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In Vitro Production and Nuclear Transfer Affect Dosage Compensation of the X-Linked Gene Transcripts G6PD, PGK, and Xist in Preimplantation Bovine Embryos[1]

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ABSTRACT

Equal expression of X-linked genes such as G6PD and PGK in females and males and the initiation of X-chromosome inactivation are critically dependent on the expression of the X-inactive specific transcript (Xist). The objective of the present study was to determine the effects of in vitro production (IVP) and nuclear transfer (NT) on the relative abundance (RA) of the X-linked transcripts G6PD, PGK, and Xist in preimplantation bovine embryos. In experiment 1, sex-determined IVP or in vivo-produced embryos were analyzed for mRNA expression of the 3 genes. The sex ratio was 36% vs. 64% in IVP blastocysts and thus deviated significantly from the expected ratio of 50% in the vivo control group. The RA of G6PD transcripts was significantly higher in female IVP embryos than in male embryos. In contrast, no significant differences were seen between in vivo-derived female embryos and their male counterparts. At the morula stage, female IVP embryos transcribed significantly more PGK mRNA than did male embryos. However, blastocysts did not exhibit significant differences in PGK transcripts. No differences were observed for in vivo-derived embryos with regard to the RA of PGK transcripts. The RA of Xist mRNA was significantly higher in all female embryos than in their male counterparts. In experiment 2, IVP, in vivo-developed, NT-derived, and parthenogenetic embryos carrying two X chromosomes of either maternal and paternal origin or of maternal origin only (parthenogenotes) were analyzed for the RA of the 3 genes. In NT-derived morulae, the RA of G6PD transcripts was significantly increased compared with their IVP and in vivo-generated counterparts. G6PD transcript levels were significantly increased in IVP blastocysts compared with in vivo-generated and parthenogenetic embryos. At the morula stage, PGK transcripts were similar in all groups, but the RA of PGK transcripts was significantly higher in IVP blastocysts than in their in vivo-generated, parthenogenetic, and NT-derived counterparts. The RA of Xist was significantly elevated in NT-derived morulae compared with IVP, in vivo-generated, and parthenogenetic embryos. NT-derived blastocysts showed an increased Xist expression compared with that of IVP, in vivo-generated, and parthenogenetic embryos. Results of the present study show for the first time that differences in X-chromosome-linked gene transcript levels are related to a perturbed dosage compensation in female and male IVP and female NT-derived embryos. This finding warrants further studies to improve IVP systems and NT protocols to ensure the production of embryos with normal gene expression patterns.

early development, embryo, gene regulation, in vitro fertilization

INTRODUCTION

Dosage compensation for X-linked genes between males and females is achieved by transcriptional inactivation of one of the two X chromosomes [1]. The Xist (X-inactive specific transcript) plays a crucial role during X-chromosome inactivation [2]. Xist is exclusively transcribed from the inactive X chromosome [3, 4]. Xist was detected for the first time in bovine 2-cell stages, and X-chromosome inactivation, indicated by late replication, is first observed at the early blastocyst stage [5].

Despite recent improvements in bovine in vitro production (IVP) systems, IVP embryos display marked differences compared with their in vivo counterparts (for reviews, see [6–8]. The in vitro culture system has profound effects on mRNA expression patterns of developmentally important genes [9–11]. Furthermore, sex-related growth differences exist in some in vitro culture systems, resulting in a faster development of male bovine embryos [12, 13]. A higher percentage of male embryos reached the expanded blastocyst stage [14]. This difference in developmental rates between male and female embryos has been attributed to differences in metabolic activity of X-linked enzymes involved in energy metabolism [15]. The housekeeping X-linked gene glucose-6-phosphate dehydrogenase (G6PD) has been considered a likely candidate for involvement in sex differences [16]. An important role of the enzyme is to detoxify oxygen radicals [17]. Similarly, phosphoglycerate kinase (PGK), a key enzyme in glycolysis, is also encoded from the X chromosome. Its expression has not yet been studied in bovine embryos.

Somatic nuclear transfer (NT) has been successful in a variety of species, albeit with low success rates [18]. The successful development of NT-derived embryos requires that the transferred nucleus be reprogrammed to establish the temporal, spatial, and quantitative well-orchestrated gene expression pattern necessary for undisturbed development. NT also is associated with deviations from the normal expression pattern of several genes [19, 20]. The effects of NT on the expression of X-linked genes have not yet been investigated.

The objective of the present study was to unravel the effects of IVP and NT on the expression pattern of the 3 X-linked gene transcripts G6PD, PGK, and Xist in single embryos employing a semiquantitative reverse transcription (RT) polymerase chain reaction (PCR) assay [20]. In the first experiment, female and male IVP or in vivo-produced embryos were analyzed for the relative abundance (RA) of the 3 genes. In the second experiment, parthenogenetic and female NT-derived embryos were employed to study potential alterations in the expression pattern of the 3 genes. Parthenogenetic embryos were included in the present investigation because they are a useful model for studying the contribution of maternal and paternal genomes on embryonic gene expression and development [21–23].

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MATERIALS AND METHODS

IVP of Bovine Embryos

Bovine embryos were produced as previously described [10]. Ovaries from a local slaughterhouse were transported to the laboratory in Dulbecco PBS (D6650; Sigma Chemical Co., St. Louis, MO) at 25–30°C. Cumulus-oocyte complexes (COCs) were isolated via slicing [24]. Category I COCs (with a homogenous, evenly granulated cytoplasm possessing at least 3 layers of compact cumulus cells) and category II COCs (with less than 3 layers of cumulus cells or partially denuded but also with a homogenous, evenly granulated cytoplasm [25]) were pooled in TCM-air (TCM 199 contains L-glutamine and 25 mM Hepes [Sigma] supplemented with 22 μg/ml pyruvate, 350 μg/ml $NaHCO_3$, 50 μg/ml gentamicin, and 0.1% BSA [fraction V, A9647; Sigma]).

For maturation in vitro, TCM 199 containing L-glutamine and 25 mM Hepes served as the basic medium; 1 ml was supplemented with 22 μg pyruvate, 2.2 μg $NaHCO_3$, and 50 μg gentamicin. For oocyte maturation, 1 ml of this medium was supplemented with 10 IU eCG and 5 IU hCG (Suigonan; Intervet, Tönisvorst, Germany) and 0.1% BSA-FAF (A7030; Sigma). COCs were divided in groups of 20–25, transferred into 100-μl maturation drops under silicone oil, and cultivated in a humidified atmosphere composed of 5% CO_2 in air at 39°C for 24 h.

Following in vitro maturation, COCs were rinsed in fertilization medium (Fert-TALP supplemented with 6 mg/ml BSA) and fertilized in Fert-TALP containing 10 μM hypotaurine (Sigma), 1 μM epinephrine (Sigma), 0.1 IU/ml heparin (HHE; Serva, Heidelberg, Germany), and 6 mg/ml BSA. Frozen semen from a bull with proven fertility for in vitro fertilization (IVF) was used. For IVF, semen was prepared by the modified swim-up procedure [26, 27]. Semen was thawed in a waterbath at 37°C for 1 min. After swim-up separation in Sperm-TALP containing 6 mg/ml BSA 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 sperm/ml. Fertilization was achieved during a 19-h coincubation under the same temperature and gas conditions as described for maturation.

Modified synthetic oviduct fluid (SOF) supplemented with BSA-FAF was employed for in vitro culture [28]. Presumptive zygotes were transferred into 30 μl of SOF after complete removal of the adhering cumulus cells by repeated pipetting. For culture, a mixture of 5% O_2, 90% N_2, and 5% CO_2 (Air Products, Hattingen, Germany) in Modular incubator chambers (615300; ICN Biomedicals, Aurora, OH) was used. The embryos were checked for first cleavage at 30 or 40 h postfertilization (see *Experimental Design*). Two-cell-stage embryos of each group were pooled, placed in new medium, and cultured up to the morula (Day 6 after insemination) or blastocyst stage (Day 7/8 after insemination). Embryos that had not cleaved after 40 h were discarded from this study. After washing 3 times in PBS containing 0.1% polyvinyl alcohol (PVA), all embryos were stored individually at −80°C in a minimum volume (≤5 μl) of medium until used.

In Vivo Production of Bovine Embryos

Holstein-Friesian donor cows were superovulated with a single i.m. injection of 3000 IU eCG (Intergonan; Intervet) between Days 9 and 13 of the estrus cycle followed 48 h later by administration of cloprostenol (Estrumate; Essex, Munich, Germany). When estrus was detected 48 h later, the donors were inseminated twice at an interval of 12 h with semen from the same bull used for IVF. On Day 7 after insemination, embryos were recovered by nonsurgical flushing of the uterine horns with 300 ml PBS plus 1% newborn calf serum employing established procedures. Only embryos of morphologic grades I and II [29] were included in this study.

After washing 3 times in PBS containing 0.1% PVA, all embryos were stored individually at −80°C in a minimum volume (≤5 μl) of medium until used.

Generation of NT-Derived and Parthenogenetic Embryos

Media. For enucleation and NT, cytochalasin B (7.5 μg/ml) was added to TCM-air. For fusion, 0.285 M mannitol containing 0.1 mM $MgSO_4$ and 0.05% BSA was used.

In vitro maturation of oocytes. Collection and in vitro maturation were carried out as described above. Eighteen to 20 h after the onset of maturation, the adhering cumulus cells were completely removed by vortexing COCs in 0.1% hyaluronidase (Sigma) in Ca- and Mg-free PBS for 2 min. Matured oocytes were selected by the presence of a visible extruded first polar body and randomly used for activation or NT.

Generation of NT Embryos

Primary cell lines. Fetal fibroblasts were obtained by explant culture from a 61-day-old female bovine fetus after evisceration and decapitation. Adult fibroblasts were established from ear skin samples of an adult female animal collected from an abattoir. Fetal and adult tissue was cut into small pieces and dispersed in 0.1% trypsin. Explants were maintained in Dulbecco modified Eagle medium-F12 and trypsinized at 90% confluency. The harvested cells were reconstituted at a concentration of 1×10^6 cells/ml and then either frozen in 10% dimethyl sulfoxide or returned to culture. The fibroblasts used for NT in these experiments were from passages 2–4 and were induced to enter a period of quiescence (presumptive G_0) by serum starvation for 7 days (0.5% fetal calf serum).

NT protocol. Oocytes were enucleated by aspirating the first polar body and the MII plate. Oocytes were stained in TCM-air containing 5 μg/ml Hoechst 33342 and 7.5 μg/ml cytochalasin B. Enucleation was confirmed by brief exposure of the karyoplast to ultraviolet light. Immediately before injection, a suspension of the donor cells was prepared by standard trypsinization. The cells were pelleted and resuspended in TCM-air and remained in this medium until injection. A single cell was sucked into a 30-μm (outer diameter) pipette and carefully transferred into the perivitelline space of the recipient oocyte. Reconstructed embryos were electrically fused at 26 h after onset of maturation. Cell fusion was induced with 1 or 2 DC pulses of 0.7 kV for 30 μsec each generated by an electrofusion machine (CFA 400; Kruess, Hamburg, Germany). Fusion was assessed 30 min later by light microscopy.

Activation and culture of parthenogenetic and reconstructed embryos. At 27 h after onset of maturation, the reconstructed embryos and the parthenogenetic controls were chemically activated by incubation in 5 μM ionomycin (Sigma) in TCM 199 for 5 min followed by a 3- to 4-h incubation in 2 mM 6-dimethylaminopyridine (6-DMAP; Sigma) in TCM 199 at 37°C. After washing 3 times, the embryos were cultured in SOF for 7 days as described above.

Determination of the Relative Abundance of Developmentally Important Gene Transcripts in Bovine Embryos

$Poly(A)^+$ RNA was isolated from single blastocysts as described recently [9] and was used immediately for RT, which was carried out in a total volume of 20 μl using 2.5 μM random hexamers (Perkin-Elmer, Vaterstetten, Germany). Prior to RNA isolation, 1 pg of rabbit globin RNA (BRL, Gaithersburg, MD) was added as an internal standard. The reaction mixture consisted of 1× RT buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3; Perkin-Elmer), 5 mM $MgCl_2$, 1 mM of each dNTP (Amersham, Brunswick, Germany), 20 IU RNase inhibitor (Perkin-Elmer), and 50 IU murine leukemia virus reverse transcriptase (Perkin-Elmer). The mixture was overlaid with mineral oil to prevent evaporation. The RT was carried out at 25°C for 10 min and 42°C for 1 h, followed by a denaturation step at 99°C for 5 min and flash cooling on ice. PCR was performed with cDNA equivalents (Table 1) from different embryos generated in different IVP or NT runs and 50 fg of globin RNA in a final volume of 50 μl of 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl; Gibco BRL, Eggenstein, Germany), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1 μM of each sequence-specific primer (globin: 0.5 μM) using a thermocycler (PTC-200; MJ Research, Watertown, MA). To ensure specific amplification, a hot-start PCR protocol was employed by adding 1 IU *Taq* DNA polymerase (Gibco) at 72°C. PCR primers were designed from the coding regions of each gene sequence using the OLIGO program. The sequences of the primers used, the annealing temperatures, the fragment sizes, and the sequence references are summarized in Table 1.

The PCR program employed an initial step of 97°C for 2 min and 72°C for 2 min (hot start) followed by different numbers of cycle (see Table 1) of 15 sec each at 95°C for DNA denaturation, 15 sec at different temperatures for annealing of primers, and 15 sec at 72°C for primer extension. The last cycle was followed by a 5-min extension at 72°C and cooling to 4°C. As negative controls, tubes were prepared in which RNA or reverse transcriptase was omitted during RT (data not shown). The PCR for the gene of interest and globin (internal standard) were performed in separate tubes.

The RT-PCR products were subjected to electrophoresis on a 2% agarose gel in 1× TBE buffer (90 mM Tris, 90 mM borate, 2 mM EDTA, pH 8.3) containing 0.2 μg/ml ethidium bromide, which was added to the running buffer to get the same concentration as in the gel. The image of each gel was recorded using a CCD camera (Quantix; Photometrics, München, Germany) and the IP Lab Spectrum program (Signal Analytics Corp., Vienna, VA). The intensity of each band was assessed by densi-

TABLE 1. Primers used for PCR of bovine embryo gene transcripts.

Genes	Primer sequences and positions	Annealing temperature (°C)	No. cycles	Embryo equivalent	Fragment size (bp)	Sequence references, EMBL accession no.
Globin						
5′ (241–260)	GCAGCCACGGTGGCGAGTAT	60	27	(0.05)	257	[59], X04751
3′ (555–657)	GTGGGACAGGAGCTTGAAAT					
G6PD[a]						
5′	CGCTGGGACGGGGTGCCCTTCATC	64	31	0.2	347	[60], NM000402
3′	CGCCAGGCCTCCCGCAGTTCATCA					
PGK[a]						
5′	TTATTGGTGGTGGAATGGCTTTTAC	59	36	0.5	357	[61], V00572
3′	GGACCATTCCACACAATCTGCTTAG					
Xist[a]						
5′ (1010–1053)	GCATAGCACCTCGCTACCGTCTCT	67	37	0.5	358	Jacobsen and Wilkins, unpublished observations; AF104906
3′ (1364–1387)	AACGATCATCTGCGATCCATTCTG					
Y-Chromosome specific						
5′	CCTCCCCTTGTTCAAACGCCCGGAATCATT	60	33	Morula	210	[62], PCT WO 86/07095
3′	TGCTTGACTGCAGGGACCGAGAGGTTTGGG		32	Blastocyst		
Cow specific						
5′	AGGTCGCGAGATTGGTCGCTAGGTCATGCA	60	33	Morula	300	[62], PCT WO 86/07095
3′	AAGACCTCGAGAGACCCTCTTCAACACGT		32	Blastocyst		

[a] The primer pair to detect these mRNAs was first designed from the given heterologous sequence, the product was sequenced, and the resulting cow-specific sequence was used to create the primer pair employed to detect the transcript of interest.

tometry using an image analysis program (IP Lab Gel). The relative amount of the mRNA of interest was calculated by dividing the intensity of the band for each developmental stage by the intensity of the globin band for the corresponding stage. Experiments were repeated with 8 embryos for each mRNA.

For each pair of gene-specific primers, semilog plots of the fragment intensity as a function of cycle number were used to determine the range of numbers of cycles over which linear amplification occurred, and the number of PCR cycles was kept within this range [9]. Because the total efficiency of amplification for each set of primers during each cycle is not known, such assays can only be used to compare relative abundances of 1 mRNA among different samples [30].

To circumvent the problem of differences in the RA of the transcripts being due to different numbers of blastocysts analyzed, the necessary number of replicates for a high enough repeatability of the assay was calculated. The average repeatability (precision) of the assay ranged from 0.60 to 0.70. Therefore, 8 replicates were included.

Sex Determination of the Embryos

DNA from each embryo was prepared using the Microcon YM-100 (42412; Millipore, Eschborn, Germany) columns according to the manufacturer's instructions with minor modifications. The supernatant from the RNA extraction was diluted with 200 μl H_2O and centrifugation for 10 min. Another 200 μl H_2O was then added to the column, which was then centrifuged for 8 min. Subsequently, the column was turned around and centrifuged at 350 × *g* for 2 min. One half of the resulting volume (~20 μl) was used for PCR. PCR was performed in a final volume of 50 μl of 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of the Y-chromosome-specific primer and 0.03 μM of the cow-specific primer, and 1 IU *Taq* DNA polymerase (Gibco). The PCR program employed an initial step of 97°C for 2 min followed by 32 (blastocysts) or 33 (morulae) cycles (see Table 1) of 15 sec each at 95°C for DNA denaturation, 15 sec at 60°C for annealing of primers, and 15 sec at 72°C for primer extension. The last cycle was followed by a 5-min extension at 72°C and cooling to 4°C. Electrophoresis and visualization of the PCR products were conducted as described above. At least 15 embryos were sexed within each group.

Experimental Design

In the first experiment, female and male in vivo-generated and IVP bovine morulae and blastocysts, which had cleaved at 30 or 40 h after insemination, were analyzed for the RA of the three X-linked gene transcripts G6PD, PGK, and Xist. The sex of the embryos was determined employing PCR analysis with Y-chomosome-specific primers. IVP embryos were divided into 2 groups according to the timing of the first cleavage to investigate how the sex ratio and the RA of the three X-linked genes are affected by an early or late cleavage.

In the second experiment, special attention was paid to embryos carrying two X chromosomes. Xist and G6PD and PGK transcript levels were detected by semiquantitative RT-PCR analysis from NT-derived and parthenogenetic embryos.

Statistical Analysis

RAs were analyzed using the SigmaStat 2.0 (Jandel Scientific, San Rafael, CA) software package. After testing for normality (Kolmogorov-Smirnov test with Lilliefor correction) and testing for equal variance (Levene Median test), an ANOVA followed by multiple pairwise comparisons using the Tukey test was employed. Sex ratios were compared with the expected ratio of 50% using chi-square analyses. Differences at $P \leq 0.05$ were considered significant.

RESULTS

In Figure 1 are photographs of representative gels from semiquantitative RT-PCR analysis of Xist and G6PD and PGK transcripts and the corresponding globin bands in diploid parthenogenetic and IVP (first cleavage after 30 or 40 h) bovine morulae and in vivo-generated and NT-derived blastocysts employing either fetal or adult donor cells. PCR results of gender determination are also shown.

Experiment 1

Sex ratio of embryos. At the morula stage, the sex ratio did not differ significantly among the different groups (cleavage 30 h after insemination: 53% female vs. 47% male; 40 h after insemination: 57% female vs. 43% male; in vivo: 53% female vs. 47% male). At the blastocyst stage, the female:male ratio was significantly altered when compared with the expected ratio of 50% in the IVP groups (30 h: 36% vs. 64%, 40 h: 38% vs. 62%). No significant difference was found for the in vivo group (53% vs. 47%).

Expression of G6PD, PGK, and Xist in IVP and in vivo-produced female and male bovine morulae and blasto-

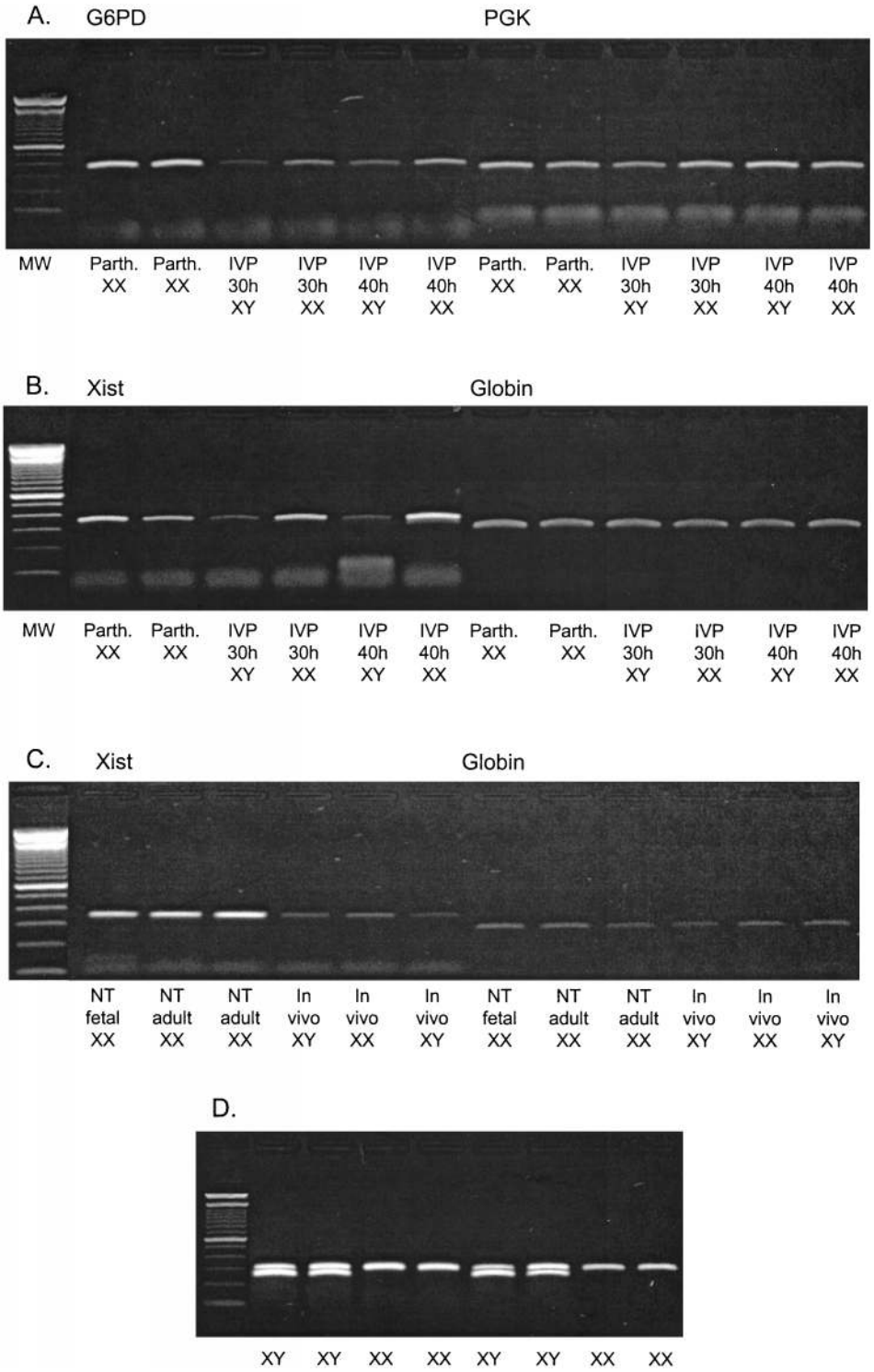

FIG. 1. Photographs of representative gels produced from semiquantitative RT-PCR analysis of parthenogenetic and IVP bovine morulae and in vivo-generated and NT-derived blastocysts employing either fetal or adult donor cells. PCR results for gender determination are also shown. **A**) Analysis of G6PD and PGK transcripts in diploid parthenogenetic and male and female IVP morulae that have cleaved the first time after either 30 h or 40 h. **B**) Expression pattern of Xist and the globin standards. **C**) Analysis of Xist and the globin standards in NT-derived blastocysts using either fetal or adult donor cells and in vivo-generated male and female embryos. **D**) PCR analysis of the sex of the embryos. Two fragments resulted from both the Y-chromosome-specific and cow-specific primers, indicating that a male embryo was analyzed, whereas a female embryo only gave 1 fragment after PCR analysis using the cow-specific primer pair.

cysts. No significant differences were determined in the RA of G6PD and PGK transcripts with respect to the occurrence of the first cleavage. Therefore, the results of both groups were combined. The RA of G6PD transcripts was significantly higher in female than in male IVP embryos at the morula and blastocyst stages (Fig. 2A). In contrast, no significant differences were seen between female and male in vivo-derived embryos (Fig. 2A). A significantly higher expression of G6PD transcripts was detected for IVP than for in vivo-derived female blastocysts (Fig. 2A).

For PGK transcripts, a significant difference was determined between female and male IVP morulae, but the level of expression was similar in female and male IVP blastocysts (Fig. 2B). No differences were observed between female and male in vivo-generated embryos (Fig. 2B). A significant increase in PGK transcripts was detected between IVP female blastocysts and their in vivo-developed counterparts (Fig. 2B).

The RA of Xist mRNA was significantly higher in all female embryos compared with their male counterparts (Fig. 2C). Furthermore, the only significant difference between female embryos cleaving after either 30 h or 40 h

was determined for Xists at the morula and blastocyst stage. Female morulae that had developed from early cleavage (30 h after insemination) showed a significantly lower RA than did female morulae originating from late cleavage (40 h after insemination). The opposite result was found for female blastocysts (Fig. 2C).

Experiment 2: Expression of G6PD, PGK, and Xist in IVP, In Vivo-Developed, Parthenogenetic, and NT-Derived Female Bovine Morulae and Blastocysts

The results of this experiment are shown in Figure 3. Because no significant differences were found for the RA of G6PD and PGK transcripts in NT-derived embryos employing either fetal or adult donor cells, the results of these 2 groups were combined. The RA of G6PD transcripts was significantly increased in NT-derived morulae compared with IVP and in vivo-generated embryos. At the blastocyst stage, G6PD transcript levels were significantly higher for IVP embryos than for in vivo-generated and parthenogenetic embryos (Fig. 3A).

No significant differences were observed in any group for PGK transcripts at the morula stage. In contrast, the RA of PGK transcripts was significantly higher in IVP blastocysts than in their in vivo-generated, parthenogenetic, and NT-derived counterparts (Fig. 3B).

Xist levels were significantly elevated in NT-derived morulae using adult donor cells compared with IVP, in vivo-generated, and parthenogenetic embryos. NT-derived morulae employing fetal donor cells showed a significantly higher expression than did IVP (first cleavage after 30 h), in vivo-generated, and parthenogenetic embryos (Fig. 3C).

At the blastocyst stage, a significant difference was seen between NT-derived embryos employing either fetal or adult donor cells, with an increased Xist expression in embryos reconstructed with adult donor cells. NT-derived embryos using adult cells as donors showed an increased expression compared with that of IVP (first cleavage after 40 h), in vivo-generated, and parthenogenetic embryos. Furthermore, Xist expression was significantly lower in in vivo-generated blastocysts than in IVP (first cleavage after 30 h) and NT-derived embryos employing fetal donor cells (Fig. 3C).

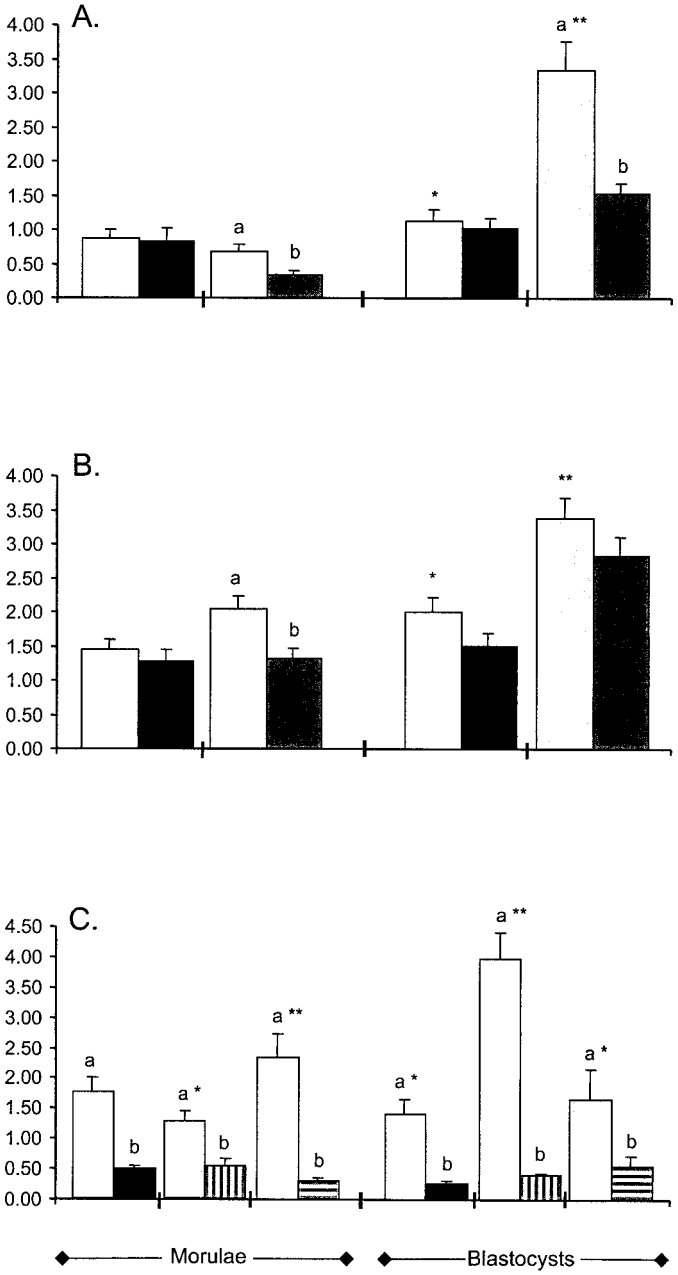

FIG. 2. Expression pattern of G6PD transcripts (**A**), PGK transcripts (**B**), and Xists (**C**) in female (open or light gray bars) and male (solid or dark gray bars) in vivo-generated (open, solid bars) or IVP (light, dark gray bars) morulae and blastocysts. **C**) The IVP group is divided into 2 subgroups according to the time of first cleavage, after 30 h (vertical stripes) or 40 h (horizontal stripes). Bars with different superscripts within each treatment group differ significantly (a:b; *:**; $P \leq 0.05$).

DISCUSSION

IVP and cloning have resulted in a high proportion of embryonic, fetal, and neonatal abnormalities summarized under the term large offspring syndrome [31]. One current hypothesis is that IVP and cloning result in aberrant expression patterns of developmentally important genes [8]. Recently, we have shown that gene expression patterns in preimplantation bovine embryos are altered by in vitro culture conditions, including the basic culture system, the protein supplementation [9, 10], and the NT protocol [20]. The results of the present study indicate that IVP and NT lead to alterations in dosage compensation of X-linked gene expression, which in turn could result in deviations from the normal sex ratio. The present study is the first to investigate the relative abundance of the three X-linked gene transcripts G6PD, PGK, and Xist in single preimplantation bovine embryos generated in vivo or in vitro, by NT, or by parthenogenetic activation. The semiquantitative RT-PCR assay associated with sex determination provides a powerful tool for investigating alterations in gene expression patterns in individual embryos of known sex. The DNA used for sexing was extracted from the supernatant of the RNA isolation buffer, which has the advantage that no biopsy has to be taken. This rules out the possibility that the gene expression pattern is altered by the manipulation procedure.

Culture systems have been shown to affect the sex ratio of the developing embryo; male embryos develop faster, and a higher percentage reach the blastocyst stage [12, 14, 32]. The present data are consistent with these results but are in contrast to those of other reports [33, 34]. No difference in the sex ratio was seen with regard to the time point of the first cleavage, e.g., 30 or 40 h postinsemination, which is consistent with some previous findings [32] but in contrast to other results [35]. The female:male ratio was

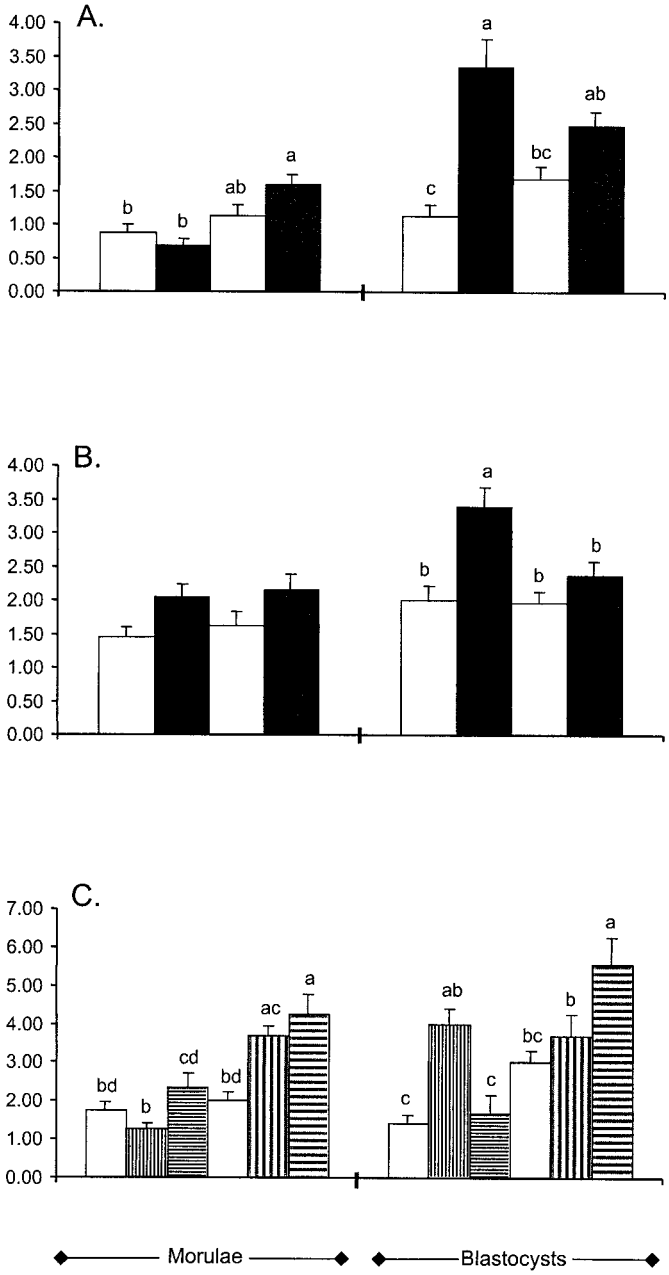

FIG. 3. Expression pattern of G6PD transcripts (**A**), PGK transcripts (**B**), and Xists (**C**) in morulae and blastocysts (carrying two X chromosomes) generated in vivo (open bars), in vitro (solid bars), after parthenogenetic activation (light gray bars), or after cloning (dark gray bars). **C**) The IVP group is divided into 2 subgroups according to the time of first cleavage, after 30 h (thin vertical black stripes) or 40 h (thin horizontal black stripes). The results for the NT-derived embryos are divided according to the use of either fetal (thick vertical gray stripes) or adult (thick horizontal gray stripes) cells. Bars with different superscripts within each treatment group differ significantly (a:b:c:d $P \leq 0.05$).

not altered in in vivo-derived embryos, confirming the results of earlier studies [36].

Female embryos have the potential to produce double the amount of X-linked enzymes relative to the male embryos, which have only one X chromosome [37]. In the present study, we demonstrated that G6PD transcripts were increased 2-fold in IVP female embryos compared with their male counterparts, indicating that in female embryos both X chromosomes were active [38, 39]. Analysis of in vivo-generated embryos showed a similar expression pattern for G6PD in female and male morulae and blastocysts, suggesting that dosage compensation for G6PD expression had not occurred in IVP embryos.

In the present study, a significant difference was detected between female and male IVP morulae in the expression pattern of PGK transcripts, suggesting that dosage compensation for PGK was delayed at this point in development. Obviously, the PGK locus was already inactivated in in vivo-derived morulae and blastocysts and IVP blastocysts. In the mouse, male and female embryos initially expressed Xist RNA. However, this expression was followed by a preferential reduction of Xist RNA in male embryos, indicating that dosage compensation for the X chromosome requires downregulation of Xist RNA in male embryos, whereas in female embryos stable Xists are produced [40]. When this subtle regulation is perturbed, a difference in the timing of locus inactivation on the X chromosome may result.

Murine Xists may be involved in the process of cis-inactivation of neighboring cells [41]. In the mouse, the paternal X chromosome is first inactivated in trophectodermal cells, whereas in the embryonic ectoderm the inactivation is random, on either the paternal or maternal X chromosome, and occurs 2 days later in development [42]. In bovine embryos, Xist mRNA is first detectable at the 2-cell stage, whereas X-chromosome inactivation starts at the early blastocyst stage [5]. The reverse RA of Xist in morulae and blastocysts developed from early and late division embryos (e.g., first cleavage 30 h or 40 h after insemination) may be explained by the presence of predominantly early blastocysts in the 30-h blastocyst subgroup, in which the newly differentiated trophoblast starts intense Xist RNA production. In contrast, morulae derived from the 40-h subgroup likely represent late morula stages in which the first differentiation with an onset of Xist production has begun. We detected Xists in both male and female embryos, indicating expression from the maternal allele as well. Similar findings have been reported for human [43, 44] and mouse [45, 46] embryos. Another explanation for the apparent presence of Xists in male embryos is that the RT-PCR product obtained was not Xist but rather its antisense, Tsix [47]. Tsix is a 40-kilobase RNA originating downstream of Xist and is transcribed across the entire Xist locus. It is expressed from both X chromosomes [48] and was first detected in murine blastocysts, and only the maternal allele is transcribed. Tsix is thought to play a crucial role in Xist regulation of expression in cis and regulating imprinted X-chromosome inactivation in extraembryonic tissues [49]. Xists were significantly increased in all female embryos compared with their male counterparts, which could be attributed to a different stability of Xists [45, 46]. Prior to X-chromosome inactivation, Xist RNA with a short half-life is expressed from both alleles and accumulates only at the site of transcription. The stabilization and spread of Xist mRNA in cis from the inactivation center along the entire length are correlated with genetic silencing of the X chromosome. In murine female blastocysts, most trophectodermal cells transcribe stable Xist RNA [50]. Furthermore, during early cleavage stages Xist is expressed from both the paternal and the maternal X chromosome in human and murine embryos [43, 45]. All analyzed gene transcripts have shown a significant increase in IVP female blastocysts compared with their in vivo counterparts. This finding supports the hypothesis that IVP systems alter the well-orchestrated expression pattern of developmentally important gene transcripts [8]. The absence of the X-chromosome in-

activation effect of the Xists in spite of its abundance in the bovine IVP embryos suggests that timing and magnitude of Xist expression alteration are not necessarily correlated with a tightly controlled inactivation process.

Female NT-derived and parthenogenetic embryos were analyzed to obtain better insight into dosage compensation in reconstructed embryos. Treatment with 6-DMAP after bovine oocyte activation induces pronuclear formation and results in the formation of a diploid parthenote [51]. Cytogenetic examination of the ploidy of the parthenogenotes analyzed in this study revealed that they contained 2 sets of chromosomes (data not shown). Adverse effects of parthenogenesis may be due to overexpression of X-linked genes [22]; dosage compensation occurs at a later stage of development in mouse parthenotes [52, 53]. In parthenogenetic mouse embryos, only 1 maternal X chromosome usually is inactivated [54]. Overexpression of X-linked genes with regard to G6PD, PGK, and Xists could not be detected in bovine parthenogenetic morulae and blastocysts, indicating that parthenogenetic development up to the blastocyst stage was comparable to that of normally fertilized embryos. Parthenogenetic embryos can also be used to isolate potentially imprinted genes because these embryos will be deficient in transcripts that must be expressed from the paternal genome [23, 55].

Recently, we found an aberrant expression pattern in NT-derived embryos with respect to genes involved in stress adaptation, trophoblastic function, and DNA methylation [20]. Aged or more differentiated donor nuclei may be expected to increase the incidence of abnormal reprogramming [56]. In the present study, no differences were found among the NT-derived embryos using either fetal or adult donor cells with regard to expression levels of G6PD and PGK transcripts. However, the RA of Xists was significantly elevated in NT-derived blastocysts employing adult donor cells compared with those employing fetal cells, indicating an incomplete reprogramming. This higher Xist RA may be due to an aberrant reactivation of the silent X chromosome. In somatic cells used for NT, one X chromosome is inactivated and needs to be reactivated in the reconstructed embryo. Reactivation of the inactive X chromosome usually takes place in primordial germ cells [57]. Experiments monitoring X-chromosome inactivation in cloned mouse embryos revealed that epigenetic marks can be removed and reestablished on either X chromosome during cloning [58].

The present study adds new information to recent observations [9, 10, 20] by demonstrating that IVP and NT affect dosage compensation of X-linked genes in female and male bovine embryos. This finding warrants further studies to improve IVP systems and NT protocols to ensure the production of embryos with normal gene expression patterns.

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