (Gordon and Lu, 1990), but also in vitro maturation conditions are not equivalent to the in vivo process (Greve, 1987; Hyttel, 1989). Consequently, some oocytes of our experiments have not acquired their developmental competence. Although only morphologically normal oocytes were selected, 36% of them, whether immature, mature, or fertilized, had an abnormal mRNA content (nul or heterogeneous). Crozet et al. (1986) and Fair et al.

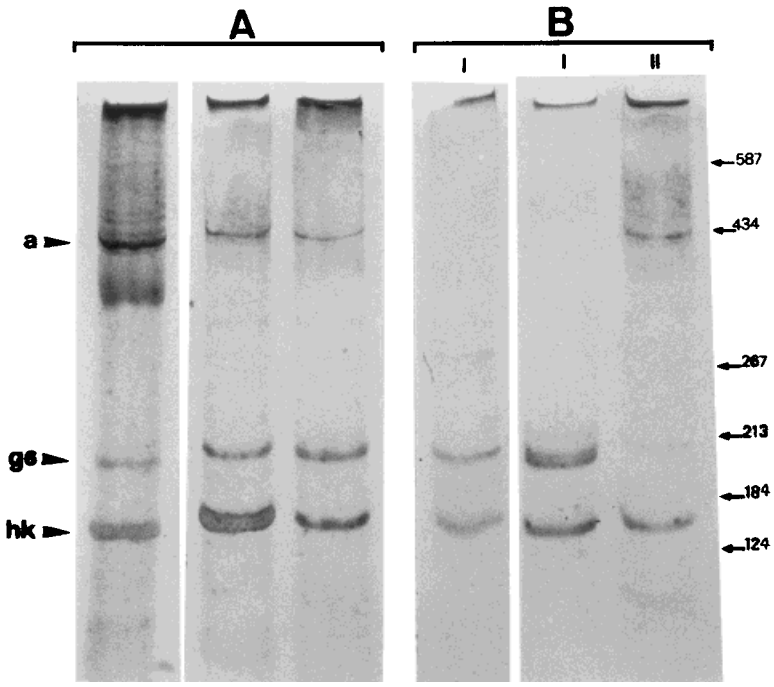

Fig. 4. PCR Products obtained with three different pairs of primers after individual RNA extraction and RT-PCR on two-cell embryos. PCR product for actin (**a**), for Glucose-6-phosphate dehydrogenase (g6) and for hexokinase (hk). **A** shows three positive embryos with a PCR signal for the three primers pairs; **B** shows heterogeneous embryos with either a good signal for G6PDH while the actin band is absent (G type, or I), or a nice signal for actin while the G6PDH band is weak (A type, or II). Hexokinase signal is always present here.

(1995) showed that small size oocytes or those derived from small size follicles (which is correlated) had a high RNA synthesis, whereas larger oocytes exhibited little or no RNA activity. The first ones probably have not yet stored the needed amount of RNA to ensure further development. This could corroborate our observation.

After the selection of the two-cell embryos at 30 HPI, described as the best ones (Grisart et al., 1994), the percentage of embryos with an abnormal RNA content decreased from 36% to 20%. Although not significant, this can suggest a correlation between embryo quality and mRNA content. As embryonic development reaches the four- and eight-cell stages, the percentages rise to 40% and 53%, respectively. An exaggerated degradation of maternal mRNA could be a possible explanation. In cattle embryos, total protein synthesis gradually declines from the zygote stage to reach a minimum at the eight-cell stage (Frei et al., 1989). In the mouse, this protein decline is correlated with a sudden loss of maternal mRNA (Bachvarova et al., 1985). For the bovine embryo, the major zygotic gene activation (ZGA) starts at the eight-cell stage (King et al., 1988; Kopecny et al., 1989; Barnes and Eyestone, 1990). Both the increase in embryonic mRNA and the selective degradation of maternal mRNA are necessary for a correct maternal-zygotic transition (MZT) (reviewed by Telford et al., 1990), and this critical step is often associated with early developmental arrest (Eyestone and First, 1991). About 60% of the selected two-cell embryos will become blastocysts, but 40% of them will block earlier, mainly at the 5–8 cell stages. The 40% and 53%, respectively, of four- and eight-cell embryos with an abnormal mRNA content could constitute that population of blocking embryos. When zygotic transcription is prevented by α-amanitin, some embryos will reach the 9–16-cell stage, but not any further (Barnes and First, 1991). It could explain the remaining 33% of nul and heterogeneous embryos at the 16-cell stage, whereas very few of them (11%) are found at the morula stage. Only embryos with sufficient maternal mRNA and a correct MZT could reach this critical developmental step. Reaching the blastocyst stage seems to be a new challenge, at least in our in vitro conditions, where the medium is unchanged for all the culture period. At the blastocyst stage, the number of positive embryos decreases and patterns of expression for some genes, mainly actin and hexokinase, fall. Such RNA degradation for a cellular component as ubiquitous as actin is unexpected when both the cell number and the net weight of the embryo should be increasing. It is also surprising to see a decrease in hexokinase mRNA when glucose consumption should be higher to ensure the cavitation process. mRNA degradation could be due to nonoptimal in vitro conditions. Similarly, Wrenzycki et al. (1996) have reported that the connexin 43 transcript is missing in in vitro produced bovine blastocysts, whereas this transcript is present at previous embryonic stages and in blastocysts produced in vivo.

We should note that due to a high percentage of embryos with an abnormal mRNA content during the

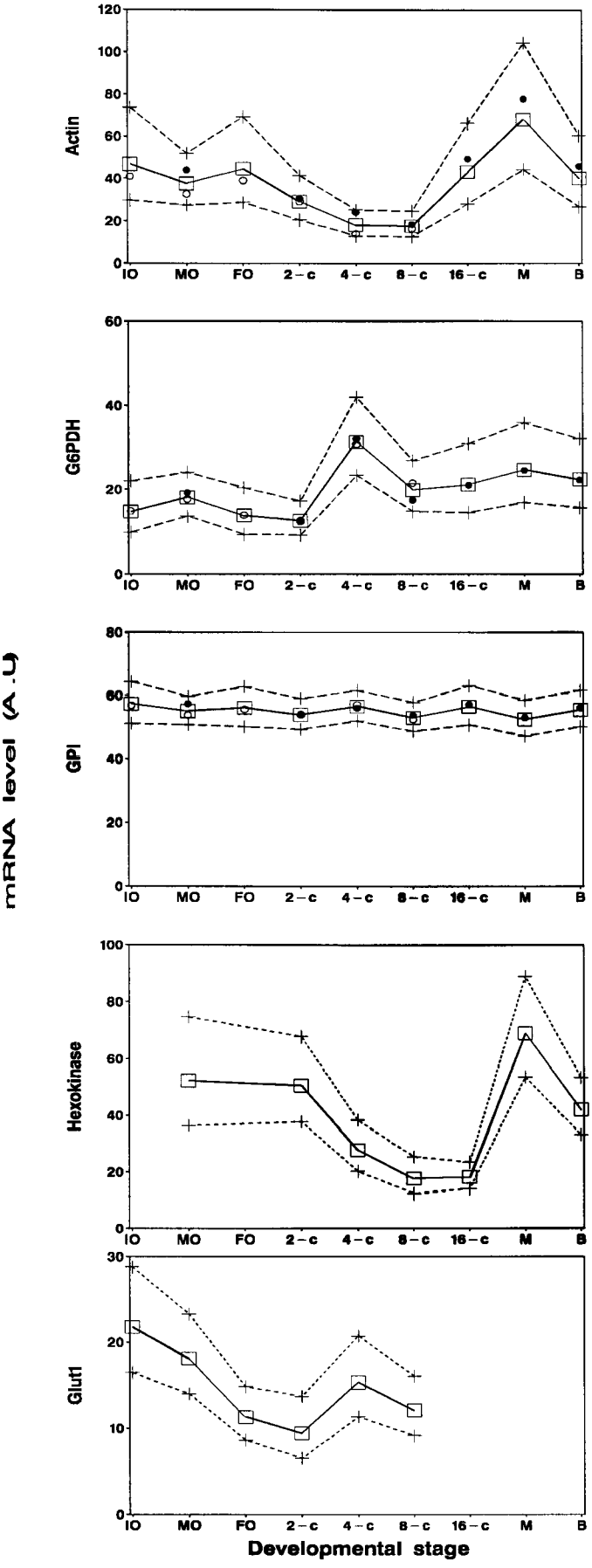
mRNA level (A.U)
Actin
G6PDH
GPI
Hexokinase
Glut1
IO
MO
FO
2-c
4-c
8-c
16-c
M
B
Developmental stage

Fig. 5.

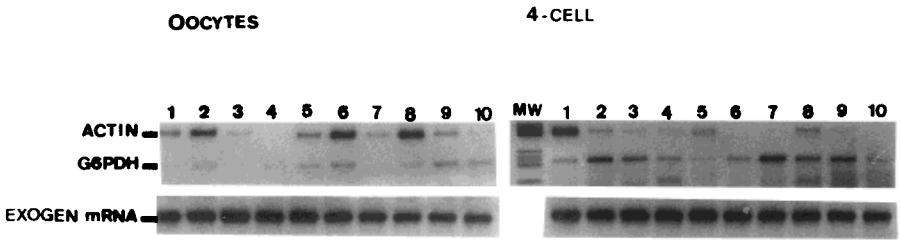

Fig. 6. PCR products obtained for actin and G6PDH transcripts, after individual RNA extraction and reverse transcription as described in Materials and Methods, on 10 immature oocytes and 10 embryos at the four-cell stage picked at 45 HPI. For each sample, results of the amplification on the exogenous added polyARNA are also reported. The mean value of the PCR product obtained on G6PDH transcript is clearly more elevated for the four-cell embryo than for the immature oocyte.

4-, 8- and 16-cell stages, patterns of expression designed from pools of embryos will often reveal an exaggerated mRNA decay during these stages.

The degradation rate of RNA messengers can vary according to the mRNA species (Paynton et al., 1988). Both the mRNA structure and its intracellular environment will influence the turnover (reviewed by Ross, 1996). Furthermore, Ho et al. (1994) showed that for mouse embryos, NaCl concentration in the culture medium has a different influence on the stability of various mRNAs before the morula stage. Surprisingly, in the present study some embryos displayed a strong signal for hexokinase, GPI, or G6PDH mRNAs, whereas the actin mRNA signal, chosen as a reference for embryo quality, was very weak, or even absent. Since actin mRNA is ubiquitous, its stability must be more quickly affected by suboptimal conditions than other genes. G6PDH and GPI transcripts are the most stable in delayed or blocked embryos. GLUT-1 is the first signal to disappear, but as its initial molecule count is unknown, its early disappearance could be due to unstability or scarcity.

Bovine β-actin mRNA is reduced by half during the early stages, then shows a sharp rise simultaneously with the ZGA. A similar pattern is reported for the mouse with a drop during oocyte maturation up to the end of the two-cell stage, followed by a sharp rise, also coincident with ZGA (Bachvarova et al., 1989; Taylor and Piko, 1990). According to the technique used, the range of this drop varies (Paynton et al., 1988; Temeles et al., 1994; Rhambatla et al., 1995). The increase in β-actin mRNA after the ZGA could reflect a higher cell number, but also a higher number of transcripts per cell (Heikinheimo et al., 1995). The low level of actin at the blastocyst stage is unexplained. We have already proposed the hypothesis that our in vitro conditions are not optimal for blastocyst survival.

The transcription of enzymes implicated in glucose metabolism may be discussed in terms of their chronological appearance in glucose consumption. Glucose is known to enter into preimplantation mouse embryos through facilitative diffusion mediated by the GLUT proteins (Gardner and Leese, 1988). RT-PCR on mouse embryos have demonstrated the presence of the GLUT-1 isoform throughout preimplantation development and the GLUT-2 isoform after the eight-cell stage (Hogan et al., 1991). Some of our unpublished results demonstrate that GLUT-1 transcript is also present throughout preimplantation development in cattle, but we have semiquantitative data only up to the eight-cell stage. GLUT-1 mRNA level is decreased by half during maturation and fertilization. This can be explained by the general degradation of maternal mRNA initiated during oocyte maturation, which affects 50% of some mRNAs (Paynton, 1988). The low level of remainining mRNA could account for the weak glucose uptake during the early stages as described by Rieger (1992). It will be interesting to obtain semiquantitative data after the major resumption of the embryonic genome. In the mouse, GLUT-1 expression increases sharply between the two-cell and the blastocyst stage (Morita et al., 1994), but here again this increase is far lower for in vitro produced blastocysts. As the bovine sequence of GLUT-2 isoform is unknown and not well conserved between species, we were unable to check its presence.

Intracellular glucose is phosphorylated by hexokinase. The temporal pattern of hexokinase shows a sharp decrease from the four-cell stage up to the 16-cell stage, followed by a strong increase at the morula stage coincident with the second increase in glucose consump-

Fig. 5. Temporal pattern of expression for several genes during bovine oocyte maturation, fertilization, and preimplantation embryonic development (IO: immature oocyte, MO: mature oocyte, FZ: fertilized zygote, 2-c, 4-c, 8-c, 16-c: 2-cell, 4-cell, 8-cell, 16-cell, M: morula and B: blastocyst). Oocytes or embryos were individually RT-PCR as described in Materials and Methods. After gel separation, signals corresponding to PCR products were quantified by densitometric scanning of the digitized image. Calibration was done according to a standard included in each gel and efficiency of RNA extraction and RT was controlled by the amplification of the reference RNA. Results are integrated OD. Final data are expressed as the mean and confidence interval for the mean ($P = 0.95$). For Actin, G6PDH, and GPI, there were two experiments pooled, or an average of 18 individual values for each developmental stage. Black and white circles are used to plot the means corresponding to the two separate experiments. Preliminary tests have showed no experiment-treatment interaction so that a restricted model excluding the corresponding terms was used to estimate a common curve (squares). For hexokinase and GLUT-1, there were only one experiment and an average of 13 individual values at each stage. Consequently for hexokinase, there were no data on immature and fertilized oocyte, whereas GLUT-1 pattern of expression is not going further the eight-cell stage.

tion (Rieger et al., 1992). A similar dramatic increase for the enzyme activity at the morula stage has been reported for the mouse (Hooper and Leese, 1988; Ayabe et al., 1994) and human embryos (Martin et al., 1993) but in these studies, the activity was low from the oocyte to the morula stage, whereas we showed here the presence of maternal transcripts for hexokinase, which gradually disappears before being replaced by zygotic mRNA. A recent study (Houghton, 1996) also reported the presence of HK transcript throughout mouse preimplantation development. Either these maternal transcripts are not translated into proteins, or their corresponding activity is too low to be detected. This enzyme also can be inhibited by cellular compartmentalization (Miccoli et al., 1996).

Glucose-6-phosphate can be transformed into glycogen, metabolized through the pentose-phosphate pathway (PPP), or through glycolysis. We have no information about glycogen formation, but we have investigated the PPP activity according to the expression of G6PDH, which catalyzes the first and irreversible step of this pathway. Our temporal expression pattern for G6PDH does not correspond to the protein activity pattern described for other mammalian species. In mice, G6PDH activity drops suddenly upon ovulation (De Schepper et al., 1985), then stabilizes up to the eight-cell stage and again falls abruptly afterward (Leese, 1987), whereas a more gradual decline was observed for human embryos (Martin et al., 1993). This down regulation seems to be due to a decrease in the number of molecules rather than to a change in kinetic properties (De Schepper et al., 1993), and according to the same study, embryonic transcription of this X-linked enzyme is unlikely as the distribution of its activity was not bimodal, whereas females should express twice as many molecules as males. Our results do not show any decrease in G6PDH transcript while development proceeds, but underline a significant temporary increase at the four-cell stage before the major ZGA. As our reverse transcription is done with oligo dT, changes in the amount of PCR products can correspond to a change in transcript abundance, but also to a change in the polyadenylation state. Polyadenylation can modify the stability and the rate of translation of the mRNA. This kind of regulation has been described for the cdk4 gene in the mouse embryo (Moore et al., 1996). The G6PDH gene is described as a "sentinel" for oxidative stress, leading rapidly to the generation of NADPH for maintenance of the cellular redox state. This adaptive response is mainly due to an increase in transcription, mRNA stability, and the rate of protein synthesis (reviewed by Kletzien et al., 1994). So the increase in PCR product detected here could be due to changes in mRNA polyadenylation due to suboptimal in vitro conditions. Experience with α-amanitin, which prevents zygotic transcription, could test this hypothesis. In addition, it has been reported that for in vivo produced bovine embryos, the activity of the PPP increases threefold after 24 hr of culture and is significatively higher for poor quality embryos (Javed and Wright, 1990).

For the glycolytic pathway, phosphofructokinase (PFK) is the key enzyme, but its activity is mostly regulated at a posttranslational level with an allosteric control by ATP and several other metabolites. Glucose-phosphate-isomerase (GPI) catalyzes the interconversion of the glucose-6-phosphate and fructose-6-phosphate, a reaction near to equilibrium. In the mouse, the transition from oocyte-coded to embryo-coded GPI has been extensively studied by exploiting its numerous genetic variants. The oocyte-coded GPI activity is stable until the eight-cell stage and then declines, whereas embryonic GPI can be detected at the early morula stage (West et al., 1986). For both mouse and human embryos, a high level of GPI activity is maintained during the early preimplantation period, but then falls to a minimum at the blastocyst stage in mice. There is no evidence of such a decline in humans (West et al., 1989). In the present study, the temporal pattern of GPI transcripts is quite stable according to developmental stage. This could be due, as in mice, to a high stability of the maternal transcripts, but also to a gradual and balanced substitution by embryonic transcripts.

In conclusion, RT-PCR studies have indicated that some bovine oocytes and embryos have an abnormal mRNA content. Their distribution, according to developmental stages or kinetics of cleavage, suggests a correlation between mRNA content and developmental capacities. Increase of hexokinase mRNAs at the morula stage could be responsible for the increase in glucose consumption described at compaction, whereas the low level of GLUT-1 mRNA after oocyte maturation is perhaps the reason for a low glucose uptake during the first cleavages. Increase in G6PDH PCR products at the four-cell stage could be an adaptative process to culture conditions. The absence or drop in intensity for several transcripts in blastocysts probably suggests that our in vitro culture conditions are nonoptimal.

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REFERENCES

Arnold SF (1971): The theory of linear models and multivariate analysis. New York: Wiley & Sons.

Ayabe T, Tsutsumi O, Taketani (1994): Hexokinase activity in mouse developed in vivo and in vitro. Human Reprod 9:347–351.

Bachvarova R (1985): Gene expression during oogenesis and oocyte development in the mammal. In Browder L.W. (ed): "Developmental Biology: A Comprehensive Synthesis, Vol. 1. Oogenesis." New York: Plenum Press, pp 453–524.

Bachvarova R, Cohen EM, De Leon V, Tokunaga K, Sakiyama S, Paynton BV (1989): Amounts and modulation of actin mRNAs in mouse oocytes and embryos. Development 106:561–565.

Barbehenn EK, Wales RG, Lowry OH (1974): The explanation for the blockade of glycolysis in early mouse embryos. Proc Natl Acad Sci USA 71:1056–1060.

Barnes FL, Eyestone WH (1990): Early cleavage and the maternal zygotic transition in bovine embryos. Theriogenology 33:141–152.

Barnes FL, First NL (1991): Embryonic transcription in in vitro cultured bovine embryos. Mol Reprod Dev 29:117–123.

Barnett DK, Bavister BD (1996): What is the relationship between the metabolism of preimplantation embryos and their developmental competence? Mol Reprod Dev 43:105–133.

Boado RJ, Pardridge WM (1991): Molecular cloning of the bovine blood-brain barrier glucose transporter cDNA and demonstration of phylogenetic conservation of the 5′ untranslated region. Mol Cell Neurosci 1:224–232.

Bolton VN, Hawes SM, Taylor CT, Parsons JH (1989): Development of spare human preimplantation embryos in vitro: An analysis of the correlations among gross morphology, cleavage rates, and development to the blastocyst. J In Vitro Fertilization and Embryo Transfer 6:3035.

Brackett B, Zuelke K (1993): Analysis of factors involved in the in vivo production of bovine embryos: Theriogenology 39:43–64.

Chen EY, Cheng A, Lee A, Kuang W, Hillier L, Green P, Schlessinger D, Ciccodicola A, D'Urso M (1991): Sequence of human glucose-6-phosphate dehydrogenase cloned in plasmids and a yeast artificial chromosome (YAC). Genomics 10:792–800.

Crozet N, Kanka J, Motlik J, Fulka J (1986): Nucleolar fine structure and RNA synthesis in bovine oocytes from antral follicles. Gamete Res 14:65–73.

Degen JL, Neubauer MG, Friezner Degen SJ, Seyfried CE, Morris DR (1983): Regulation of protein synthesis in mitogen-activated bovine lymphocytes: Analysis of actin-specific and total mRNA accumulation and utilization. J Biol Chem 258:12153–12162.

De Schepper GG, Van Noorden CJF, Koperdraad F (1985): A cytochemical method for measuring enzyme activity in individual preovulatory mouse oocytes. J Reprod Fertil 74:709–716.

De Schepper GG, Vander Perk C, Westerveld A, Oosting J, Van Noorden CJF (1993): In situ glucose-6-phosphate dehydrogenase activity during development of pre-implantation mouse embryos. Histochemical J 25:299–303.

Eyestone WH, First NL (1991): Characterization of developmental arrest in early bovine embryos cultured in vitro. Theriogenology 35:612–624.

Fair T, Hyttel P, Greve T (1995): Bovine oocyte diameter in relation to maturational competence and transcriptional activity. Mol Reprod Dev 42:437–442.

Freeman DH Jr (1987): "Applied Categorical Analysis." New York: Marcel Dekker.

Frei RE, Schultz GA, Church RB (1989): Qualitative and quantitative changes in protein synthesis occur at the 8–16 cell stage of embryogenesis in the cow. J Reprod Fertil 86:637–641.

Gardner DK, Leese HJ (1988): The role of glucose and pyruvate transport in regulating nutrient utilization by preimplantation mouse embryos. Development 104:423–429.

Gordon I, Lu K (1990): Production of embryos and its impact on livestock production. Theriogenology 33:77–87.

Greve T, Bousquet T, King WA, Betteridge VJ (1984): In vitro fertilization and cleavage of in vivo matured bovine oocytes. Theriogenology 22:151–165.

Griffin LD, MacGregor GR, Muzny DM, Harter J, Cook RG, McCabe ERB (1989): Synthesis and characterization of a bovine hexokinase 1 cDNA probe by mixed oligonucleotide primed amplification of cDNA using high complexity primer mixtures. Biochem Med Metabol Biol 41:125–131.

Grisart B, Massip A, Dessy F (1994): Cinematographic analysis of bovine embryo development in serum-free oviduct-conditioned medium. J Reprod Fertil 101:257–264.

Grizzle JE, Starner CF, Koch GG (1969): Analysis of categorical data by linear models. Biometrics 25:489–504.

Heikinheimo O, Lanzendorf SE, Baka SG, Gibbons WE (1995): Cell cycle genes c-mos and cyclin B1 are expressed in a specific pattern in human oocytes and preimplantation embryos. Mol Human Reprod 10:699–707.

Ho Y, Doherty AS, Schultz RM (1994): Mouse preimplantation embryo development in vitro: effect of sodium concentration in culture media on RNA synthesis and accumulation and gene expression. Mol Reprod Dev 38:131–141.

Hogan A, Heyner S, Charron MJ, Copeland NG, Gilbert DJ, Jenkins NA, Thorens B, Schultz GA (1991): Glucose transporter gene expression in early mouse embryos. Development 113:363–372.

Hooper MK, Leese HJ (1989): Activity of hexokinase in mouse oocytes and preimplantation embryos. Biochem Soc Trans 17:546–547.

Houghton FD, Sheth B, Moran B, Leese HJ, Fleming TP (1996): Expression and activity of hexokinase in the early mouse embryo. Mol Human Reprod 2:793–798.

Hyttel P, Greve T, Callesen H (1989): Ultrastructural aspects of oocyte maturation and fertilization in cattle. J Reprod Fertil 38:35–47.

Javed MH, Wright RW (1991): Determination of the pentose phosphate and Embden-Meyerhof pathway activities in bovine embryos. Theriogenology 35:1029–1037.

King WA, Niar A, Chartrain I, Betteridge KJ, Guay P (1988): Nucleolus organizer regions and nucleoli in preattachment embryos. J Reprod Fertil 82:87–95.

Kletzien RF, Harris PKW, Foellmi LA (1994): Glucose-6-phosphate dehydrogenase: a housekeeping enzyme subject to tissue-specific regulation by hormones, nutrients, and oxidant stress. FASEB J. 8:174–181.

Kogure K, Shinohara Y, Terada H (1993): Evolution of the type II hexokinase gene by duplication and fusion of the glucokinase gene with conservation of its organization. J of Biol Chem 268:8422–8424.

Kopecny V, Fléchon JE, Camous S, Fulka J (1989): Nucleogenesis and the onset of transcription in the eight-cell bovine embryo: fine-structural autoreadiographic study. Mol Reprod Dev 1:79–90.

Leese HJ (1987): Non-invasive methods for assessing embryos. Hum Reprod 2:435–438.

Leese HJ (1995): Metabolic control during preimplantation mammalian development. Human Reprod Update 1:63–72.

Martin KL, Hardy K, Winston RML, Leese HJ (1993): Activity of enzymes of energy metabolism in single human preimplantation embryos. J Reprod Fertil 99:259–266.

McCullagh P, Nelder JA (1983): "Generalize Linear Models." London: Chapman and Hall.

McKiernan SH, Bavister BD (1994): Timing of development is a critical parameter for predicting successful embryogenesis. Human Reprod 9:2123–2129.

Miccoli L, Oudard S, Sureau F, Poirson F, Dutrillaux B, Poupon MF (1996): Intracellular pH governs the subcellular distribution of hexokinase in a glioma cell line. Biochem J 313:957–962.

Moore GD, Ayabe T, Kopf GS, Schultz RM (1996): Temporal patterns of gene expression of G1-S cyclins and cdks during the first and second mitotic cell cycles in mouse embryos. Mol Reprod Dev 45:264–275.

Morita Y, Tsutsumi O, Oka Y, Taketani Y (1994): Glucose transporter GLUT1 mRNA expression in the ontogeny of glucose incorporation in mouse preimplantation embryos. Biochem Biophys Res Commun 199:1525–1531.

Nudel U, Zakut R, Shani M, Neuman S, Levy Z, Yaffe D (1983): The nucleotide sequence of the rat cytoplasmic b-actin gene. Nucleic Acids Res 11:1759–1771.

Olszanska B, Borgul A (1993): Maternal RNA content in oocytes of several mammalian and avian species. J Experimental Zool 265:317–320.

Paynton BV, Rempel R, Bachvarova R (1988): Changes in state of polyadenylation and time course of degradation of maternal mRNAs during oocyte maturation and early embryonic development in the mouse. Dev Biol 129:304–314.

Rhambatla L, Patel B, Dhanasekaran K, Latham KE (1995): Analysis of G protein a subunit mRNA abundance in preimplantation mouse embryos using a rapid quantitative RT-PCR approach. Mol Reprod Dev 41:314–324.

Rieger D, Loskutoff NM, Betteridge KJ (1992): Developmentally related changes in the uptake and metabolism of glucose, glutamine and pyruvate by cattle embryos produced in vitro. Reprod Fertil Dev 4:547–557.

Ross J (1996) Control of messenger RNA stability in higher eukaryotes. TIG 12:171–174.

Taylor KD, Piko L (1990): Quantitative changes in cytoskeletal b- and g-actin mRNAs and apparent absence of sarcomeric actin gene transcripts in early mouse embryos. Mol Reprod Dev 26:11–121.

Telford NA, Watson AJ, Schultz GA (1990): Transition from maternal to embryonic control in early mammalian development: A comparison of several species. Mol Reprod Dev 26:90–100.

Temeles GL, Ram PT, Rothstein JL, Schultz RM (1994): Expression patterns of novel genes during mouse preimplantation embryogenesis. Mol Reprod Dev 37:121–129.

Thompson JG, Partridge J, Houghton FD, Cox CI, Leese HJ (1996): Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. J Reprod Fertil 106:299–306.

Van Langendonckt A, Donnay I, Schuurbiers N, Auquier P, Carolan C, Massip A, Dessy F (1997): Effects of supplementation with fetal calf serum on development of bovine embryos in synthetic oviduct fluid medium. J Reprod Fertil 109:87–93.

Van Soom A, Van Vlaenderen I, Mahmoudzadeh R, Dluyker H, de Kruif A (1992): Compaction rate of in vitro fertilized bovine embryos related to the interval from insemination to first cleavage. Theriogenology 38:905–919.

Walker JI, Faik P, Morgan MJ (1990): Characterisation of the 5′ end of the gene for human glucose phosphate isomerase (GPI). Genomics 7:638–643.

West JD, Leask R, Green JF (1986): Quantification of the transition from oocyte-coded to embryo-coded glucose phosphate isomerase in mouse embryos. J Embryol Exp Morphol 97:225–237.

West JD, Flockhart JH, Angell RR, Hillier SG, Thatcher SS, Glasier AF, Rodger MW, Baird DT (1989): Glucose phosphate isomerase activity in mouse and human eggs and pre-embryos. Human Reprod 4:82–85.

Wrenzycki C, Carnwath JW, Niemann H (1996): Expression of the gap junction gene connexin43 (CX43) in preimplantation bovine embryos derived in vivo or in vitro. Theriogenology 41:251.

Williams SA, Birnbaum MJ (1988): The rat facilitated glucose transporter gene. J Biol Chem 263:19513–19518.

Zollo MZ, D'Urso M, Shlessinger D, Chen EY (1993): Sequence of mouse glucose-6-phosphate dehydrogenase cDNA. DNA seq 3:319–322.