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Nuclear control of early embryonic development in domestic pigs

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In mammals, growing oocytes have characteristically high levels of RNA synthesis. After the initiation of meiosis, that is germinal vesicle breakdown, this RNA synthesis ceases. Although there is limited evidence for RNA synthesis by the zygote, significant amounts of RNA synthesis do not occur until a species-specific cell stage. In pigs, significant amounts of mRNA synthesis cannot be detected before the four-cell stage. There appear to be three qualitatively different periods of transcription during the four-cell stage. The first occurs during a short (<2 h) G1 phase. The second, occurs after completion of DNA synthesis (S phase) about 16 h after cleavage to the four-cell stage, and the third occurs about 24 h after cleavage to the four-cell stage. Correlated with these changes in RNA synthesis are changes in nucleolar morphology, amino acid transport characteristics, protein production, mitochondrial morphology, and metabolism of the embryo. The mechanisms that regulate initiation of RNA synthesis in early mammalian embryos appear to repress transcription. A state of transcription permissiveness then follows that sets into motion the differentiation programme.

Introduction

A complete understanding of the molecular events that occur during embryogenesis is essential not only for reducing the loss of conceptuses, but also for its application to newly emerging biotechnologies, such as cloning by nuclear transfer, production of transgenic animals, manufacture of chimaeras and *in vitro* maturation, fertilization and development. Early mammalian development has been best characterized in mice. This is largely due to economic considerations, such as costs of animals, housing, and super-ovulatory drugs. In addition, the supply of embryos from other species is limited by low ovulation and conception rates as well as long generation intervals. The mouse model has been valuable for providing direction for initial studies in other mammalian species; however, as will be discussed, it falls short of providing a sound basis of information for pigs. Previous reviews have focused on the mouse model and provided a species comparison (Prather and First, 1988; Telford *et al.*, 1990); this review will focus on molecular events that occur during embryogenesis in mice and pigs with a comparison with other species where appropriate.

Development of pig embryos immediately after fertilization is independent of embryonic mRNA synthesis. During the four-cell stage, the pig embryo first begins to produce significant amounts of mRNA. As the embryo develops to the blastocyst stage, a variety of metabolic changes occur that result in the establishment of the primary tissues. The first nuclear events probably establish the entire differentiation programme and may be partially responsible for the asynchrony seen in development at elongation. Thus these early differentiation events may have a profound influence upon embryonic survival.

Early Embryonic Cell Cycles

The duration of early embryonic cell cycles has previously been determined by collecting embryos at various times after the onset of oestrus or after hormonal stimulation. The rationale for this type of experimental design was the inability of zygotes placed *in vitro* to develop beyond the four-cell stage, i.e. they stopped developing or 'blocked' at the four-cell stage (Davis, 1985). Culture conditions have been described that permit development from the zygote to blastocyst (reviewed by Reed *et al.*, 1992; Petters, this supplement). Pig embryos do not seem to be sensitive to glucose or phosphate (Hagen *et al.*, 1991; Petters *et al.*, 1990) as are the embryos of hamsters (Schini and Bavister, 1988), mice (Chatot *et al.*, 1989) and cattle (Ellington *et al.*, 1990). Our studies used Whitten's medium to obtain high rates of development of zygotes to the blastocyst stage. Beckmann *et al.* (1990) showed development to term of zygotes cultured to blastocyst in Whitten's medium.

The duration of the early cell cycles might be regulated by the swine leucocyte antigen (SLA) haplotype. This gene is similar to the preimplantation embryonic development (*PED*) gene in mice which maps to the Qa-2 region of the major histocompatibility complex (MHC) (Tian *et al.*, 1992). It is thought that the MHC antigen is linked to the cell surface via glycosylphosphatidylinositol. Such a linkage may provide a mechanism for a transmembrane signal, as removal of the linkage slows development. In miniature pigs $SLA^{a/a}$ and $SLA^{c/c}$ embryos develop faster than do the $SLA^{d/d}$ embryos (Ford *et al.*, 1988). Such genetic differences in domestic pigs may account for some of the variation observed in the timing of blastocyst formation and elongation.

The duration of the one-cell stage, i.e. the first cell cycle, has been estimated as 15 h (Hunter, 1974). The duration of the two-cell stage, i.e. the second cell cycle, has been estimated as about 7 h *in vivo* (Hunter, 1974). However, *in vitro*, the two-cell stage is about 14 h (Stumpf *et al.*, 1992). The Feulgen's reaction was used to quantitate the amount of DNA present at various times after cleavage to the two-cell stage. Most of the cell cycle at the two-cell stage is composed of a DNA synthesis phase (S). It is difficult to conclude whether there is a gap phase 1 (G1) or gap phase 2 (G2) owing to the low sensitivity of the procedures. In contrast to the two-cell stage, the four-cell stage *in vitro* is about 50 h (Schoenbeck *et al.*, 1992). The four-cell stage has a G1 phase of less than 2 h and a DNA synthesis phase of 14–16 h, followed by a G2 phase of about 34 h. This G2 phase seems excessive and may be an *in vitro* induced artefact. In mice, *in vitro* conditions, mainly temperature variations, have been shown to result in protracted G2 phases, while not affecting the S phase (Smith and Johnson, 1986). The estimated duration of the four-cell stage *in vivo* is 27 h (Hunter, 1974).

Although the Feulgen's stain (which quantitates DNA) provided some very important basic information regarding the S phase, it did not provide precise timing as to the onset of DNA synthesis. Culture in the presence of [^{3}H]thymidine followed by autoradiography (which indicates active DNA synthesis) was attempted; however, silver grains were never observed over the nuclei of two- or four-cell stage embryos that, based on the Feulgen data, should have been in S phase. In contrast, silver grains could routinely be observed over nuclei from blastocyst stage embryos (Fig. 1). Further precursor transport experiments determined that very little [^{3}H]thymidine was transported across the plasma membrane of the two- or four-cell stage pig embryos. This is in contrast to the situation in mice (Luthardt and Donahue, 1973; R. A. Schoenbeck, M. S. Peters and R. S. Prather, unpublished) and rabbits (Collas *et al.*, 1992), in which [^{3}H]thymidine readily crosses the plasma membrane. It should be noted that some [^{3}H]thymidine can apparently cross the plasma membrane of pig zygotes (Laurincik *et al.*, 1993).

The duration of the eight-cell stage *in vivo* has been estimated as about 15 h (Hunter, 1974). *In vivo*, compaction begins at the late eight-cell stage (about 49 h after ovulation) and blastocyst formation occurs at the 32-cell stage (about 92 h after ovulation; Hunter, 1974).

Ribonucleic Acid in the Early Pig Embryo

Maternal message

During oocyte growth and development, in mice, all classes of RNA are transcribed and accumulate as stable mRNA (Ebert *et al.*, 1984). During meiotic maturation about one third of the polyadenylated

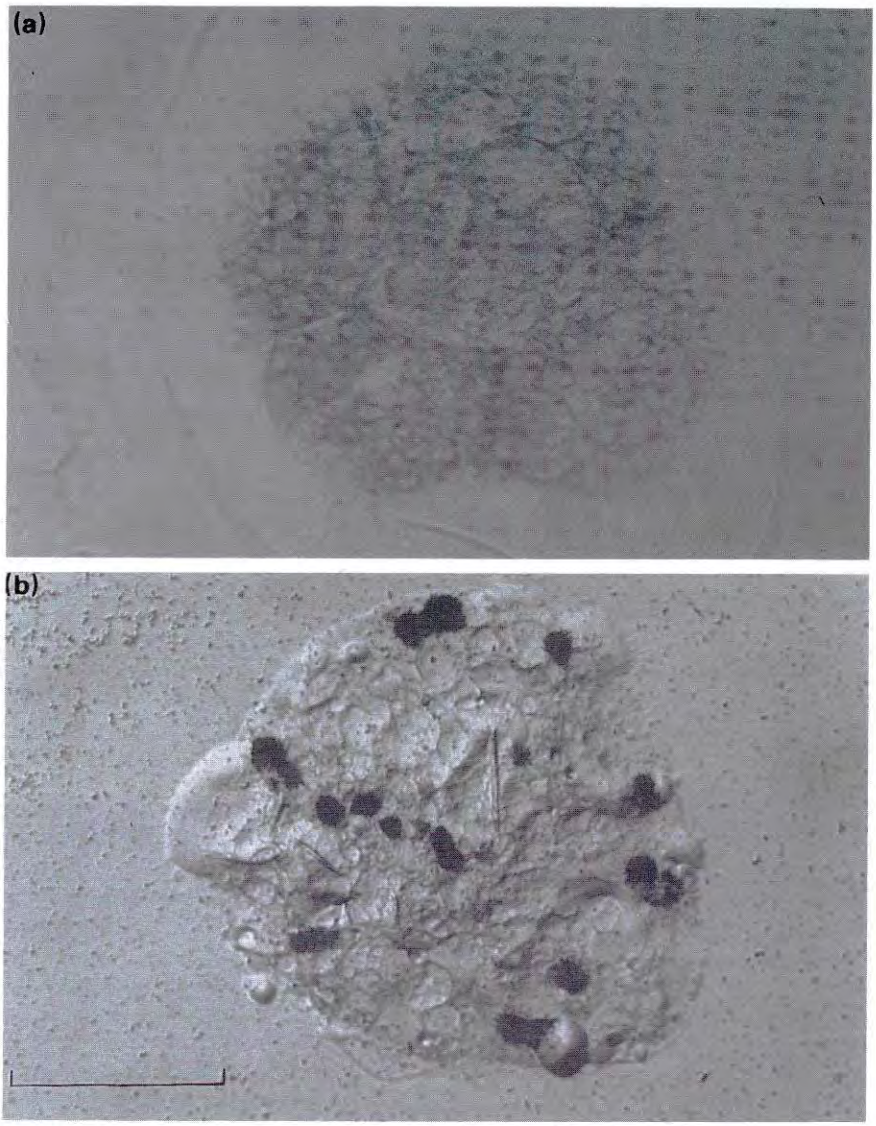

Fig. 1. Thymidine incorporation is shown. Pig embryos were cultured in the presence of 1 μmol [^{3}H]thymidine l^{-1} for 3 h during the time at which DNA synthesis was occurring during the four-cell stage and at the early blastocyst stage. The embryos were then subjected to autoradiography. (a) A four-cell stage embryo with no silver grains is shown, similar results were obtained with eight-cell stage embryos (not shown). In contrast, (b) shows silver grains over a number of nuclei of a blastocyst stage embryo. Silver grains were also obtained over nuclei with mouse pronuclear, two- and four-cell stage embryos (not shown).

RNA is either deadenylated or degraded (Bachvarova, 1981). During the two-cell stage, another 70% of the total polyadenylated and 90% of the histone and actin mRNA are degraded (reviewed by Schultz, 1986). This parallels the degradation seen with exogenously injected globin (Brinster *et al.*, 1980), conalbumin and ovalbumin mRNA (Ebert *et al.*, 1984). In addition, much of the remaining mRNA is in a form that cannot be translated. This may be due to a variety of factors. Fertilization causes polyadenylation, and polyadenylation may be necessary for ribosome association and subsequent translation. In sea urchins, RNA is uncapped before fertilization, but becomes capped at fertilization and is thus made

available for processing (Caldwell and Emerson, 1985). In *Xenopus* mRNA–protein interactions may occur until specific cell stages, at which time the mRNA and proteins dissociate and are free to act upon the nucleus. Thus the RNA–protein complex may be able to regulate both transcription (the released protein interacting with the chromatin) and translation (the released mRNA interacting with the ribosomes; Crawford and Richter, 1987).

Indirect measurements in sheep, cows and pigs also suggest a fall in availability of maternal message at a species-specific cell stage. The amount of protein produced, as measured by incorporation of L-[^{35}S]methionine by the eight-cell stage cow embryo is 68% of that of the zygote stage (Frei *et al.*, 1989), whereas in sheep there is a dramatic decrease of 95% during the eight-cell stage (Crosby *et al.*, 1988). In pigs, there is a 68% fall in protein produced from the zygote to the early four-cell stage and a 72% fall from the early four-cell stage to the late four-cell stage (Jarrell *et al.*, 1991). Although these data suggest a reduction in availability of mRNA, they may simply be the result of differential L-[^{35}S]methionine transport (see below). Thus the question of availability of mRNA remains unanswered.

RNA synthesis

In a variety of species, early embryo development is characterized by cell cycles that have back-to-back mitosis and S phases. This allows no gap phases for RNA polymerases to bind to the DNA for transcription to occur (Rivier and Rine, 1992). Thus early cleavage stages are characterized by the absence of RNA synthesis (Telford *et al.*, 1990). Although RNA synthesis does occur during oocyte growth, it ceases at germinal vesicle breakdown. A few reports suggest that a very small amount of RNA synthesis may occur in zygotes (mouse: Clegg and Piko, 1982; human: Tesarik and Kopecny, 1989) and the bovine two- and four-cell stage (Viuff *et al.*, 1992). The synthesis of significant amounts of RNA does not resume until a species-specific cell stage. In mice, the onset of synthesis of significant amounts of RNA occurs in the G1 phase of the two-cell stage. At this time heat shock proteins (Bensuade *et al.*, 1993), as well as a developmentally regulated transcription-requiring complex (Conover *et al.*, 1991), are transcribed and subsequently translated. In pigs, transcription appears to begin during the four-cell stage, as evidenced by changes in nucleolar morphology, structure of the nuclear matrix, sensitivity to *in vitro* conditions, qualitative changes in protein synthesis, sensitivity to α-amanitin and [^{3}H]uridine incorporation (see below).

Direct measurement of [^{3}H]uridine incorporation would be an obvious method for determining when RNA synthesis occurs in pigs. Although no incorporation can be detected during the zygote or two-cell stage, incorporation can be detected at the four-cell stage and increases 1000 fold by the blastocyst stage (Freitag *et al.*, 1991). However, these data should be interpreted with caution since we (R. S. Prather, M. M. Sims and N. L. First, unpublished) have been unable to detect significant amounts of [^{3}H]uridine transport in two-cell pig embryos (Fig. 2). It should be noted that although we used 1.6 μmol [^{3}H]uridine l^{-1}, Freitag *et al.* (1991) used 9 μmol [^{3}H]uridine l^{-1}. Similar results of poor uridine transport also occur in mice (Clegg and Piko, 1982) and hamsters (Seshagiri *et al.*, 1992). Thus it appears from the above data that the mechanism responsible for significant amounts of [^{3}H]uridine transport in pigs first occurs during the four-cell stage. If little or no transport occurs at an earlier stage, then measures of incorporation are meaningless.

It should be mentioned that [^{3}H]uridine incorporation into the nuclei of two-cell stage pig embryos was reported by Hyttel *et al.* (1993). These data are difficult to reconcile with the previously reported information. It is possible that Hyttel *et al.* (1993) detected rRNA or tRNA synthesis because using identical procedures with bovine embryos labelling was not blocked by α-amanitin (Viuff *et al.*, 1992). It is also possible that early pig embryos are synthesizing precursor mRNA as in early rabbit embryos (see discussion in Telford *et al.*, 1990), or that individual mRNA transcripts are not completely transcribed before mitosis occurs as in *Drosophila* (Thummel, 1992).

Nucleolar morphology and rRNA production

The morphology and function of nucleoli change during embryogenesis in many species. In mice, the germinal vesicle oocyte synthesizes rRNA. However, rRNA synthesis ceases at germinal vesicle

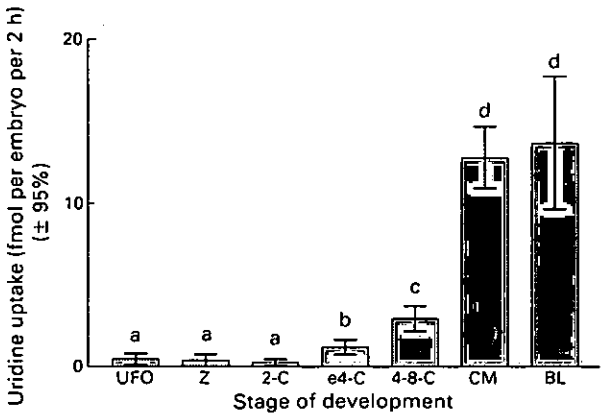

Fig. 2. Uptake of [5,6-^{3}H]uridine during pig embryogenesis. UFO: unfertilized oocyte; Z: zygote; 2-C: two-cell stage; e4-C: early four-cell stage; 4-8-C: late four-cell stage and eight-cell stage; CM: compact morula; BL: blastocyst were cultured in 1.6 μmol [5,6-^{3}H]uridine l^{-1} for 2 h, then rinsed and radioactivity was measured. A pool of one to six oocytes per embryo was used per determination. Five to 20 determinations were made per cell stage. Confidence intervals (95%) were constructed about each mean using the Student's *t* test. Very little uptake was observed at the unfertilized oocyte and two-cell stage. The amount of uptake observed at the zygote stage was not different from zero. Columns with different letters above are significantly different ($P < 0.05$). Uptake increases dramatically between the eight-cell stage and the compact morula stage (R. S. Prather, M. M. Sims and N. L. First, unpublished).

breakdown. Ribosomal RNA production cannot be detected at the pronuclear stage. The first positive detection of rRNA production is at the late two-cell stage (Piko and Clegg, 1982). The detection of rRNA production is correlated with the ultrastructure of the nucleoli. Nucleoli active in transcription, or definitive nucleoli, are characterized as being reticulated, having fibrillar cores and are surrounded by fibrillo-granular cortices (Hillman and Tasca, 1969). In mice, the oocyte at the germinal vesicle stage has definitive nucleoli, whereas after germinal vesicle breakdown, fertilization and pronuclear formation, there are no definitive nucleoli (Hillman and Tasca, 1969). The prenucleoli that appear at the pronuclear stage are composed of fine fibrils in a spherical centre surrounded by an agranular zone (Hillman and Tasca, 1969). Early in the first cell cycle prenucleoli are numerous and congregate during interphase, forming a single prenucleolus within each pronucleus prior to nuclear envelope breakdown (Prather *et al.*, 1990). At the late two-cell stage, a single fibrillar prenucleolus begins to reticulate. This pattern of prenucleoli reticulating to form definitive nucleoli repeats itself at the four-cell stage forming two definitive nucleoli. By the eight-cell stage all nucleoli are reticulated, and thus are definitive nucleoli (Hillman and Tasca, 1969).

In pig embryos, definitive nucleoli are present in germinal vesicle oocytes (Norber, 1972; Crozet *et al.*, 1981), but absent in pronuclear and two-cell stage embryos (Norberg, 1970, 1973a; Szollosi and Hunter, 1973). Four-cell stage nucleoli begin to associate with chromatin and reticulate (Norberg, 1970; Hyttel and Niemann, 1990) and become definitive nucleoli by the eight-cell stage (Norberg, 1973b; Tomanek *et al.*, 1989). Tomanek *et al.* (1989) have shown that pig embryos do incorporate [^{3}H]uridine into nucleoli at the five- to eight-cell stage, but do not state whether the nucleoli that incorporate the uridine are four-cell stage blastomeres or eight-cell stage blastomeres. Their results should be interpreted with caution since uridine appears to be poorly transported in the early embryo (see above), and thus may not be readily available for incorporation into newly formed rRNA at earlier stages.

Qualitative changes in protein synthesis

Qualitative changes in protein synthesis are seen during preimplantation embryo development in cows (four- to eight-cell stage: Frei *et al.*, 1989; Barnes and First, 1991), sheep (eight- to 16-cell stage: Crosby *et al.*, 1988) and humans (four- to eight-cell stage: Braude *et al.*, 1988; Artley *et al.*, 1992). Many of these changes in protein profiles are sensitive to α-amanitin, which suggests embryonically directed transcription.

In pigs, the first qualitative change in protein production occurs at fertilization (Jarrell *et al.*, 1991) and is the result of the dephosphorylation of a 26 kDa protein (Ding *et al.*, 1992). The next major qualitative change occurs during the four-cell stage and is sensitive to α-amanitin (Jarrell *et al.*, 1991). This qualitative change appears to occur between 12 and 16 h after cleavage (Schoenbeck *et al.*, 1992) and corresponds well to the beginning of G2 (see above). These qualitative changes can be blocked with the addition of α-amanitin, and thus suggest transcriptional dependence. Unfortunately, none of these proteins has yet been identified.

In addition to the qualitative changes seen during the four-cell stage, further changes in the types of protein produced are detected as the embryo undergoes compaction and blastocoel formation (Jarrell *et al.*, 1991).

Small nuclear ribonuclear proteins

Small nuclear ribonuclear proteins (snRNPs) are responsible for processing pre-RNA into RNA. The more abundant snRNAs (U1, U2, U3, U4, U5, U6) are found in the nuclei of early mouse embryos (Lobo *et al.*, 1988; Dean *et al.*, 1989; Prather *et al.*, 1990) and U2 snRNAs are found in the nuclei of early bovine embryos (Watson *et al.*, 1992). These snRNAs are a carryover from maternally derived stores created during oogenesis. During the early cleavage period, the relative abundance of U1, U2, U3 and U6 does not increase uniformly (Lobo *et al.*, 1988; Dean *et al.*, 1989; Prather *et al.*, 1990). A mouse monoclonal antibody (Y12) that precipitates the B and D core proteins of the snRNP, however, does not recognize antigens in early mouse and pig embryogenesis. In mice, the Y12 antigen is absent from pronuclei, but present in germinal vesicle and interphase nuclei at, and beyond, the two-cell stage (Stricker *et al.*, 1989; Dean *et al.*, 1989). Similarly, in cattle embryos, a marked increase in the labelling of nuclei with the Y12 antibody occurs coincidentally with the onset of RNA synthesis at the eight- to 16-cell stage (Kopecny *et al.*, 1991; Kopecny and Fakan, 1992).

In pigs, the Y12 antigen is absent from both pronuclei and two-cell stage nuclei, but present in germinal vesicle and interphase nuclei at, and beyond, the four-cell stage (Prather and Rickords, 1992). The appearance of the Y12 antigen in mice and pigs at the two- and four-cell stage, respectively, is sensitive to α-amanitin. Thus, the appearance of the Y12 antigen is correlated with mRNA production and processing in mouse embryos and expected mRNA production and processing in pig embryos. However, cycloheximide treatment within 6 h after cleavage to the four-cell stage cannot block the appearance of Y12 antigen in late four-cell stage pig embryos, suggesting that the Y12 antigen might be maternally inherited, and its appearance may simply be due to its stabilization in the nuclear matrix at the onset of RNA production (Prather and Rickords, 1992). Alternatively, if there is a G1 phase, then mRNA and protein may have been produced before the addition of cycloheximide. This observation needs to be resolved. Similar changes in the nuclear matrix of mice are known to occur during the two-cell stage (Prather and Schatten, 1992, unpublished).

Other changes that suggest new RNA synthesis in pigs

Other indicators suggest new RNA synthesis at the four- to eight-cell stage. These include the detection of male specific antigens on the cell surface of eight-cell stage embryos (White *et al.*, 1987), and the change in glucose metabolism (Flood and Wiebold, 1988) and β-galactosidase activity. The activity of β-galactosidase is low at the two- to four-cell stage, but increases 75-fold by the blastocyst stage (Murnane *et al.*, 1990). Unfortunately these investigators did not separate the two-cell data from the four-cell data. As an aside, this endogenous β-galactosidase activity may adversely affect gene transfer experiments that rely upon β-galactosidase activity as a marker.

An *in vitro* induced artefact has been shown to occur at the cell stage where embryos from other species first begin producing RNA. This is the 'block' to development. In pigs this 'block' occurs during the four-cell stage (reviewed by Davis, 1985; Telford *et al.*, 1990). Interestingly, the *in vitro* induced 'block' occurs in G2 of the cell cycle in pigs (Eyestone *et al.*, 1986) after the qualitative change in protein profiles, (R. S. Prather, unpublished) as in mice (Bolton *et al.*, 1984). This sensitivity to *in vitro* conditions may be due to a deficiency in the mechanism for p34cdc2 protein kinase activation (Aoki *et al.*, 1992) or overall protein synthesis rate (Anbari and Schultz, 1993). Thus the sensitivity to *in vitro* conditions is correlated with the cell stage mRNA production.

Spermine and spermidine synthesis are necessary for blastocoel formation in both mice and pigs. Spermine and spermidine interact with chromatin and affect differentiation in animals and plants (Heby, 1981). Inhibition of synthesis of polyamines with methylglyoxal-bis guanylhydrazone delays the normal uptake of [^{3}H]uridine in mice (Alexandre and Gueskins, 1984) and reversibly delays formation of the blastocyst in both mouse and pig embryos (Alexandre and Gueskins, 1984; Lane and Davis, 1984; Zwierzchowski *et al.*, 1986).

A final indicator that newly transcribed mRNA is necessary for development is the ability of α-amanitin to block cleavage. If pig zygotes, two-cell or early four-cell stage embryos are placed in α-amanitin, development does not proceed to the eight-cell stage (Jarrell *et al.*, 1991; Schoenbeck *et al.*, 1992; R. S. Prather, unpublished). However, if four-cell stage embryos are placed in medium containing α-amanitin 24 h after cleavage to the four-cell stage, development will proceed to the eight-cell stage (Schoenbeck *et al.*, 1992). This finding suggests that, although some new transcription occurs before 16 h after cleavage to the four-cell stage, transcription necessary for cleavage to the eight-cell stage occurs only 24 h after cleavage to the four-cell stage. Likely candidates for the message produced more than 24 h after cleavage may include cyclin B or cdc25 (the *Drosophila string* gene).

Thus, it appears that germinal vesicle stage oocytes synthesize all classes of RNA. This transcription ceases at germinal vesicle breakdown and does not resume until the four-cell stage. Maternal RNA present at the early four-cell stage may be degraded. This degradation, in concert with post-translational modifications and newly synthesized RNA results in a qualitative change in protein production. The onset of RNA synthesis is correlated with changes in structure of the nucleolus and nuclear matrix, overall cellular metabolism and a sensitivity to conditions *in vitro*.

Amino acid transport in mice and pigs

Amino acid transport is facilitated by specific transporters within the plasma membrane of the cell. Different transport systems in the same cell type can be distinguished on the basis of their different, although sometimes overlapping, substrate selectivities. These transport systems have been classified according to their preference for anionic, cationic or zwitterionic amino acids and whether they are dependent upon external sodium. In the early mouse and rat embryo, the detection of some of these systems is developmentally regulated (Van Winkle, 1992). Although there are at least six systems for amino acid transport in mice that change in activity as preimplantation development proceeds, this discussion will focus upon only four of these systems.

The first transport systems to be discussed transport zwitterionic amino acids. System L is sodium-independent and selects for bulky zwitterionic amino acids such as leucine. System $B^{o,+}$ is sodium-dependent and transports a broad scope of cationic, zwitterionic and bicyclic amino acids. In mice, the activity of system L gradually declines as the oocyte matures, is fertilized and develops to the blastocyst stage (Van Winkle *et al.*, 1990a). In contrast, system $B^{o,+}$ is not detected in unfertilized oocytes or zygotes. A small amount of activity can be detected at the two-cell stage (the time at which the mouse embryo begins producing is own RNA) and increases by the eight-cell stage. By the time the embryo develops to the blastocyst stage, the activity of system $B^{o,+}$ increases tenfold over the eight-cell stage (Van Winkle *et al.*, 1990b).

As in mice, system L appears to be present in pig oocytes and early blastocysts (Prather *et al.*, 1993a). However, in direct opposition to the order of developmental expression in mice, system $B^{o,+}$ appears to be present in the oocyte, but not the blastocyst (Prather *et al.*, 1993a). Thus, although mice provided a starting point for studies in pigs, the developmental regulation of system $B^{o,+}$ is exactly the opposite of

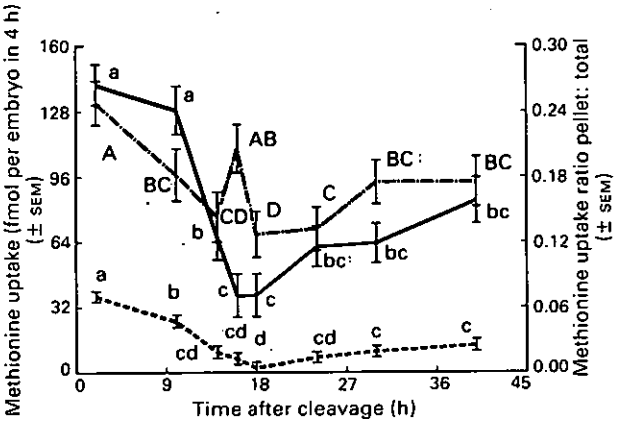

Fig 3. Methionine uptake and incorporation (adapted from Schoenbeck *et al.*, 1993). Embryos were observed every 2 h for cleavage to the four-cell stage. At various times after cleavage, ten embryos were labelled with L-[^{35}S]methionine for 4 h. The mean (± SEM) of uptake (——) and incorporation into protein (-----) are indicated (differences within uptake or within incorporation are denoted with different superscripts; $^{a,b,c,d}P < 0.05$). The ratio of methionine uptake (pellet:total) is indicated (± SEM) (——). Values with different superscripts are significantly different ($P < 0.05$).

what was expected. In pig oocytes and blastocysts, there is no evidence for the presence of systems A, ASC or $b^{0,+}$. We will be testing the hypothesis that system $B^{0,+}$ activity disappears during the four-cell stage, when a dramatic decline in L-[^{35}S]methionine uptake has been observed 16 h after cleavage to the four-cell stage (Fig. 3).

The next systems to be discussed are involved in transport of anionic amino acids. In the mouse system, x_c^-, a sodium-independent transporter, is present in the oocyte and its activity diminishes as the embryo develops (Van Winkle *et al.*, 1992). The other transporter is system X_{AG}^-, a sodium-dependent transporter, which is not detectable until after the eight-cell stage (Van Winkle *et al.*, 1991). In contrast to the situation in mice, system x_c^- cannot be detected in pig oocytes, and it was not detected in blastocyst stage embryos (Prather *et al.*, 1993b). However, system X_{AG}^-, although not detectable in oocytes, was detectable in early and hatched blastocysts (Prather *et al.*, 1993b). Thus of the four systems characterized to date only two, system L and system X_{AG}^-, are expressed in a developmental fashion similar to that in mice.

Mitochondrial changes in early development

The morphology of mitochondria differ depending upon the cell type, some are thin and filamentous, whereas others are spherical or highly branched. The mitochondria of early embryos of most domestic animals contain few cristae. In pig embryos, mitochondria assume a cortical location early in development, but become dispersed within the blastomeres as the embryo develops to the blastocyst stage. From the oocyte to eight-cell stage, mitochondria occur in small aggregates, and these aggregates are not seen in the trophoblast or inner cell mass of blastocysts. The aggregates, although appearing to be composed of many mitochondria, are in fact usually composed of a few mitochondria with a complex structure (Krause *et al.*, 1992). Development of cristae in the mitochondria occurs as the pig embryo develops to the blastocyst stage (Hyttel and Niemann, 1990; Krause *et al.*, 1992). These mitochondrial changes during preimplantation development are temporally correlated with changes in glucose metabolism, respiration and RNA synthesis.

Maternal message degradation and a possible G1 phase during the four-cell stage

The evidence discussed above suggests that a dramatic change in mRNA production occurs at 14–16 h after cleavage to the four-cell stage, and that mRNA necessary for development beyond the four-cell stage was produced after 24 h after cleavage to the four-cell stage. These data call for two periods of G2-directed mRNA synthesis (S phase is completed by 16 h after cleavage). However, in an additional experiment, embryos were added to medium containing α-amanitin at various times after cleavage to the four-cell stage, followed by labelling 24 h after cleavage to the four-cell stage with L-[^{35}S]methionine. Uptake of L-[^{35}S]methionine is lowered 24 h after cleavage if the embryos are added to the α-amanitin more than 4 h after cleavage to the four-cell stage (Schoenbeck *et al.*, 1992). A possible explanation for this observation is the production of mRNA that signals the destruction of the maternal mRNA that code for amino acid transport proteins. In addition to the absolute uptake of L-[^{35}S]methionine, the production of maternal type proteins can be maintained if the embryos are cultured in α-amanitin (Schoenbeck *et al.*, 1992). This result again suggests that mRNA production is necessary for the degradation of these maternal type RNAs that code for these proteins. These two experiments suggest that mRNA production occurs during a G1 phase of the four-cell stage.

In mice, a degradation of maternally derived mRNA occurs during the two-cell stage (see above). Similarly, in cows and sheep, it has been suggested that a decrease in availability of mRNA occurs during the stage at which the embryo begins RNA production (see above). Since both the amount of L-[^{35}S]methionine taken up by the four-cell stage pig embryo and the ratio of uptake to incorporation changes as the embryo progresses through the four-cell stage, it is likely that both degradation of pre-existing transcripts occurs as well as a decrease in the transport of L-[^{35}S]methionine across the plasma membrane.

Another suggestion of the degradation of maternally derived RNA comes from the loss of the maternal complement of A/C type nuclear lamins after the two-cell stage in mice (Schatten *et al.*, 1985) the four-cell stage in pigs (Prather *et al.*, 1989a) and the eight- to 16-cell stage in cows (Prather *et al.*, 1989a). If zygotic production of mRNA coding for the A or C lamins occurs, then lamins A and C should be detectable after the embryo begins producing its own RNA. Thus it appears that mRNA produced during G1 of the four-cell stage pig embryo directs the destruction of maternally derived mRNA.

Mechanisms regulating the onset of RNA synthesis

The overall regulation of the initiation of RNA synthesis in the early embryo still needs to be determined. The regulation of the timing of specific events in differentiation has generally been referred to as due to an intrinsic 'time clock'. This timing mechanism can be reset by nuclear transfer to an enucleated oocyte (Prather and Robl, 1991). Results from some previous studies using *Xenopus* embryos have suggested that the initiation of RNA synthesis is due to the change in the nuclear:cytoplasmic ratio; however, this needs to be re-evaluated (Lund and Dahlberg, 1992). There is also some evidence that cyclin B mRNA may be partially responsible for initiating transcription (Weeks *et al.*, 1991). In other species, such as *Drosophila*, the duration of the cell cycle may reflect the time necessary for transcription to be completed, i.e. as the duration of the cell cycle increases then the genes that are physically longer are more likely to complete transcription before mitosis (Thummel, 1992). Unique combinations of these early genes could then act to promote expression of late genes.

In the mouse embryo, many events occur at a fixed time after fertilization. Transcription is independent of DNA synthesis (Poueymirou and Schultz, 1987), cell division (Petzoldt, 1984) and the nuclear: cytoplasmic ratio (Petzoldt and Muggleton-Harris, 1987). Enhancers are needed in the early mouse embryo to facilitate transcription (Martinez-Salas *et al.*, 1989), owing to a general repression of transcription in the early zygote (Bonnerot *et al.*, 1991) followed by a transcription permissive state (Latham *et al.*, 1992). Initiation of transcription probably depends upon a cAMP-dependent protein kinase (PK-A) (Poueymirou and Schultz, 1989; Manejwala *et al.*, 1991).

Factors that prevent transcription in the early mammalian embryo may include competition for promoter regions between DNA and RNA polymerases (Rivier and Rine, 1992), or between transcription factors and histone structure (Workman and Kingston, 1992). The latter explanation is supported by the

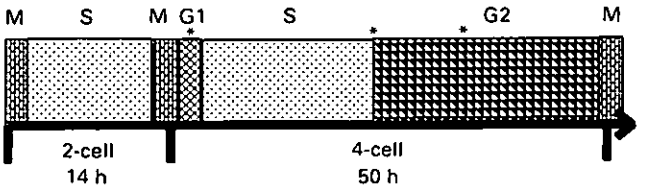

Fig. 4. An overview of the two- and four-cell stage pig embryo *in vitro* (M: mitosis; G1: gap phase 1; G2: gap phase 2; S: DNA synthesis). *Denotes α-amanitin-sensitive event.

observation that there is a different type of DNA packaging, somatic histone H1 is not present, in the mouse zygote and two-cell stage embryo compared with other stages of development (Clark *et al.*, 1992).

In comparison, very little is known about initiation of transcription and the regulation of developmental events in domestic pigs. It is known that some developmental events can be reprogrammed by nuclear transfer such as the timing of blastocoel formation (Prather *et al.*, 1989b), the developmental appearance of an snRNP epitope (Prather and Rickords, 1992) and the disappearance of lamins A/C (Prather *et al.*, 1989a). If the mouse is a suitable model for initiation of transcription for pigs, then it appears that there may be a multitude of factors that prevent transcription in the zygote and two-cell stage pig embryo, followed by a transcription permissive period during the four-cell stage.

Conclusions

Pig zygote and two-cell stage embryos do not appear to synthesize significant amounts of mRNA and thus must rely upon maternally derived mRNA to direct development. The first indication of mRNA synthesis occurs during the G1 phase of the four-cell stage, which is followed by 14–16 h of DNA synthesis. At the start of G2, a major qualitative change in protein synthesis occurs that is sensitive to α-amanitin. Another α-amanitin-sensitive event occurs beyond 24 h after cleavage to the four-cell stage. These three periods of mRNA synthesis during the four-cell stage may (1) cause degradation of pre-existing mRNA, (2) cause a major onset of embryonically directed protein synthesis, and (3) result in transcripts necessary for cleavage to the eight-cell stage, respectively (see Fig. 4). A few characteristics that may be unique to the early pig embryo compared with many other mammals are (1) glucose and phosphate are not detrimental to development; (2) thymidine is transported across the plasma membrane very poorly; (3) the developmental appearance of amino acid transport systems is different; and (4) mRNA synthesis begins during the four-cell stage. The mechanisms regulating the timing of these events has yet to be elucidated.

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