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Nuclear Transfer Protocol Affects Messenger RNA Expression Patterns in Cloned Bovine Blastocysts

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ABSTRACT

The successful production of embryos by nuclear transfer (NT) employing cultured somatic donor cells depends upon a variety of factors. The objective of the present study was to investigate the effects 1) of two different activation protocols, 2) the use of quiescent or nonquiescent donor cells (G_0 or G_1 of the cell cycle), and 3) passage number of donor cells on the relative abundance (RA) of eight specific mRNAs (DNA methyltransferase, DNMT; mammalian achaete-scute homologue, Mash2; glucose transporter-1, Glut-1; heat shock protein 70.1, Hsp; desmocollin II, Dc II; E-cadherin, E-cad; interferon tau, IF; insulin-like growth factor 2 receptor, Igf2r) in single blastocysts employing a semiquantitative reverse transcription-polymerase chain reaction assay. The results were compared with those for their in vitro (IVP)- and in vivo-generated noncloned counterparts. In experiment 1, employing either FBA (fusion before activation) or AFS (fusion and activation simultaneously) to generate NT blastocysts, Hsp mRNAs were not found in NT embryos from either protocol, whereas Hsp transcripts were detectable in IVP embryos. The relative abundance (RA) of IF transcripts was significantly increased in the AFS and IVP groups compared to the FBA treatment. In experiment 2, the use of either G_0 or G_1 donor cells to produce cloned embryos both significantly reduced the relative amount of DNMT transcripts and significantly increased the RA of Mash2 compared to the IVP embryos. In addition, IF transcript levels were significantly elevated in NT blastocysts employing G_1 donor cells for NT compared to IVP embryos and those generated using G_0 cells. In experiment 3, donor cells, either from passsage 5/6 or 8, were employed for NT. DNMT transcripts were significantly decreased, whereas Mash2 transcripts were significantly increased in both NT groups compared to their IVP counterparts. The amount of IF mRNA was significantly higher in P8-derived than in P5/6 and IVP embryos. In experiment 4, the RA of DNMT transcripts was decreased in in vivo-derived blastocysts compared to those produced in vitro. Mash2 expression was increased in in vivo embryos and those IVP embryos produced in medium containing Sigma BSA. The RA of Hsp was higher in IVP embryos produced in serum containing medium than in those produced in Sigma BSA or in vivo. In vivo embryos and those produced in Life Technologies BSA had the lowest expression of IF transcripts. Expression of all other genes was not affected by variation in NT methodology or IVP culture systems throughout experiments 1–4. In conclusion, depending on steps of the cloning procedure NT-derived embryos display marked differences from their IVP- and in vivo-derived counterparts. An aberrant expression pattern in NT embryos was found with respect to genes thought to be involved in stress adaptation, trophoblastic function, and DNA methylation during preimplantation development.

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INTRODUCTION

Cloning of bovine embryos via nuclear transfer (NT) employing fetal or adult somatic donor cells has made great progress during the past few years. However, a high rate of embryonic, fetal, and neonatal abnormalities has been consistently observed that are summarized under the term large offspring syndrome [1–7].

The production of viable offspring derived from NT depends upon the successful combination of a great variety of factors including oocyte quality and age, the reconstruction process, the culture conditions, as well as the cell cycle stages of both the donor and recipient cells [8]. Fusion before activation (FBA) has resulted in a significantly higher proportion of reconstructed embryos developing [6, 9] and cloned calves born [2]. NT protocols employing donor cells from either quiescent cultures induced to exit the cell cycle and enter G_0 by serum starvation are generally thought to be superior with regard to the production of reconstructed viable blastocysts and offspring [10]. However, donor cells from actively multiplying cultures yielded viable cloned offspring as well [2]. Donor cells from different passage numbers in culture and even from senescent cells have resulted in live offspring [10, 11].

For successful development of the reconstructed embryo, the transferred nucleus must be reprogrammed to establish the temporal, spatial, and quantitative well-orchestrated expression pattern correlated with normal development. This reprogramming of a nucleus constitutes a structural remodelling and is associated with fundamental changes in genomic activity [12]. The first most obvious morphological change observed in the transplanted nuclei is a rapid swelling caused by the presumptive exchange of both acidic and basic proteins between donor nuclei and cytoplasm [13]. Ultrastructural studies of bovine NT-derived embryos revealed complete reprogramming as indicated by the activity of the nuclear envelope and transcription of heterogenous nuclear RNA [14], whereas only partial reprogramming of the nucleolar fine structure was observed [15–17]. The molecular mechanism(s) underlying reprogramming are largely unknown at present [18, 19]. Epigenetic DNA modifications, such as DNA methylation [20] and changes in chromatin configuration [21] as well as genetic imprinting [22] have been proposed as likely candidates. Endogenous genes such as the transcription factor Oct-4, fibroblast growth $factor_2$ and gp 130, TEC-3 antigen, and three metabolic enzymes were found to be expressed in a similar manner throughout the development of bovine nonmanipulated and NT embryos [23–25], indicating a full reprogramming. A similar mRNA profile has been detected between in vivo- and NT-generated embryos employing the differential display-reverse transcription-polymerase chain reaction (RT-PCR) technology [26]. However,

altered levels of transcripts were found for several growth factor and cytokine genes [24, 27, 28]. An abnormal expression of developmentally important genes is suspected to have detrimental effects on embryonic development. The expression pattern of bovine NT-derived embryos for a set of marker genes has not yet been determined in relation to targeted modifications of the NT procedure.

The objective of the present study was to determine the relative abundance of gene transcripts in reconstructed blastocysts derived from distinct modifications of the cloning protocol and to compare their expression profile with that of in vitro (IVP)- and in vivo-derived embryos. Employing a sensitive semiquantitative RT-PCR assay [29], we have analyzed the effects of different fusion and activation protocols, the use of G_0 versus G_1 donor cells, as well as passage number of cultured cells on the relative abundance (RA) in single blastocysts of eight specific mRNAs. These mRNAs were selected for the present investigation to gain the maximum possible insight into crucial steps in early cloned embryo development and included genes involved in compaction and cavitation (desmosomal glycoprotein desmocollin II, Dc II; and cell adhesion protein E-cadherin, E-cad), metabolism (glucose transporter-1, Glut-1), stress (heat shock protein 70.1, Hsp), early differentiation and trophoblastic function (interferon tau, IF; and mammalian achaete-scute homologue, Mash2), growth factor signalling (insulin-like growth factor 2 receptor, Igf2r); and DNA methylation (DNA methyltransferase, DNMT). Recently, we have shown that the expression pattern of a set of marker genes indicative for normal embryo development is altered by the presence of serum in the medium [29]. Furthermore, the basic culture medium was found to have profound effects on the transcriptional activity of bovine embryos [30]. By including IVP- and in vivo-derived embryos we intended to gain insight into the transcriptional normality of the cloned embryos.

MATERIALS AND METHODS

Generation of NT Blastocysts: In Vitro Maturation (IVM) of Oocytes

Oocytes obtained from slaughterhouse ovaries were matured in vitro using methods described previously [5] and divided into those used for cloning or in vitro fertilization (IVF, see below). Briefly, cumulus-oocyte complexes (COCs) were aspirated and collected into Hepes-buffered TCM199 (H199; Life Technologies, Auckland, New Zealand). Only COCs with a compact, nonatretic cumulus oophorus-corona radiata and a homogenous ooplasm were selected for IVM. Ten COCs were matured in 50-μl droplets of medium overlaid with paraffin oil. The maturation medium comprised TCM199 supplemented with 10% fetal calf serum (FCS) (Life Technologies), 10 μg/ml ovine FSH (Ovagen; ICP, Auckland, New Zealand), 1 μg/ml ovine LH (ICP), 1 μg/ml estradiol (Sigma, St. Louis, MO), and 0.1 mM cysteamine (Sigma). Dishes were cultured at 38.5°C in a humidified 5% CO_2 in air atmosphere. After maturation, oocytes were prepared for NT by totally removing the cumulus-corona by vortexing COCs in 0.1% hyaluronidase (Sigma) in Hepes-buffered SOF (HSOF) [31] for 3 min, followed by three washes in H199 + 10% FCS.

Nuclear Transfer with Granulosa Cells

Primary cell lines. Two primary cell lines were used during the course of these experiments. Both were established from mural granulosa cells collected by aspirating ovarian antral follicles. One cell line, denoted EFC isolated from a Friesian dairy cow has been reported recently [5], while the other cell line denoted J1 was isolated from a Jersey heifer.

Media. Matured oocytes, cytoplasts, and reconstructed embryos were either held or manipulated in H199-based media for the period following maturation and up to the time of fusion. Following this point, reconstructed embryos were cultured in media based on bicarbonate or Hepes-buffered versions of AgResearch synthetic oviduct fluid medium (AgR SOF; which is a modified formulation to that described earlier [32] and is commercially available from AgResearch, Hamilton, New Zealand). AgR SOF-medium was essentially as described previously [32] with the addition of 10 μM 2,4-dinitrophenol from Days 4 to 5 onward to act as an uncoupler of oxidative phosphorylation. Approximately 24% of the reconstructed embryos develop to blastocysts when the medium is supplemented with Life Technologies BSA, and up to 36% blastocysts can be obtained when Sigma BSA is employed.

Nuclear transfer procedure. The basic NT procedures were carried out essentially as described previously [5] with some modifications. Briefly, oocytes matured for 18–19 h were enucleated by aspirating the first polar body and the MII plate. Enucleation was confirmed by visualizing the karyoplast, while still inside the pipette, under UV light after previously staining oocytes with 5 μg/ml Hoechst 33342 (Sigma). Donor cells were induced to enter a period of quiescence (presumptive G_0) by serum starvation for 10–14 days or were used in G_1 of the cell cycle. G_1 cells were obtained by first picking mitotic cells off glass coverslips individually with a micropipette. Mitotic cells were allowed to cleave in the presence of H199 + 10% FCS, and single diploid cells were fused to cytoplasts within 1–3 h postmitosis. With the cells used in these experiments, this timing was prior to entry into the S-phase as indicated by negative BrdU incorporation in control subsets of the cells. Recipient cytoplasts were dehydrated in H199 containing 10% FCS and 5% sucrose. This medium was also used for micromanipulation. After injection of the donor cell, the reconstructed embryos were rehydrated and electrically fused to cytoplasts between 22 and 25 h after onset of maturation. Fusion was induced with two DC pulses of 2.25–2.50 kV/cm for 15 sec each at room temperature in a mannitol-based medium [5].

Activation. Two activation strategies were compared: 1) FBA and 2) activation and fusion simultaneously (AFS). In the FBA treatment, reconstructed embryos were activated 3–5 h after successful fusion. With AFS, reconstructed embryos were activated 15 min after delivery of the electrical pulses used for cell fusion. Activation was chemically induced in both AFS and FBA groups using the same methods. Reconstructed embryos were incubated in 30-μl drops of 5 μM ionomycin (Sigma) in HSOF + 1 mg/ml fatty acid-free (FAF) BSA (Sigma) for 4 min at 37°C. After washing, embryos were cultured for 4 h in 2 mM 6-dimethylaminopurine (Sigma) in AgR SOF + 10% FCS.

In vitro culture. Culture of the reconstructed embryos was performed in 20-μl drops of AgR SOF containing 8 mg/ml BSA overlaid with paraffin oil. During the course of these experiments, NT embryos were cultured in AgR SOF supplemented with two alternative sources of BSA, namely FAF BSA from Sigma or Life Technologies. Whenever possible, groups of five embryos were cultured together. Embryos were cultured in a humidified modular incubator chamber (ICN Biomedicals, Aurora, OH) at 38.5°C in a 5% CO_2, 7% O_2, 88% N_2 gas mix. On Days 4–5 (Day 0 = day of NT and day of in vitro fertilization), morula-stage embryos were transferred to fresh 20-μl drops of AgR SOF containing 10 μM 2,4-dinitrophenol acting as an uncoupler of oxidative phosphorylation [31]. On Day 7 blastocysts that were considered morphologically viable [33] were washed three times in PBS containing 0.1% polyvinyl alcohol (PVA), snap frozen individually in a minimal volume (5 μl or less) of medium, and stored at −80°C for subsequent gene expression analyses.

In Vitro Production of Control Blastocysts

IVP1 and IVP2. IVM was performed as described above. For IVF, spermatozoa were prepared from frozen-thawed semen obtained from sires characterized for IVF; a Friesian sire to serve as a control for the EFC clones and a Jersey sire to match the J1 cell line. Motile sperm were collected following centrifugation at approximately 700 × *g* for 30 min at room temperature after layering upon a discontinuous Percoll gradient (45%/90%). Insemination with a final concentration of 2×10^6 sperm/ml was performed in 50 μl of fertilization medium (a modified Tyrode albumin lactate pyruvate [TALP]) [34] in microdrops under oil for 24 h under the same atmospheric conditions as described for oocyte maturation. Following fertilization and removal of the cumulus cells, embryos were cultured under the same conditions as described above with AgR SOF being supplemented with either Sigma or Life Technologies BSA for the IVP1 and IVP2 culture systems, respectively.

IVP3. Bovine embryos were produced as described recently [30]. Cumulus-oocyte complexes were isolated via slicing [35] and category I and II COCs [36] pooled in TCM-air (TCM 199 containing L-glutamine and 25 mM Hepes [Sigma] supplemented with 22 μg/ml pyruvate, 350 μg/ml $NaHCO_3$, 50 μg/ml gentamicin, and 10% heat-inactivated [30 min at 56°C] estrous cow serum [ECS, collected on the first day of standing estrus]).

For maturation in vitro, TCM-air was supplemented with 1 μg/ml es-

TABLE 1. Primers used for PCR

Genes	Primer sequences and positions	Annealing temperature (°C), cycle number, and embryo equivalent	Fragment size (bp)	Sequence references (EMBL accession no.)
Globin[a,b]	5′ primer (241–260) = GCAGCCACGGTGGCGAGTAT 3′ primer (555–657) = GTGGGACAGGAGCTTGAAAT	60 × 27 (0.05)	257	[69] (X04751)
Glucose transporter-1 (Glut-1)[a]	5′ primer (1609–1638) = AGGAGCTGTTCCACCCCCTGGGAGCTGACT 3′ primer (1906–1935) = TGTGGGTGAAGGAGACTCTGGCTGATAAAA	59 × 32 0.1	327	[70] (M60448)
Heat shock protein 70.1 (Hsp)[a]	5′ primer (1861–1890) = AAGGTGCTGGACAAGTGCCAGGAGGTGATT 3′ primer (2319–2348) = ACTTGGAAGTAAACAGAAACGGGTGAAAAA	59 × 36 0.2	488	[71] (U09861)
Mammalian achaete-scute homologue (Mash2)[a,c]	5′ primer = CGCTGCGCTCGGCGGTGGAGTA 3′ primer = GGGACCCGGGCTCCGAGCTGTG	67 × 37 0.5	210	[72] (U77629)
DNA methyltransferase (DNMTI)[a,c]	5′ primer = CGCATGGGCTACCAGTGCACCTT 3′ primer = GGGCTCCCCGTTGTATGAAATCT	60 × 34 0.1	312	[73] (X63692)
Desmocollin II (Dc II)[b]	5′ primer (2085–2109) = CTCCTGGCGATGACAAAGTGTATTC 3′ primer (2503–2527) = GCCGATCCTCTTCCTTCGTAGTTAT	57 × 31 0.1	443/397	[74] (M81190)
Interferon tau (IF τ)[b]	5′ primer (420–423) = GCTATCTCTGTGCTCCATGAGATG 3′ primer (755–778) = AGTGAGTTCAGATCTCCACCCATC	57 × 35 0.4	359	[75] (G163764)
E-cadherin (E-cad)[b]	5′ primer (pos. 1486–1515) = CTCAAGCTCGCGGATAACCAGAACAAAGAC 3′ primer (pos. 1785–1814) = AGGCCCCTGTGCAGCTGGCTCAAATCAAAG	55 × 32 0.3	332	[76] (X06339)
Insulin-like growth factor 2 (Igf2r)[b]	5′ primer (4799–4823) = CGCCTACAGCGAGAAGGGGTTAGTC 3′ primer (5067–5091) = AGAAAAGCGTGCACGTGCGCTTGTC	62 × 33 0.1	293	[77] (J03527)

[a,b] These genes were analyzed within one blastocyst.
[c] The primer pair to detect these mRNAs was first designed from the given heterologous sequence, the product was sequenced, and the resulting bovine-specific sequence was used to create the primer pair employed to detect the transcript of interest.

tradiol-17β (Serva), 0.5 μg/ml FSH (Folltropin; Vetrepharm, London, Ontario, Canada), 0.06 IU/ml hCG (Ekluton; Vemie, Kempen, Germany). Cumulus-oocyte complexes were divided into groups of 20–25, transferred into 100-μl maturation drops under silicone oil, and cultured for 24 h in a humidified atmosphere of 5% CO_2 in air at 39°C.

Following IVM, 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), and 6 mg/ml BSA (Sigma). Frozen semen from one bull with proven fertility in IVF was used. The semen was prepared by the modified swim-up procedure [37, 38]. Briefly, 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* at 37°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 occurred during a 19-h culture under the same temperature and gas conditions as described for maturation above.

After removal of all of the adhering cumulus cells by repeated pipetting, presumptive zygotes were transferred into 30 μl of SOFaa supplemented with 10% ECS (estrus cow serum) [31]. Culture was accomplished in a humidified atmosphere of 7% O_2, 88% N_2, and 5% CO_2 (Air Products, Hattingen, Germany) at 39°C in Modular incubator chambers (ICN Biomedicals). Embryos were cultured continuously in this medium up to the blastocyst stage on Day 7 and were individually snap frozen for subsequent analyses.

In Vivo Production of Bovine Embryos

Holstein Friesian donor cows were superovulated with a single i.m. injection of 3000 IU eCG (Intergonan; Intervet, Tönisvorst, Germany) between Days 9 and 13 of the estrus cycle followed 48 h later by an injection of cloprostenol (Estrumate; Essex, Munich, Germany). When estrus was detected approximately 48 h after cloprostenol, the donors were inseminated twice at an interval of 12 h with semen from the same bull as used for IVF for IVP3. On Day 7 after insemination, embryos were recovered by nonsurgical flushing of the uterine horns with 300 ml PBS supplemented with 1% NBCS (newborn calf serum) and individually snap frozen for future use. Only blastocysts with morphological grades I and II [33] were included in this study.

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

Poly(A)$^+$ RNA was isolated from single blastocysts as described recently [28] and was used immediately for reverse transcription (RT) that was carried out in a total volume of 20 μl using 2.5 μM random hexamers (Perkin-Elmer). 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 reaction was carried out at 25°C for 10 min, 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 as described in Table 1 from individual embryos generated in different IVP culture systems, NT experiments and in vivo production as well as 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 PTC-200 thermocycler (MJ Research, Watertown, MA). To ensure specific amplification, a hot start PCR was employed by adding 1 IU Taq DNA polymerase (Gibco) at 72°C. The PCR primers were designed from the coding regions of each gene sequence using the OLIGO program. The sequences of these primers, the annealing temperatures, the fragment sizes, and the sequence references have been 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 cycle numbers (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 the RT reaction.

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 EtBr. The image of each gel was recorded using a charge-coupled device camera (Quantix, Photometrics, München, Germany) and the IP Lab Spectrum program (Signal Analytics Corporation, Vienna, VA). The intensity of each band was assessed by densitometry 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.

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 cycle number over which linear amplification occurred and the number of PCR cycles was kept within this range [29]. Because the total efficiency of amplification for each set of primers during each cycle is not known, such an assay can only be used to compare relative abundances of one mRNA among different samples [39].

To circumvent the problem that the differences in the relative abundance of the transcripts were due to different cell numbers of the blastocysts analyzed, the number of replicates was calculated to get an acceptable repeatability of the assay (0.90). The average repeatability (precision) of the assay varied from 0.60 to 0.70. A total of eight replicates was performed for each specific gene transcript from an individual embryo. This allowed calculation of statistically significant differences between treatment groups for each transcript.

Experimental Design

In the first experiment, the relative abundance of the eight gene transcripts was compared in cloned bovine blastocysts that were reconstructed employing two different activation strategies. When fusion was induced before activation (FBA), the reconstructed embryos were activated 3–5 h postfusion. Fusion and activation occurred simultaneously (AFS), when reconstructed embryos were activated 15 min after delivery of the electrical pulses used for fusion. In both groups, fusion was induced between 22 and 25 h postmaturation. Cells from the follicular cell line EFC between passages 4 to 10 of culture and synchronized in G_0 by serum deprivation for 10–14 days were used as donors. Nuclear transfer embryos were cultured in AgR SOF with Sigma BSA. Blastocysts produced following IVF (IVP1) as described above served as controls.

In the second experiment, the relative amount of the same eight gene transcripts was determined in cloned bovine blastocysts produced using either G_0 or G_1 donor cells from the J1 follicular cell line. Cultured cells were used for NT between passages 5 to 8 of culture. Cells were used in G_0 following 11–15 days of culture in low serum-containing medium, whereas G_1 cells were fused within 1–3 h postmitosis. These embryos were all reconstructed by FBA treatment. The third experiment employed J1 donor cells from different passages numbers (P5/6 versus P8), and the embryos were reconstructed by FBA. In experiments 2 and 3, embryos were cultured in AgR SOF with Life Technologies BSA and in vitro fertilized embryos (IVP2) cultured in the same medium served as controls.

In the fourth experiment, the relative abundance of the eight gene transcripts in blastocysts produced in the IVP1 and IVP2 culture systems were compared to an alternative in vitro embryo production system (IVP3) and to in vivo-generated blastocysts. Cloned embryos and embryos derived from IVP1 and IVP2 were generated at the AgResearch, Ruakura laboratories and analyzed for mRNA expression in the Mariensee laboratory. In vivo and IVP3 embryos were produced and analyzed in the Mariensee lab. The IVP3 embryos were included as an additional control to cope with potential effects from the different origin of the embryos with regard to donor animals, slaughterhouse collection, etc.

Statistical Analysis

Data 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 Tukey test was employed to determine differences between embryos generated employing different activation protocols, cell cycle stages, passage numbers, and culture systems. Differences of $P \leq 0.05$ were considered to be significant.

RESULTS

Representative gel photos of mRNA expression patterns in cloned, IVP-, or in vivo-produced blastocysts from the various experiments are shown in Figure 1. The relative abundance of gene transcripts is described below.

Experiment 1

The effects of the two different activation protocols, either FBA or AFS, on the relative abundance of eight gene transcripts in cloned bovine blastocysts compared to their in vitro-generated counterparts (IVP1) are summarized in Figure 2. Hsp mRNA was not detectable in the two groups of NT blastocysts, whereas Hsp expression was found in IVP embryos. Furthermore, the RA of IF transcripts was significantly increased in the AFS and IVP groups compared to the FBA treatment. No significant differences were detected for the other mRNAs.

Experiment 2

The alterations in mRNA abundance due to the cell cycle stage of the donor cells used for NT (G_0 or G_1) compared to IVP embryos from the same culture system are shown in Figure 3. The relative amount of the DNMT transcript was significantly reduced in both NT-embryo groups compared to the IVP embryos, whereas the RA of Mash2 was significantly increased in the NT embryos. In addition, IF transcript levels were significantly elevated in blastocysts employing donor cells in G_1 for NT compared to IVP embryos and those reconstructed using donor cells in G_0. The other genes were not altered in their expression pattern.

Experiment 3

The different passage number of donor cells, either P5/6 or P8, used to reconstruct embryos affected the expression pattern of the eight analyzed genes in a similar manner as the donor cells at G_0 or G_1 in experiment 2 (data not shown). DNMT transcripts were significantly decreased, whereas Mash2 transcripts were significantly increased in both NT groups compared to their IVP counterparts and the amount of IF mRNA was significantly higher in P8-derived than in P5/6 and IVP embryos. No effects were seen on the expression pattern for the other genes.

Experiment 4

The effects of three different culture systems on the RA of eight gene transcripts in IVP blastocysts were compared with in vivo-derived counterparts (Fig. 4). The relative abundance of DNMT transcripts was significantly decreased in in vivo-derived embryos compared to the in vitro-generated ones. The RA of Mash2 transcripts was significantly increased in in vivo-generated embryos compared to embryos grown in serum (IVP3) and AgR SOF enriched with Life Technologies BSA (IVP2). In addition, the RA

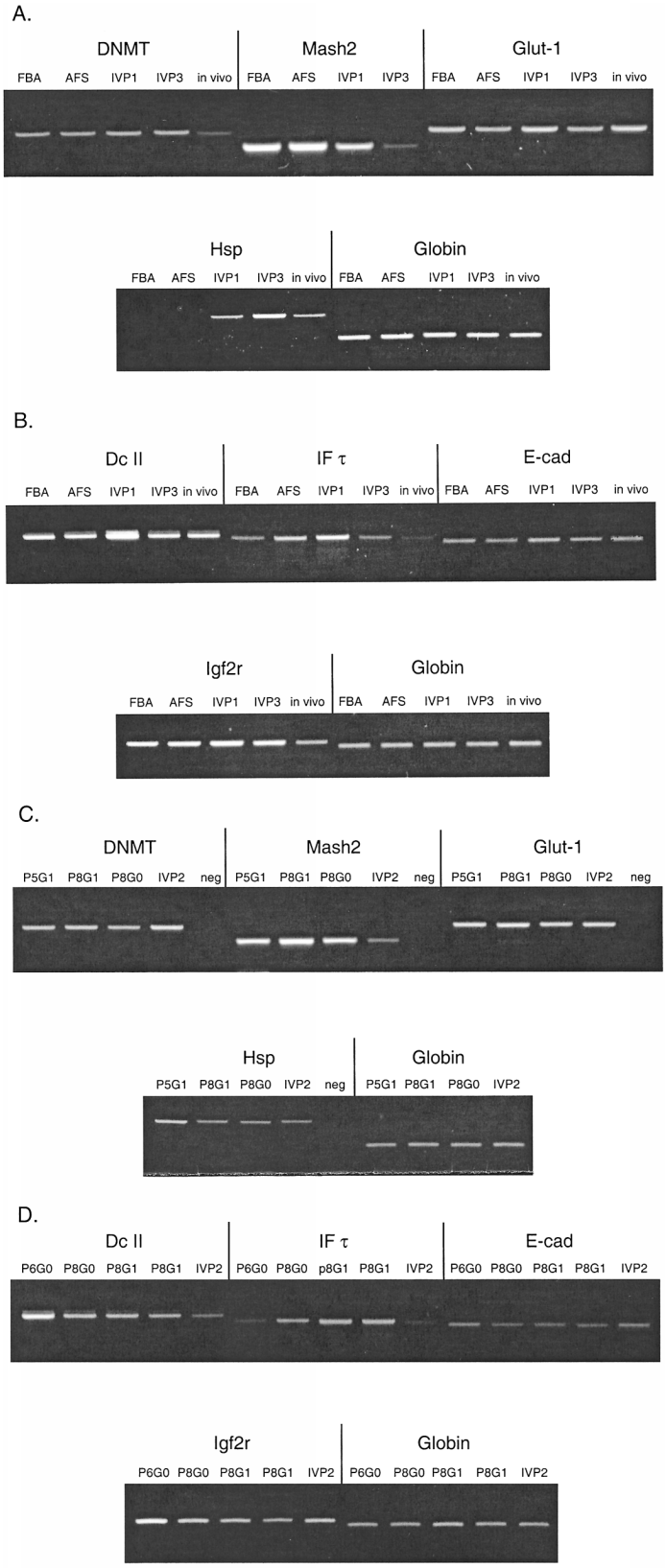

FIG. 1. Representative gel photographs of a semiquantitative RT-PCR analysis in cloned, in vivo-, or in vitro-produced bovine blastocysts. **A**) DNMT, Mash2, Glut-1, and Hsp transcripts as well as the globin standards in NT embryos employing different activation protocols and in IVP (IVP1 and IVP3)- and in vivo-derived embryos. **B**) Expression patterns of Dc II, IF, E-cad, Igf2r, and the globin standards are shown. **C**) A gel photo of the analysis of DNMT, Mash2, Glut-1, and Hsp transcripts as well as the globin standards in NT embryos using quiescent or nonquiescent donor cells and donor cells in different passage numbers and in vitro-produced embryos (IVP2). **D**) Dc II, IF, E-cad, Igf2r fragments, and the globin standards.

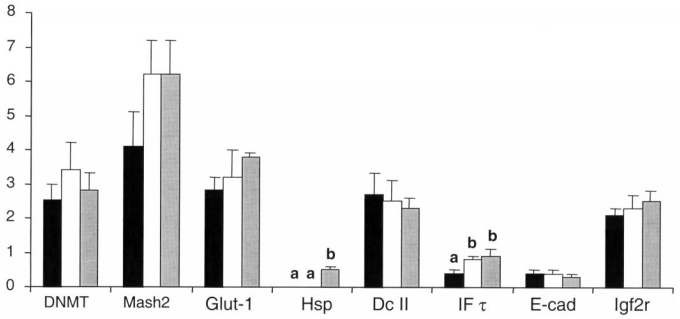

FIG. 2. Effects of the two different activation protocols, either FBA (black bars) or AFS (open bars), on the relative abundance of eight gene transcripts (means ± SEM) in cloned bovine blastocysts compared to their in vitro-generated counterparts (gray bars; IVP1). Bars with different superscripts within each gene transcript differ significantly (a:b $P \leq 0.05$).

of this transcript was significantly higher in blastocysts grown in AgR SOF supplemented with Sigma BSA (IVP1) than in SOF supplemented with serum (IVP3). The amount of Hsp transcript was significantly higher in embryos grown under serum-enriched conditions (IVP3) compared to in vivo-derived embryos and those generated employing Sigma BSA as a protein source (IVP1). The RA of IF transcripts was significantly elevated in embryos grown in Sigma BSA (IVP1)- or serum (IVP3)-enriched medium compared to embryos grown in vivo and using Life Technologies BSA (IVP2). No significant differences were detected for the other gene transcripts.

DISCUSSION

In the present study, we have selected the timing of fusion and activation, use of either quiescent (G_0) or nonquiescent (G_1) cells, as well as the passage number of donor cells as important steps in the NT protocol to investigate their effects on the genomic activity of NT-derived blastocysts. By fusing the cells prior to artificial activation, the donor cells are exposed over an extended period to cytoplasmic factors present in the recipient oocyte, and this may facilitate reprogramming [40]. It had been hypothesized that the chromatin of quiescent (G_0) cells may be more susceptible to those structural changes after NT that are associated with reprogramming of gene expression [8]. Aged or more differentiated donor nuclei used for NT have been suspected to increase the frequency of abnormal reprogramming [41]. In the study reported here, the expression pattern of eight gene transcripts thought to play a cru-

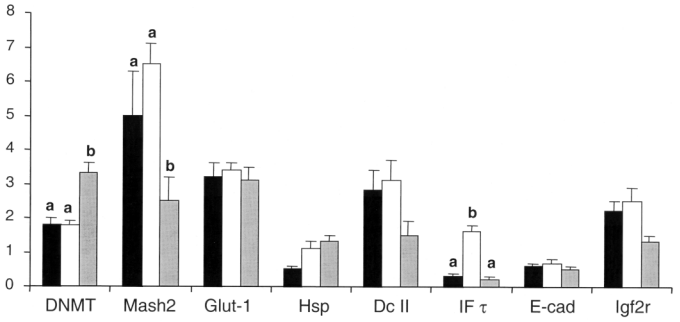

FIG. 3. Alterations in mRNA abundances (means ± SEM) attributed to two different cell cycle stages, either G_0 (black bars) or G_1 (open bars) donor cells, used for NT compared to IVP embryos (gray bars; IVP2). Bars with different superscripts within each gene transcript differ significantly (a:b $P \leq 0.05$).

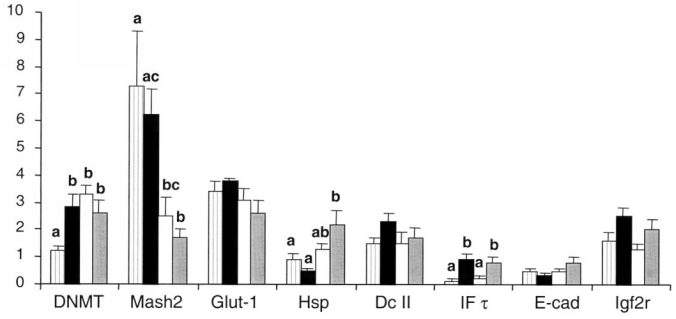

FIG. 4. Effects of three different culture systems (IVP1, black bars; IVP2, open bars; IVP3, gray bars) on the RA of eight gene transcripts (means ± SEM) in in vitro-produced blastocysts compared with their in vivo-derived counterparts (lined bars). Bars with different superscripts within each gene transcript differ significantly (a:b:c $P \leq 0.05$).

cial role in preimplantation development was determined in reconstructed blastocysts and their transcriptional activity was compared with that of in vitro- and in vivo-derived embryos. Recently, it had been shown that the basic culture medium and supplementation with serum significantly affected the relative abundance of Glut-1, Hsp, Dc II, IF τ, and E-cad [29, 30]. Blastocysts were chosen for the present analysis because early reprogramming is controlled by post-translational events inherited from the mature cytoplasm of the recipient oocyte. The major activation of the bovine embryonic genome commences at the 8- to 16-cell stage [42], and the reprogramming is then completed by the embryo itself [43, 44]. To better understand reprogramming and to separate the effects of NT from those of suboptimal culture conditions (the NT embryos have been cultured for the same period employing the same culture conditions as their IVP counterparts), in vivo-derived embryos were included in this study. Many differences have been found between in vivo- and in vitro-derived embryos [45–47]. It should be born in mind that in vivo embryos were collected after superovulatory treatment and may therefore not fully resemble those derived from a single embryo collection. However, the developmental capacity of transferable bovine embryos is not affected by superovulatory treatments and the ovarian responses as shown by high pregnancy rates and the delivery of a high proportion of normal calves [48, 49]. As recently reported [30], the results of experiment 4 show that the culture system can affect the expression pattern of developmentally important genes. However, the differences do not detract from the validity of the present findings made for cloned embryos. They were compared with IVP embryos produced in the same laboratory as well as IVP- and in vivo-derived ones from the laboratory in which the cloned embryos were analyzed for gene expression.

Our results show that distinct steps in the NT protocol affect the relative abundance of several of the tested genes thought to play a crucial role in the understanding of abnormal development after NT. An interesting observation of our study was the inhibitory effects of the activation protocols of the NT procedure on the expression of Hsp transcripts. Hsp has been identified as a sensitive indicator of stress in bovine preimplantation embryos [29, 30]. However, further experiments are needed to determine how the activation protocol affects the stress response of NT embryos and how persistent the lack of Hsp expression is. Speculatively, the failure to detect Hsp transcripts could indicate the inability of NT embryos to cope with an adverse environment and thus contribute to the impaired viability of offspring derived from NT embryos.

In the present study, IF τ transcription in embryos generated with the FBA treatment was similar to that of in vivo-derived blastocysts and significantly reduced compared to AFS and the IVP1 control blastocysts. The highest relative amount of IF τ was found in NT embryos produced with G_1 donor cells that had not been serum starved or from cells in an advanced passage number (P8). It has been shown that early and high expression of IF τ indicates poor quality of the bovine embryo [29, 30, 50]. This is consistent with the findings of this study and supported by data whereby development to the blastocyst stage is greater with FBA compared to AFS [6, 9]. Furthermore, initial results on the post-transfer viability of cloned embryos produced using G_0 cells indicate that higher pregnancy rates can be obtained with those produced with G_1 cells (unpublished data).

DNA methylation in mammals is thought to be essential in the regulation of transcription in embryonic development, involved in X chromosome inactivation as well as cell differentiation and imprinting, and usually is associated with gene silencing [51]. The three possible methylation types, i.e., loss, gain, and allelic change in methylation, were observed in mouse fetuses derived from embryonic stem cells [52]. DNMT1 is the most extensively studied and abundant DNA methyltransferase and is thought to be responsible for copying methylation patterns following DNA synthesis [53]. Disruption of the DNMT1 gene in mice resulted in abnormal imprinting, embryonic lethality, and greatly reduced levels of DNA methylation [53]. DNMT transcription had not been investigated in bovine embryos until now. Compared to IVP control blastocysts, the RA of DNMT transcripts was significantly decreased in NT embryos independent of whether G_0 or G_1 donor cells were used and irrespective of passage number. Interestingly, only a low level of DNMT transcription was found for in vivo-derived embryos. This suggests that the IVP process in general has more severe effects on DNMT-1 expression than the aspects of the NT protocol examined here. Recently, it has been shown that DNMT levels were significantly downregulated in cultured fibroblasts that were in G_0/G_1 of the cell cycle [54]. Low-level overexpression of DNMT1 resulted in cellular transformation, while a dramatically elevated expression was detrimental to cells [55, 56]. Abnormally high DNMT transcript levels could be correlated with methylation errors even in de novo methylation that would then be copied after cell division [57]. If persistent, this could lead to epigenetic changes suspected to be associated with the abnormalities seen in offspring after IVP and NT [19, 47].

The mouse Mash2 gene encodes a transcription factor required for development of trophoblast progenitors. A null mutation in Mash2 blocks the formation of the spongiotrophoblast. As a result, the chorioallantoic circulation of the placenta is not established and mutant embryos die at around 10 days of embryonic development [58]. The Mash2 gene is paternally imprinted in mice [58]. Mash2 transcription had not been investigated in bovine embryos until now. The relative amount of Mash2 mRNA was significantly elevated in NT embryos in experiments 2 and 3 compared to their IVP2 counterparts. However, in vivo-generated embryos also showed a relatively high level of Mash2 transcription. Mash2 is highly expressed in diploid trophoblast cells of the postimplantation mouse embryo but only transiently expressed at low levels in the embryo prop-

er [59]. Recently, it has been shown that mechanisms other than DNA methylation, such as allele-specific chromatin conformation, may be involved in the maintenance of parental origin-specific expression of Mash2 [60].

Surprisingly, expression of one of the primary candidate genes regulating fetal growth, Igf2r, was not altered in NT- and IVP-derived embryos. Igf2r mRNA expression has been determined throughout bovine preimplantation development in vitro [61] and down-regulates insulin-like growth factor (IGF)-II activity by binding, internalizing, and degradation of the ligand [62]. The Igf2r gene is paternally imprinted in mice [58]. Mutant mice expressing increased levels of IGF-II exhibited body weights that are 125–135% that of normal mice [62, 63]. Recently, it had been shown that the levels of IGF-II mRNA were significantly elevated in bovine fetuses originating from IVP embryos as compared with the in vivo counterparts [64].

In the present study, NT embryos showed a deviation in expression pattern with respect to DNMT and Mash2 mRNA when compared to their IVP counterparts, but this expression was similar to that of in vivo-derived embryos. Moreover, expression of Mash2 and IF τ, i.e., genes that are exclusively expressed in the trophectoderm, the precursor of the placenta, was heavily affected by the cloning procedure. These results support the hypothesis that deviation from normal placentation is a major cause of pregnancy loss after transfer of cloned and IVP embryos [5, 65–67].

In conclusion, the present results demonstrate for the first time that modifications of the NT protocol can alter the expression pattern of developmentally important genes in NT-derived embryos compared to their IVP and in vivo-derived counterparts. An aberrant expression pattern in NT embryos was found for genes thought to be involved in stress adaptation, trophoblastic function, and DNA methylation during preimplantation development. These early deviations in gene expression patterns seen in NT-derived embryos warrant further investigations in postimplantation and neonatal development to understand the causative mechanism of the abnormalities and elevated mortality after transfer of NT- and IVP-derived embryos. Results of this study show that mRNA phenotyping provides a useful tool to unravel effects of NT at the molecular level. By employing in vitro and in vivo control embryos it is possible to optimize the NT protocol and to ensure production of embryos with a normal gene expression profile. This will likely aid to reduce the incidence of the large offspring syndrome and should increase the proportion of viable calves. The recent development in cDNA array technology that allows the simultaneous determination of several thousands of gene transcripts will provide a useful tool for such studies [68].

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