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GENE EXPRESSION IN THE BOVINE PREIMPLANTATION EMBRYO

BY

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ABSTRACT

The timing of a critical event in early bovine embryogenesis, the activation of the embryonic genome, was analyzed by examination of quantitative and qualitative patterns of RNA and protein synthesis.

Initial experiments were performed on mouse embryos in order to establish that the methods used produced reliable results.

To assess the relative rates of translation and transcription, the in vitro uptake and incorporation of [^{35}S]-methionine and [^{3}H]-uridine, respectively, by embryos at different stages of development were measured.

Qualitative patterns of protein synthesis were analyzed by one-dimensional polyacrylamide gel electrophoresis of [^{35}S]-labelled proteins from single embryos. A range of stages of development was examined to allow identification of stage-dependent shifts in protein synthetic patterns.

Total nucleic acid was extracted from groups of eggs and early embryos, and either resolved electrophoretically and subjected to Northern transfer, or appplied directly to nylon membranes. These samples were then hybridized to a radiolabelled fragment of the human 28S rRNA gene. By comparison of the levels of hybridization to the probe with that of a standard RNA preparation, the amount of total RNA present in the eggs or embryos was calculated.

The data from the analysis of mouse embryos indicated that

the murine embryonic genome is activated at the 2-cell stage, which is in agreement with the findings of other researchers.

Examination of the patterns of protein synthesis by bovine embryos revealed that the 1-cell unfertilized to 4-cell stages of development produce similar qualitative patterns. There was no detectable incorporation of [^{3}H]-uridine by bovine embryos up to the 16-cell stage of development, and levels of incorporation of [^{35}S]-methionine by single bovine embryos were found to decrease until the 8-16-cell stage. At the 8-16-cell stage, a number of changes in the qualitative pattern of protein synthesis are evident. These include the disappearance of a band at 35 kD (putative lactate dehydrogenase) and the transient appearance of a prominent band at 27 kD. A new qualitative pattern of protein synthesis (including a strong band of putative actin of molecular weight 43 kD) is established after the 16-cell stage, which persists until the late blastocyst stage. Rates of both transcription and translation increase dramatically between the 16-cell and the blastocyst stage. Furthermore, it was estimated that a single bovine blastocyst contains over 14 times as much RNA as a post-ovulatory oocyte. As an aggregate, this evidence suggests that the bovine embryonic genome is activated at the 8-cell stage.

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INTRODUCTION

A phenomenon without equal in the biological world is the transformation of the unicellular zygote into a highly organized multicellular embryo. While there are interspecies differences in the timeframe within which this occurs, the general features of fertilization, migration into the uterus and implantation at the blastocyst stage are similar for all mammals.

A pivotal event in early mammalian embryogenesis is the transfer of developmental control from the maternal to the zygotic genome. This event is essential for normal development to continue, and marks the beginning of the metabolic activation of the embryo. Information obtained from studies on RNA and protein synthesis in the embryos of the mouse (reviewed by Schultz, 1986a,b) and those of other mammals such as the sheep (Crosby et al., 1988) and the human (Braude et al., 1988) suggest that this switch in developmental control is exerted at the level of transcription. In order to contribute to the knowledge regarding RNA and protein synthetic events in embryogenesis, an investigation has been carrried out on the patterns of gene expression during early cleavage stages in the bovine embryo.

Until recently, bovine embryology has received only scant attention. There are a number of reasons for the deficit of research in this area. The most obvious are the high costs of the purchase and maintenance of large domestic animals, and the practical problems of keeping and handling them. The long time periods required for

superovulation (at least 20 days) and the long gestation (280 days) of the cow lead to experiments of long duration. Furthermore, since virtually all surgical methods of embryo collection and transfer for cattle have ceased, research on bovine embryos normally encountered in the oviduct (Days 1-4) has been limited (Newcomb, 1982).

Recent success with a number of novel techniques, however, has focussed attention on the study of the early bovine embryo. One of these procedures, the in vitro maturation and fertilization of the bovine oocyte, followed by the culture in vitro to the blastocyst stage of the resultant zygote, has been successfully carried out in a number of laboratories (for example Stubbings et al., 1988; Goto et al., 1988). The use of nuclear transfer for the cloning of animals has also been carried out with some degree of success with embryos of both the bovine (S.Willadsen, personal communication) and the sheep (Smith and Wilmut, 1988). Both of these techniques have the as-yet unrealized potential for the production of a very large number of offspring from a single animal.

Due to the potential practical value of the afore-mentioned techniques, it is desirable that an effort be made to improve their efficiency. In order to do so, our current knowledge regarding the physiology and molecular biology of the bovine embryo must be increased. The metabolism of the embryo, for example, must be studied in order that the in vitro culture conditions essential to both of these techniques may be optimized. Indicators of periods at which pivotal developmental events occur must also be identified, as these

periods may represent times at which the embryo is particularly sensitive to environmental influences (analogous to the 2-cell "block" in the mouse embryo [Goddard and Pratt,1983]). Any special metabolic requirements of the embryos may then be met in order to ensure that the embryos can continue to develop through these stages.

The present study of the patterns of protein and RNA synthesis was undertaken in order to fill some of these gaps in the current understanding of the molecular biology of the early bovine embryo. The animal which has traditionally been used for such studies is the mouse. It has a number of advantages over other mammals which may be used in experimental embryology. The principle ones are its high fecundity, short generation interval, and low cost (Hogan et al., 1986). In addition to these, there are also the benefits derived from the well-established status of the mouse as a research animal. For example, the techniques for the recovery, manipulation and culture of preimplantation embryos have been established, and a large number of genetically distinct strains, often with biochemically or physiologically defined characteristics, are readily available (Hogan et al., 1986). As a result of these advantages, the vast majority of the investigations on the events occuring during the first few cleavage stages in mammalian embryos have been carried out on mouse embryos. Theoretical and methodological approaches pertaining to the molecular biology of the murine embryo were applied by the author first to the mouse and then in parallel studies

to the cow embryo, and therefore these will be reviewed briefly here.

In the mouse oocyte, all classes of RNA are synthesized and accumulated during development up to the period of meiotic maturation. This includes ribosomal RNA (rRNA), which accounts for 60 - 70% of the total RNA content of mouse oocytes and embryos up to the blastocyst stage, and also polyadenylated RNA (Piko and Clegg, 1982). During meiotic maturation, however, the total RNA content of mouse oocytes decreases by 20% (Bachvarova et al., 1985). It has been shown that the qualitative pattern of protein synthesis changes markedly during meiotic maturation of mouse oocytes in vitro (Schultz and Wassarman, 1977). Virtually all of the changes observed take place after the breakdown of the oocyte's germinal vesicle (GV). The changes also take place in anucleate oocyte fragments during culture in vitro (Schultz et al., 1978). This evidence would suggest that these changes are mediated at the post-transcriptional and/or post-translational levels.

The newly-ovulated mouse egg contains approximately 0.35 ng of total RNA. Of this, the ribosomal RNA has been calculated to comprise 64%, tRNA somewhat less than 40%, and polyadenylated RNA accounts for 6.6% of the total (Piko and Clegg, 1982). Nearly all of the proteins synthesized in the 1-cell fertilized embryo are also synthesized in the oocyte, although some changes in the pattern of protein synthesis are associated with fertilization (Schultz et al., 1979). Differential mRNA activation, differential polypeptide turnover, and post-translational modifications are largely responsible

for the changes in the polypeptide synthetic profile which occur. There also appear to be fertilization-independent, fertilization-dependent and fertilization-accelerated changes in polypeptide synthesis during the first cleavage, all of which are involved in the transient appearance of a protein complex with a molecular mass of 35 kD during the early 2-cell stage (Howlett and Bolton, 1985).

While the pool of maternal informational macromolecules in the egg serves to control the period of development of the mouse embryo up to the 2-cell stage, transcription from the zygotic genome is necessary for normal development to continue from this stage onward. Some of the lines of experimentation which have led to this conclusion are (see Schultz, 1986b for review): (1) 30 to 40% of the bulk maternal RNA, 70% of the total poly(A)+ RNA, and as much as 90% of the histone and actin mRNA is degraded within 24 hours of fertilization. The synthesis of RNA, on the other hand, increases markedly up to the late 2-cell stage such that the synthesis of all classes of RNA is readily detectable by this time. The rate of RNA synthesis increases from 1.25 pg/cell/hour at the 2-4-cell stage to 2.5 pg/cell/hour at the 8-cell stage, and by the blastocyst stage, the rate has reached 5.0 pg/cell/hour (Clegg and Piko, 1977); (2) The first appearance of functional nucleoli with fibrillar centers and cytochemically-demonstrable nucleolus organizer regions (NORs) at the 2-cell stage correlates well with the biochemical detection of the beginning of rRNA synthesis at this stage (Engel et al., 1977); (3) The rate of protein synthesis remains at a relatively low level from fertilization to the 8-cell stage. Once the 8-cell stage is reached,

there is a progressive increase in synthetic rate accompanying the transition of the morula to the blastocyst, with protein synthesis increasing approximately 7-fold between the 8-cell and blastocyst stages (Abreu and Brinster, 1978). The synchronous increase in the synthesis of both RNA and protein after the 2-cell stage is probably due to the transcription and translation of further quantities of the same species of mRNAs which are synthesized after the 2-cell stage of cleavage (Braude, 1979); (4) A number of changes in the polypeptide synthetic pattern occur as fertilized eggs develop to the 2-cell stage (see above). Since these changes also occur in physically enucleated eggs or in eggs treated with the transcriptional inhibitor α-amanitin , they appear to be regulated at the post-transcriptional level, relying on the use of maternal transcripts. Many of the changes in the polypeptide synthetic profile in the late 2-cell stage, however, appear to be dependent upon putative mRNA synthetic events (Flach et al., 1982). For example, a complex of polypeptides of MW 67,000 to 70,000 appears at the early 2-cell stage of mouse development. Since these proteins do not appear in fertilized eggs cultured to the 2-cell stage in the presence of α-amanitin, they are likely produced as a result of new transcription. These proteins represent heat shock proteins hsp 68 and hsp 70 (Schultz, 1986b). By the late 2-cell stage, most of the "maternal" proteins (i.e. coded for by maternal messages) are no longer being synthesized by the embryo, and many new autoradiographic bands, including the ones mentioned above, are apparent (Flach et al., 1982; Van Blerkom and Brockway, 1975). These protein patterns persist largely unchanged

up to the blastocyst stage; (5) Fertilized eggs treated with transcriptional inhibitors such as α-amanitin and actinomycin D are markedly inhibited in development beyond the 2-cell stage; (6) Genetic variants or proteins have been used to advantage to identify paternal gene expression in embryos. Using crosses of appropriate mouse lines, paternally-derived *B*-glucoronidase (Wudl and Chapman, 1976) and *B*2-microglobulin (Sawicki et al., 1982) were both detected by the late 2-cell stage; (7) When embryos from certain mouse strains are cultured in vitro from the 1- or 2-cell stages, an arrest in development is observed. This phenomenon is referred to as the '2-cell block' (Goddard and Pratt, 1983), and alludes to a critical stage occuring after the first division during which the embryos appear to be particularly sensitive to culture conditions. As this block has been observed in other mammalian species at a stage corresponding to the activation of the embryonic genome (for example at the 4-cell stage in the human embryo [Braude et al., 1988], and at the 8-cell stage in the sheep [Crosby et al., 1988]), it may coincide with an overall change in the metabolic activity of the embryo.

In this respect, it is interesting to note that the mouse embryo also experiences a switch in its energy substrate preference during the first few cleavage divisions. Ooyctes and embryos up to the 4-cell stage require pyruvate. After the third cell division, glucose becomes an optional energy source for developing embryos (Leese and Barton, 1984). By the blastocyst stage, the preference of the embryos for glucose has become well-established. Research has

shown that there is also a blockade to glycolysis in the early human embryo which is removed with development (Wales et al., 1987).

In summary, the work discussed above indicates that the transition from maternal to embryonic control in the mouse embryo occurs at the 2-cell stage. It also indicates that a block to in vitro development is linked to this transition and may serve to mark a similar transition in a number of other mammalian species.

While there are compelling reasons for the use of the mouse as an experimental model in these studies, caution must always be exercised when making extrapolations to other species. There can be no replacement for knowledge obtained by direct experimentation on the system in question. This was stated well by Fehilly and Willadsen (1986): "Indeed, there is no particular reason to believe that mouse embryos are more similar to cow embryos or human embryos than mice are to cattle or human beings".

The overall diameter of the bovine oocyte is 150 to 190 μm, including a zone pellucida with a thickness of approximately 12 to 15 microns (Linares and King, 1980). The human oocyte is somewhat smaller, with a diameter of 140 μm (Tesarik et al., 1986), while the mouse embryo has a diameter of only 70 μm (Abramczuk and Sawicki, 1974). The diameter of the bovine embryo remains virtually unchanged from the oocyte to the blastocyst stages (Lindner and Wright, 1983). Immature oocytes in early antral follicles of cattle have been shown to be actively engaged in rRNA

and hnRNA synthesis by autoradiography after a short pulse of [^{3}H]-uridine (Crozet et al., 1986).

Maturation of the oocyte is accompanied by nucleolar compaction, which leads to the formation of electron-dense nucleoli. This compaction is associated with an impairment of rRNA synthesis which persists until the nucleoli become re-vacuolated at the 8-cell stage (Camous et al., 1986). Similarly, in human embryos, it is only at the early 8-cell stage that the nucleoli become fully functional and capable of rRNA synthesis and processing (Tesarik et al., 1988). This association of nucleolar compaction with an impairment of rRNA synthesis has also been reported in pig oocytes (Crozet et al., 1981) and in human oocytes (Tesarik et al., 1983) and is probably indicative of a period of storage of RNA. From autoradiographic studies of [^{3}H]-uridine labelled oocytes, Crozet et al. (1986) found that nucleoplasm labelling persisted in cow oocytes from large antral follicles, indicating that some RNA synthesis occurs even at this late stage.

Maturation of bovine oocytes in vivo is stimulated by a surge of leutinizing hormone (LH) in cattle. In vitro, it can be induced by a range of conditions (see, for example, Sirard et al., 1988). The set of nuclear and cytoplasmic changes associated with maturation are completed by about 20 hours after the LH peak in vivo (Hytell et al., 1986). Protein synthesis is required in at least four distinct stages of bovine oocyte maturation, with new classes of proteins being synthesized at these stages. These stages are: germinal vesicle (GV) breakdown, chromatin condensation, metaphase I, and also at some

time during the ensuing 12 hours before meiosis I is completed (Sirard et al., 1988).

Following maturation, the oocyte is released from the follicle, and it does not complete meiosis until the necessary interaction with sperm cells has taken place (Shea, 1981). Fertilization may be accomplished by natural mating or, more commonly in experimental programs, by artificial insemination. In vivo, heat is shown approximately 24 hours prior to ovulation, with the time of ovulation being used to define the time at which fertilization is expected to occur (Hytell et al., 1988). The age of the embryo is calculated by the number of hours or days post-estrus. If fertilization fails to occur, no cleavage takes place and, generally, the oocytes degenerate over a period of several days and the ooplasm may undergo fragmentation (Shea, 1981). A sequence of nuclear and cytoplasmic changes is associated with fertilization (Hytell et al., 1988). About 28 hours after estrus, the first cleavage division of the zygote occurs.

After fertilization, a transition from small, dense nucleoli to large, more compact ones occurs. Ultrastructural observations of early bovine embryos (King et al., 1988) have shown that, at the 2-cell stage, the nucleoli are small, and are composed of a dense fibrillar core with a low affinity for silver nitrate. At the late 8-cell stage the nucleoli are larger and ring-shaped, with silver being incorporated into one or two NORs. The NORs are the sites of rRNA synthesis and thus their appearance suggests that active RNA synthesis from the 18S and 28S rRNA genes has resumed by the late 8-cell stage. The nucleoli of morulae and blastocysts are large and

possess several fibrillar centres, a prominent granular component, and numerous silver deposits.

There are other lines of evidence which indicate that the bovine embryonic genome becomes active between the late 8-cell and early 16-cell stages. For example, there is a block to in vitro culture of embryos past this stage, analogous to the '2-cell block' observed in the mouse (Thibault, 1966). This block to development occurs in both species at about the same time that the NORs first become transcriptionally active (King et al., 1988). Furthermore, when early embryos of the cow were cultured in vitro in a medium enriched by [^{3}H]-uridine, first incorporation was observed in cow embryos only in the late 8-blastomere stage (Kopecny et al., 1985; Camous et al., 1986). This incorporation only took place after nucleolar vacuolation in the late 8-cell embryo.

Bovine embryos that have passed the 8-cell stage in vivo can be more readily cultured in vitro for several days than can earlier embryos, and they can undergo apparently normal compaction, blastulation and hatching (Wright and Bondioli, 1981). It has been suggested by King et al. (1988) that a criterion for such successful in vitro culture is nucleoli which, morphologically and physiologically, can be distinguished as being sites for active rRNA gene transcription, such that their activity can be maintained in vitro without the presence of factors presumed to be essential for their activation.

By Day 7 after estrus, there are from 80 to 120 cells in a typical bovine embryo (Betteridge and Flechon, 1988). These cells secrete

fluid in sufficient quantities to form a visible cavity or blastocoel. The development, expansion and accumulation of fluid in the blastocoel depends upon the development of junctional complexes and mechanisms for transport of fluid and ions across the first organized epithelial tissue in the embryo, the trophectoderm (Linares and King, 1980). Pivko et al.(1986) found a distinct difference between the labelling intensity with [^{3}H]-uridine between the nuclei of the trophoblast cells and those of the inner cell mass (ICM) of early bovine blastocysts, with the cells of the ICM being labelled more heavily. This was interpreted as a very early metabolic sign of the divergent differentiation of the two types of embryonic cells.

There is often a high degree of variability in morphological development and embryo quality between embryos obtained from a single flush. Studies of bovine blastocysts suggest that there is a good correlation between their morphological appearance and their viability, which can be measured in terms of cytogenetic normality (King et al., 1987), cellular normality (Chartrain and Picard, 1988), or, most importantly, embryo survival after transfer (Shea, 1981; Lindner and Wright, 1983). The following set of criteria for the discrimination between superior and inferior embryos is generally accepted for blastocysts collected from Days 7 to 11 (the most common period for collection prior to transfer; Shea, 1981; Linares and King, 1980). Embryos are judged to be of good morphological quality when they are spherical and symmetrical in shape, with a well-defined inner cell mass (ICM). An embryo is classified as 'fair' when the embryo is somewhat assymetric in shape, and when there

are some extruded and/or fragmented blastomeres present outside of the main cell mass. A poor embryo may possess some or all of the following characteristics: severe retardation in development; a significant number of extruded or degenerating cells; cellular debris in the perivitellin space; uneven blastomere size; and a noticeable withdrawl of the cell mass from the inside of the zona pellucida.

Shea's results (1981) indicate that embryos which are retarded in their development generally display evidence of degeneration, and only 44% of such embryos result in pregnancy after transfer, compared to 71% of embryos of good quality. Furthermore, it has been found that there is a relationship between glucose consumption and blastocyst viability, with zero or low glucose consumption when embryonic development is limited, and accelerated glucose metabolism in rapidly expanding blastocysts (Renard et al., 1980).

Thus signs of degeneration or delayed morphological differentiation may reflect altered embryonic metabolism, which in turn impairs further development. Yet some embryos which are judged to be superior fail to yield a pregnancy while poorly rated embryos have been known to produce a high pregnancy rate (Shea, 1981). Thus reliable criteria have yet to be developed as a means of predicting the viability of embryos during early development.

From Days 7 to 17, the embryo progresses from the early blastocyst stage through a period of expansion, hatching and pre-attachment elongation. This phase of development sets domestic animals apart from laboratory species and man, in which hatching and "true" implantation occur more or less simultaneously

(Betteridge and Flechon, 1988). Endoderm cells spread out from beneath the ICM at about Day 8 and, by Day 10, completely line the trophectodermal cells that surround the blastocoel, thereby forming the trophoblast. Mesodermal cells, which also differentiate from the ICM, begin to migrate out between the trophectoderm (TE) and the endoderm at about Day 14 or 16, and as they do so, separate into two layers. The outer layer of mesoderm lines the TE to form the chorion, while the inner layer covers the endoderm to constitute the wall of the yolk sac (Betteridge and Flechon, 1988). Hatching, the process of the expansion of the blastocyst out of the protective covering of the zona pellucida, begins around Day 9, followed by a period of rapid elongation that usually begins between Days 12 and 14 (Shea, 1981). By Day 16 the embryos may be over one hundred times their original length.

The peri-implantation period (Days 14-17; Betteridge and Flechon, 1988) has been the focus of much of the research undertaken on the bovine embryo due to the fact that the physiological activities of the embryo before attachment are critical for the maternal recognition of pregnancy. One of the many proteins which are produced and secreted by peri-implantation bovine embryo and which play important roles in the establishment and maintenance of pregnancy in the cow is bovine trophoblast protein-1 (bTP-1) (Godkin et al., 1988). Alpha-fetoprotein (AFP; Janzen et al., 1982) is also produced in large quantities by peri-implantation embryos, but its production is not initiated by events associated with implantation and thus it is not believed to play a direct role in dam-

embryo interactions. There is also a wide range of as-yet unidentified proteins which are produced by the bovine conceptus (see Godkin et al., 1988 for a survey).

The bulk of the knowledge compiled to date on the molecular biology of the early bovine embryo concerns qualitative aspects of nucleogenesis and RNA synthesis. Yet neither quantitative studies of protein and RNA synthesis nor an examination of the qualitative patterns of protein synthesis has been carried out on the bovine embryo. Thus the present studies on bovine embryos were designed to aid in the identification of the stages at which major shifts in gene expression, as manifested as changes in the patterns of RNA and protein synthesis, occur. The specific objectives were to:

1. establish that previously-published techniques for the examination of protein and RNA synthetic patterns in preimplantation mouse embryos could be used by the author to produce repeatable and reliable data. This would serve both to demonstrate the usefulness of these techniques, and would allow comparison of results from the embryos of the mouse, the cow, and a number of other mammals.
2. establish that, in the hands of the author, these techniques could be applied to single embryos. This is particularly important in work with cattle as the value of each embryo makes it important to maximize the amount of information derived from all specimens. Furthermore, this allows variation between individuals to be

examined separately from variation between different stages in development.

3. identify the characteristic qualitative patterns of proteins produced during specific stages of early bovine embryo development. This served to demonstrate the presence or absence of proteins which can be used as markers for the different stages in development, including that of genome activation.

4. examine overall patterns of protein and RNA synthesis in preimplantation bovine embryos. As the activation of the zygotic genome is associated with marked changes in the metabolism of the mammalian embryo, these patterns were compared to those obtained by other researchers. The data were used to synthesize a chronology of the major transcriptional and translational events occuring during bovine embryo preimplantation development.

MATERIALS AND METHODS

I. Procurement of embryos

A. Mouse Embryos

Female, random-bred Swiss albino CD1 mice (Charles River Breeding Laboratories) of six to eight weeks of age were used for these studies. They were maintained on a schedule of 12 hours of light, from 7:00 to 19:00 hours. Superovulation was induced by intraperitoneal (i.p.) injection with 7.5 IU of pregnant mare serum gonadotropin (PMSG; Folligon, Invert. Cambridge, U.K.) followed 48 hours later with an i.p. injection of 7.5 IU of human chorionic gonadotropin (HCG, Sigma). Injections of PMSG were administered between 14:00 and 16:00 hours, resulting in ovulation at about 4:00 a.m. the following day. Females used for recovery of fertilized eggs and embryos were single-mated overnight with CD1 males. The presence of a copulation plug the following morning served as the criterion for insemination, and thus that day was designated as Day One of pregnancy.

For the recovery of fertilized or unfertilized eggs, females were sacrificed on Day One at approximately 16 hours post-HCG, and their oviducts were removed. The eggs were dissected from the ampullary segment of the oviduct and freed from cumulus cells by exposure to hyaluronidase (355 U/ml; ovine hyaluronidase, Gibco) in M2 medium (Whittingham, 1971) containing 0.4% (w/v) bovine serum albumin (BSA; Pentex Bovine Albumin Crystalline, Miles Laboratories Inc.) for 5 - 10 minutes at room temperature. Medium

containing eggs and cumulus cells was passed through a filter made with Nitex monofilament screen mesh (B. and S.H. Thompson, St. Laurent, Quebec). The smaller cumulus cells passed through the mesh with repeated media washes. The screen was then inverted and the eggs collected with fresh medium in a clean glass culture dish. Egg preparations were freed from any remaining clumps of contaminating cumulus cells by several transfers to fresh M2 medium using a pulled Pasteur pipette.

Two-cell, four-cell, non-compacted eight-cell and early morula stage embryos were recovered at 42-44, 54-56, 66-68 and 74-76 hours post-HCG, respectively, by flushing M2 medium through the fimbriated end of the oviduct with a syringe and #30G needle (Becton, Dickinson and Co.). Blastocyst stage embryos were recovered by flushing uteri from females at 92-94 hours post-HCG with medium. Embryos were washed by several transfers through fresh medium and morphologically abnormal embryos or stage-retarded embryos were discarded.

Figure 1 shows mouse 1-cell unfertilized eggs and 8-cell embryos. All of the embryos were highly synchronous, and also generally of good morphological quality. Any embryos which were clearly abnormal were discarded.

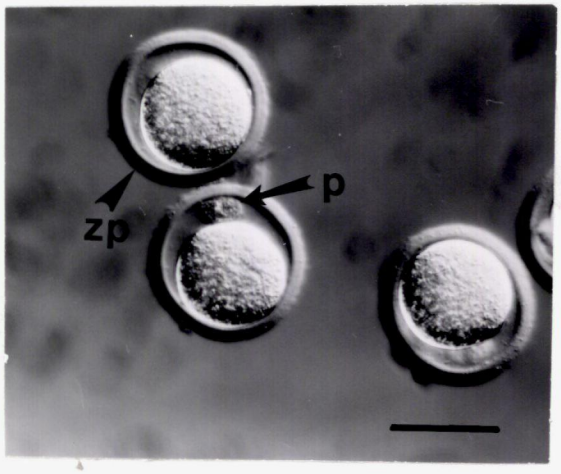
Embryos to be used for RNA extraction were placed in microfuge tubes with approximately 5 µl of M2 medium and stored at -70°C.

Figure 1

Mouse Embryos at Different Stages of Development

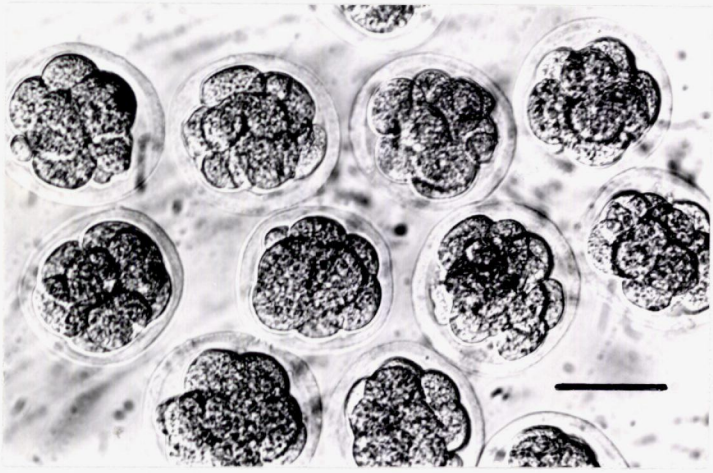
Stages are as follows (scale bars = 50 μm; all photographs taken with a Leitz Fluovert inverted microscope [West Germany]):

A. 1-cell unfertilized eggs (one with a visible polar body) at 16 hours post-hCG



p = polar body; zp = zona pellucida

B. 8-cell embryos at 68 hours post-hCG



B. Cow Embryos

Embryos were either obtained from a number of donor cows maintained at the University of Calgary's Animal Resource Centre (ARC), or were donated by Steen Willadsen at Alta Genetics Incorporated (AGI) from an experimental donor cow pool. The cows used were from a variety of breeds, including Holstein, Charolais, Hereford, and crossbred animals.

Superovulation of the animals kept at the ARC was induced in the following manner: 11 days after estrus, each animal received an intramuscular (i.m.) injection of 3 mls (1000 U/ml) pregnant mare serum gonadotropin (PMS - Laboratorio Elea S.A.C.I.F., Buenos Aires, Argentina). Three days later, 2-3 mls (250 μg/ml) prostaglandin (PG) (cloprostenol - Estrumate, Coopers, Willowdale, Ontario, Canada) was administered, followed 1.5 to 2 days later by estrus. The animals were bred using artificial insemination 12 and again 24 hours after the onset of estrus. The ages of the embryos were calculated from days or hours after the onset of estrus.

Immature follicular oocytes (to be referred to henceforth as "F.O.'s") were obtained from ovaries collected very shortly after slaughter at a local abattoir. The ovaries were transported to the Health Sciences Centre (HSC) at the University of Calgary where the follicles were aspirated with a 16-gauge needle attached to a 5 ml syringe, followed by washing and sorting of the F.O.'s in TLH medium (HEPES-buffered Tyrodes; Bavister et al., 1983).

Post-ovulatory oocytes (to be referred to henceforth as "P.O.'s") and embryos to be collected on Days 1 to 4 after estrus were

recovered surgically at AGI. A salpingectomy was performed by a veterinarian who first inserted one hand inside the vagina of the cow and made an incision in the dorsal side of this organ. This allowed the surgeon to reach over and 'stuff' the ovarian bursa back into the vagina. The oviduct and ovary were then severed from the vagina using a specially-designed stainless steel instrument, and the oviduct and ovary were carefully withdrawn from the vagina. Each oviduct was flushed with 15 mls of PB-1 medium (Whittingham and Wales, 1969) supplemented with 4 mg/ml bovine serum albumin (BSA; Fraction V, Sigma) and 10% inactivated calf serum (Gibco, Grand Island, New York). The embryos or eggs were recovered using a pulled Pasteur pipette. The adhering cumulus masses on the P.O.'s were removed by passing them up and down a fine-bore pulled Pasteur pipette. The eggs were then transferred into a microfuge tube with a small quantity of PB-1.

Embryos to be recovered from cows on Day 5 or later were collected using a standard non-surgical procedure (Elsden et al., 1976) at AGI. Following palpation of the ovaries to determine the number of corpa lutea and thus the number of embryos which could be recovered, a Folley catheter was inserted into the uterus. Each uterine horn was flushed with 250 mls of PB-1. The fluid was collected in a glass bottle and passed through a plankton filter with a 75 μm mesh (Em Con). The embryos were collected and placed in a 5 ml plastic tube or an 0.5 ml microfuge tube and stored at room temperature. The tubes were then wrapped in several layers of paper towelling and transported to the HSC. Each embryo or group of embryos was assigned a number for the purposes of record-keeping.

The amount of time between recovery of the eggs and their arrival at the HSC varied between one and six hours. Embryos to be used for RNA extraction were transferred through several washes of M2 medium and then placed in microfuge tubes with approximately 5 µl of M2 medium. They were immediately placed at -70°C.

Figure 2 shows bovine embryos at different stages of development. It should be noted that, unlike the case with mouse embryos, both polar bodies are rarely visible in bovine embryos. Figure 3 shows two examples of the morphological heterogeneity encountered among embryos at the same stage of development. The late morulae shown in Figures 3A and B were obtained from the same flush of the same cow on Day 5.5. The two blastocysts shown in Figures 3C and D were both obtained from cows flushed on Day 7.

II. <u>In Vitro</u> Labelling of Embryos with [^{35}S]-Methionine

Labelling of embryos was carried out using the method employed by Hahnel et al. (1986) with minor modifications. Embryos were transferred into 2-3 mls of M2 medium and incubated at 37°C for one hour. The embryos were then placed in a prewarmed 50 µl drop of M2 medium containing either 0.83 µM L[^{35}S]-methionine (1253 Ci/mmol on July 27, 1987 - Amersham Oakville, Ontario, Canada) or 0.75 µM L[^{35}S]-methionine (1330 Ci/mmol on January 18, 1988 - Amersham). The incubation mix contained 40 µl of M2 medium, 5 µl of 2 X M2 medium, and 5 µl of [^{35}S]-methionine, and was submerged under silicon oil (Dow Corning Corp.) in a plastic dish (Falcon). The embryos were incubated at 37°C

Figure 2

Bovine Oocytes and Embryos at Different Stages of Development

Stages are as follows:

A. Immature F.O. surrounded by a large mass of cumulus cells,

B. P.O. obtained approximately 12 hours after ovulation,

C. One 8-cell and one 8-16-cell embryo obtained on Day 5,

D. Late morula obtained on Day 7,

E. Hatched blastocyst obtained on Day8.

Abbreviations used are: cc, cumulus cell mass; c, cumulus cell; zp, zona pellucida; bc, blastocoel; ICM, inner cell mass; t, trophectoderm. The scale bar = 100 μm (applies to all panels). All photomicrographs were taken with a Leitz Fluovert inverted microscope.

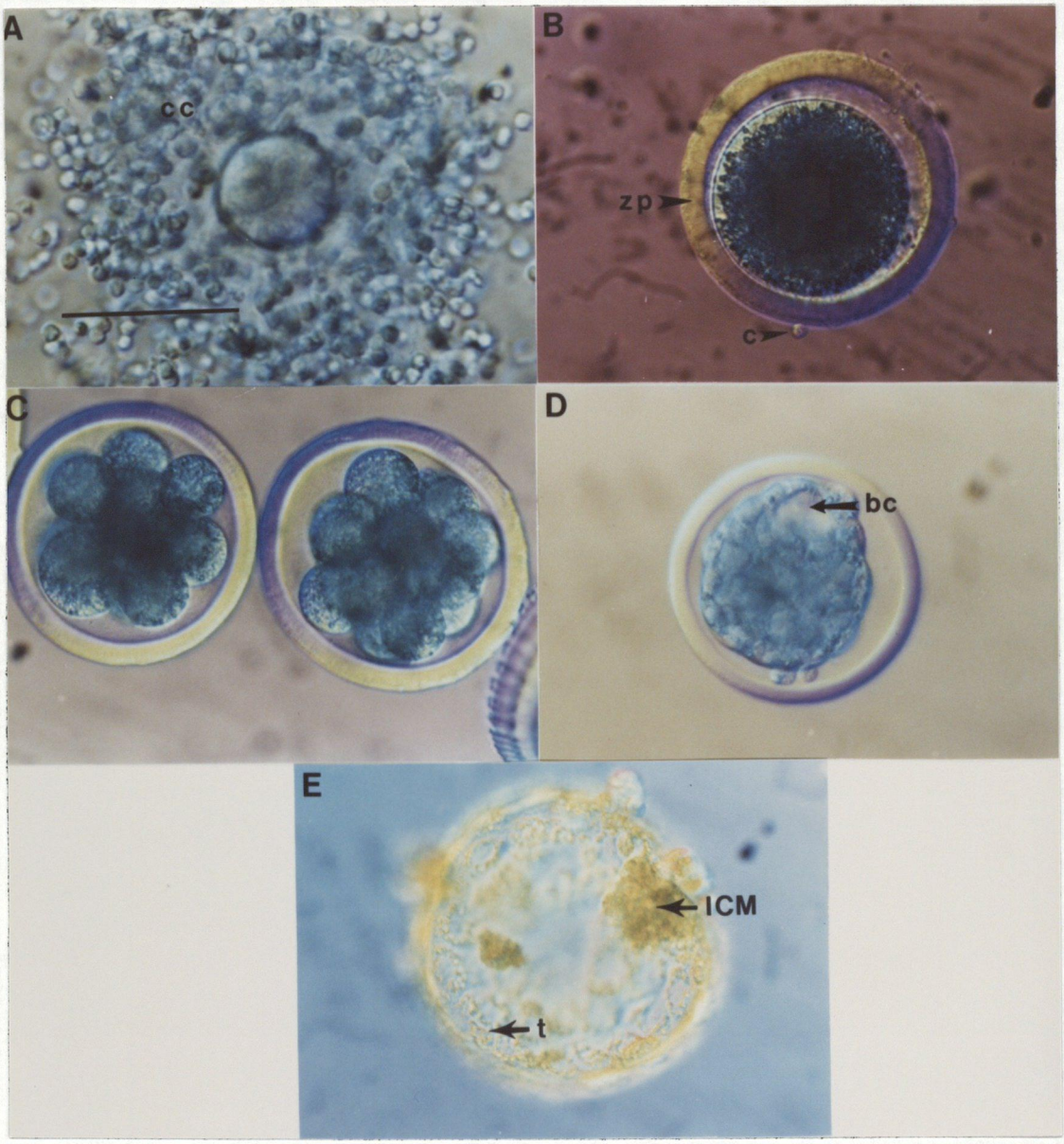

A
cc
B
zp
c
C
D
bc
E
ICM
t

Figure 3

Bovine Blastocysts of Differing Morphological Quality

Embryos shown are as follows:

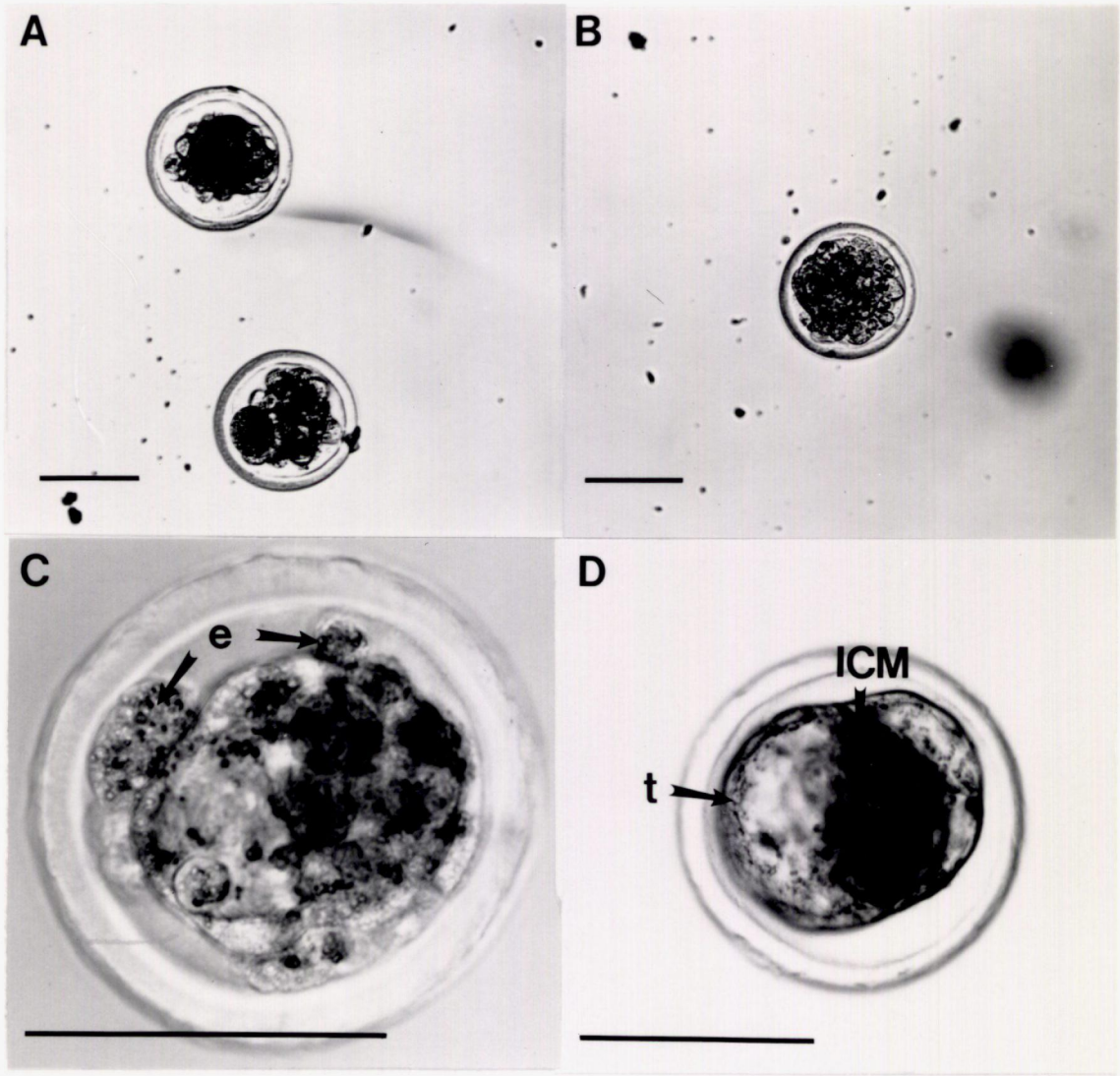
A. Blastocysts collected at Day 5.5 (#48 [top], 49[bottom]),

B. Blastocyst collected from same cow as for A at Day 5.5 (#50),

C. Blastocyst collected at Day 7 (#25),

D. Blastocyst collected at Day 7 (#24).

Abbreviations used: ICM, inner cell mass; t, trophectoderm, e, extruded cells. Scale bars = 100 μm. All photographs taken with a Leitz Fluovert inverted microscope.


A
B
C
e
D
ICM
t

in a humidified atmosphere of 5% CO_2 for two hours and then rapidly washed three times in 4 ml aliquots of fresh, ice-cold medium free of radioactivity, with a final wash in a 4 ml aliquot of phosphate buffered saline (PBS) to remove protein present in the medium. Washed individual (bovine) or groups (mouse) of eggs or embryos were placed, with a small amount of PBS, in microfuge tubes, and they were disrupted by adding 10 - 25 µl of SDS-dissociation buffer (62.5 mM Tris-HCl, pH 6.8, containing 2% w/v SDS, 10% v/v glycerol and 5% v/v 2-mercaptoethanol). The samples were stored at -70°C.

Samples were thawed and then placed in boiling water for 5-10 minutes, followed by quenching on ice for approximately 5 minutes. Uptake of L[^{35}S]-methionine by individual eggs or embryos was assayed by spotting an 0.5, 1.0 or 2.0 µl aliquot from each lysate onto each of two Whatman GF/C glass fibre filters. The amount of lysate remaining was then measured in order to calculate the original volume. The filters were dried at room temperature, and one replicate, to be used for calculation of incorporation of isotope into the endogenous TCA-precipitable (protein) pool, was then washed with 20 ml of ice-cold 10% trichloroacetic acid (TCA) and 10 mls of ice-cold absolute ethanol and dried at room temperature. The other replicate was used to measure total uptake of the label into the endogenous free amino acid pool of the embryos.

The filters were placed in plastic or glass scintillation vials (Fisher Scientific), and 7 mls of scintillation cocktail ('Econofluor-2' or 'Aquasol-2', New England Nuclear, Lachine, Quebec, Canada) was added prior to counting using a Beckman LS 6800 counter.

The efficiency of counting of [^{35}S]-methionine using 'Econofluor-2' or 'Aquasol-2' was calculated to be 77.6% and 75.3%, respectively. The incorporation rate in number of counts per minute (cpm)/embryo/hour was calculated by taking into account the total number of embryos in each labelling group, the total volume of the lysate, and the length of the labelling period, and then adjusted for the amount of decay of the isotope by multiplying the number of cpm incorporated by the reciprocal of the fraction of the original specific activity of the isotope which remained at that time (as shown in specification sheets supplied by the manufacturer with each batch of radioactive precursor). This was then converted to number of Curies (Ci) incorporated/embryo/hour by first converting the number of cpm to disintegrations per minute (dpm) using the efficiencies of counting (above), and then by using the conversion factor 1 μCi = 2.22 X 10^6 dpm. The number of Ci incorporated was converted to number of fmoles incorporated by using the value of the specific activity of the [^{35}S]-methionine (in Ci/mmole) as given in the manufacturer's specification sheets for each batch of radioactive precursor.

III. One-Dimensional SDS-Polyacrylamide Gel Electrophoresis of Proteins

The patterns of proteins produced by mouse or bovine embryos at different stages of development were examined by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) carried out on a Biorad Protean vertical

slab gel electrophoresis unit according to the method of O'Farrell (1975). 12 µl samples of the SDS-dissociated extracts were loaded onto 12% slab gels that were 0.75 mm thick. The amount of radioactivity loaded per lane varied from 20,000 cpm for samples from bovine trophectoderm to 800 cpm from single mouse embryos. Samples within a single gel, however, contained approximately the same amount of radioactivity to facilitate comparisons.

The molecular weight (MW) markers used were either [^{14}C]-methylated markers (1.11 X 10^7 dpm/10 µl; Amersham; myosin, 200 kD; phosphorylase b, 92.5 kD; bovine serum albumin (BSA), 69 kD; ovalbumin, 46 kD; carbonic anhydrase, 30 kD; and lysozyme, 14.4 kD) or non-radioactive markers (Pharmacia, Uppsala, Sweden; phosphorylase b, 94 kD; BSA, 67 kD; ovalbumin, 43 kD; carbonic anhydrase, 30 kD; trypsin inhibitor, 20.1 kD; lysozyme, 14.4 kD).

If non-radioactive MW markers were used, following electrophoresis, the gels were stained with Coomassie blue (0.05% Coomassie blue - R250, 10% acetic acid, and 25% isopropanol) for 30 to 60 minutes, and then destained and fixed (in 10% ethanol, 10% acetic acid) for at least 4 hours. If radioactive MW markers were employed, the gels were simply fixed overnight (in 30% methanol, 10% glacial acetic acid).

The gels were then treated with 'En^{3}hance' (New England Nuclear, Lachine, Quebec, Canada) for 30 to 60 minutes, rinsed with distilled water, soaked in 3% glycerol for 30 to 60 minutes, and then dried. The gels were then exposed to X-ray film (Kodak X-Omat AR-F) for autoradiography at -70°C. Exposure times varied from 2 days (20,000 cpm/lane) to over 50 days (800 cpm/lane).

IV. In Vitro Labelling of Embryos with [^{3}H]-Uridine or [^{3}H]-Adenosine

Embryos were transferred using a pulled Pasteur pipette from unlabelled M2 medium into a pre-warmed 50 µl drop of M2 medium containing 5.13 µM [5,6-^{3}H]-uridine (1.0 mCi/ml, 39 Ci/mmol; Amersham) or 5.0 µM [2,5',8-^{3}H]-adenosine (1.0 mCi/ml, 40 Ci/mmol; Amersham). The incubation mix contained 30 µl of M2 medium, 10 µl of 2 X M2 medium, and 10 µl of isotope. The embryos were incubated in a plastic dish (Falcon) under silicon oil at 37°C in a humidified atmosphere of 5% CO_2 air for 2 hours. Mouse embryos were labelled in groups (9-10/group at the 8-cell stage; 2-4/group at the blastocyst stage). The bovine embryos were labelled singly (with the exception of a group of three 16-cell stage embryos; see Results, Table 5).

All of the bovine embryos at the morula to blastocyst stage which were labelled with [^{3}H]-uridine were obtained from AGI. They had been cultured from the early cleavage stages to the blastocyst stage by incubation in a block of agar in the oviduct of a ewe for four days. Three of the blastocysts were physically damaged and were cultured in M2 medium overnight at 37°C. Another three blastocysts were manipulated by either making a tear in, or completely removing, their zona pellucidae prior to introducing the blastocysts into the labelling medium. Another three were treated by injecting the labelling medium directly into the blastocoel. The concentration of the injected labelling solution for this last group of embryos was 400, rather than 200, µCi/ml.

Following the labelling period, the embryos were rapidly washed four times in 4 ml aliquots of fresh, ice-cold M2 medium free of radioactivity. Washed single embryos or groups of embryos were placed in microfuge tubes, and were disrupted by adding 10 - 25 µl of SDS-dissociation buffer. The samples were stored at -70°C. Samples were later thawed, and lysate was spotted onto glass fibre filters and treated as for [^{35}S]-methionine-labelled samples in order to assess uptake and incorporation of the precursors into the endogenous free nucleotide and RNA pools, respectively.

The counting efficiency of [^{3}H] was found to be 36% with 'Econofluor-2' and 41% using 'Aquasol-2'. The number of cpm of [^{3}H]-uridine incorporated/embryo/hour was calculated and then converted to number of pmol incorporated/embryo/hour in the same manner as for labelling with [^{35}S]-methionine, with the exception of the omission of the compensation for the decay of the isotope.

V. Preparation of Total RNA from Bovine Brain

Fresh bovine brain was obtained at a local slaughterhouse. A small piece of brain tissue from a freshly-slaughtered cow was placed in a polyethylene bag. The bag was completely sealed and immediately immersed in an absolute ethanol-dry ice slurry. The sample was then transported to the HSC and either used immediately or stored at -70°C. A piece of tissue estimated to weigh only a few grams was chipped from the frozen sample and ground to a fine powder using a mortar and pestle (stored at -70°C prior to use) and liquid nitrogen as the coolant. The resultant powder was transferred

into an ice-cold 15 ml Corex tube, and 10 mls of ice-cold RNA extraction solution (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5, 5 mM $MgCl_2$) was added per 1 gram (estimated) of tissue. The solution was mixed to homogeneity by inversion. The tube was then spun at 3500 rpm at 4°C for 10 minutes. The supernatant was transferred to another 15 ml Corex tube. 20% SDS (w/v) was added to a final concentration of 1%, and 0.5 M EDTA was added to a final concentration of 5 mM. The solution was mixed well, and the proteins were then removed by extraction first with phenol (water-saturated and neutralized; Bethesda Research Laboratories [BRL]), then with a 1:1 solution of phenol:SEVAG (24:1 chloroform:isoamyl alcohol), and finally at least once with SEVAG alone. When a clear supernatant was obtained, it was transferred by pipette into a siliconized 15 ml or 30 ml Corex tube. Two volumes of ice-cold absolute ethanol were added, and the RNA was allowed to precipitate at -20°C for at least five hours.

In order to pellet the RNA, the sample was spun at 15,000 rpm for 20 minutes (for 15 ml Corex tubes) or at 10,000 rpm for 40 minutes (for 30 ml Corex tubes) at 4°C. The supernatant was carefully poured off, and the pellet was dried under vacuum. The pellet was resuspended in 15 - 100 µl sterile water and transferred to a microfuge tube.

The concentration of the RNA was determined by diluting a 2 µl aliquot 1:250 with water, and then measuring the optical density (O.D.) of the sample at 260 nm and 280 nm using a Beckman Model 35 UV spectrophotometer. When the ratio of the O.D.260/O.D.280 readings is 2.0, an O.D. reading of 1.0 at a wavelength of 260nm

corresponds to an RNA concentration of 40 mg/ml (Maniatis et al., 1982). After the RNA concentration in the solution was determined, it was stored at -70°C.

The degree of degradation of the RNA was examined by running a 5 µg aliquot of each sample on a vertical 1% agarose-formaldehyde gel as described by Maniatis et al.(1982). Figure 4 shows samples of bovine brain total RNA (Lane 2) and adult mouse brain total RNA (Lane 3; kindly supplied by G. A. Schultz; concentration = 1.4 mg/ml) with an RNA ladder (BRL; Lane 1). As can be seen, the 28S rRNA bands are readily identifiable, and a large portion of this RNA appears to be intact.

VI. Preparation of Total RNA from Mouse and Bovine Embryos

Total nucleic acid was extracted from samples of 1 to 40 mouse embryos, from groups of 1 to 13 bovine P.O.'s, or from single bovine embryos according to the method of Braude and Pelham (1979). A microfuge tube containing the eggs or embryos was removed from storage at -70°C and placed on ice. 20 µl phenol, 5 µl yeast tRNA (nuclease-free, 2.5 mg/ml, BRL) and 20 µl extraction buffer (0.2 M NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4) was immediately added. The sample was then vortexed vigorously for 20 seconds, and transferred by capillary action into a 50 µl baked capillary pipette (Fisher Scientific). One end was sealed with a thickness of approximately 3 mm of PVC putty. The phases were separated by centrifugation for one minute at 5000 rpm in a hemotocrit centrifuge (ReadACrit;

Figure 4

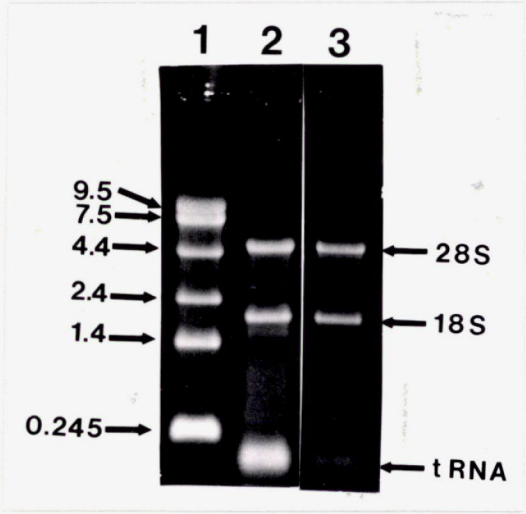
Total RNA from Bovine and Mouse Brain

Lane 1: 9 µg RNA size standards (BRL)

Lane 2: 10 µg total bovine brain RNA.

Lane 3: 3.0 µg total mouse brain RNA (courtesy of Dr.G.A.Schultz)

The sizes of the bands in the RNA ladder (in kB) are indicated on the left side of the Figure, while the positions of the 28S rRNA, 18S rRNA and tRNA bands in the bovine and mouse RNA samples are indicated on the right-hand side of the Figure.



ClayAdams, Parsipanny, New Jersey).

The capillary tube was scarred and broken just above the phenol:aqueous interface, and the aqueous phase was carefully transferred into a sterile microfuge tube. 60 µl of cold, abolute ethanol was added, the solution was mixed well, and it was placed at -20°C for at least 5 hours.

To recover the RNA, the microfuge tube was centrifuged at 15,000 rpm at 4°C for 30 minutes. The supernatant was carefully drawn off using a pulled Pasteur pipette, and then the pellet was dried under vacuum. The RNA was resuspended in 10 to 100 µl of sterile water and stored at -70°C. The efficiency of recovery of total RNA using this procedure, as calculated by Dr. Gil Schultz, was 69.2 ± 2.2%.

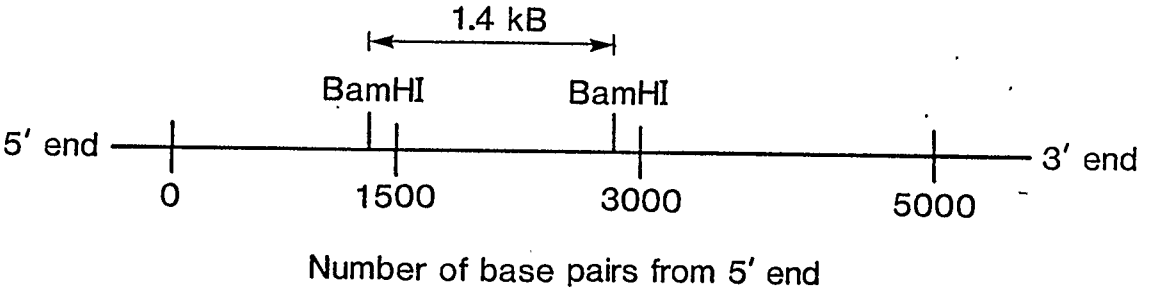
VII. Synthesis of a 28S rRNA Nick-Translated Probe

The plasmid pABB was kindly supplied by Dr. Jim Sylvester of the University of Pennsylvania. As shown in Figure 5, this plasmid contains a 1.4 Kb BamHI restriction fragment from the middle of the human 28S rRNA gene cloned into the BamHI site of pBR322 (Gonzalez et al., 1985). It was used directly to synthesize a [^{32}P]-labelled, nick-translated probe against mouse and bovine 28S rRNA. The probe was synthesized using the method of Maniatis et al. (1982) with some modifications. The reaction mixture consisted of: 50 mM Tris-Cl, pH 7.2, 10 mM $MgSO_4$, 0.5 mM dithiothreitol (DTT), 10 ng BSA (Pentax Fraction V, Sigma), 10.0 µg of pABB DNA, 0.4 nmoles each of dATP, dGTP and dTTP (Pharmacia), 33.3 pmoles of

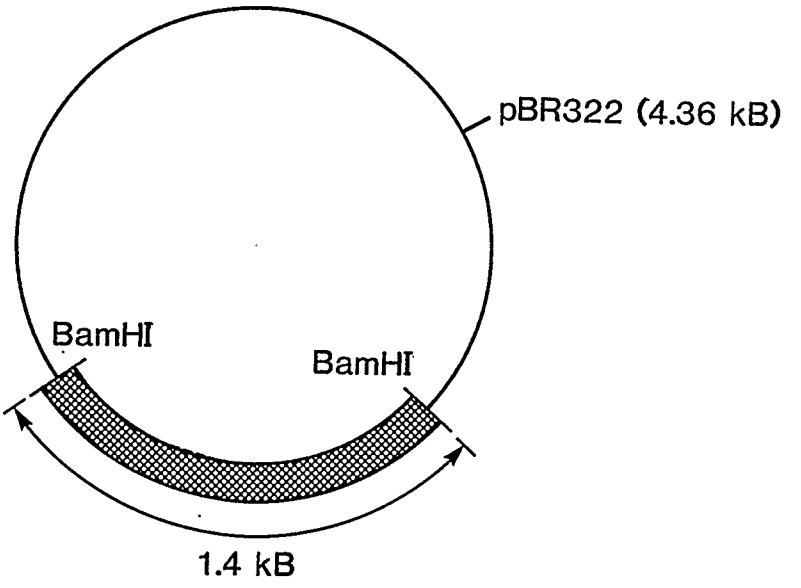
Figure 5

Structure of the Plasmid pA_{BB}

A. Diagram showing the 1.4 kB fragment of the human 28S rRNA gene which is contained within pA_{BB} (from Gonzalez et al.,1985)



B. Schematic representation of pA_{BB} (5.76 kB)



[^{32}P]-dCTP (10 mCi/ml, 3000 Ci/mmole, Amersham), 0.1 mg of DNAse I (Pharmacia), and 10 U of DNA polymerase I (Pharmacia). The first seven ingredients were mixed well before the DNA polymerase was added. The final mixture (having a volume of 20 µl) was then mixed again before being placed at 13°C for a period of 30 to 60 minutes. 1 µl aliquots were withdrawn at 10-minute intervals and spotted on to glass fibre filters for the measurement of TCA-precipitable counts. When the reaction was judged to have reached its peak (usually after about 30 minutes), it was stopped by adding 2 µl of 0.5 mM EDTA. The probe was then separated from unincorporated nucleotides by passing the reaction mixture through a 5 ml Sephadex G-75 column and collecting the fastest-eluting radioactive component. This solution was then subjected to ethanol precipitation for a period of at least 2 hours at -20°C. An average specific activity of 5 X 10^5 cpm/ng DNA was obtained. The probes were stored at -20°C until use (usually within 7 days).

VIII. Synthesis of 28S rRNA Riboprobes

The plasmid pGA$_{BB}$, which serves as a transcription vector for the 1.4 kB fragment of the human 28S rRNA gene, was constructed by inserting the 1.4 kB BamHI fragment of pA$_{BB}$ into the multiple cloning site (MCS) of pGEM-1 (Promega Biotec; kindly supplied by the lab of Dr. Gil Schultz) in the standard manner (Maniatis et al., 1982; see Appendix One). pGA$_{BB}$ was linearized by restriction digestion with either HindIII (Pharmacia) or EcoRI (Boehringer Mannheim), and was transcribed using T7 or Sp6 RNA polymerase (Promega

Biotec), respectively with the RNA being labelled by supplying [^{35}S]-uridine as a nucleotide precursor. The riboprobes were synthesized in the following manner: 1 µg of linearized pGA$_{BB}$ DNA was incubated for 30 minutes to one hour at 38 - 40°C in a transcription reaction containing 47 mM Tris pH 7.5, 7 mM $MgCl_2$, 2.5 m M spermidine, 12 mM NaCl, 12 mM dithiothreitol (DTT), 0.6 mM each of GTP, ATP and CTP, 14 µM unlabelled UTP, 125 µCi of [^{35}S]-uridine 5'-[α-thio]-triphosphate (1240-1380 Ci/mmol; Amersham), 20 units of RNasin (Promega Biotec), and 10 units of T7 or Sp6 RNA polymerase (Promega Biotec).

The reactions were stopped by chilling on ice and two 1.0 µl aliquots were removed and spotted on glass filters to measure total and TCA-precipitable radioactivity. The amount of RNA transcribed and its specific activity was calculated. An average specific activity of 2.0 X 10^5 cpm/ng RNA was obtained. The probes were stored at -70°C until required (2 days to 2 months later).

All of the riboprobes were tested against dot blots of approximately 5 µg of total RNA from bovine brain to detect hybridization (see Figure 16, Appendix One).

IX. Northern and Slot Blot Analysis of Total RNA from Bovine and Mouse

The nick-translated probes and riboprobes made from pAB B and pGA$_{BB}$, respectively, were used to probe Northern and slot blots of mouse and bovine RNA. Northern blots were prepared by electrophoretically separating defined quantities of total RNA from

both mouse and bovine brain, oocytes and/or embryos on vertical denaturing 1% agarose-formaldehyde gels in the manner described by Maniatis et al. (1982). A typical gel contained both: 1. known quantities of total RNA from brain tissue; and 2. total RNA extracted from a series of pools of embryos; for both mouse and bovine samples. A gel containing 10 µg yeast tRNA (BRL) and 10 µg of bovine RNA was also run and ethidium bromide-stained in order to demonstrate the relative positions of the 28S rRNA, 18S rRNA, and tRNA bands as well as to test the level of hybridization of tRNA to the probes used (see Results).

The gels were run at 170 volts for 2 hours, with 1 X MOPS (20mM MOPS, 5mM sodium acetate, 1mM EDTA, pH 7.0) as the running buffer. After electrophoresis, the gels containing the mouse and bovine RNA samples were subjected to capillary blotting on to 'Nytran' nylon membrane (Schleicher and Schuell, Keene, New Hampshire) using the manufacturer's protocol. This blotting was carried out for 12 - 17 hours with 10 X SSC (1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0) as the transfer buffer. The membrane was then dried at room temperature and baked for 2 hours at 60 - 80°C.

Slot blots were also prepared, by blotting various quantities of total RNA from mouse and bovine brain and oocytes and embryos on to 'Nytran' using a Biorad slot-blot apparatus. These membranes were baked for 2 hours at 60 - 80°C.

Following baking, the blots were sealed in plastic and 5 - 15 mls of prehybridization solution was added. Different prehybridization and hybridization solutions were used for either

the nick-translated probes or the riboprobes. The same solution was used for both prehybridization and hybridization of blots with riboprobes. This solution contained: 50% formamide (MCB), 5 X SSC, 0.1% SDS, 1 mM EDTA. 0.01 mg/ml yeast tRNA (BRL), and 2.5% dextran sulfate.

The prehybridization solution for use with nick-translated probes contained: 2 X STE (20 mM Tris-Cl, 0.2 M NaCl, 2 mM EDTA, pH 8.0), 10 X Denhardt's, 2 mg poly A (Sigma), 4 mg yeast tRNA (BRL), 1.5 mg sheared salmon sperm DNA, 0.2% SDS, and 2.5% dextran sulfate. The hybridization solution was identical to that for prehybridization, with the exception of the omission of the salmon sperm DNA. The blots were prehybridized for at least 2 hour at 65°C (for nick-translated probes) or 58°C (for riboprobes) in a shaking water bath.

Following prehybridization, the blots were exposed to the radioactive probes at a concentration of approximately 1 X 10^6 cpm/ml (or 4-8 ng radiolabelled probe/ml) of hybridization solution. The probe was added to 1 ml of hybridization solution in a microfuge tube. The solution was boiled for 5 minutes, and then quenched on ice for 5 - 10 minutes. This solution was then added to the bag containing the blots, the bag was sealed, and hybridization was carried out for 12 - 17 hours at the same temperature as for the prehybridizations.

The blots were then removed from the hybridization bags and were subjected to a series of washes of increasing stringency. In the case of blots probed with nick-translated DNA, the series of washes used were:

1 X (2 X STE, 0.1 % SDS) briefly at room temperature

2 X (2 X STE, 0.1 % SDS) for 10 minutes at 60°C

2 X (1 X STE, 0.1 % SDS) "

2 X (0.5 X STE, 0.1 % SDS) "

2 X (0.2 X STE, 0.1 % SDS) "

In the case of blots probed with radioactively-labelled RNA, the conditions for washing were as follows:

1 X (5 X SSC, 0.1 % SDS) briefly at room temperature

2 X (5 X SSC, 0.1 % SDS) for 20 minutes at 68 - 70°C

4 X (0.1 X SSC, 0.1 % SDS) " "

After completion of the washes, the blots were dried at room temperature and then subjected to autoradiography with pre-flashed X-ray film (Kodak X-Omat AR-F) at -70°C. The exposure times varied from 12 hours to 4 days.

The autoradiographs were developed and the relative amount of hybridization of each of the samples with the probe was measured by densitometric scanning using either the "Ultroscan XL" (LKB - Bromma) laser scanner in the lab of Dr. Mark Bisby or a scanning system consisting of a densitometric analysis system using a video camera and a Coreco frame grabber with an IBM XT computer system developed by W.G. Tatton and his colleagues. Linear regression analysis of the data from the Northern blot and plotting of the curves was carried out using the 'Macintosh' "Cricket Graph" software package. By comparison of the embryo samples with the standards, the absolute amounts of total RNA present in bovine and mouse eggs or embryos was determined. The slot blots were also

scanned to yield data on the relative amounts of total RNA contained within bovine P.O.'s and embryos at different stages of development.

RESULTS

I. In Vitro Labelling of Embryos with L[^{35}S]-Methionine

For the purpose of determining the relative rates of protein synthesis by embryos at different stages of development, the rate of incorporation of radiolabelled L-methionine into the intracellular TCA-precipitable pool of single embryos was measured. This precursor (along with leucine) is one of the most useful amino acids to employ in such studies, as the embryonic endogenous pools of these two amino acids are both small from the egg to the blastocyst stage (Schultz et al., 1981). This means that these pools are readily saturable with label, and the intra- and extra-cellular pools of label approach the same specific activities after short labelling periods. Furthermore, the kinetics of uptake and incorporation of methionine and leucine have been shown to be similar, at least during early mouse embryonic development (Kaye et al., 1982), and thus data obtained from the use of either may be compared.

A. Mouse Data

Table 1 shows the values obtained for the uptake and incorporation of L[^{35}S]-methionine by mouse embryos at different stages of development. The rate of incorporation of label at the 1-cell unfertilized stage was set at 1.00. This provided a simple means of clearly showing the effects of fertilization and the first few

cleavage divisions upon the relative rate of incorporation by the embyos.

Table 1

Uptake and Incorporation of L[^{35}S]-Methionine into Mouse Oocytes and Embryos at Different Stages of Development

		Incorporation	
Stage	Uptake[a]	Absolute[b]	Relative[c]
1-cell unfert'd. (n=15)	21,984 ± 1479	2,327 + 248	1.00
1-cell fert'd. (n=5)	21,240 ± 1619	2,198 ± 438	0.92
2-cell (n=16)	17,730 ± 440	1,662 ± 132	0.69
8-cell (n=8)	25,250 ± 2687	1,759 ± 215	0.73
16-32-cell (n=7)	47,464 ± 13,667	3,886 ± 1443	1.62
Blastocyst (n=5)	259,466 + 8,559	13,957 ± 496	5.82

[a]Uptake is presented in (#cpm [^{35}S]-methionine in the TCA-soluble pool)/embryo/hour ± S.D.

[b]'Absolute' rate of incorporation is presented in (#cpm [^{35}S]-methionine incorporated into the TCA-insoluble pool)/embryo/hour ± S.D.

[c]'Relative' rate of incorporation was determined by setting the absolute rate of incorporation of label at the 1-cell unfertilized stage to 1.0, and by determining the values of the other incorporations with respect to this number.

As can be seen from this Table, the relative rate of incorporation declines by 31% from the 1-cell unfertilized stage until the 2-cell stage. It then increases over 8-fold from the 2-cell stage until the late blastocyst, reaching a value of 5.82 by that stage.

B. Bovine Data

Table 2 shows the rates of incorporation of L[^{35}S]-methionine by bovine F.O.'s, P.O.'s, and embryos at different stages of development.

Table 3 shows the average and relative rates of incorporation of L[^{35}S]-methionine into the bovine specimens at each stage of development. As for the mouse embryos, the rate of incorporation of [^{35}S]-methionine into the P.O. was set at 1.0, and all the other rates were calculated relative to it.

These Tables show that there is an extremely high rate of incorporation of [^{35}S]-methionine into immature F.O.'s which are surrounded by a mass of cumulus cells. The Tables also show that the rate of incorporation of [^{35}S]-methionine declines between the F.O. and the 8-cell stage, after which time it increases again until, at the blastocyst stage, the rate of incorporation is almost eight times as at the P.O. stage.

Note that in the case of the F.O. samples, both the labelling and measurement of incorporation of radiolabelled methionine was carried out with the cumulus mass still attached to the oocyte and thus the rates of incorporation reflect the activity of both the large number of cumulus cells in addition to that of the oocyte itself. It should also be noted that embryos having very low rates of incorporation were not included in the Tables, as they are likely to be abnormal (i.e. either severly retarded in development or morphologically highly abnormal). For example, the blastocysts #48 and #49, which were found to incorporate 8,416 and 6,758 cpm

Table 2

Absolute Rates of Incorporation of L[^{35}S]-Methionine into Bovine Oocytes and Embryos at Different Stages of Development

Stage	Embryo #[a]	Incorporation (cpm/embryo/hr)[b]
F.O.	150	233,516
	151	222,516
	152	142,779
P.O.	1*	2,244
	2*	2,256
	3*	1,947
	4*	3,524
	13*	4,316
	14*	2,408
	15*	4,516
	21*	5,679
	51*	6,209
	53*	5,761
1-Cell Fertilized	41*	3,744
	42*	2,041
	43*	1,467
	111	2,928
	112	2,909
	113	3,339
	119	4,757
	120	2,298
	121	3,310
2-Cell	46*	3,097
	114	2,312
	115	3,319
	116	2,016
4-Cell	117	2,186
	118	2,077
8-Cell	95	1,494
	96	1,325
8 - 16-Cell	17*	3,129
	97	4,090
	98	2,561
16 - 32-Cell	102	3,128
	103	4,772
Morula(32-48-Cell)	12*	19,709
	160	9,488
Blastocyst	24*	57,138
	25*	13,824
	50*	45,939
	75*	20,988
	144	21,584
	145	30,817
	146	28,438
	147	10,830
	148	24,075

[a] An * beside an embryo indicates that it was labelled in 0.83 μM [^{35}S]-methionine; all other embryos were labelled in 0.75 μM [^{35}S]-methionine for two hours at 37°C.

[b] The decrease in the specific activity of the isotope with time was corrected for by multiplying the #cpm counted by the inverse of the fraction of the original specific activity remaining at that time.

Table 3

Average and Relative Rates of Incorporation of L[^{35}S]-Methionine into Bovine Oocytes and Embryos at Different Stages of Development

Stage	Avg. Incorporation (#cpm inc'd/e/hr + SD)	Relative Incorporation
F.O.	199,573 ± 28,586	51.36
P.O.	3,886 ± 1,638	1.00
1-Cell Fert'd.	2,977 ± 976	0.77
2-Cell	2,686 ± 621	0.69
4-Cell	2,477	0.63
8-Cell	1,410	0.36
8-16-Cell	3,260 ± 773	0.84
16-32-Cell	3,950	1.02
32-48-Cell	14,599	3.98
Blastocyst	28,181 ± 14,917	7.68

[^{35}S]-methionine, respectively, into the endogenous TCA-precipitable pool/hour were not included in the displayed values.

II. One-Dimensional SDS-Polyacrylamide Gel Electrophoresis of [^{35}S]-Labelled Proteins from Embryos

A. Mouse Data

Figures 6 and 7 show the patterns of proteins synthesized by mouse embryos at the 1-cell fertilized to blastocyst stages of development. Band 'a', with molecular weight 79 kD, is present in significant quantities from the 1-cell embryo to the blastocyst stage, with an increase in intensity after the 2-cell stage to become one of

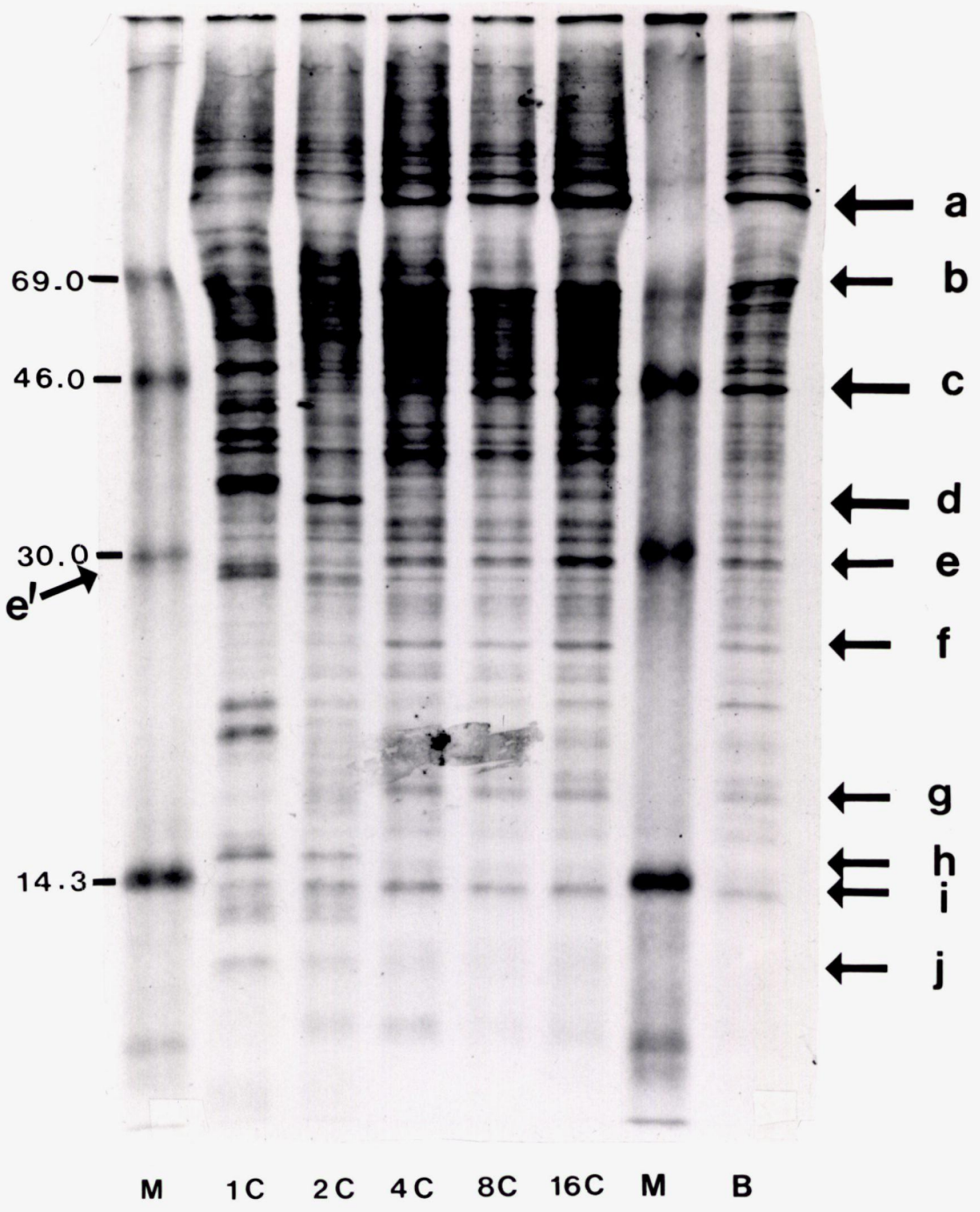
Figure 6

One-Dimensional SDS-Polyacrylamide Gel of [^{35}S]-Labelled Proteins from Groups of Mouse Embryos at Different Stages of Development

Groups of mouse embryos at different stages of development (5 X 1-cell fertilized, 23 X 2-cell, 27 X 4-cell, 19 X 8-cell, 17 X 16-cell, and 20 X blastocyst) were collected and labelled with [^{35}S]-methionine followed by disruption with SDS-dissociation buffer (see Materials and Methods). Aliquots, each containing 10,000 cpm, from each of these groups of embryos were loaded into a polyacrylamide gel and subjected to electrophoresis, followed by fixation, treatment with 'En^{3}hance', and autoradiography. Exposure time to X-ray film (Kodak X-Omat) at -70^{o}C was 7 days.

The samples included in the gel are: M, molecular weight markers (Amersham); 1C, one-cell fertilized eggs; 2C, 2-cell embryos; 4-C, four-cell embryos; 8C, 8-cell embryos; 16C, 16-cell embryos; and B, blastocysts.

The molecular weights (in kD) of the proteins in the marker lane are displayed to the left of the gel, while the letters to the right of the gel ('a' to 'j'; 'e" to the right) indicate protein bands which are prominent an/or which vary in intensity across the stages of mouse embryo development (see also Figure 7).



7 – Day Exposure

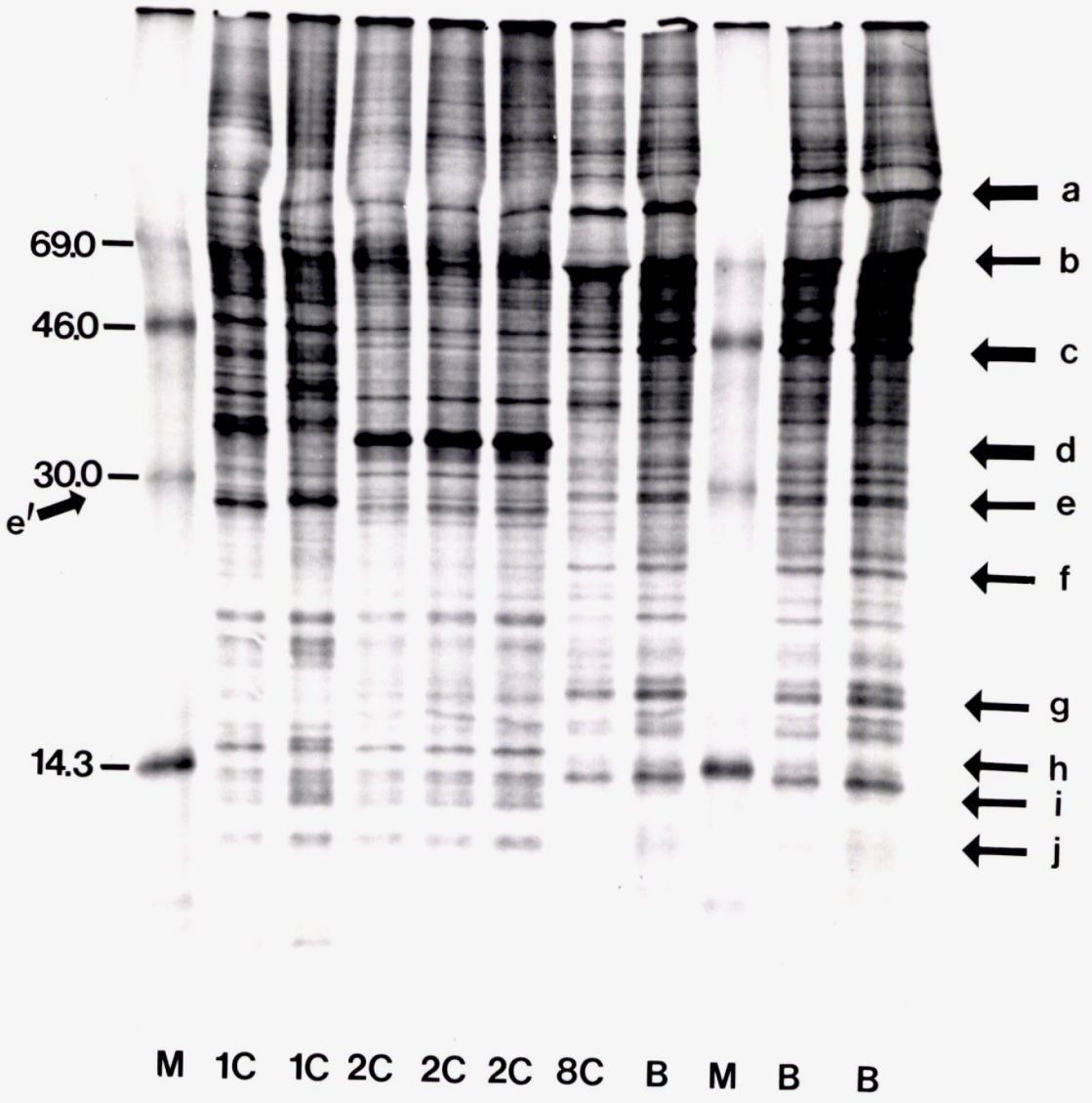
^{35}S-Methionine

Figure 7

One-Dimensional SDS-Polyacrylamide Gel of [^{35}S]-Labelled Proteins from Single Mouse Embryos at Different Stages of Development

Single mouse embryos were labelled with [^{35}S]-methionine and lysed with SDS-dissociation buffer. Aliquots of 800 cpm from single embryos at different stages of development were then loaded into a 12% SDS-polyacrylamide gel and subjected to electrophoresis followed by fixation, treatment with 'En^{3}hance', and autoradiography (see Materials and Methods). Exposure time to X-ray film (Kodak X-Omat) at -70°C was 51 days.

Labelling of the Figure is as for Figure 6.



51-Day Exposure

the two most prominent bands (along with band 'c') by the blastocyst stage. Band 'b', corresponding to a molecular weight of 70 kD, is also present at all stages of development, yet while it is relatively faint at the early stages of development, it becomes prominent after the 2-cell stage, and is one of the darkest bands at the blastocyst stage. Band 'c', with a molecular weight of 43 kD, is present only faintly at the 1-cell fertilized and 2-cell stages, increasing in intensity after the 2-cell stage to become one of the two most prominent bands by the blastocyst stage. Band 'd', with a molecular weight of 35 kD, is very prominent only at the 2-cell stage (see especially Figure 7), with no apparent synthesis before or after this stage. Band 'e', actually a doublet corresponding to molecular weights of approximately 28 - 29 kD, only becomes visible after the 2-cell stage. Band 'e", with a molecular weight of 28 kD is only present in one-cell fertilized samples (note that this band seems to be present in the 1-cell embryo in Lane 2 of Figure 7, but not in the 1-cell embryo in Lane 3 of Figure 7). Band 'f', with molecular weight 24 kD, appears (in only very small quantities) at the 2-cell stage, but it becomes far more visible by the 4-cell stage. Band 'f' continues to be produced up to the blastocyst stage. Band 'g', with a molecular weight of 17.4 kD, is visible after the 4-cell stage and becomes quite prominent by the blastocyst stage (see especially Figure 7). It cannot be determined from these gels whether it is synthesized prior to the 4-cell stage. Band 'h', with a molecular weight of 15 kD, appears only to be synthesized at the 1-cell and 2-cell stages. Band 'i', actually a small group of bands corresponding to molecular weights of 12.9 to 14.1 kD, shows expression of the entire group of bands at the 1-cell and

2-cell stages. After the 2-cell stage, only the top band in this group, with a molecular weight of approximately 14 kD, is expressed. This band becomes prominent by the blastocyst stage. Band 'j', with an apparent molecular weight of 11.5 kD, appears only to be expressed prior to the 4-cell stage.

B. Bovine Data

Figure 8 shows an autoradiograph of a one-dimensional SDS-polyacrylamide gel containing proteins synthesized by a number of single bovine P.O.'s. Figure 9 is an autoradiograph of a one-dimensional polyacrylamide gel of proteins from bovine embryos at the one-cell fertilized to the blastocyst stage of development.. Figure 10A displays the patterns made by autoradiography, and Figure 10B shows the Coomassie blue-stained pattern, of a one-dimensional gel containing both non-radioactive, as well as [^{14}C]- or [^{35}S]-labelled samples.

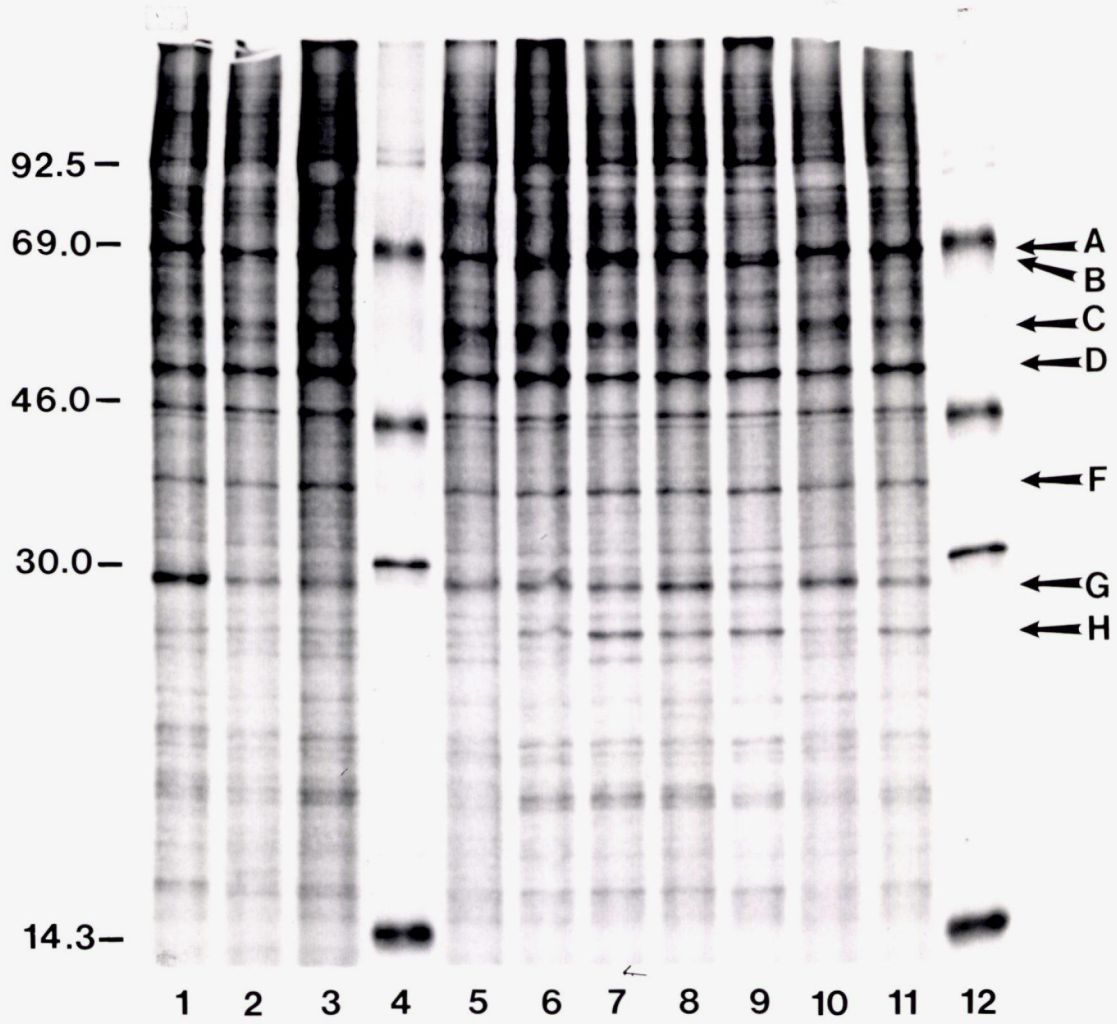
It should be noted that a band corresponding to a molecular weight of 53 kD appears in Lanes 2, 4, and 6 of the Coomassie-stained gel. It is very intense in Lane 4, and corresponds to a gap in the autoradiographic bands seen in this lane in Figure 10A. This band is likely to represent a contaminant, as it is present in quantities which far exceed the maximum total quantity of protein present in single embryos.

Figure 8

One-Dimensional SDS-Polyacrylamide Gel of [^{35}S]-Labelled Proteins from Single Bovine Post-Ovulatory Oocytes

Single bovine P.O.'s were labelled with [^{35}S]-methionine and lysed with SDS-dissociation buffer. Aliquots of 1800 cpm from single P.O.'s were then loaded on to a 12% SDS-polyacrylamide gel and subjected to electrophoresis followed by fixation, treatment with 'En^{3}hance' and autoradiography (see Materials and Methods). Exposure time to X-ray film (Kodak X-Omat) at -70^{o}C was 22 days.

The samples included in the gel are: Lanes 4 and 12: molecular weight markers (Amersham); all other lanes: single bovine P.O.'s. The molecular weights of the proteins in the marker lanes are indicated to the left of the gel in kD, while the letters A to H, to the right, indicate protein bands which are prominent and/or which vary in intensity across the stages of development (see also Figures 9 and 10).



22-Day Exposure

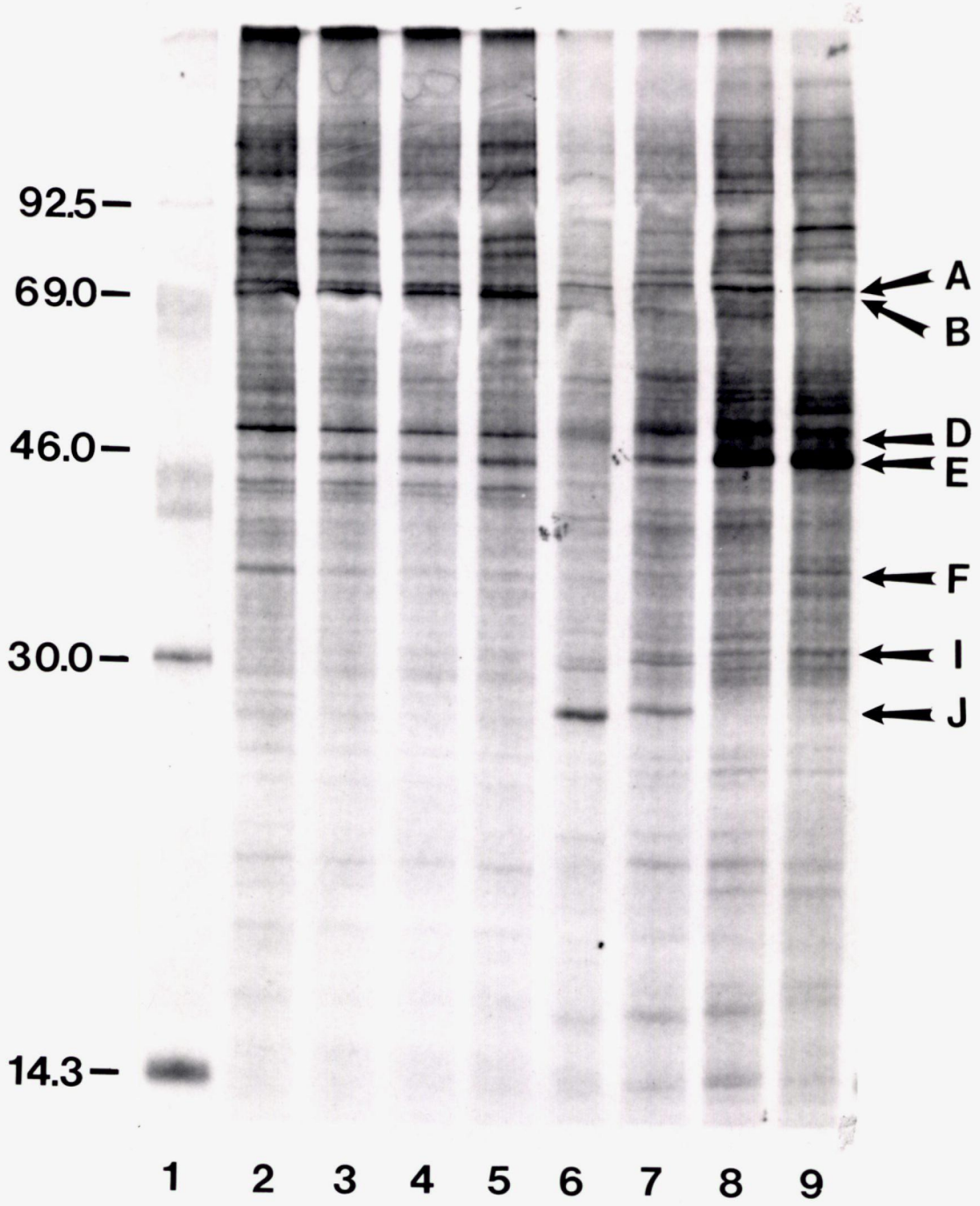
Figure 9

One-Dimensional SDS-Polyacrylamide Gel of [^{35}S]-Labelled Proteins from Single Bovine Embryos at Different Stages of Development

Bovine embryos from the 1-cell fertilized to the blastocyst stage were labelled with [^{35}S]-methionine and then lysed with SDS-dissociation buffer (see Materials and Methods). Aliquots containing 1000 cpm from single embryos at different stages of development were loaded on a 12% SDS-polyacrylamide gel and subjected to electrophoresis followed by fixation, treatment with 'En^{3}hance', and autoradiography. Exposure time to X-ray film (Kodak X-Omat) at -70^{o}C was 30 days.

The samples contained in the gel are as follows: Lane 1, molecular weight markers (Amersham); Lane 2, one-cell fertilized egg; Lane 3, 2-cell embryo; Lane 4, 2-3-cell embryo; Lane 5, 4-cell embryo; Lane 6, 8-cell embryo; Lane 7, 8-16-cell embryo; Lane 8, 16-32-cell embryo; Lane 9, Day 7 blastocyst.

The molecular weights (in kD) of the proteins in the marker lane appear to the left of the gel, while the letters to the right of the gel indicate protein bands which are prominent and/or which vary in intensity across the different stages of development (see also Figures 8 and 10).



30-Day Exposure

Figure 10

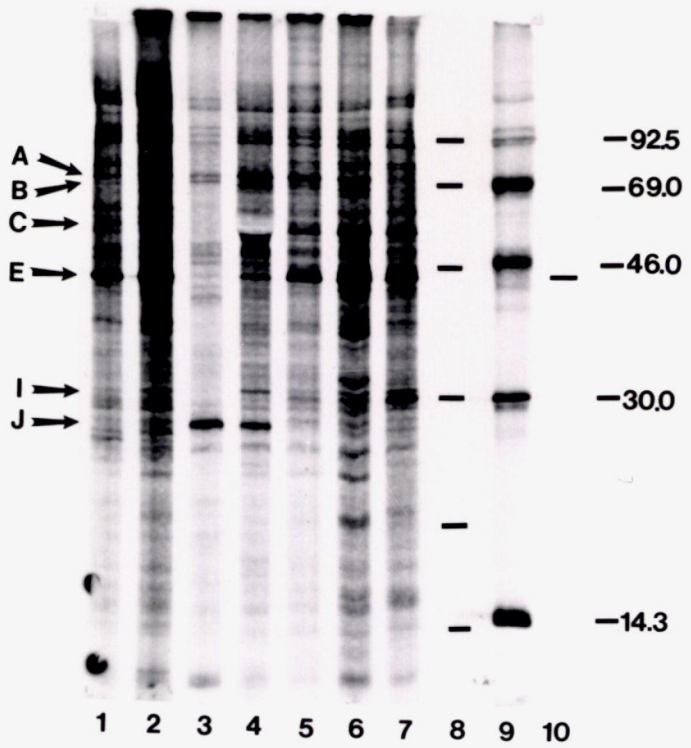
One-Dimensional SDS-Polyacrylamide Gel of [^{35}S]-Labelled Proteins from Single Bovine Oocytes and Embryos at Different Stages of Development

Single bovine F.O.'s, P.O.'s, and embryos from the 8-cell to the blastocyst stage, as well as trophectoderm from a Day 13 expanded blastocyst were labelled with [^{35}S]-methionine and subsequently lysed with SDS-dissociation buffer (see Materials and Methods). Aliquots, containing approximately 4000 cpm each, were loaded on a 12% SDS-polyacrylamide gel. A sample of pure, non-radioactive actin was also loaded onto the gel, as were both radioactive (Amersham) and 'cold' (Pharmacia) MW markers. The gel was subjected to electrophoresis, followed by staining with Coomassie blue, fixation, treatment with 'En^{3}hance', and autoradiography (see Materials and Methods). Exposure time to X-ray film (Kodak X-Omat) at -70°C was 14 days.

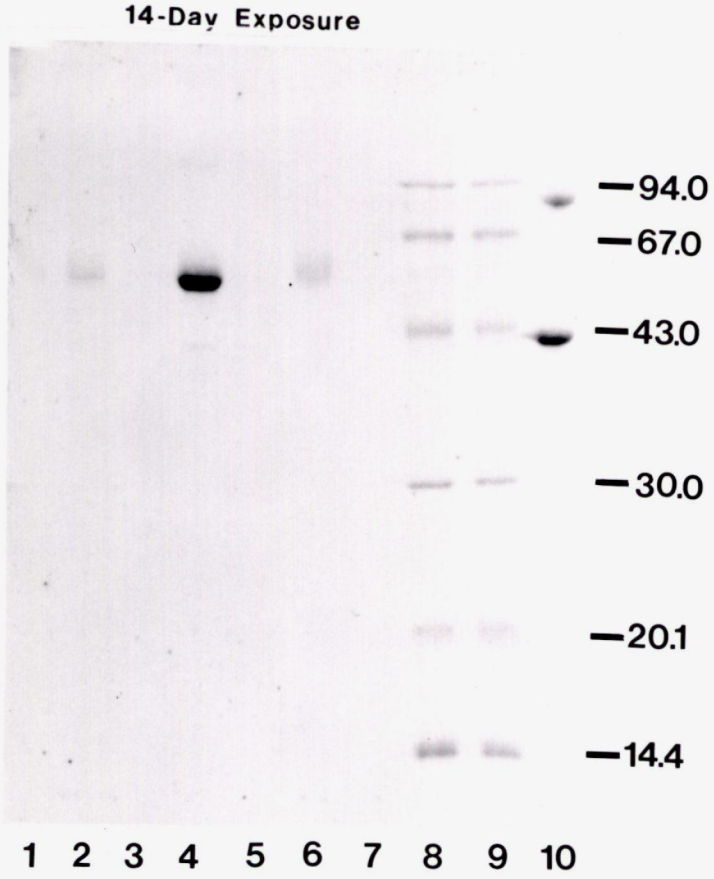
Figure 10A is a photograph of the autoradiograph, while Figure 10B is a photograph of the Coomassie-stained gel. The samples contained in the gel are as follows: Lane 1, F.O. with a large mass of adhering cumulus cells; Lane 2, same as Lane 1; Lane 3, P.O.; Lane 4, 8-cell embryo; Lane 5, Day 6 late morula (32-64-cell); Lane 6, Day 7 blastocyst; Lane 7, trophectoderm from a Day 13 hatched blastocyst; Lane 8, Pharmacia MW standards; Lane 9, both Pharmacia and Amersham MW standards; Lane 10, 'pure' actin (MW 42 kD; band at approx. 80 kD is an unidentified contaminant).

The labelling of the Figure is for Figures 8 and 9, with the sizes in kD of the Amersham MW standards being indicated in Figure 10A, and the sizes of the Pharmacia MW standards being indicated in Figure 10B.

A


A
B
C
E
I
J
92.5
69.0
46.0
30.0
14.3
1 2 3 4 5 6 7 8 9 10

B


14-Day Exposure
94.0
67.0
43.0
30.0
20.1
14.4
1 2 3 4 5 6 7 8 9 10

Examination of the patterns of proteins produced by bovine F.O.'s, P.O.'s, and embryos at different stages of development reveals many inter-stage differences in expression of individual protein bands. For the sake of brevity, these changes will not be listed. They can be summarized, however, by noting that the protein bands produced fall into five major categories: 1. bands which are specific to P.O.'s (bands 'G' and 'H'); 2. those which are only synthesized between the P.O. and 8-cell stages ('B' and 'F'); 3. a band which is only expressed at the 8-16-cell stages ('J'); 4. those bands which demonstrate increased synthesis after the 8-16-cell stage ('C', 'E' and 'I'); and 5. those bands which appear to be synthesized at relatively constant levels throughout development ('A' and 'D').

III. Uptake of [^{3}H]-Uridine by Embryos

Despite the fact that [^{3}H]-adenosine has been shown by previous researchers to be taken up by mouse embryos at about a 1000-fold higher rate than [^{3}H]-uridine (Clegg and Piko, 1977), the latter substrate was employed by the author for the investigation of RNA synthesis in mouse and bovine embryos. One of the advantages of [^{3}H]-uridine for these studies is that its incorporation is relatively uncomplicated. Unlike [^{3}H]-adenosine, which is readily taken up by both RNA and DNA, over 90% of [^{3}H]-uridine is taken up into RNA (Clegg and Piko, 1977). Furthermore, none of the [^{3}H]-uridine is taken up into the polyA tracts of mRNA molecules, or into the 3'-termini of tRNA molecules. (Clegg and Piko, 1977). Many studies on

RNA synthesis using in situ hybridization also employ [^{3}H]-uridine as the label, and thus for the purposes of the comparison of those studies with the present one (see Discussion), it was decided to employ the same substrate.

A. Mouse Data

Table 4 shows the rates of incorporation of [^{3}H]-uridine by groups of mouse embryos as measured at different stages of development.

Table 4

Incorporation of [^{3}H]-Uridine into Mouse Embryos At Different Stages of Development

Stage	#cpm inc'd/embryo/hr.	# pmoles inc'd/embryo/hour
8-Cell	12.2	3.5×10^{-4}
16-32-Cell	524.0	1.5×10^{-2}
32-64-Cell	1558.0	4.5×10^{-2}

There is a very low rate of incorporation of label into the 8-cell mouse embryos. The rate of incorporation increases over 40-fold between the 8-cell and 16-32-cell (morula) stages, and 3-fold between the morula and blastocyst (32-64-cell) stages. Thus the overall rate of incorporation of [^{3}H]-uridine into the intracellular RNA pool of mouse embryos increases over 120-fold between the 8-cell and the blastocyst stages.

B. Bovine Data

Table 5 shows the rates of incorporation of [^{3}H]-uridine or [^{3}H]-adenosine into bovine embryos at different stages of development. The rate of incorporation was measured separately for each embryo, except for three 16-cell embryos, which were treated as a group. Comments are given for each embryo, to indicate unusual treatment, either at AGI or in the hands of the author (see Materials and Methods).

Table 5

Incorporation of [^{3}H]-Uridine or [^{3}H]-Adenosine into Bovine Embryos at Different Stages of Development

Embryo Type	Embryo #	Comments	#cpm inc'd/e/hr
4-cell	89	Normal embryo	background*
4-cell	90	"	background*
3 X 16-cell	157	Normal embryos	21
Morula - Blast.	130	Cultured in Sheep 4 days	297
"	131	"	685
"	132	"	357
"	133	Zona Punctured	775
"	134	"	287
"	135	Zona Missing	719
Blastocyst	107	Cultured in sheep 4 days	187
"	108	"	157
"	109	"	422
"	110	"	332
Blastocyst	136	Cavity Injected	447
"	137	"	170
"	138	"	108

* designates embryos which were labelled with 5 μM [^{3}H]-adenosine; all the rest were labelled with 5.13 μM [^{3}H]-uridine

As shown in Table 5, there was no detectable incorporation of [^{3}H]-adenosine by bovine 4-cell embryos, and only a low level of incorporation of [^{3}H]-uridine by 16-cell embryos. By the morula to blastocyst and blastocyst stages, however, the average rate of incorporation was calculated to be 380 $\pm$ 222 cpm or (1.1 $\pm$ 0.65) X 10^{-2} pmol [^{3}H]-uridine per embryo per hour.

IV. Quantitation of Total RNA from Mouse and Bovine Eggs and Embryos

The method used to quantitate RNA in bovine and mouse embryos was the densitometric scanning of autoradiographs of total RNA probed with radiolabelled human 28S rRNA. As ribosomal RNA comprises approximately 70% of the total RNA in embryos (Piko and Clegg, 1982), and as 28S rRNA constitutes 67% of rRNA (Lewin, 1985), approximately 50% of the total RNA from mammalian embryos is present as 28S rRNA. 28S rRNA therefore, as a highly abundant RNA species, was chosen for use as a probe for the quantitation of the total amount of RNA present in mouse and bovine embryos at different stages of development. This RNA species has been been shown to be effective for the quantitation of RNA in mammalian eggs by G.A. Schultz et al. (data not shown).

The plasmids pA$_{BB}$ and pGA$_{BB}$ served as templates for the synthesis of nick-translated probes and riboprobes for this RNA, respectively. Riboprobes were synthesized as, theoretically, RNA:RNA hybridization provides a higher sensitivity of detection than does DNA:RNA hybridization (Casey and Davidson, 1977).

The video densitometric analysis system proved to be the most useful for the comparative analysis of samples on Northern blots or slot blots, as it allowed the whole sample, rather than just a small section of it, to be included in the analysis.

A. Northern Blot Analysis

Figure 11A shows an ethidium-bromide stained formaldehyde-agarose gel containing samples of total bovine brain RNA (Lane 2), yeast tRNA (Lane 1), and an RNA ladder (BRL; Lane 3). This gel, when compared to Figure 11B serves to show that the position of the bands visible in the autoradiograph of the Northern blot do indeed correspond to the 28S rRNA bands. This gel also contained an extra lane of yeast tRNA which, instead of being stained, was transferred onto a 'Nytran' membrane and hybridized to a [^{35}S]-labelled riboprobe made from pGA$_{BB}$ (see Materials and Methods). There was no detectable hybridization of the tRNA with the probe (data not shown), and thus it could be expected that the tRNA did not add to the background of the autoradiographs of the Northerns or slot blots.

Figure 11B is a photograph of an autoradiograph of the Northern blot of total RNA from bovine and mouse embryos and brain which was probed with a [^{35}S]-labelled riboprobe. A small amount of degradation was present in the RNA samples. The most degraded specimens are the bovine P.O. total RNA samples, and the least degraded are the mouse 8-cell embryo total RNA specimens. The degradation was taken into account by attempting to include in

Figure 11

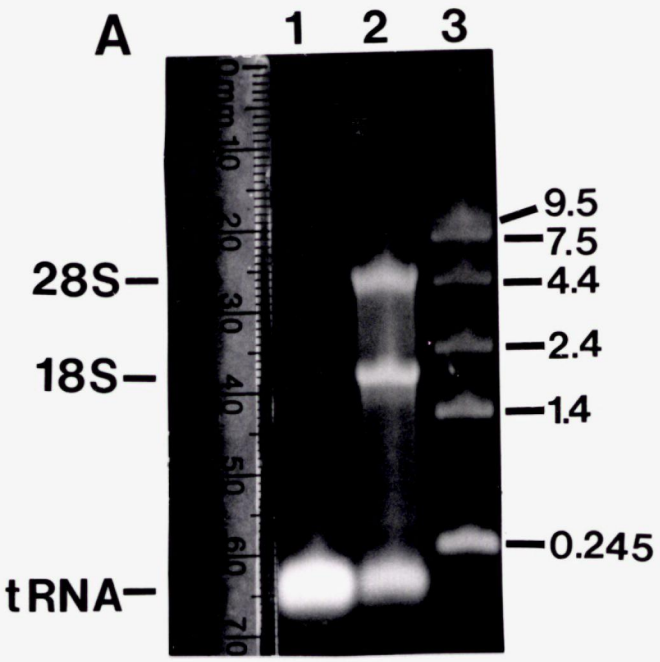
Northern Blot Analysis of Total RNA from Mouse and Bovine Brain Tissue, Eggs and Embryos

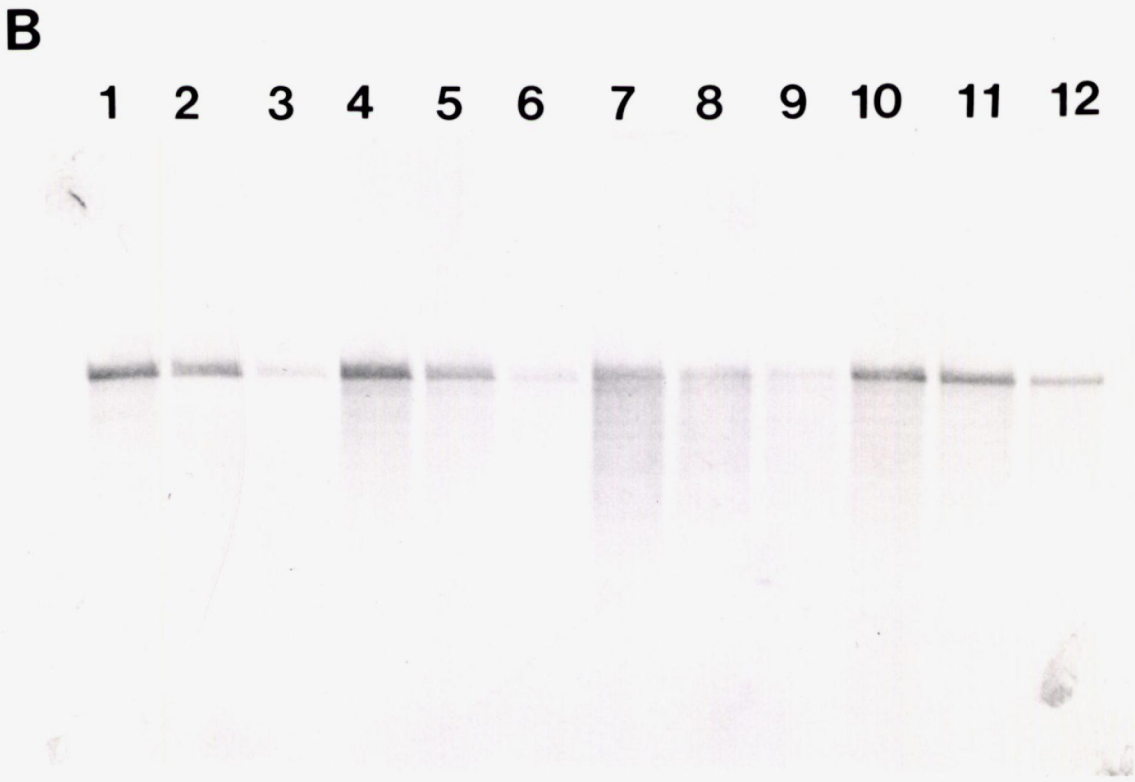
Total RNA was extracted from groups of bovine and mouse P.O.'s or embryos, as well as from bovine and mouse brain tissue. These RNAs were then subjected to Northern blotting. The resultant blots were hybridized to a riboprobe for 28S rRNA followed by several different exposures to preflashed X-ray film (Kodak X-Omat) at -70°C (see Materials and Methods).

Figure 11A displays an ethidium-bromide-stained 1% formaldehyde-agarose gel containing the following samples: Lane 1, 10 μg yeast tRNA (BRL); Lane 2, 10 μg bovine brain total RNA; Lane 3, 3 μg RNA size markers (BRL). The size of the bands in the RNA ladder (in kB) are indicated on the right-hand side of the gel. The positions of the 28S and 18S rRNA, as well as the tRNA bands, are indicated to the left of the gel.

Figure 11B is a photograph of an autoradiograph of the Northern blot obtained after a 17-hour exposure to pre-flashed film. The samples in the lanes are as follows:

Lane #	Contents
1	total RNA from 5 X 8-cell mouse embryos
2	total RNA from 2 X 8-cell mouse embryos
3	total RNA from 1 X 8-cell mouse embryos
4	4 ng total RNA from adult mouse brain
5	2 ng total RNA from adult mouse brain
6	0.75 ng total RNA from adult mouse brain
7	total RNA from 6 X bovine P.O.'s
8	total RNA from 3 X bovine P.O.'s
9	total RNA from 1 X bovine P.O.
10	4 ng total RNA from bovine brain
11	2 ng total RNA from bovine brain
12	0.75 ng total RNA from bovine brain


A
1
2
3
28S
18S
tRNA
9.5
7.5
4.4
2.4
1.4
0.245


B
1
2
3
4
5
6
7
8
9
10
11
12

the scans all of the RNA which hybridized to the probe, including the downstream 'trails' of degraded material.

Linear regression curves were obtained from the densitometric scanning of the autoradiograph shown in Figure 11B. From this analysis, the quantity of total RNA present in an 8-cell mouse embryo was calculated to be: 0.31 ng from the 1 X 8-cell sample (Figure 11B, Lane 3), 0.84 ng from the 2 X 8-cell sample (Lane 2), and 0.501 ng from the 5 X 8-cell sample (Lane 1). Thus the average value ($\pm$ S.D.) obtained for the total RNA content of an 8-cell mouse embryo was 0.55 $\pm$ 0.27 ng. Correcting this for the efficiency of extraction of 69% (see Materials and Methods), this becomes 0.80 $\pm$ 0.39 ng.total RNA per 8-cell mouse embryo.

The amount of total RNA present in a single bovine P.O. was calculated to be: 0.47 ng from the 1 X P.O. sample (Figure 11B, Lane 9), 0.89 ng from the 3 X P.O. sample (Lane 8), and 0.79 ng from the 6 X P.O. sample (Lane 7). The average of these values is 0.72 $\pm$ 0.22 ng which, after correction for the efficiency of extraction, becomes 1.04 $\pm$ 0.32 ng total RNA per single bovine P.O.

B. Slot Blot Analysis

Figure 12A is an autoradiograph obtained of a slot blot which was probed with a [^{32}P]-labelled nick-translated probe synthesized from the plasmid pA$_{BB}$, followed by a 24-hour exposure to pre-flashed X-ray film (Kodak X-Omat) at -70^{o}C. The quantities of brain total RNA were to serve as standards in the quantitation of the amounts of total RNA present in the embryos. The contents of

Figure 12

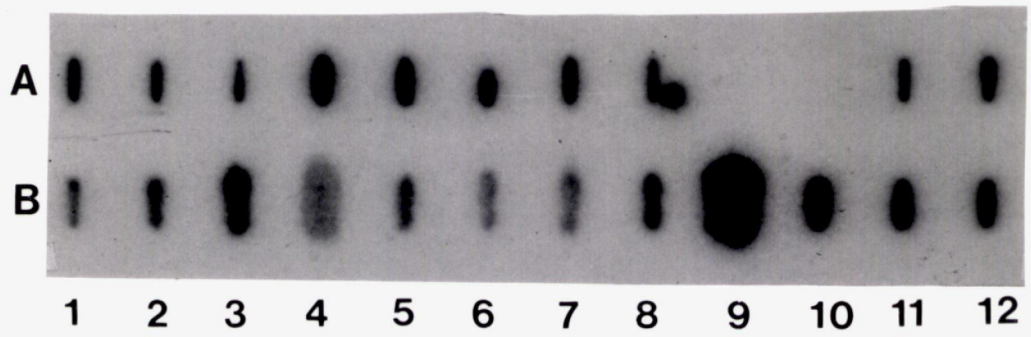
Slot Blot Analysis of Total RNA from Mouse and Bovine Brain and Embryo or Oocyte Samples

Figure 12A is a photograph of an autoradiograph of one of several slot blots obtained using nick-translated pABB as the probe. It contains the following samples:

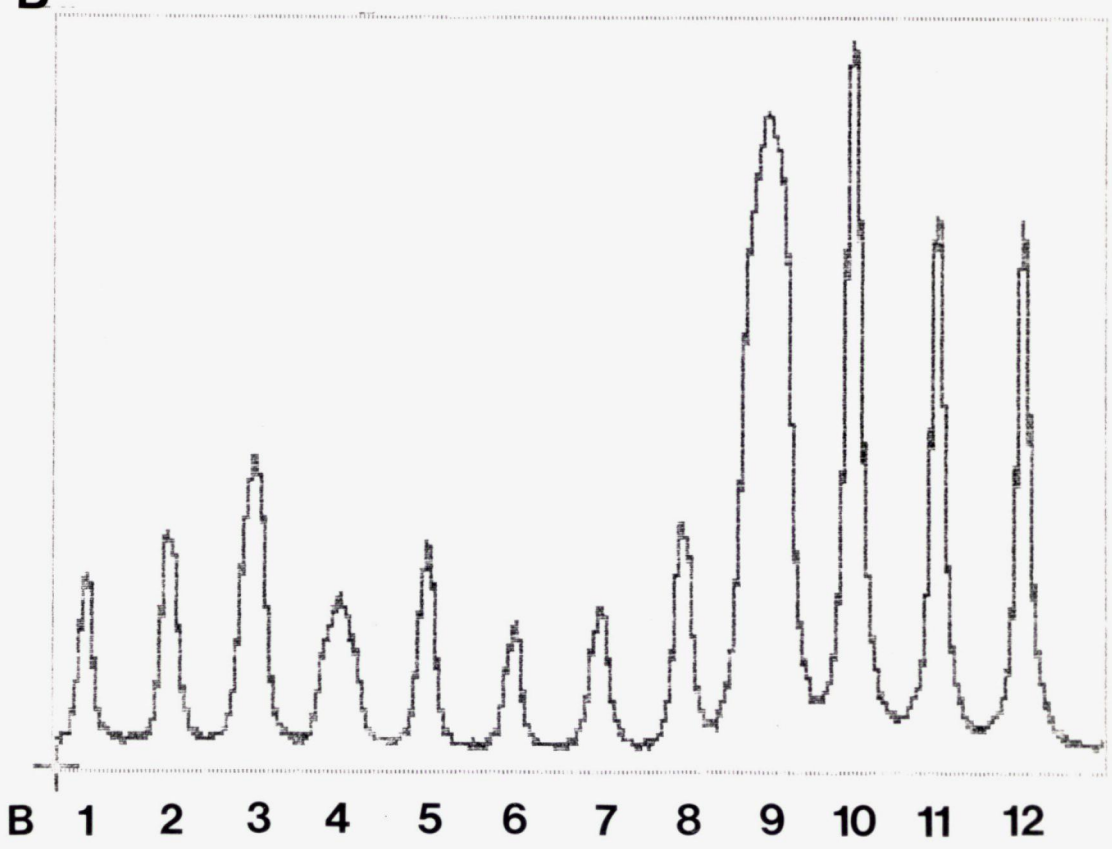
Slot	Sample
A 1	Total RNA from 4 X 8-cell mouse embryos
2	Total RNA from2 X 8-cell mouse embryos
3	Total RNA from 1 X 8-cell mouse embryos
4	2 ng total RNA from mouse brain
5	1 ng total RNA from mouse brain
6	0.75 ng total RNA from mouse brain
7	0.5 ng total RNA from mouse brain
8	0.25 ng total RNA from mouse brain
9	empty
10	empty
11	0.25 ng total RNA from bovine brain
12	0.5 ng total RNA from bovine brain
B 1	Total RNA from 1 X bovine P.O.
2	Total RNA from 1 X bovine P.O.
3	Total RNA from 1 X bovine P.O.
4	Total RNA from 4 X bovine P.O.'s
5	Total RNA from 1 X bovine P.O.
6	Total RNA from 1 X bovine egg (fert'd)
7	Total RNA from 1 X bovine egg (fert'd)
8	Total RNA from 1 X 2-cell bovine embryo
9	Total RNA from 1 X bovine blastocyst
10	2 ng total RNA from bovine brain
11	1 ng total RNA from bovine brain
12	0.75 ng total RNA from bovine brain

Figure 12B shows a densitometric scan (using 'Ultroscan XL') of Row B from this slot blot.

A



B



each slot are listed in the text for this Figure.

Figure 12B shows a densitometric scan across Row B of the slot blot shown in Figure 12A. As can be seen, there was some 'spreading' of some of the specimens, especially the bovine blastocysts (for example slot B9).

The relative amounts of RNA present in bovine embryos at different stages of development were determined by direct comparison of the values obtained from densitometric scanning using the video analysis and the 'Ultroscan XL' systems. A total of three different blots were examined, including the one shown in Figure 12A (data from other two slot blots not shown). By comparison of the data from all three blots, Table 6, which lists the RNA contents of bovine embryos at different stages of development, could be constructed.

Table 6

Relative and Absolute Quantities of Total RNA Present in the Bovine Post Ovulatory Oocyte. and in Embryos at Different Stages of Development

Stage	Relative Qty. Total RNA[a]	Absolute Qty. RNA (ng)
P.O.	1.0	1.04
1-Cell Fert'd	1.0	1.04
2-4-Cell	0.93	0.97
Blastocyst	7.0 - 10.0	7.3 - 10.4

[a] Quantity total RNA present in P.O. set at 1.0; all other values calculated relative to this.

The quantity of total RNA present in the bovine embryo decreases slightly between the P.O. and the 2-4-cell stages of

development. By the blastocyst stage, however, the quantity of RNA has increased significantly to 7.3 - 10.4 ng total RNA per embryo. This pattern is similar to the changes in RNA content found in mouse eggs, with high relative quantities of RNA in blastocysts compared to eggs (Piko and Clegg, 1982). Furthermore, the measurement of incorporation of [^{3}H]-uridine into bovine embryos, showing high rates of incorporation by the morula and blastocysts stages, indicates that large quantities of RNA are being synthesized at this time. This would lead to the relatively high quantities of RNA which are shown in Table 6.

DISCUSSION

I. Mouse Results

A. In Vitro Labelling of Embryos with [^{35}S]-Methionine

Table 7 provides a comparison of the rates of uptake and incorporation of this label by mouse embryos as determined in this study and by Hahnel et al. (1986) using a nearly identical technique (the sole difference being that Hahnel et al. labelled their embryos in a total volume of 0.5 - 1.0 ml, while the author labelled the embryos under oil in a volume of 50 µl).

The relative rates of incorporation follow similar patterns in both sets of results, with incorporation decreasing to less than 70% of that of the 1-cell unfertilized egg rate by the 2-cell stage, and increasing thereafter to over 500% by the blastocyst stage. It is due to the similarities in these patterns of incorporation that the author had confidence in the use of this technique for the measurement of the relative rates of incorporation of [^{35}S]-methionine by embryos at different stages of development. These results are consistent with the activation of the murine embryonic genome at the 2-cell stage, as reported in the literature (Schultz, 1986a), which results in a rapid increase in the rate of uptake of [^{35}S]-methionine as the rate of protein synthesis increases.

Table 7

Rates of Uptake and Incorporation of L[^{35}S]-Methionine by Mouse Embryos at Different Stages of Development as Compared to Data of Hahnel et al. (1986)

	R.E.Frei			Hahnel et al.(1986)		
	Uptake[a]	Incorporation		Uptake[a]	Incorporation	
Stage of Embryo		Absolute[b]	Relative[c]		Absolute[b]	Relative[c]
1-Cell Unfert'd.	21,984 ± 1,479	2,327 ± 248	1.00	18,706 ± 1,004	7,414 ± 644	1.00
1-Cell Fert'd.	21,240 ± 1,619	2,198 ± 438	0.92	-------	-------	----
2-Cell	17,730 ± 440	1,662 ± 132	0.69	15,840 ± 399	4,677 ± 233	0.63
8-Cell	25,250 ± 2,687	1,759 ± 215	0.73	20,214 ± 117	5,314 ± 329	0.72
16-32-Cell	47,764 ± 13,667	3,886 ± 1,143	1.62	-------	-------	----
Blastocyst	259,466 ± 8,559	13,957 ± 496	5.82	209,783 ± 5,692	52,097 ± 5,814	7.03

[a] #cpm [^{35}S]-methionine incorporated into the TCA-soluble pool/embryo/hour after a 2-hour incubation at 37°C (±S.D.)

[b] #cpm [^{35}S]-methionine incorporated into the TCA-insoluble pool/embryo/hour ± S.D.

[c] Rate of absolute incorporation at the 1-cell unfertilized stage set at 1.0; all other rates determined relative to this

B. Patterns of Protein Synthesis on One-Dimensional SDS-Polyacrylamide Gels

Examination of the two one-dimensional 12% polyacrylamide gels containing mouse embryo proteins presented in Figures 6 and 7 reveals several striking changes in the patterns of proteins produced by mouse embryos between the 1-cell fertilized and the blastocyst stage of development. There is a two-step transition in the patterns of proteins produced as development progresses. The first step occurs between the 1-cell and 2-cell stages, with the appearance of a new set of protein bands, along with a drop in prominence, or outright disappearance, of a number of other bands. The second step takes place between the 2-cell and the 4-8-cell stages of development. This step represents an even more definitive change in the pattern of proteins produced. A large number of protein bands disappear, and a new set of protein bands take their place. This new pattern of bands is persistent and thus from the 4-cell to the blastocyst stage, the pattern of protein synthesis by mouse embryos remains remarkably constant.

This general pattern of changes in the protein profiles of mouse embryos at different stages agrees with previous findings (Van Blerkom and Brockway, 1975). This technique provides a reliable method for the analysis of the synthesis of proteins from single embryos. Furthermore, it can be said that the analysis of single embryos is preferable to the grouping of embryos for examination in one-dimensional gels, as this allows true inter-embryo comparisons to be made.

C. *In Vitro* Incorporation of [^{3}H]-Uridine by Embryos

The rates of incorporation determined from the labelling of embryos at the 8-, 16-32- and 32-64-cell stages were shown in Table 4. Table 8 compares this data with results obtained by Clegg and Piko (1977) under identical conditions.

Table 8

Measurements Obtained by Clegg and Piko (1977) and R.E. Frei (Present Study) of Incorporation of [^{3}H]-Uridine by Mouse Embryos at Different Stages of Development

	#pmoles incorporated/embryo/hour	
Stage	R.E. Frei	Clegg and Piko
2-Cell	--	1.6×10^{-5}
4-Cell	--	5.5×10^{-5}
8-Cell	3.5×10^{-4}	5.5×10^{-4}
Morula - Early Blast.	1.5×10^{-2}	0.75×10^{-2}
Expanded Blast.	4.5×10^{-2}	1.9×10^{-2}

There is over a 100-fold increase in incorporation obtained by Clegg and Piko from the 2-cell to the blastocyst stage. There is also good agreement between the two sets of values over the range where they coincide. Despite the fact that Piko and Clegg's (1977) values at the morula - early blastocyst and expanded blastocyst stages were about 50% of those obtained by the author at the same stages, there exists in both sets of data a synchronous increase in the rate of incorporation between the earliest and latest stages measured.

Piko and Clegg (1977) also determined the absolute rates of RNA synthesis at different times during the course of development of preimplantation mouse embryos. They found that the rate of RNA synthesis increased from about 3.75 pg/embryo/hour in the late two to early four-cell embryo to about 40 pg/embryo/hour in the eight-cell embryo, about 300 pg/embryo/hour in the morula - early blastocyst, and finally again about 300 pg/embryo/hour in the late blastocyst.

Therefore increases in the rate of incorporation of [^{3}H]-uridine are reflected in increases in the absolute rates of RNA synthesis in early mouse embryos, at least until the early blastocyst stage, when the RNA synthetic rate on a per-cell basis reaches a maximum. This pattern of incorporation of [^{3}H]-uridine by mouse embryos agrees with earlier studies (Mintz, 1964; Monesi and Molinaro, 1971) showing the first detectable incorporation of labelled precursors into RNA in the nucleus at the 4-cell stage, and a sharp increase in the rate of incorporation from the 8-cell stage to the blastocyst stage.

D. Quantitation of Total RNA in Mouse Embryos

The total RNA content of mouse 8-cell embryos was estimated by densitometric determination of the relative amount of hybridization of an [^{35}S]-labelled 28S rRNA riboprobe to oocyte or embryo RNA and to RNA standards. The value obtained from densitometric scanning of the Northern blot shown in Figure 11B was 0.80 $\pm$ 0.39 ng total RNA per 8-cell mouse embryo.

The amounts of total RNA in mouse eggs at different stages of development have been determined by Piko and Clegg (1982) using absorbance at 260 nm of phenol-extracted RNA subjected to alkaline hydrolysis. A value of 0.69 $\pm$ 0.07 ng total RNA per 8-cell mouse embryo was obtained by these researchers. This is well within a single standard deviation of the average value obtained by this author for total RNA in mouse embryos at this stage.

II. Bovine Results

The bovine, in its natural state, is usually a mono-ovulate. Yet superovulation is now a routine procedure and, as a result, the majority of the embryos which are employed in scientific studies are produced by this method. Thus the question arises of whether embryos which are produced by natural means and by superovulation are truly comparable. Most of the evidence which has been produced to date suggests that these two types of embryos may, for most intents and purposes, be considered to be identical. The ranges of development encountered among embryos of any particular age from both unstimulated and superovulated cows usually overlap (Betteridge et al., 1980), despite clear evidence that supplementary progesterone treatment accelerates embryonic growth in cattle (Garrett et al., 1987). Furthermore, the transfer of embryos from superovulated donors to synchronous recipients results in 55% pregnancies, which is only slightly lower than the 70% pregnancies from unstimulated donors (Shea, 1981). Thus, unless

and until sufficient research on singly ovulated embryos proves otherwise, both types of embryos may be considered to be identical.

One of the major qualitative differences observed between the embryos obtained from superovulated mice and cows was the degree of heterogeneity within the embryo population. Embryos obtained from mice were generally developmentally synchronous and of good morphological quality. Due to the relatively large numbers available for study, the variation within the population was decreased by discarding any mouse embryos which were morphologically abnormal or retarded in development. The data obtained from the labelling studies reflected this homogeneity, with relatively small amounts of variation between embryos which were subjected to identical treatments.

By contrast, the population of bovine embryos used in these experiments was quite heterogeneous, in terms of both morphology and developmental synchrony. This heterogeneity is commonly encountered within populations of bovine embryos (see, for example, Shea, 1981; Linares and King, 1980). It was increased in these studies, however, by the necessity of inclusion into the experimental population of virtually every embryo which was made available to the author.

Two typical examples of morphological heterogeneity were shown in Figure 3. The three embryos shown in Figures 3A and 3B were all obtained from the same cow which was flushed on Day 5.5. Based on the criteria for embryo quality outlined in the Introduction, the embryos in Figure 3A (#48 and #49) are of poor quality, while the embryo in Figure 3B (#50) is of good quality. Similarly, the

embryos shown in Figures 3C (#25) and 3D (#24) were obtained from cows flushed on Day 7. Embryo #25 can be classified as poor, while embryo #24 is an excellent specimen.

Both morphological heterogeneity and developmental asynchrony were particularly noticeable among embryos at later stages of development (i.e. 16-cell stage and later). This was reflected in the results of the labelling studies, as the embryos of poor quality generally incorporated far fewer counts of [^{35}S]-methionine per hour than did embryos of good quality. Table 2 demonstrated that blastocyst #25 had a far lower rate of incorporation of [^{35}S]-methionine than did #24. Similarly, the rates of incorporation for embryos #48 and #49 were 8,416 and 6,758 cpm/hour, respectively (data not shown in Table 2), while the rate of incorporation of label by embryo #50 was 45,939 cpm/hour. This demonstrates clearly that there is a positive correlation between morphological embryo quality and the level of metabolic activity as measured by incorporation of radiolabelled precursors. Furthermore, there were also very high standard deviations obtained from the results with cow embryos relative to those obtained with mouse embryos. This was likely due either to the variable quality of the bovine embryos, or simply to some as-yet uncharacterized metabolic factor which contributes to the greater homogeneity among the mouse embryos.

A. In Vitro Labelling with [^{35}S]-Methionine

The absolute and relative rates of incorporation of this precursor into the TCA-precipitable pools of bovine embryos are shown in Tables 2 and 3, respectively. There is a 64% decrease in incorporation between the P.O. stage and the 8-cell stage, followed by a 21-fold increase in rate by the blastocyst stage. The volume of the embryo remains constant during this period (Betteridge and Flechon, 1988). If these rates are recalculated on a per-cell basis, they are found to increase only modestly, from about 176 cpm [^{35}S]-methionine incorporated/cell/hour at the 8-cell stage to approximately 280 cpm incorporated/cell/hour at the blastocyst stage.

In order to compare these data with that obtained by other investigators, Table 9 and Figure 13 were constructed. It should be noted that only the results of groups number 4 and number 5 took into account the specific activity of the endogenous methionine pool.

It is evident from these data that the measurement of the incorporation of [^{35}S]-methionine without the parallel measurement of the specific activity of the endogenous pool gives a good approximation of the relative rates of protein synthesis. It is also clear from all of the mouse data that there is an increase in the incorporation of precursor between the 2-cell and the 8-cell stages, while the bovine data indicates that this increase is not initiated until after the 8-cell stage in these embryos.

Table 9

Comparison of the Rates of Incorporation of Radiolabelled Methionine or Leucine into the TCA-Insoluble Pools of Mouse or Bovine Embryos at Different Stages of Development

Stage	Rate of Incorporation in Each Study[a]													
	1		2		3		4 *		5 *		6		7	
	Relative[b]	Absolute[c]	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.
1-Cell Unfert'd.	1.00±0.42	1.80	1.00	4.0	1.00	25.0	1.00	51.0	1.00	87.7	1.00	1.98	1.00	1.02
1-Cell Fert'd.	0.77±0.25	1.32	-	-	1.10	27.7	0.92	47.0	0.79	69.6	1.77	3.50	0.92	0.93
2-Cell	0.69±0.16	1.24	0.63	2.15	0.45	12.5	0.81	40.9	0.75	65.7	0.78	1.54	0.69	0.70
4-Cell	0.55±0.02	0.99	-	-	-	-	-	-	-	-	-	-	-	-
8-Cell	0.36±0.03	0.65	0.72	2.90	2.56	64.0	2.36	120.0	-	-	1.71	3.40	0.73	0.74
8-16-Cell	0.84±0.20	1.51	-	-	-	-	-	-	-	-	-	-	-	-
16-32-Cell	1.02±0.30	1.84	-	-	-	-	-	-	-	-	-	-	1.62	1.65
Mid Morula	3.76±1.86	6.77	-	-	-	-	-	-	-	-	-	-	-	-
Blastocyst	7.25±3.84	13.10	7.00	28.1	5.82	145.4	7.38	375.2	5.75	504.3	2.50	4.95	5.82	5.91

[a]Studies: 1=R.E.F. - bovine (used [^{35}S]-methionine); 2 = Hahnel et al.(1986 - used [^{35}S]-methionine); 3 = Merz et al.(1981 - used [^{35}S]-methionine); 4 = Abreu and Brinster (1978 - used [^{3}H]-leucine); 5 = Brinster, Wiebold and Brunner (1976 - used [^{3}H]-leucine); 6 = Petzoldt, Hoppe and Illmensee (1980 - used [^{35}S]-methionine); 7 = R.E.F. - mouse (used [^{35}S]-methionine).

[b]Rate of absolute incorporation at the 1-cell unfertilized stage set at 1.0 for each study; all other rates determined relative to this (note: only for study 1 [R.E.F. - bovine] were the relative rates given ± S.D., due to space limitations)

[c]Absolute rate of incorporation given in #fmol amino acid incorporated/embryo/hour

* Rates calculated after measurement of the specific activity of the endogenous amino acid pools of the embryos

Figure 13

Rates of Incorporation of Radiolabelled Methionine or Leucine into the TCA-Insoluble Pools of Mouse or Bovine Embryos at Different Stages of Development

The values for relative rates of incorporation of radiolabelled precursors into the TCA-insoluble pools of mouse or bovine embryos are represented in this graph. The stage of development is represented on the X-axis, while the relative rates of incorporation (in a semi-log scale) are plotted on the Y-axis.

The data for each curve was taken from the following studies:

_____ = Present Study - Bovine

___ ___ = Present Study - Mouse

o o o o = Hahnel et al. (1986) - Mouse

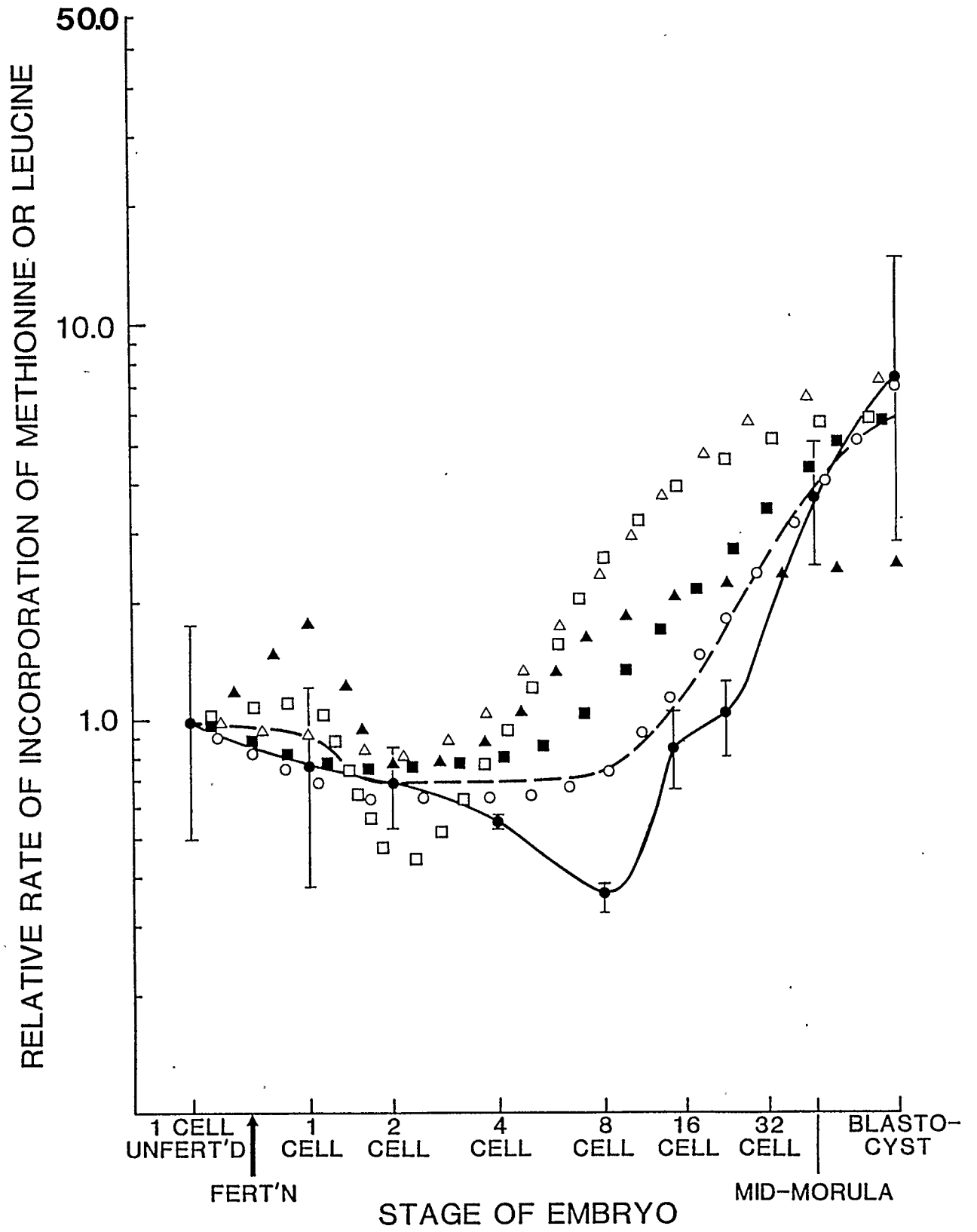
□ □ □ □ = Merz et al. (1981) - Mouse

△ △ △ △ = Abreu and Brinster (1978) - Mouse

■ ■ ■ ■ = Brinster, Wiebold and Brunner (1976) - Mouse

▲ ▲ ▲ ▲ = Petzoldt, Hoppe and Illmensee (1980) - Mouse

The ranges are indicated for the bovine data points.


50.0
10.0
1.0
RELATIVE RATE OF INCORPORATION OF METHIONINE OR LEUCINE
1 CELL UNFERT'D
1 CELL
2 CELL
4 CELL
8 CELL
16 CELL
32 CELL
BLASTO-CYST
FERT'N
MID-MORULA
STAGE OF EMBRYO

It is interesting to note that the sheep embryo, the genome of which is activated at the 8-cell stage (Crosby et al., 1988), shows the same timing of lowest incorporation of [^{35}S]-methionine (at the 8-cell stage) as does the bovine embryo. This indicates that the timing of de novo transcription and translation from the bovine embryonic genome is likely to be delayed with respect to that of the mouse, while it is similar to that of the sheep embryo, occurring between the third and the fourth cleavage divisions.

B. Patterns of Protein Synthesis on One-Dimensional SDS-Polyacrylamide Gels

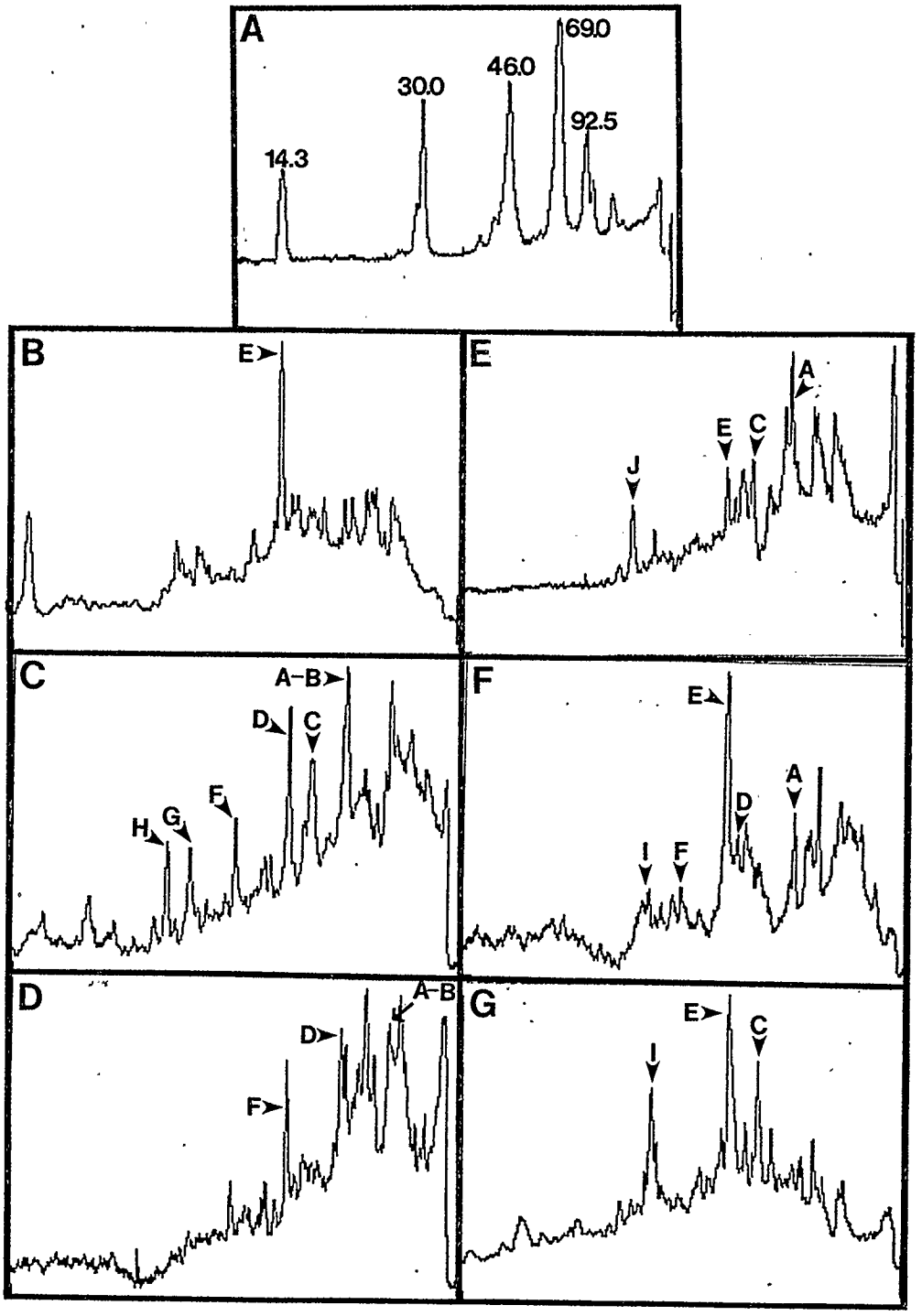
Figure 14 consists of a series of densitometric scans of one-dimensional gels of proteins from bovine embryos at different stages of development. This Figure highlights some of the prominent features of the protein profiles, including: 1. the presence of strong 'E' bands in the F.O., the 8-cell embryo, the blastocyst and the TE sample; 2. the P.O.-specific expression of bands 'G' and 'H'; 3. the presence of the 'F' band at the P.O. and 1-cell fertilized stages and again, to a lesser extent, in the blastocyst but not in the TE sample; 4. the apparent expression of band 'J' only at the 8-cell stage; 5. the absence of band 'B' from the 8-cell stage onward and of band 'A' in the TE sample ; 6. the presence of band 'C' in the P.O. and then once again from the 8-cell stage onward until it is present strongly in the blastocyst and TE samples. Note that band 'C' is not seen in the blastocyst profile displayed in Panel F of Figure 14, but is evident in the blastocyst present in Lane 6 of Figure 10; and finally, 7. the

Figure 14

Densitometric Scans of One-Dimensional Protein Patterns from a Bovine Follicular Oocyte, from a Bovine Post-Ovulatory Oocyte, and from Bovine Embryos at Different Stages of Development

Densitometric scans (using the "Ultroscan XL" system) were produced from different lanes of the autoradiographs shown in Figures 8, 9 and 10. Panel A is a scan of the Amersham MW markers from Figure 10 (Lane 9); Panel B is a scan of the protein bands from the F.O. in Lane 1 of Figure 10; Panel C is from the P.O. in Lane 7 of Figure 8; Panel D is a scan of the bands from the 1-cell fertilized egg in Lane 2 of Figure 9; Panel E represents the protein bands from the 8-cell embryo in Lane 4 of Figure 10; Panel F is from the blastocyst in Lane 9 of Figure 9; and Panel G is a scan of Lane 7 of Figure 10, which contains a sample of trophectoderm from a hatched blastocyst recovered on Day 13.

The molecular weights of marker proteins represented in Panel A are shown above each peak (in kD). The identity of the major bands present in the scans represented in Panels B to G are indicated by the letters 'A' to 'J' (see Figures 8, 9 and 10).


A
14.3
30.0
46.0
69.0
92.5
B
E
C
A–B
D
C
F
E
H
G
F
D
A–B
F
D
G
E
C
J
E
C
A
J
F
E
D
A
I
F
I

appearance of band 'I' at the blastocyst stage and even more strongly in the TE sample.

There are significant differences between the proteins synthesized by Day 13 trophectoderm and the blastocysts examined. Since neither an isolated ICM nor an entire embryo from Day 13 was analyzed in this manner, however, it is not possible to determine whether there are TE- or ICM- specific proteins synthesized by the blastocyst at this stage of development.

The overall pattern of proteins produced by bovine embryos at different stages of development has some striking similarities and differences from that produced by mouse embryos. The strong similarity is the production of a fairly similar set of proteins from the P.O. stage until some time during the first few cleavage divisions of the embryo (up to the 2-cell stage in the mouse embryo, and the 8-cell stage in the bovine). There is then a brief transition period during which a particular cell stage (the 2-cell stage in the mouse and the 8-cell stage in the bovine) produces at least one prominent band which is not synthesized at any other time between fertilization and blastocyst formation, followed by a shift to a pattern of bands which remains constant up to the blastocyst stage. The striking difference between the mouse and the bovine is the timing of these transitions. While the 2-cell stage is the pivotal one in the case of mouse embryogenesis, it is the 8-cell stage which appears to fill an analogous role in bovine embryo development.

In summary, there are three different patterns of protein synthesis which are expressed as the bovine embryo proceeds through development. The first, which for the purpose of this

discussion shall be designated as "#1", is present from the P.O. until the 4-cell stage (Figure 9). The second "#2", is evident only at the 8-16-cell stages, and is marked by the transient appearance of a protein band of MW 27 kD. The third, "#3", is produced from the 16-32-cell stage onward, and is characterized by a very different set of proteins than were evident before the 8-cell stage.

It is interesting to note that studies on early human embryos (Braude et al., 1988) and early sheep embryos (Crosby et al., 1988) also demonstrated a major change in the pattern of proteins produced by embryos, with this change taking place shortly after the activation of the embryonic genome. This occurred at the 4-8-cell stage in the human embryo, and at the 8-16-cell stage in the sheep. It was found that a fairly uniform set of proteins was produced between the 1-cell unfertilized stage and the 4-cell stage in the case of human embryos or the 8-cell stage in the case of sheep embryos, after which time a new set of proteins was produced. Both studies showed that this transition coincided with the activation of transcription from the embryonic genome under investigation. It was pointed out by Braude et al. (1988) that a high incidence of spontaneous cleavage arrest also occurs around the time of gene activation in the mouse and the pig.

Evidence from the examination of the patterns of proteins produced by bovine embryos at different stages of development, taken together with the observation that a block to cleavage in vitro occurs at the 8-cell stage in bovine embryos (Thibault, 1966), strongly suggests that the 8-cell stage in the bovine conceptus is

analogous to the 2-cell stage in the mouse embryo, the 4-cell stage of the human embryo, and the 8-cell stage of the sheep embryo.

Some of the bands found to be produced by bovine embryos at different stages of development were also found in the patterns produced by mouse embryos. It is tempting to speculate on the identity of some of these common protein bands. It should be noted, however, that this must be done with caution, as only a definitive test such as Western blotting using protein-specific antibodies can be used to positively identify proteins.

One such prominent protein band is band 'E' (MW 43 kD) which is evident in both bovine F.O.'s as well as in the blastocysts of both the bovine and the mouse (band 'c' in the mouse gels; Figures 6, 7, 9 and 10). A polypeptide with a similar molecular weight has been observed to be a major product of synthesis by follicular cells from embryos of the human (Schultz et al., 1988), the mouse (Van Blerkom and Brockway, 1975), and the sheep (Moor and Osborn, 1983). Thus this band appears to be a reliable marker for the presence of follicular cells in oocyte preparations. This band has also been shown by other investigators to be prominent at the blastocyst stage in the mouse (Van Blerkom and Brockway, 1975), the sheep (Crosby et al., 1988), the human (Braude et al., 1988), as well as in the rabbit, gerbil, rat and hamster (Norris et al., 1985).

From appproximately the 8-cell stage onward in mouse embryos, and after the 16-32-cell stage in cattle embryos (Betteridge and Flechon, 1988) a morphological reorganization occurs (i.e. compaction), along with the formation of tight intercellular contacts. As development and differentiation continue, further morphogenetic

events take place. The cytoskeleton (which includes the proteins B-actin [MW 42 kD] and tubulin [MW 55 kD]) is known to play a key role in all of these events (Darnell et al., 1986).

The cytoskeletal protein B-actin has been shown to be produced in significant quantities by mouse follicular oocytes (Van Blerkom and Brockway, 1975). During oocyte maturation and fertilization, the rate of synthesis of actin declines. Its synthesis increases 90-fold between the 1-cell and the blastocyst stages, representing 3.7% of the total protein synthesis at the latter stage (Abreu and Brinster, 1978). All of this evidence, along with the parallel migration of band 'E' and the purified actin sample (see Figure 10) seems to indicate that this band is composed of B-actin.

Similarly, band 'C' (MW approximately 57 kD) becomes prominent by the blastocyst stage and is one of the strongest bands in the TE sample (see Figure 10). It is also prominent in proteins produced by the blastocysts of the mouse (Van Blerkom and Brockway, 1975), sheep (Crosby et al., 1988), human (Braude et al., 1988), rat, gerbil and hamster (Norris et al., 1985). It has been shown that the rate of synthesis of tubulin (MW 55 kD) increases 14-fold between the 8-cell stage and the blastocyst stage in the mouse embryo, at which time it comprises 2% of the total protein synthesis (Abreu and Brinster, 1978). Thus, it is probable that band 'C' is, indeed, tubulin.

Finally, band 'F' (MW 35 kD), which is prominent in the bovine P.O.'s examined (Figure 8), declines in prominence after fertilization until it is only faintly visible at the 4-cell stage (Figure 9). A band of identical molecular weight is also found in unfertilized eggs of the

human (Braude et al., 1988), mouse (Van Blerkom and Brockway, 1975), and the rabbit (Van Blerkom and Manes, 1974) and, in each case, the band disappears after the major qualitative shift in protein synthesis during the first few cleavage divisions. The most likely identity of the major protein in this band is lactate dehydrogenase (LDH). Its synthesis is known to constitute 2 - 5% of the total in fully-grown mouse oocytes, with a 7-fold decrease in rate during oocyte maturation, followed by further decreases during early development (Schultz, 1986). These changes in its rate of synthesis reflect the use of pyruvate as an energy source instead of glucose during oocyte and early embryo development. The strong presence of this band in the bovine P.O.'s examined and a sharp decline in its intensity after fertilization correlate well with the typical pattern of the switch in energy substrate use during early development (Leese and Barton, 1984).

C. In Vitro Labelling of Embryos with [^{3}H]-Uridine

As shown in Table 5, there was no detectable incorporation of label by single bovine embryos at the 4-cell stage and only a small amount of incorporation by a group of three 16-cell embryos. Furthermore, treatments such as the rupture or removal of the zona pellucida (embryos #133, 134 and 135) or direct injection of the label into the blastocoel (embryos #136, 137 and 138) did not have an appreciable effect on the rate of incorporation of this precursor by these embryos.

The rate of incorporation at the 16-cell stage of development was found to be 21 cpm [^{3}H]-uridine/embryo/hour or 6.1 X 10^{-4} pmoles/embryo/hour. By the morula-blastocyst stage of development, this rate has increased 18-fold to 380 $\pm$ 220 cpm/embryo/hour or (1.1 $\pm$ 0.65) X 10^{-2} pmoles/embryo/hour. This finding supports the work undertaken by other researchers showing that the initial incorporation of [^{3}H]-uridine by bovine embryos only occurs in the late 8-cell stage (Camous et al., 1986).

Table 10 compares the data obtained by the author on mouse and bovine embryos with that obtained by Clegg and Piko (1977; see also Table 9 above).

Table 10

Rates of Incorporation of [^{3}H]-Uridine into Mouse and Bovine Embryos at Different Stages of Development

	#pmol inc'd/e/hour at following stages:				
Organism	1-cell Fert.	4-Cell	8-Cell	16-32-Cell	Blast.
Mouse[a]	2 X 10^{-5}	6 X 10^{-5}	6 X 10^{-4}	1 X 10^{-2}	2 X 10^{-2}
Mouse[b]	---	---	4 X 10^{-4}	2 X 10^{-2}	5 X 10^{-2}
Bovine[c]	---	bkg.[d]	---	6 X 10^{-4}	1 X 10^{-2}

[a] Clegg and Piko (1977)
[b] Present study
[c] Present study
[d] Labelled with [^{3}H]-adenosine - background counts only detected

The rates of incorporation of nucleotide precursor into the 8-cell mouse embryo is very close to that at the 16-cell stage of the bovine embryo. By the blastocyst stage, there is a much higher rate of incorporation of precursor into both mouse and bovine embryos,

indicating that the embryos are actively synthesizing RNA at this stage.

The rate of incorporation of [^{3}H]-uridine into blastocysts is somewhat lower in the bovine than in the mouse. This may reflect an inter-species difference in the efficacy of [^{3}H]-uridine use in studies on RNA synthesis, a delay in the timing of maximization of the rate of RNA synthesis in bovine embryos with respect to mouse embryos, or a physiological difference in rate of RNA synthesis between mouse and bovine embryos.

D. Quantitation of Total RNA in Bovine Post-Ovulatory Oocytes by Northern Analysis

The value obtained by Piko and Clegg (1982) for the RNA content of an unfertilized mouse egg is 0.35 $\pm$ 0.03 ng. Thus the value of 1.04 $\pm$ 0.32 ng estimated for the RNA content of a bovine P.O. is three times larger than this value. The ratio of the values of total RNA per mouse and bovine P.O. is not as great, however, as the ratio of their volumes. The volume of a mouse egg is 1.8 X 10^5 μm^3 (Piko and Clegg, 1982) , while that of a bovine embryo is 2.15 X 10^6 μm^3, giving a ratio of 1:12.

Table 11 provides a comparison of the amounts of total RNA present in eggs of three different mammals.

Table 11

RNA Contents of Eggs from the Mouse, Rabbit and Bovine[1]

Species	Egg Diameter (μm)	Egg Volume (μm^3)	RNA Content Per Egg (ng)	RNA Content Per μm^3 (fg)
Mouse	70	1.8×10^5	0.35	2.0
Rabbit	125	1.0×10^6	6.0	6.0
Bovine	160	2.15×10^6	1.04	0.49

[1] All of the data for the mouse and the rabbit is from Piko and Clegg (1982) except the RNA content of rabbit egg, which comes from Schultz (1975); bovine data from present study

Comparison of the RNA content per μm^3 of mouse, rabbit and bovine eggs shows that the bovine value is significantly lower than for the other species, while the quantity for the rabbit is three times higher than that for the mouse. The meaning of this data is unclear at the present time.

E. Slot Blot Analysis of the Relative Amounts of Total RNA in Bovine Post-Ovulatory Oocytes and Embryos

It was found that the RNA content of bovine embryos decreased only slightly between the 1-cell and the 2-4-cell stages. By the blastocyst stage, however, a 7- to 10-fold increase in the amount of total RNA present per embryo was observed. From the value for the quantity of total RNA present in a single P.O. obtained by the author, there can be calculated to be roughly 7.3 - 10.4 ng of total RNA per bovine blastocyst. This supports the results obtained by the author for the labelling of bovine embryos with [^{3}H]-uridine,

which showed a large increase in its rate of incorporation into embryos between the 16-cell and the blastocyst stage. This increase in the rates of transcription would be expected to lead to an increase in the RNA content of the embryos.

High relative amounts of total RNA in blastocysts as compared to oocytes have also been found in the mouse (Piko and Clegg, 1982) and the rabbit (Manes, 1969), as have high rates of RNA synthesis after the first few cleavage divisions in these species.

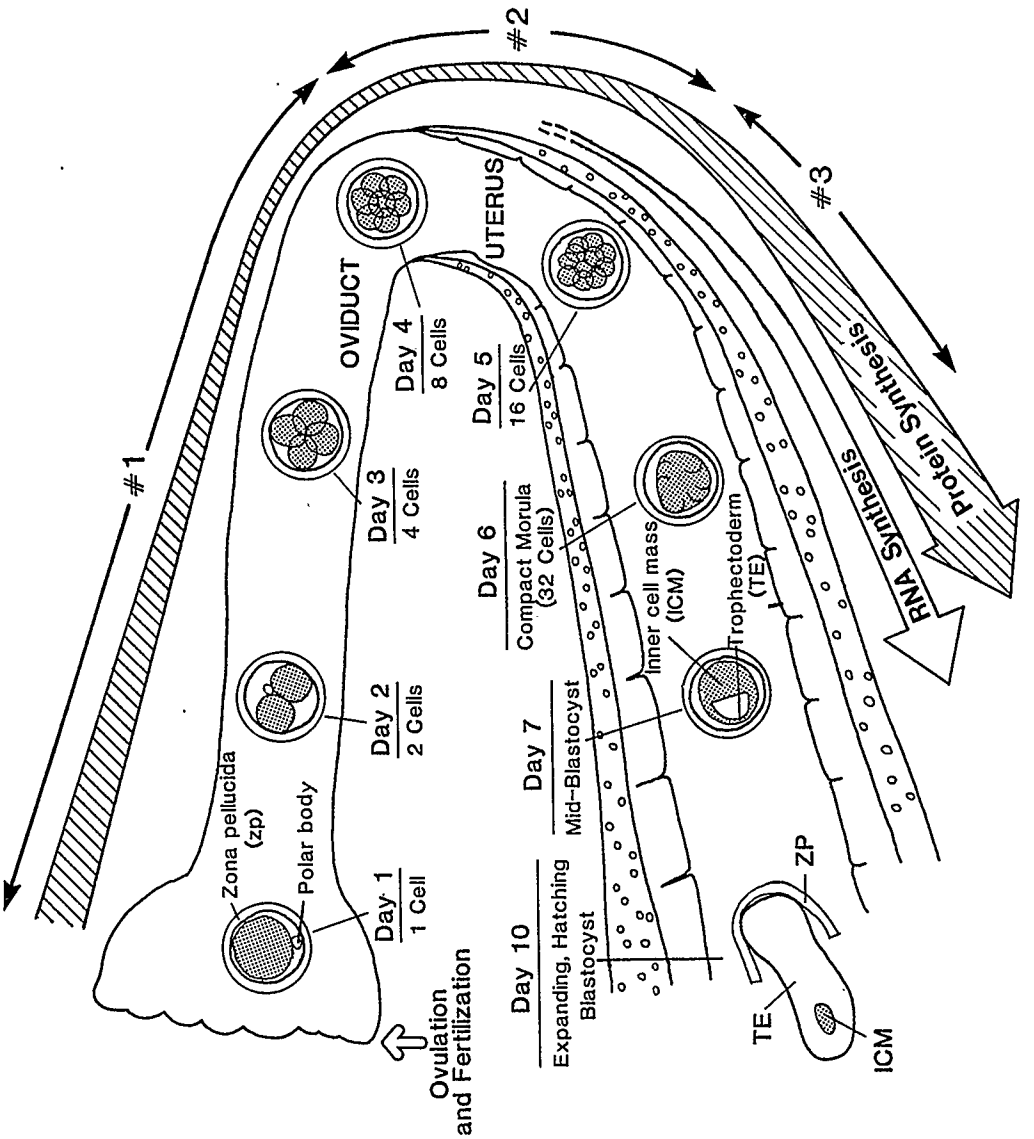
The results obtained from the examination of the bovine embryo are summarized in Figure 15. As can be seen, the 8-16-cell stage represents the metabolic "turning point" of early development. Translational levels reach a minimum during this period, a transient qualitative pattern of protein synthesis (#2) is synthesized by the embryos, and de novo transcription begins sometime shortly after this stage. All of this evidence strongly suggests that bovine embryonic genome is activated between the third and fourth cleavage divisions.

The activation of the bovine embryonic genome appears to occur two cleavage divisions later than in the mouse embryo (2-4-cell stage; Schultz, 1986) or the human embryo (4-8-cell stage; Braude et al., 1988), but at the same stage as in the sheep embryo (Crosby et al., 1988). Before any sweeping conclusions may be made, however, several points must be kept in mind. One is that, while much of the data for the mouse and the bovine has been obtained from embryos which were cultured in vivo until the time of experimentation, human experimental embryos are always cultured

Figure 15

Schematic Presentation of Patterns of RNA and Protein Synthesis in the Preimplantation Bovine Embryo

The locations of preimplantation bovine embryos from the 1-cell to the blastocyst stage of development in the bovine reproductive tract are illustrated. The movement of embryos from the fimbria to the body of the uterus is represented schematically, as the movement is not continuous in vivo. In addition, the ages of the embryos at each stage is only an approximation, as the actual timing of the cleavage divisions varies considerably over the first 8 days of development. The relative rate of protein synthesis at different stages of development is illustrated by the thickness of a cross-hatched band to the right of the reproductive tract. The rate of RNA synthesis is indicated by the relative thickness of a clear band. The times at which the three different qualitative patterns of protein synthesis (#1, #2 and #3) are produced by the embryos are indicated by arrows beside the band representing the rate of protein synthesis.


#1
#2
#3
OVIDUCT
UTERUS
Zona pellucida (zp)
Polar body
Day 1
1 Cell
Day 2
2 Cells
Day 3
4 Cells
Day 4
8 Cells
Day 5
16 Cells
Day 6
Compact Morula (32 Cells)
Day 7
Mid-Blastocyst
Day 10
Expanding, Hatching Blastocyst
Inner cell mass (ICM)
Trophectoderm (TE)
Ovulation and Fertilization
RNA Synthesis
Protein Synthesis
ZP
TE
ICM

in vitro, as they are usually 'extras' from in vitro fertilization programs (Braude et al., 1988). There is reason to believe that significant differences exist between natural and in vitro culture conditions, and until these are completely identified, it cannot be assumed that embryos react identically to in vivo and in vitro conditions. Furthermore, it is obvious that there is a large number of inter-species differences between the mouse, the cow and the human and since only a very small fraction of these would be revealed by this analysis, it cannot be assumed that a complete picture of the processes influencing development has been formulated.

Tables 12 and 13 were constructed in order to place the data on the timing of the activation of the genomes of different species in context with information on general development. The mouse, the rabbit, the human and the cow represent diverse reproductive strategies. While the mouse has a short (4-day) estrus cycle, the rabbit does not undergo estrus, with ovulation occurring in response to copulation. Both the mouse and the rabbit produce large litters (Catchpole, 1977). The bovine has long estrus cycles (Catchpole, 1977), as does the human (Mahesh, 1979) and both species usually produce one offspring at a time (Catchpole, 1977).

As shown by Table 12, the mouse and the rabbit both have short gestation periods. The size and the RNA contents of their eggs differ significantly, however. The RNA content of the rabbit egg is by far the highest among these four species. The cleavage rate of the rabbit embryo is also the most rapid, as the blastocyst is composed of between 5,000 and 10,000 cells at 6.5 days after ovulation (Manes

Table 12

Comparison of Size and RNA Content of Eggs and Developmental Rates of Embryos from the Mouse, Rabbit, Bovine and Human

Species	Diameter of Egg (μm)[1]	RNA Content of Egg (ng)[2]	Time Post-Ovulation at the Following Stages (hrs):[3] 2-Cell	4-Cell	16-Cell	Blastocyst	Total Gestation (days)[4]
Mouse	70	0.35	30	45	64	82	19-20
Rabbit	125	6.00	18	22	40	100	30-32
Human	140	0.43	36	48	72	96	252-274
Bovine	160	1.04	24	50	96	168	275-290

[1]Data from: mouse - Abramczuk and Sawicki (1974); rabbit - Hartman (1929); human - Tesarik et al. (1986) bovine - Linares and King (1980)

[2]Data from: mouse - Piko and Clegg (1982); rabbit -Schultž(1975); human - Schultz, G.A., unpublished results; bovine - present study

[3]Data from: mouse - Piko and Clegg (1977); rabbit - Maurer (1978);human - McLaren (1982); bovine - Betteridge and Fléchon (1988)

[4]Data from: McLaren (1982)

Table 13

Patterns of RNA and Protein Synthesis in Embryos of the Mouse, Rabbit, Human and Bovine

Stage of Dev't.:	1-Cell				2-Cell				16-Cell				Blastocyst			
Species:[1]	M	R	H	B	M	R	H	B	M	R	H	B	M	R	H	B
Rel. RNA Content:[2]	1.0	1.0	N.A.	1.0	0.69	1.4	1.0	0.97	1.97	1.7	0.25*	N.A.	4.2	3.45	0.58**	14.5
RNA Synthesis:[3]	-	-	-	N.A.	-	+	-	N.A.	+++	+	++*	-	+++	+++	+++**	+++
Protein Pattern:[4]	Early	Early	Early	Early	Mid	Early	Early	Early	Late	Mid	N.A.	Mid	Late	Late	Late	Late

[1] M = mouse; R = rabbit; H = human; B = bovine

[2] Data from: mouse - Piko and Clegg (1982 - based on ng total RNA; amount present at 1-Cell set at 1.0; other amounts calculated relative to this value); rabbit - Manes (1969 - based on #ng total RNA; amount present at 1-Cell set at 1.0); human - Tesarik et al. (1986 - based on #ribosomes - number present at 1-Cell set at 1.0; other numbers calculated relative to this value; * = 8-cell stage human embryo; ** = early morula); bovine - present study

[3] Data from: mouse - Monesi and Molinaro (1971 - in situ hybridization with ^{3}H-uridine) and Clegg and Piko (1977 - #pmol ^{3}H-uridine incorporated/embryo/hour); rabbit - Manes (1969 - #cpm ^{3}H-uridine incorporated/embryo/hour); human - Tesarik et al. (1986 - in situ hybridization with ^{3}H-uridine); bovine - present study

[4] 'Early', 'Mid' and 'Late' refer to patterns #1, #2 and #3, respectively from 1-dimensional PAGE of ^{35}S-labelled proteins from bovine embryos (present study), and analogous stages in mouse, rabbit and human embryos; data from: mouse - Van Blerkom and Brockway (1975); rabbit - Van Blerkom and Manes (1974); human - Braude et al. (1988); bovine - present study

N.A. = Data not available

and Daniel, 1969). The eggs of the bovine and the human have relatively low RNA contents, and slow rates of development.

Table 13 indicates that the mouse embryo undergoes the most rapid transition to a high rate of transcription and the production of a 'late' pattern of protein synthesis. The human embryo, the genome of which is not activated until the 4-8-cell stage (Braude et al., 1988), is rapidly synthesizing RNA by the 8-cell stage. Bovine and rabbit embryos, on the other hand, do not produce high levels of transcription until the blastocyst stage. Note that transcription is detected at the 2-cell stage in the rabbit embryo, but at levels which are not considered to be physiologically significant (Manes, 1969).

While no clear-cut pattern emerges from these data, several general conclusions can be drawn. The embryos of the mouse and the rabbit, which both develop rapidly, also undergo genomic activation relatively early, at about 40 hours post-ovulation. Furthermore, while the maternal RNA present in the mouse egg is only necessary to support development to the 2-cell stage, the rabbit embryo is dependent on maternal RNA until the 8-cell stage, and must also support a very rapid series of cell divisions. This may explain the large population of RNA present in the rabbit egg. The human and the cow embryos, on the other hand, undergo delayed genomic activation, approximately 50 hours post-coitum in the human embryo, and 80 hours post-coitum in the bovine embryo, and their overall rates of development are lower. The bovine egg also possesses a higher RNA content than that of the human which, in an analogous situation to the rabbit, may serve to sustain metabolism

for a prolonged period, to the 8-16-cell stage, rather than the 4-8-cell stage in the case of the human embryo.

Thus the mouse and the rabbit are adapted to high rates of reproduction, with large litters being produced in rapid succession. The human and the cow, on the other hand, invest more energy in embryonic development, with single offspring being produced after long gestation periods. There appears to be a delay in the activation of the embryonic genome associated with these prolonged gestation periods.

Conclusions

The experimental data obtained in these studies on the preimplantation bovine embryo allow the following conclusions to drawn:

1. It has been shown that it is possible to derive meaningful molecular biological information from the analysis of single mouse or bovine embryos. Rates of incorporation of radiolabelled methionine and uridine are high enough in bovine embryos to allow significant differences in rates at different times in development to be measured. Single embryos also provide sufficient quantities of radiolabelled proteins to allow clear qualitative analysis of patterns of synthesis on one-dimensional SDS-polyacrylamide gels.

2. A clear correlation has been established between morphological and metabolic heterogeneity in populations of bovine embryos. Embryos judged from visual inspection to be of poor quality were found to have far lower rates of incorporation of radiolabelled RNA

or protein synthetic precursors than embryos of good quality. When the results from individual embryos are grouped for analysis, this heterogeneity is amplified and is manifested in the large standard deviations obtained for the rates of incorporation of these precursors.

3. Specific patterns of protein and RNA synthesis have been identified which support the premise that the genome of the bovine embryo is activated between the 8- and the 16-cell stages of development. High rates of transcription and translation are evident after the 16-cell stage, and the total RNA content of the bovine embryo increases significantly between the 1-cell stage and the blastocyst. Furthermore, the analysis of the qualitative patterns of protein synthesis have revealed that the 8-16-cell stage represents a transitional period between the 'early' (1-cell to 4-cell stages) and 'late' (morula stage onward) stages of bovine embryo development.

It is clear, then, that the stage at which the zygotic genome is activated represents the initiation of the 'metabolic program' which carries the embryo through the course of its development. The evidence would suggest that this activation entails the selective expression of a particular set of genes. This selectivity is manifested, for example, in the shift in the qualitative pattern of protein synthesis between the 4-cell and the morula stage in the bovine embryo. A question which remains unanswered is to what extent the genome of the embryos is affected by this process. Is this metabolic activation associated with an irreversible "reprogramming" of the genome, or is does it cause only more superficial changes?

Nuclear transplantation studies with mouse embryos indicate that isolated blastomeres from embryos at the 2-cell stage or later, when transferred into the cytoplasm of an enucleated zygote, cannot support continued development (McGrath and Solter, 1984). In contrast, Steen Willadsen (personal communication) with work on bovine embryos, and Smith and Wilmut (1988), with work on sheep embryos, have shown that single cells isolated from the ICMs of blastocysts can support continued development when they are transferred into enucleated 1-cell embryos. It is clear, therefore, that at least some bovine and sheep ICM cells are totipotent or are capable of being reprogrammed by the zygotic ooplasm.

The implication is that cells from post-activation-stage sheep and bovine embryos possess fundamentally different qualities from post-activation mouse embryo blastomeres. In mouse embryos, the initiation of transcription from the embryonic genome appears to be linked to an irreversible decrease in its potency. Some portion of the cells of sheep and cow embryos, by contrast, retain a capability for totipotency for an as-yet-undetermined period of time after transcription from the embryonic genome has been initiated. Thus in mouse embryos, the programs of differentiation and genomic activation appear to be coordinately regulated. In the embryos of sheep and cows, however, they function independently.

Further experimentation will help to elucidate the exact mechanisms by which these two programs operate as well as the reasons for the differences between the mouse and other species. Examination of the patterns of RNA and protein which are produced by nuclear transplantation embryos would be useful tools to

investigators in the same manner that they proved useful to this author in the examination of bovine embryos. Whatever the findings are, it is clear that McGrath and Solter (1984) were mistaken when they declared "that the cloning of mammals by simple nuclear transfer is biologically impossible". An exciting era in animal embryology has begun in which the only limitations are those imposed by the imaginations of the investigators.

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APPENDIX ONE

pGEM-1, a recombinant, dual-promoter vector designed for the transcription of either strand of DNA inserted into the MCS by either the Sp6 or the T7 RNA polymerase promoters (Melton et al., 1984), is shown in the left panel of Figure 16A. Samples of both pABB (Figure 5, Materials and Methods; kindly supplied by Jim Sylvester of the University of Pennsylvania) and pGEM-1 (kindly supplied by G.A. Schultz) DNA were linearized with the restriction endonuclease BamHI (Boehringer Mannheim) (see right panel of Figure 16A), and the 1.4 kB fragment from pABB was gel-isolated and cloned in to the BamHI site of pGEM-1 in the standard manner (Maniatis et al., 1982).

The resultant plasmid, named pGABB, is shown in the left panel of Figure 16B. Its length is approximately 4.4 kB and, as shown in the left and right panels of Figure 16B, it contains the 1.4 kB fragment of the human 28S rRNA gene within the BamHI site of the MCS of pGEM-1, flanked by the Sp6 and T7 RNA polymerase promoters.

In order to test the direction of transcription of pGABB for the synthesis of anti-sense 28S rRNA, riboprobes were synthesized using both the T7 and the Sp6 RNA polymerase promoters of this plasmid. The resultant probes were then hybridized to dot blots of 5 μg of bovine brain total RNA (see Materials and Methods). The results indicate that the riboprobe transcribed from the T7 RNA polymerase promoter consists of anti-sense 28S rRNA, while the riboprobe synthesized from the Sp6 promoter is the sense RNA and hence does not hybridize to bovine total RNA.

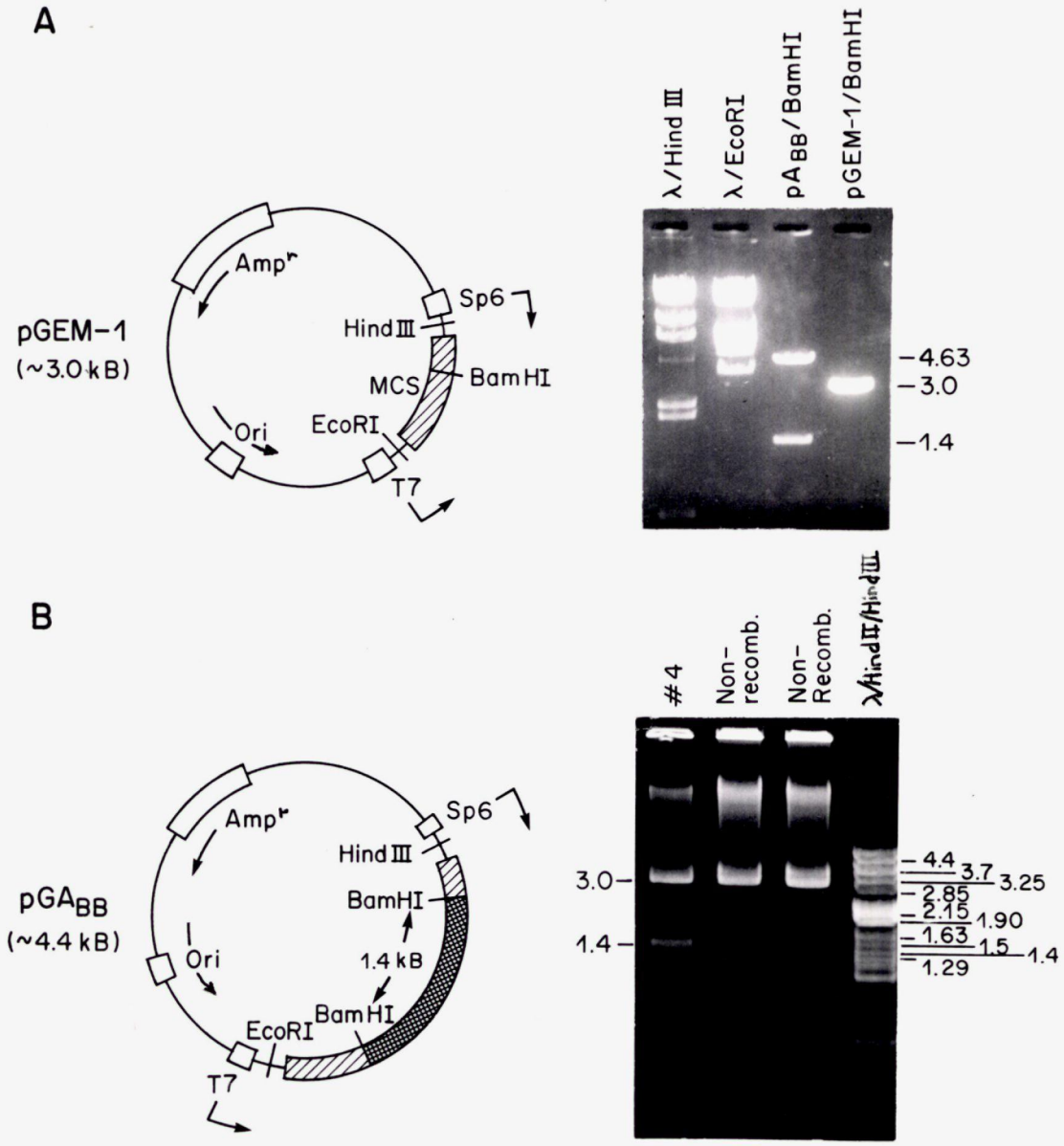
Figure 16

Construction of pGA$_{BB}$ and Test for Direction of Transcription Producing Anti-Sense 28S rRNA

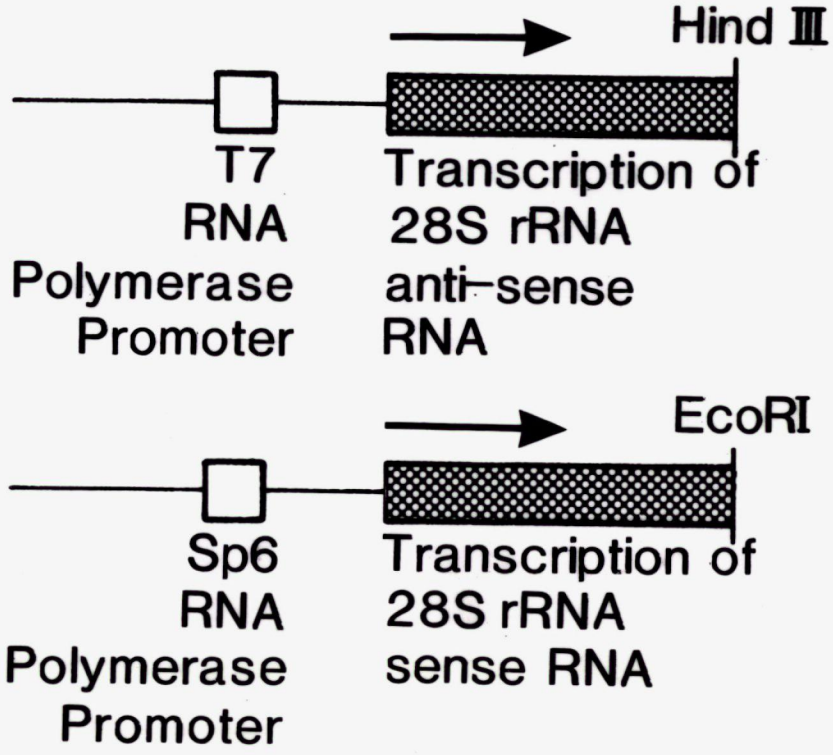
The left side of panel A shows the structure of the plasmid pGEM-1. It contains an origin of replication (Ori), a gene encoding ampicillin resistance (Amp^r), a multiple cloning site (MCS), with promoters for both the T7 and Sp6 RNA polymerases flanking it. The direction of transcription from each promoter is indicated by arrows. The right side of panel A is the ethidium bromide-stained gel pattern of pA$_{BB}$ and pGEM-1 DNAs restricted with BamHI, with EcoRI- and HindIII-digested λ DNAs (Pharmacia) as size markers (sizes of markers indicated in kB to the right of the gel). The positions of the 4.63 and 1.4 kB fragments of pA$_{BB}$ and the 3.0 kB linearized pGEM-1 are indicated (in kB) on the right side of the gel.

The left side of panel B is a schematic diagram of the recombinant plasmid pGA$_{BB}$, which is 4.4 kB in length. The right side of panel B is the ethidium bromide-stained gel pattern of BamHI-restricted pGA$_{BB}$ (Lane 1; indicated as "#4"; Lanes 2 and 3 contain restriction digests of non-recombinant plasmids), with HindII- and HindIII-restricted λ DNA supplying size markers (sizes of markers are indicated in kB to right of gel). The positions of the 3.0 and 1.4 kB fragments from the BamHI-digestion of pGA$_{BB}$ and pGEM-1 are indicated to the left of the gel (in kB).

The left side of panel C depicts the direction of transcription of HindIII- and EcoRI-linearized pGA$_{BB}$ DNA from the T7 and Sp6 RNA polymerase promoters, respectively. The right side of panel C shows the autoradiographs of dots of 5 μg of total bovine brain RNA probed with [^{35}S]-labelled riboprobes transcribed from either the Sp6 (top) or T7 (bottom) promoters of pGA$_{BB}$.


A
pGEM-1
(~3.0 kB)
Amp^r
Sp6
Hind III
MCS
BamHI
Ori
EcoRI
T7
λ/Hind III
λ/EcoRI
pA_BB/BamHI
pGEM-1/BamHI
4.63
3.0
1.4
B
pGA_BB
(~4.4 kB)
Amp^r
Sp6
Hind III
BamHI
Ori
1.4 kB
EcoRI
BamHI
T7
#4
Non-recomb.
Non-Recomb.
λ/Hind III/Hind III
3.0
1.4
4.4
3.7
3.25
2.85
2.15
1.90
1.63
1.5
1.4
1.29

C


Hind III
T7
RNA
Polymerase
Promoter
Transcription of
28S rRNA
anti-sense
RNA
EcoRI
Sp6
RNA
Polymerase
Promoter
Transcription of
28S rRNA
sense RNA