Characterization of Deoxyribonucleic Acid Synthesis and the Transition from Maternal to Embryonic Control in the 4-Cell Porcine Embryo[1]

R.A. SCHOENBECK, M.S. PETERS, L.F. RICKORDS, T.T. STUMPF, and R.S. PRATHER[2]

Department of Animal Sciences, University of Missouri-Columbia, Columbia, Missouri 65211

ABSTRACT

These studies were conducted to identify the point during the 4-cell stage at which the porcine embryo begins to control development. Reproductive tracts of gilts were flushed 48 h after the onset of estrus to obtain 1- and 2-cell embryos. To determine the duration of the 4-cell stage in vitro, development of 29 embryos was timed from cleavage to the 4-cell stage and from cleavage to the 8-cell stage. The average duration of the 4-cell stage was 50.5 h. The duration of the 4-cell stage was positively correlated ($p < 0.01$) with culture time in vitro before cleavage to the 4-cell stage. DNA content was determined by using the Feulgen's reaction and quantified with micro-densitometry. Staining units (SU; density × area) were calculated at 0, 2, 4, 6, 8, 10, 12, 16, 20, 24, 30, and 36 h post-cleavage to the 4-cell stage (P4C). Results revealed a possible G_1 phase (< 2 h) with DNA synthesis starting within 2 h P4C. DNA synthesis was completed by 16 h P4C, and was followed by an extended G_2 phase. Embryos were evaluated for uptake and incorporation of [^{35}S]methionine and for qualitative changes in protein profiles specific to time points during the 4-cell stage (2, 10, 14, 16, 18, 24, 30, and 40 h P4C). Methionine uptake and incorporation into protein followed similar patterns, both decreasing until 16–18 h P4C, followed by a steady increase through the 4-cell stage. Protein profiles revealed qualitative changes beginning at 14 and 16 h P4C. Four-cell stage embryos from various time points P4C (0, 4, 8, 12, 16, 20, and 24 h P4C) were cultured in α-amanitin until 24 h P4C, when all embryos were radiolabeled. Uptake and incorporation of [^{35}S]methionine followed similar patterns, with both decreasing until 16 h P4C, followed by a steady increase through the 4-cell stage. A qualitative change began to occur when α-amanitin was added at 12 h P4C, with most qualitative changes complete by 16 h P4C. Embryos completed the third cell cycle when continuously cultured in the presence of α-amanitin from 24, 30, and 36 h P4C. These results suggest that the maternal genome controls development until 16 h P4C (after completion of DNA synthesis) and that the embryonic genome begins to control development at the start of the G_2 phase.

INTRODUCTION

Molecular changes that occur during mammalian preimplantation embryonic development have been well evaluated in the mouse, but recent information suggests that the molecular changes that occur in the cow, sheep, rabbit, and human are different from those in the mouse. Preimplantation development in mammals is characterized by a period of absence of RNA synthesis followed by the initiation of RNA synthesis at species-specific cell stages. Proteins synthesized during this time are translated from mRNA transcribed from the maternal genome. Maternally derived mRNA is thought to be degraded prior to or shortly after the transition to embryonic control of RNA production [1]. Incorporation of uridine characterizes new mRNA synthesis and the activation of the embryonic genome and transcription. This renewed incorporation of uridine is in conjunction with qualitative changes in protein profiles as seen in the mice [2–4] and cattle [5, 6].

α-Amanitin, an RNA polymerase II inhibitor, was used to identify the period of activation of the embryonic genome and the specific proteins that may be dependent upon tran-

scripts derived from the embryo. During the 4-cell stage in the pig, renewed uridine incorporation [7] and α-amanitin sensitivity occurs simultaneously with qualitative changes in protein profiles, suggesting that the maternal-to-embryonic transition occurs during the 4-cell stage [8]. Similarly, in the mouse, α-amanitin-sensitive proteins are synthesized during G_1 of the 2-cell stage [9].

Preimplantation embryos of mammalian species undergo developmental blocks in vitro if not in the correct culture medium. The stage when the block occurs has been correlated with the time when the embryonic genome takes over control of development from the maternal genome [1]. These adverse conditions then result in either impaired transmission of inherited maternal information [10] or defective activation of the embryonic genome [11]. Since the 4-cell stage porcine embryo is sensitive to in vitro development [1], as seen in the 2-cell mouse embryo, this marks a critical step in early differentiation.

The cell cycle consists of four distinct phases, mitotic phase (M), pre-DNA synthesis phase (G_1), DNA synthesis phase (S), and post-DNA synthesis phase (G_2). In a dividing cell, the M phase alternates with an interphase or growth period. Interphase is divided into two "gap" phases (G_1 and G_2) separated by DNA synthesis. DNA replication generally occupies only a fraction of the cell division cycle. Embryonic when compared to somatic cell cycles lack or have short gap phases, thus the cleavage stage cell cycles are short and dominated by S and M phases. This pattern is possible because the cleavage stage embryo is spared the need for

cytoplasmic replication by virtue of its inheritance from the oocyte.

Cell cycle phases of preimplantation embryos have been determined in several species using different methods. The Feulgen's reaction [12, 13] and [³H]thymidine incorporation [14–16] have been used to study the cell cycle of the mouse preimplantation embryo. In addition, [³H]thymidine incorporation in the rabbit [17] and DAPI fluorescent DNA stain in cattle [18] have been used to study the cell cycle. Better understanding of the cell cycle and proteins involved will allow for biotechnologies, i.e., nuclear transfer or gene integration, to be more efficiently performed. Therefore, we investigated the relationship between protein synthesis in the cell cycle (M, G_1, S, G_2) at the 4-cell stage and the appearance of specific proteins that are the products of the embryonic genome in the 4-cell stage porcine embryo.

MATERIALS AND METHODS

Oocyte and Embryo Collection

Crossbred gilts were monitored once a day for estrus by exposure to a mature boar and were artificially inseminated at 12 and 24 h post-estrus detection. Forty-eight hours after detection of the second estrus, gilts were administered 23 cc of 5% pentothal via a peripheral ear vein followed by 5% halothane/oxygen anesthesia. Oocytes, zygotes, and 2-cell embryos were collected by exposing the reproductive tract via a mid-ventral incision and flushing retrograde with modified Whitten's medium (WM: adjusted osmolarity of 240–255 mOsm) containing 0.3% BSA (Fraction V; Sigma Chemical Co., St. Louis, MO) [19]. Embryos were rinsed in WM; only normal-appearing embryos were used in the subsequent experiments.

In Vitro Culture of Embryos

Embryos were cultured in 50-µl drops of WM under 10 ml of sterile paraffin oil that had been pre-equilibrated in WM salts. Incubation was conducted at 39°C in a humidified atmosphere of 5% CO_2 in air.

DNA Quantification

Embryos were cultured in WM and observed every 2 h for cleavage to the 4-cell stage. When embryos were being observed, warm water (36–39°C) flasks were used to maintain the temperature of culture plates. Eight embryos per pig were allotted equally to 12 time points (0, 2, 4, 6, 8, 10, 12, 16, 20, 24, 30, and 36 h post-cleavage to the 4-cell stage; P4C). Sperm, in vivo-matured metaphase II oocytes, and germinal vesicle stage oocytes, with 1 C, 2 C, and 4 C, respectively (C = DNA content), were used as controls to provide DNA reference values for determining stage of cell cycle. Mature boar semen was collected and diluted 20:1 with semen extender. The diluted semen was placed on clean microscope slides and allowed to air-dry. In vivo-

matured metaphase II oocytes were recovered as described in the embryo collection procedures section. Germinal vesicle stage oocytes were aspirated from ovaries obtained from a slaughterhouse. Cumulus cells were stripped by vortexing for 5 min in Hepes-buffered Tyrode's medium (HbT) [20] containing 1 mg hyaluronidase (Sigma)/ml. Embryos and oocytes were placed in an acidic WM (pH = 2.5) for approximately 30 sec, then rinsed in Ca^{2+}/Mg^{2+}-free WM until the zonae pellucidae were removed. Embryos and oocytes were then air-dried on a clean microscope slide for 1–2 h. Fixing and staining procedures using Schiff's basic stain for the Feulgen's reaction was used as described by Bolton et al. [9]. Slides were stored at −20°C until quantitation.

When all samples had been collected, slides were removed from −20°C and allowed to warm to room temperature (25°C). Slides were analyzed using an integrated micro-densitometer (Bioquant 250-CF, New Orleans, LA) that estimates the quantity of DNA in nuclei by measuring the light absorption at a wavelength of 546 nm.

Radiolabeling of Embryos

Embryos were labeled for 4 h in 26 µl of WM containing approximately 1.15 mCi L-[³⁵S]methionine/ml (925 to 1248 Ci/mM; Amersham Corp., Arlington Heights, IL). Embryos were then successively rinsed through seven 50-µl drops of nonradioactive WM with a final rinse in HbT stock (BSA-free). Embryos were placed individually in 15 µl of SDS lysis buffer in 0.5-ml microcentrifuge tubes and stored at −70°C. Similarly, 5-µl samples were taken from the last rinse in WM and from the original radiolabeling drop to serve as a background estimate of radioactivity and to establish the total counts per minute (cpm) per fmole, respectively.

Samples were thawed and boiled for 1 min immediately prior to analysis. Three microliters were removed from each sample and placed in a 1.5-ml microcentrifuge tube. One hundred microliters of cold 5% BSA and 100 µl of cold 30% trichloroacetic acid (Fisher Scientific, Pittsburgh, PA) were sequentially added and the proteins were allowed to precipitate for at least 1 h at 4°C. Tubes were then centrifuged at 15 000 × g for 10 min at 4°C. The supernatant was aspirated and placed in a scintillation vial. The precipitate was then resuspended with 200 µl H_2O and transferred into a scintillation vial. Four milliliters of ScintiVerse BD (Fisher Scientific) was added to all scintillation vials and counted in a β-counter (LKB Wallac 1214; LKB, Gaithersburg, MD). Total uptake of radiolabeled methionine was calculated by adding acid-soluble and acid-insoluble cpm; cpm values were converted to fmoles and rates of uptake and incorporation were expressed to 3 significant digits as fmol · embryo⁻¹ · 4 h⁻¹. Samples containing lysed 4-cell embryos were refrozen until one-dimensional SDS-PAGE could be performed on the remaining volume. Differences between mean rates of uptake and incorporation of radiolabeled methio-
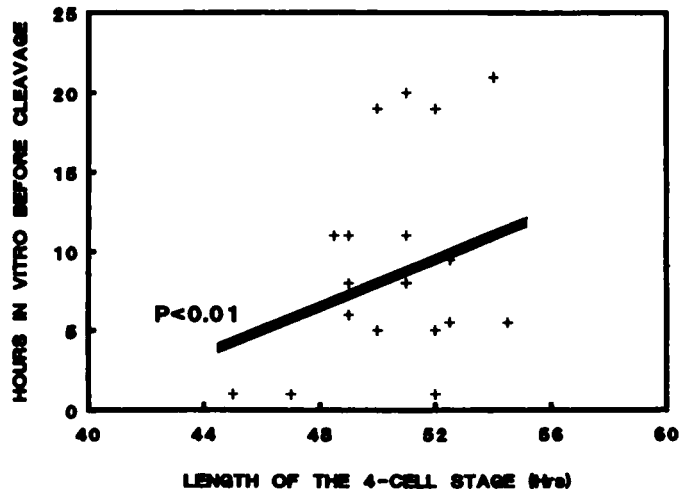
FIG. 1. Duration of the 4-cell stage in vitro. Mean 4-cell stage durations (50.5 ± 2.3 h) from 29 embryos from 3 different gilts. An increase in culture duration ($p < 0.01$) was observed with increased culture time pre-4-cell cleavage.

nine by embryos at different stages were determined using MGLH of Systat [21] with protected LSDs.

Protein Profiles

The remaining 12 μl from each sample were available for 10.0% or 12.5% one-dimensional SDS-PAGE. Prestained molecular mass markers (Rainbow Protein Molecular Weight Markers, Amersham) were run in parallel. After electrophoresis, the gels were fixed for 1 h in 30% methanol:10% acetic acid:60% H_2O, followed by a 1-h treatment with a fluorite (En^3Hance; New England Nuclear, Boston, MA), and a 30-min precipitation in water. Gels were dried and exposed to Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY) at $-70°C$ for 2–4 days.
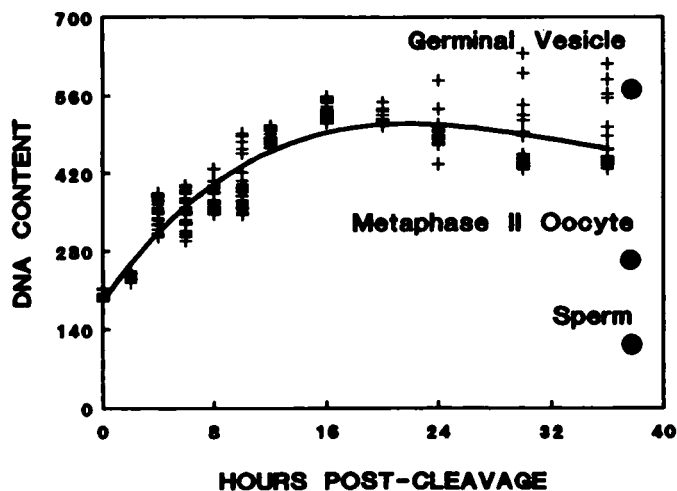

FIG. 2. DNA quantitation during the 4-cell stage. Four-cell embryo mean SUs with > 20 measurements/time point taken at 0, 2, 4, 6, 8, 10, 12, 16, 20, 24, 30, and 36 h post-cleavage to the 4-cell stage ($^{a,b,c,d,e}p < 0.01$).

α-Amanitin Culture

Four-cell embryos were cultured in the presence of 20 μg/ml α-amanitin (A-2263, Sigma). This culture was performed to establish the time point during the 4-cell stage when production of mRNA is necessary for production of stage-specific proteins and continued cell division. Ten embryos per group were placed in α-amanitin at 0, 4, 8, 16, 20, and 24 h P4C. At 24 h P4C, approximately 1 mCi/ml [35S]methionine was added to all α-amanitin-cultured embryo wells. In addition, an α-amanitin-free drop was used as a control with embryos labeled at 24 h P4C.

RESULTS

Experiment 1: 4-Cell In Vitro Culture Duration

Twenty-nine embryos from 3 gilts were collected as 1- or 2-cell embryos. Embryos were cultured in WM and observed every 2 h for cleavage to the 4- and 8-cell stages. A mean of $50.5 ± 2.3$ h was observed for the 4- to 8-cell stage. A majority of the 4-cell embryos cleaved from 48 to 53 h P4C as displayed in Figure 1. Furthermore, the duration of the 4-cell stage ($p < 0.01$) was dependent upon culture time in vitro before cleavage to the 4-cell stage.

Experiment 2: 4-Cell DNA Quantitation

Eight embryos (4 blastomeres per embryo allowed for a total of 32 possible nuclei) were observed per time point. Eighty-one percent (313/384) of all nuclei from 4-cell embryos mounted, fixed, and stained were viewable for collection of data points. Boar sperm (1C), in vivo-matured metaphase II oocytes (2C), and germinal vesicle stage oocytes (4C) controls revealed mean staining units (SUs; density × area) of 125.2, 205.9, and 586.6, respectively. These controls were used to indicate relative amounts of DNA compared to quantified DNA of 4-cell embryos. Mean arbitrary SUs at 0, 2, 4, 6, 8, 10, 12, 16, 20, 24, 30, and 36 h P4C were 200.0^a, 234.2^a, 360.9^b, 351.8^b, 370.7^{bc}, 394.4^c, 481.5^d, 530.8^e, 516.2^{de}, 497.2^{de}, 473.6^{de} and 475.35^d ($^{a,b,c,d,e}p < 0.01$; Fig. 2). This indicated a short if any G_1 phase, < 2 h, with DNA synthesis starting by approximately 2 h P4C ($p < 0.05$). DNA synthesis was completed by 16 h P4C followed by an extended G_2 phase.

Experiment 3: [35S]Methionine Labeling

Uptake and incorporation of radiolabeled methionine by porcine embryos (10 per time point) at 2, 10, 14, 16, 18, 24, 30, and 40 h P4C are displayed in Figure 3. Mean uptake of radiolabeled methionine at 2 h was 104^a fmol · embryo^{-1} · 4 h^{-1} and decreased ($^{a,b,c}p < 0.05$) to 103^a, 56.6^b, 30.9^c, 35.6^c fmol · embryo^{-1} · 4 h^{-1} at time points of 10, 14, 16, and 18 h, respectively. This decreasing trend was reversed at 24, 30, and 40 h with a mean uptake of 54.4^{bc}, 53.3^{bc}, and 71.3^{bc} fmol · embryo^{-1} · 4 h^{-1}, respectively. Incorporation of methionine into protein followed a similar pattern with
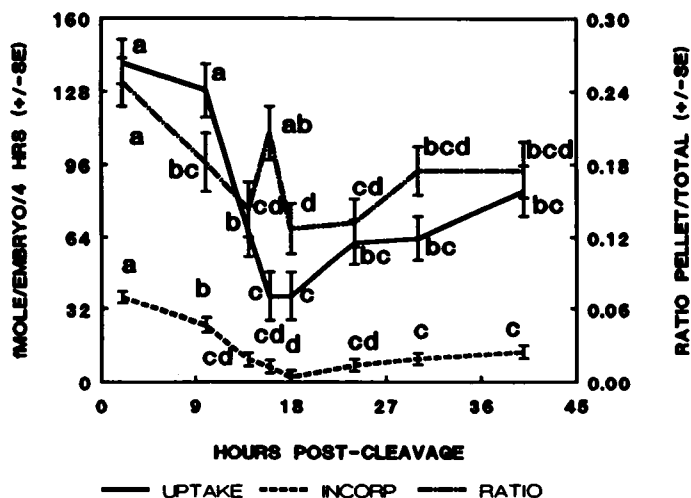
FIG. 3. [^{35}S]Methionine uptake and incorporation during the 4-cell stage. Uptake (solid line), incorporation (dotted line), and ratio (incorporation/total; solid-interrupted line) of [^{35}S]methionine (± SE) into porcine 4-cell embryos. Ten embryos/time point at 2, 10, 14, 16, 18, 24, 30, and 40 h postcleavage to the 4-cell stage were analyzed. Significant differences ($p < 0.05$) within mean uptake, incorporation, and ratio are denoted by different superscripts.

decreasing levels ([a,b,c,d]$p < 0.05$) from 2, 10, 14, 16, and 18 h with 37.2[a], 25.1[b], 9.93[cd], 6.80[cd], and 2.28[d] fmol · embryo^{-1} · 4 h^{-1}, respectively. This decreasing trend was reversed at 24, 30, and 40 h with a mean incorporation of 7.3[cd], 10.1[c], and 13.3[c] fmol · embryo^{-1} · 4 h^{-1}. A dam effect was observed ($p < 0.05$) on uptake and incorporation of [^{35}S]methionine.

Qualitative changes in protein profiles were observed from 10 to 24 h P4C as displayed in Figure 4. Specifically, at 10 h P4C, protein bands at approximately 17, 22, and 34 kDa were prominent with decreasing intensity at 14 and 16 h P4C. In addition, protein bands at approximately 26 and 42 kDa were present at 24 h P4C. A 50-kDa protein band was present at 16 h P4C with increasing intensity until 24 h P4C when it became very prominent.
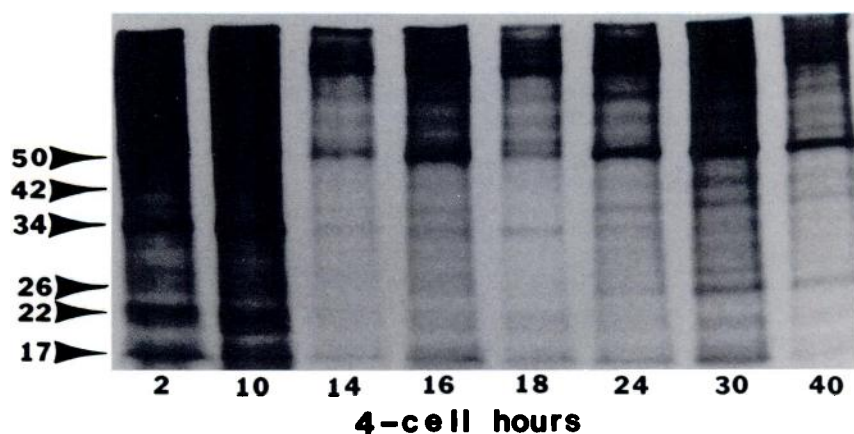
Experiment 4: α-Amanitin Culture with [^{35}S]Methionine Labeling

Uptake and incorporation of radiolabeled methionine by porcine embryos cultured in the presence of α-amanitin from various time points (0, 4, 8, 12, 16, 20, 24, and 24 h with no α-amanitin culture P4C) during the 4-cell stage are displayed in Figure 5. Mean uptake of radiolabeled methionine when α-amanitin treatment began at 0 h was 80.5[a] fmol · embryo^{-1} · 4 h^{-1} and decreased ([a,b,c,d]$p < 0.05$) to 53.7[b], 52.9[b], 43.6[bc], and 32.7[c] fmol · embryo^{-1} · 4 h^{-1} at the 4-, 8-, 12-, and 16-h time points, respectively. This decreasing trend was reversed at 20, 24, and 24 h with no α-amanitin to a mean uptake of 46.0[bc], 50.5[b], and 57.8[b] fmol · embryo^{-1} · 4 h^{-1}, respectively. Incorporation of methionine into protein followed a similar pattern with levels decreasing ([a,b,c,d]$p < 0.05$) from 0, 4, 8, 12 to 16 h with 8.93[a], 6.54[abc], 5.94[cd], 4.39[cd], and 3.55[d] fmol · embryo^{-1} · 4 h^{-1}, respectively. This decreasing trend was reversed at 20, 24, and 24 h with no α-amanitin to a mean incorporation of 5.11[cd], 6.43[b], and 7.66[b] fmol · embryo^{-1} · 4 h^{-1}. A dam effect was observed ($p < 0.05$) on uptake and incorporation of [^{35}S]methionine.

Qualitative changes in protein profiles were observed when α-amanitin was added 8–24 h P4C (Fig. 6). Specifically, addition of α-amanitin at 8 h P4C resulted in prominent protein bands at approximately 22 and 34 kDa, but intensity decreased when α-amanitin was added 12 h P4C and was no longer detectable when added at 16 h P4C. In addition, protein bands at approximately 26- and 42-kDa were not present at 24 h P4C. A 50-kDa protein band was present when α-amanitin was added 16 h P4C, with increasing intensity until 24 h P4C, when it became prominent.

Four-cell embryos (n = 55) were cultured in the presence of α-amanitin for various times and durations during the 4-cell stage. Four-cell embryos placed in WM plus α-amanitin at 0, 4, 8, 12, 16, and 20 h P4C did not proceed
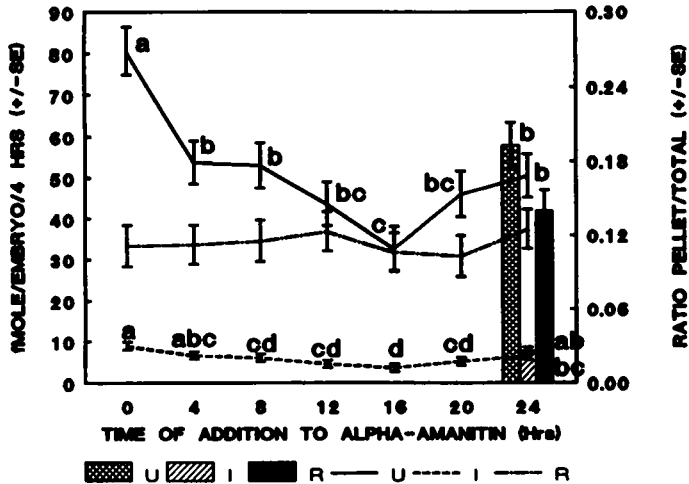

FIG. 4. Qualitative changes in protein profiles during the 4-cell stage. A single embryo was applied to each lane. Estimated molecular mass × 10^3 on the vertical axis.

FIG. 5. [³⁵S]Methionine uptake and incorporation during the 4-cell stage: α-amanitin. Uptake (solid line), incorporation (dotted line), and ratio (incorporation/total; solid-interrupted line) of [³⁵S]methionine (10 embryos/ time point) after α-amanitin culture from 0, 4, 8, 12, 16, 20, and 24 h post-cleavage to the 4-cell stage followed by labeling of all embryos at 24 h. A 24-h post-cleavage to the 4-cell stage control (no α-amanitin) was used to demonstrate uptake (crossed bar), incorporation (diagonal bar), and ratio (incorporation/total; solid bar). Significant differences (p < 0.05) within mean uptake, incorporation, and ratio are denoted by different superscripts.

to the 8-cell stage. However, 4 of 10 embryos placed in WM and α-amanitin at 24 h P4C completed the third cell cycle. All embryos placed in WM and α-amanitin at 30 and 36 h P4C proceeded to the 8-cell stage.

DISCUSSION

Early preimplantation embryo development relies on messages stored within the oocyte during oogenesis and

thus is controlled by the maternal genome, independent from the embryonic genome [22]. Several events or changes are characteristic of the transition from maternal to embryonic control of development; these include the longest preimplantation embryo stage, "in vitro culture block" stage, initiation of embryonic RNA synthesis, major changes in qualitative patterns of protein synthesis, and developmental arrest when continuously cultured in α-amanitin. Studies have implied that in the pig, the transition from maternal to embryonic control occurs sometime during the 4- to 8-cell stage [7, 8, 23, 24]. Species variations have been observed with regard to differences in developmental stages when this transition occurs and when the embryo is no longer dependent on transcripts derived from the maternal genome. In this study, we have focused specifically on the 4-cell embryo as the stage of development in pigs when the transition from maternal to embryonic control of the genome occurs and have investigated the molecular events associated with this transition.

Beckmann et al. [19] and Jarrell et al. [8] have successfully cultured pronuclear or 2-cell stage pig embryos through the 4-cell in vitro culture block to blastocysts in WM. Data presented here using WM suggest that the duration of the 4-cell stage in vitro is 50 h. The duration of the 4-cell stage in vivo has been estimated to be from 24 to 36 h [25], with variation between dams. The 4-cell stage appears to be the longest stage during porcine preimplantation embryonic development.

Smith and Johnson [13] and Barnes and Eyestone [18] showed the greatest variability in cell cycle duration in the G_2 phase for each of the first four cell cycles in mice and
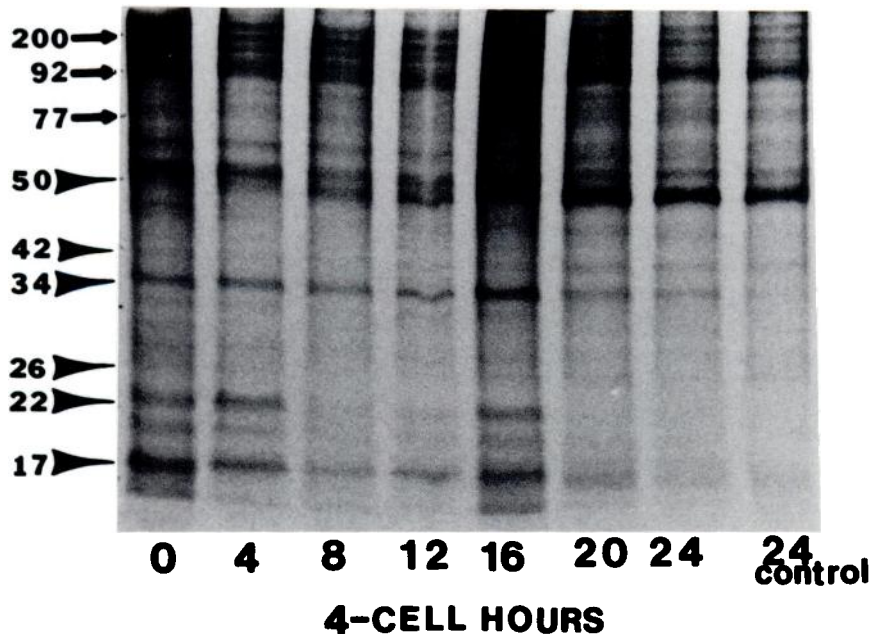

FIG. 6. Qualitative changes in protein profiles at 24 h P4C: α-amanitin. An equal number of counts were applied to each lane. Estimated molecular mass × 10³ on the vertical axis.

cattle. In the pig, variability and longer cleavage times could represent both a physiological response and an artifact due to sensitivity of embryos to environmental stress (i.e., culture medium, light exposure, temperature). Cleavage rates of mouse embryos cultured in vitro, when compared to those cultured in vivo, seem to be slower or "retarded" and were double those observed in vivo [26] and might be due to exposure to lower temperatures, light, or unfavorable culture constituents. In vitro-matured mouse embryos have lower metabolic rates as blastocysts [27] and the amount of [^{35}S]methionine taken up and incorporated in in vitro-cultured porcine blastocyst stage embryos was significantly lower than in freshly collected blastocysts, but protein profiles were not different [8]. Lowering the temperature from 37 to 20°C at any point between the S phases of the first and second cell cycles in the mouse leads to a prolonged G_2 phase [13]. When such a drop in temperature is avoided during the first and second cell cycles, cleavage times are similar to those in vivo. To help prevent this temperature decrease in the present study, warm water (36–39°C) flasks were used to maintain the temperature of culture plates.

In preliminary studies, pig embryos were labeled with [^{3}H]thymidine for 3 h during the 4-cell, 8-cell, 16-cell, and blastocyst stages. Only embryos at the 16-cell and blastocyst stages showed incorporation by autoradiography (unpublished observations). However, [^{3}H]thymidine incorporation was observed in the mouse pronuclear, 2-cell, and 4-cell stage embryos (unpublished observations and [14, 15, 29]). One possibility is that transport of thymidine is limited or nonexistent in the early cleavage stage porcine embryo. The absence of significant thymidine transport is consistent with absence of significant [^{3}H]uridine transport prior to the 4-cell stage in the pig (unpublished observations). Since incorporation of [^{3}H]thymidine was unsuccessful, we decided to pursue DNA quantification by use of the Schiff's basic DNA stain for the Feulgen's reaction.

During the species-specific maternal-to-embryonic transition, cell cycle phases vary in duration. Prior to the 8- to 16-cell stage in cattle [18], mid-blastula embryos in frogs [30], 14th interphase in Drosophila [28], and the 16- to 32-cell stage in rabbits [17], there is the absence of or a very short G_1 phase. In the 4-cell pig embryo, SUs were higher ($p < 0.05$) at the 2-h time point than at the 0-h time point. We concluded that the G_1 phase is either lacking or very short, thus DNA synthesis is initiated by < 2 h P4C. Stumpf et al. [31] showed a short possible G_1 phase (approximately 2 h) in the 2-cell pig embryo. In 2-cell pig embryos [31], the S phase is consistent in duration (approximately 14 h) with the 4-cell stage. Barnes and Eyestone [18] reported consistent S phases (8 h) for the first three cell cycles under maternal control in cattle embryos. After characterizing the cell cycle of the 4-cell porcine embryo, we selected time points that were symmetrical to the time of completion of DNA synthesis to examine qualitative and quantitative protein changes. Expression of genes unique to the embryo in

the form of mRNA synthesis and translation indicate when embryonic control of development is initiated. Changes in protein patterns of early cleavage stage embryos are presumed to reflect the transition from the maternal to embryonic genome. This occurs during the second cell cycle in mice [32, 33], the third cell cycle in humans [34] and rabbits [35], and the fourth cell cycle in sheep [36] and cattle [6, 18].

Stability of maternal mRNA is a characteristic of the maternal control period. Reduction in [^{35}S]methionine incorporation may indicate a change in message stability. A similar event occurs in the mouse at the 2-cell stage, with a decline in the amount of poly(A) RNA, suggesting a period of degradation [37]. The rate of [^{35}S]methionine uptake in 4-cell embryos decreased 70% from the early 4-cell stage until 16 h P4C, when it proceeded to increase steadily through the 4-cell stage. Protein synthesis, shown by incorporation of [^{35}S]methionine, decreased 94% from 4-cell cleavage until 18 h P4C, when it began to increase. Uptake and incorporation results were comparable in pattern but slightly higher than those published by Jarrell et al. [8]. More importantly, the ratio between incorporation and total [^{35}S]methionine decreased until 14 h P4C with a peak at 16 h P4C, and then returned to original levels. This increase could be the result of the embryonic genome initiating transcription. The decrease to and eventual rise around the critical 16-h time point suggest a degradation of maternal mRNA and the onset of transcription of embryonic mRNA. Such a maternally programmed decline in protein synthesis will result in a decrease in the number of ribosomes needed for translation. Activation of rRNA synthesis is associated with the transition from maternal to embryonic control in mice [38], humans [39], cattle [40], sheep [41], and pigs [42].

A decline in total protein synthesis occurs from pronuclear stage embryos until species-specific maternal-to-embryonic transition stages in pigs [8], sheep [36], and cattle [6]. This decrease in detectable protein synthesis may be due to decreased transport of the precursor. If the rate of protein synthesis does decrease, this could delay the accumulation of cell-cycle regulatory factors, such as cyclin proteins that may control the transition from G_2 to M [43].

Mammalian embryos cultured in the presence of α-amanitin are inhibited from expressing new transcripts during the transition from maternal to embryonic developmental control. Uptake and incorporation of [^{35}S]methionine were examined at 24 h P4C in embryos treated with α-amanitin at various time points P4C. Both uptake and incorporation decreased 60% from 4-cell cleavage until 16 h P4C, when both began to increase steadily through the 4-cell stage. Since these embryos were all labeled with [^{35}S]methionine at 24 h P4C, and early treatment with α-amanitin resulted in higher levels of uptake than later treatment, there may be RNA synthesis prior to the S phase. Although the level of [^{35}S]methionine uptake changed with the time of α-amanitin addition, the ratio resembled a flat

line with no differences ($p > 0.05$) between time points (Fig. 5). The production of new proteins (26, 42, and 50 kDa) after 16 h P4C was prevented when embryos were cultured in the continuous presence of α-amanitin, indicating these proteins were transcribed from the embryonic genome as displayed in Figure 6. Interestingly, transcription appears to be necessary for degradation of the maternal proteins as observed with qualitative protein changes in embryos cultured in α-amanitin. Coincident with the transcription needed for new embryonic proteins in cattle is the apparent transcriptionally regulated disappearance of some maternally derived proteins [18]. However, in the mouse 2-cell stage embryo, an embryonic-transcription-independent decline in proteins is observed [9,32]. Our data revealed that 40% (4/10), 100% (5/5), and 100% (5/5) embryos at 24, 30, and 36 h P4C divided to the 8-cell stage, respectively, thus indicating that by 24 h P4C, 4-cell porcine embryos have the required embryonic transcripts needed for further development.

When compared to other livestock species (cattle and sheep), pig embryos develop to blastocysts at a faster rate. Interestingly, porcine embryos also spend less time in the oviduct [25]. Correspondingly, the developmental stage when the embryos enter the uterus is comparable to the developmental stage when the transition from maternal to embryonic control occurs as seen with cattle (8- to 16-cell), sheep (8- to 16-cell), and now in the pig 4-cell embryo.

Genomic activation (during G_2) is coupled to increases in cell cycle duration as observed in Xenopus [44], Drosophila [28], cattle [18], and also in the 4-cell porcine embryo. Embryonic transcription, in pig 4-cell embryos, begins during the extended gap phase (G_2), as observed in cattle embryos [18]. Translational selectivity occurs and some maternal message fails to be translated in favor of the new embryonic message [18].

In conclusion, many crucial molecular changes take place at the 4-cell stage in porcine embryos. The 4-cell stage appears to be one of the longest cell stages of the preimplantation porcine embryo and is the developmental stage when the in vitro culture block occurs. A short G_1 phase and an extended G_2 phase would indicate that embryonic transcription might be occurring. DNA synthesis is completed by 16 h P4C and new embryonic proteins are detected during the G_2 phase. Development is dependent on these new embryonic proteins as shown by the absence of the proteins and developmental arrest at the 4-cell stage when cultured in α-amanitin. In addition, maternal-directed protein synthesis decreases from fertilization until 16 h P4C when it increases as renewed uridine and methionine incorporation reveals embryonic mRNA production.

ACKNOWLEDGMENTS

REFERENCES

1. Telford NA, Watson AJ, Schultz GA. Transition from maternal to embryonic control in early mammalian development: a comparison of several species. Mol Reprod Dev 1990; 26:90–100.
2. van Blerkom J, Brockway GO. Qualitative patterns of protein synthesis in the preimplantation mouse embryo. 1. Normal pregnancy. Dev Biol 1975; 44:148–157.
3. Clegg KB, Piko L. Size and specific activity of the UTP pool and overall rates of RNA synthesis in early mouse embryos. Dev Biol 1977; 58:76–95.
4. Prather RS, First NL. Chimerization of highly asynchronous murine blastomeres: developmental alteration? Gamete Res 1988; 19:359–367.
5. Camous S, Kopecny V, Flechon JE. Autoradiographic detection of the earliest stage of [³H]-uridine incorporation into the cow embryo. Biol Cell 1986; 58:195–200.
6. Frei RE, Schultz GA, Church RB. Qualitative and quantitative changes in protein synthesis occur at the 8- to 16-cell stage of embryogenesis in the cow. J Reprod Fertil 1989; 86:637–641.
7. Tomanek M, Kopecny V, Kanka J. Genome reactivation in developing early pig embryos: an ultrastructural and autoradiographic analysis. Anat Embryol 1989; 180:309–316.
8. Jarrell VL, Day BN, Prather RS. The transition from maternal to zygotic control of development occurs during the 4-cell stage in the domestic pig, Sus scrofa: quantitative and qualitative aspects of protein synthesis. Biol Reprod 1991; 44:62–68.
9. Bolton VN, Oades PJ, Johnson MH. The relationship between cleavage, DNA replication and gene expression in the mouse 2-cell embryo. J Embryol Exp Morphol 1984; 79:139–163.
10. Goddard MJ, Pratt HPM. Control of events during early cleavage of the mouse embryo: an analysis of the "2-cell block." J Embryol Exp Morphol 1988; 73:111–133.
11. Robl JM, Lohse-Heideman JK, First NL. Strain differences in early mouse embryo development in vitro: role of nucleus. J Exp Zool 1988; 247:251–256.
12. Howlett SK, Bolton VN. Sequence and regulation of morphological and molecular events during the first cell cycle of mouse embryogenesis. J Embryol Exp Morphol 1985; 87:175–206.
13. Smith RKW, Johnson MH. Analysis of the third and fourth cell cycles of mouse early development. J Reprod Fertil 1986; 76:393–399.
14. Snow MHL. Abnormal development of pre-implantation mouse embryos grown in vitro with [³H]thymidine. J Embryol Exp Morphol 1973; 29:601–615.
15. Luthardt FW, Donahue RP. Pronuclear DNA synthesis in mouse eggs. Exp Cell Res 1973; 82:143–151.
16. Abramczuk J, Sawicki W. Pronuclear synthesis of DNA in fertilized and parthenogenetically activated mouse eggs. Exp Cell Res 1975; 92:361–372.
17. Collas P, Balise JJ, Robl JM. Influence of cell cycle stage of the donor nucleus on development of nuclear transplantation rabbit embryos. Biol Reprod 1992; 46:492–500.
18. Barnes FL, Eyestone WH. Early cleavage and the maternal zygotic transition in bovine embryos. Theriogenology 1990; 33:141–152.
19. Beckmann LS, Cantly TC, Rieke AR, Day BN. Development and viability of one- and two-cell porcine embryos cultured through the "four-cell block." Theriogenology 1990; 33:193.
20. Bavister BD, Leibfried ML, Liebermann G. Development of preimplantation embryos of the golden hamster is a defined culture medium. Biol Reprod 1983; 28:235–247.
21. Wilkinson L. SYSTAT: The System for Statistics. Evanston, IL: SYSTAT, Inc.; 1990.
22. Johnson MH. The molecular and cellular basis of preimplantation mouse development. Biol Rev 1981; 56:463–498.
23. White KL, Anderson GB, Pashen RL. Identification of a male-specific histocompatibility protein on preimplantation porcine embryos. Gamete Res 1987; 17:107–113.
24. Prather RS, Simms MM, Maul GG, First NL, Schatten G. Nuclear lamin antigens are developmentally regulated during porcine and bovine embryogenesis. Biol Reprod 1989; 41:123–132.
25. Hunter RHF. Chronological and cytological details of fertilization and early embryonic development in the domestic pig, Sus scrofa. Anat Rec 1974; 178:169–185.
26. Bowman P, McLaren A. Cleavage rate of mouse embryos in vivo and in vitro. J Embryol Exp Morphol 1970; 24:203–207.
27. Menke TM, McLaren A. Mouse blastocysts grown in vivo and in vitro: carbon dioxide production and trophoblast outgrowth. J Reprod Fertil 1970; 23:270–275.
28. Edgar BA, O'Farrell PH. Genetic control of cell division patterns in the Drosophila embryo. Cell 1989; 57:177–187.

29. Krishna M, Generoso WM. Timing of sperm penetration, pronuclear formation, pronuclear DNA synthesis, and first cleavage in naturally ovulated mouse eggs. J Exp Zool 1976; 202:245–252.

30. Newport J, Kirschner M. A major developmental transition in early Xenopus embryos. I. Characterization and timing of cellular changes at the midblastula stage. Cell 1982; 30:675–686.

31. Stumpf TT, Schoenbeck RA, Prather RS. DNA synthesis during the porcine embryo two-cell stage. Biol Reprod 1992; 46(suppl 1):71.

32. Flach G, Johnson MH, Braude PR, Taylor RAS, Bolton VN. The transition from maternal to embryonic control in the 2-cell mouse embryo. EMBO J 1982; 1:681–686.

33. Latham KE, Garrels JI, Chang C, Solter D. Quantitative analysis of protein synthesis in mouse embryos. I. Extensive reprogramming at the one- and two-cell stages. Development 1991; 112:921–932.

34. Braude, P, Bolton V, Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. Nature 1988; 32:459–461.

35. van Blerkom J, Manes C. Development of preimplantation rabbit embryos in vivo and in vitro. II. A comparison of qualitative aspects of protein synthesis. Dev Biol 1974; 40:40–51.

36. Crosby IM, Gandolfi F, Moor RM. Control of protein synthesis during early cleavage of sheep embryos. J Reprod Fertil 1988; 82:769–775.

37. Piko L, Clegg KB. Quantitative changes in total RNA, total poly(A) and ribosomes in early mouse embryos. Dev Biol 1982; 89:362–378.

38. Hansmann I, Gebauer J, Bihl L, Grimm T. Onset of nucleolus organizer activity in early mouse embryogenesis and evidence for its regulation. Exp Cell Res 1978; 114:263–268.

39. Tesarik J, Kopecny B, Plachot M, Mandelbaum J. Activation of nucleolar and extranucleolar RNA synthesis and changes in the ribosomal content of human embryos developing in vitro. J Reprod Fertil 1986; 78:463–470.

40. Kopecny V, Flechon JE, Camous S, Fulka J. Nucleologenesis and the onset of transcription in the eight-cell bovine embryo. Mol Reprod Dev 1989; 1:79–90.

41. Calarco PG, McClaren A. Ultrastructural observations of preimplantation stages of sheep. J Embryol Exp Morphol 1976; 36:609.

42. Norberg HS. Ultrastructural aspects of the preattached pig embryo: cleavage and early blastocyst stages. A Anat Entwickl-Gesch 1973; 143:95–114.

43. Murray AW, Kirschner MW. Cyclin synthesis drives the early embryonic cell cycle. Nature 1989; 339:275–280.

44. Kimmelman D, Kirschner M, Scherson T. The events of the midblastula transition in Xenopus are regulated by changes in the cell cycle. Cell 1987; 48:399–407.