ALKALINE PHOSPHATASE ISOZYME EXPRESSION
IN PREATTACHMENT BOVINE EMBRYOS

A Thesis
Presented to
The Faculty of Graduate Studies
of
The University of Guelph

by
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In partial fulfilment of requirements
for the degree of
Doctor of Philosophy
October, 1998

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0-612-35804-6
ABSTRACT

ALKALINE PHOSPHATASE EXPRESSION
IN PREATTACHMENT BOVINE EMBRYOS

Kathryn Elizabeth McDougall
University of Guelph, 1998

Supervisors:
Dr. A.C. Hahnel
Dr. W. A. King

The majority of loss of bovine embryos occurs during preattachment development, possibly due to faulty gene expression from the embryonic genome. Markers for successful transfer of control of development from maternal to embryonic genome are lacking in cattle. The mouse is the most extensively studied mammalian embryo model, and it was found that alkaline phosphatases (AP) are expressed differentially during embryogenesis, suggesting that APs would be a good model for examining embryonic genome transcription during bovine embryogenesis. Bovine embryos were produced in vitro and in vivo, mRNA expression was determined using reverse transcription and polymerase chain reaction and protein was detected by an azo-dye coupling technique. Tissue specific AP 2 (TSAP2) mRNA was detected at the 4-cell stage in vitro and morula stage in vivo. Tissue specific AP 3 (TSAP3) mRNA was detected at the 8-cell stage in vitro and 16-cell stage in vivo. Tissue non-specific AP (TNAP) was detected only in late blastocysts, and intestinal AP (IAP) only sporadically in late blastocysts in vitro. A cyclic pattern of transcription was detected for TSAP2 and TSAP3. Examination with cell-cycle inhibitors, which act by disrupting microtubules of embryos, affected expression, while DNA polymerase inhibition did not, suggesting that these transcripts are sensitive to
microtubule status for detection, expression or storage. Embryos produced in vivo showed very low AP activity until the blastocyst stage, when activity was found only on trophectoderm cells. Embryos produced in vitro showed AP protein localized to areas of blastomere contact during early stages, then only on trophectoderm cells at the blastocyst stage. Alkaline phosphatase activity is due to maternal TNAP protein up to the 16-cell stage, with a switch to embryonic genome derived TSAP during later preattachment development. The differences in expression of TSAP2 and TSAP3 between in vitro and in vivo produced embryos and after treatment with microtubule inhibiting drugs suggest that culture does not accurately mimic the in vivo environment, and that in vitro manipulations cause changes in gene expression.
ACKNOWLEDGMENTS

First and foremost I would like to thank my advisory committee, Drs. Ann Hahnel, Allan King, Ann Gibbins and Jon LaMarre for the opportunity to work and learn under their guidance, and to Dr. King and his lab group for supplying the culture facilities in which all the bovine embryo culture was performed. Thank you to Carol Wasnidge and Liz St. John for their patience in teaching me many of the techniques that were used in this study, as well as friendship and support. I also wish to acknowledge the help of Claire Plumb and Pamela Bauer for their assistance throughout different portions of this project. A special thank you must go to Drs. Walter Johnson and Harold Moore for help in the superovulation and flushing aspects of this project, without which I could not truly appreciate the new reproductive technologies, cows, and manure for what they really are. Dr. Melissa Farquhar was infinitely patient teaching me how to use the confocal microscope, without which many of the plates for this thesis would not have been possible. I also would like to acknowledge the Natural Sciences and Engineering Research Council of Canada and OMAFRA for funding and research animals.

I also must thank my friends, Sheldon Kawarsky, Jennifer Bond, Michelle Forbes, Jim Gilmore, Margery Hay, and Cheryl Semotok for their support, Tuesday night dinners and movies, general relaxation, keeping me relatively sane, and great ideas.

Last, but certainly not least, thank you so much to my family. I owe them so much for all the help and support over the last four years. Their encouragement and support are unsurpassed, and quite often it was their enthusiasm over something that I know was only slightly understood that kept me going. My father summed up my family's views on my work when he looked at me one weekend and asked with a totally
straight face "so do molecules have DNA or does DNA have molecules?". It put my
work into perspective for me, and forced me to think about communication of science to
everyone, not just other scientists. It also told me that while completing this degree is
important, so are family and friends. Thank you.
DECLARATION OF WORK PERFORMED

I declare that with the exception of the items indicated below, all work reported in this thesis was performed by me.

The superovulation and artificial insemination of animals for the study of in vivo produced embryos was performed by Dr. Walter Johnson and Dr. Harold Moore (Department of Population Medicine, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada) and embryos less than 5 days gestational age recovered from tracts with the assistance of Dr. Ann Hahnel, Ms. Carol Wasnidge and Ms. Claire Plumb (Department of Biomedical Sciences, University of Guelph). Tracts were obtained from animals at slaughter at MGI Meat Labs (Kitchener, Ontario, Canada). Embryos older than day 5 were recovered by non-surgical flushing performed by Dr. Walter Johnson and Dr. Harold Moore. Conditions for the heat inactivation of bovine isozymes were determined by Ms. Claire Plumb.
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<th>Definition</th>
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<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>BOEC</td>
<td>bovine oviduct epithelial cells</td>
</tr>
<tr>
<td>cDNA</td>
<td>copy deoxyribonucleic acid</td>
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<tr>
<td>COC</td>
<td>cumulus-oocyte complex</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>2'-deoxynucleoside 5'-triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EAP</td>
<td>embryonic alkaline phosphatase</td>
</tr>
<tr>
<td>GCAP</td>
<td>germ-cell alkaline phosphatase</td>
</tr>
<tr>
<td>HOM</td>
<td>L-homoarginine</td>
</tr>
<tr>
<td>hpi</td>
<td>hours post insemination</td>
</tr>
<tr>
<td>IAP</td>
<td>intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>ICM</td>
<td>inner cell mass</td>
</tr>
<tr>
<td>IVC</td>
<td>in vitro culture</td>
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<tr>
<td>IVF</td>
<td>in vitro fertilization</td>
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<tr>
<td>IVM</td>
<td>in vitro maturation</td>
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<tr>
<td>LEV</td>
<td>levamisole</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MZT</td>
<td>maternal-zygotic transition</td>
</tr>
<tr>
<td>NBT</td>
<td>nitro blue tetrazolium chloride</td>
</tr>
<tr>
<td>OET</td>
<td>onset of embryonic transcription</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PHE</td>
<td>L-phenylalanine</td>
</tr>
<tr>
<td>PLAP</td>
<td>placental alkaline phosphatase</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>sdH₂O</td>
<td>single-deionized water</td>
</tr>
<tr>
<td>TNAP</td>
<td>tissue non-specific alkaline phosphatase</td>
</tr>
<tr>
<td>TSAP</td>
<td>tissue specific alkaline phosphatase</td>
</tr>
<tr>
<td>TSAP2</td>
<td>tissue specific alkaline phosphatase 2</td>
</tr>
<tr>
<td>TSAP3</td>
<td>tissue specific alkaline phosphatase 3</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>ZGA</td>
<td>zygotic genome activation</td>
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INTRODUCTION

One of the most important challenges for today's dairy and beef industries is the production of large quantities of high quality product for a minimum of cost. Consumers want high quality beef and milk, and the cost to producers is high if production is low. In the attempt to increase overall productivity and quality, the new reproductive technologies of artificial insemination, in vitro fertilization, culture of large numbers of embryos, embryo transfer and nuclear transfer are being implemented commercially in more areas of the developing world, but are not yet common place in industry today. With the exception of artificial insemination, these technologies are still not cost-effective on a large scale, and the success of these technologies is still low.

On the average North American dairy or beef cattle farm, embryonic mortality is estimated to be 30 to 40% (Zavy, 1994). Some, but not all of this, is attributable to factors such as nutrition, genetic variability, stress and overall animal health. However, reduction or elimination of these factors does not completely resolve the problem. Compound the loss of potentially high quality embryos with the cost of maintaining non-breeding or non-producing adult animals, and it becomes clear that it is in the best interests of the industry to determine what the other causes of embryonic mortality are, and to try to reduce or eliminate these causes.

In the bovine preattachment embryo, development requires that a number of genetic and morphological events occur in a strictly ordered and coordinated manner. Ovulation is followed by fertilization, a cleavage period controlled by molecules stored in the oocyte, initiation of gene expression from the embryonic genome, compaction,
cellular differentiation, blastocoel formation and embryo hatching over the course of approximately 10 days. If any of these events should not occur properly, development arrests and embryo mortality occurs. It is not clear whether gene expression and morphogenesis leading to the blastocyst stage in mammals are driven by time post fertilization ("zygotic clock") or by number of cleavages, or a combination of both. Nor do we fully understand nutritional and environmental factors important to normal development, or how manipulations used by new reproductive technologies affect gene expression. One of the limiting factors is the lack of a method to determine health status of embryos.

Most of the study of gene expression during early mammalian embryogenesis has focused on the mouse, and it was found in this species that a particular family of isozymes called the alkaline phosphatases (AP) exhibit stage-specific expression during early embryogenesis. Two members of this family are expressed in early development with one form found exclusively in the preimplantation embryo and rarely in the adult. This family of isozymes also was found to be expressed in bovine blastocysts and appeared to provide an interesting model to study early gene expression during preattachment bovine embryo development.

The intention of this thesis is to identify expression patterns of the AP family of genes in preattachment bovine embryos. The bovine embryo is an ideal tool for this study because it is an economically important species, and it is possible to produce and maintain these embryos in vitro for most of the period of preattachment development. Preattachment development spans a longer period in cows and thus allows for a larger
window of opportunity than in the mouse to study genetic and morphological changes.

Identification of a suitable family of genes to use as markers of normal bovine embryonic development, such as the APs, would allow for evaluation of gene expression status of embryos produced using the new reproductive technologies with the aim of reducing embryonic loss and increasing overall productivity in today’s cattle industry.
Embryo Production and the New Reproductive Technologies:

In Vivo:

The main focus of the new reproductive technologies involving in vivo embryo production is to increase the quality and quantity of offspring (Lohuis, 1995), while reducing rates of embryonic mortality (Armstrong, 1993). Embryo mortality in vivo is high, ranging from 35 to 45% (King, 1991). The idea behind the new reproductive technologies is to use spermatozoa from a high quality bull and fertilize high quality oocytes ultimately producing multiple high quality offspring from those two parents per year. In this way, a large number of economically valuable progeny from quality parents could be produced in a fraction of the time it would take with natural breeding (Armstrong, 1993; Zavy, 1994; Lohuis, 1995). Among those techniques used today are artificial insemination, superovulation and embryo transfer.

Artificial insemination involves collection, and usually freezing, of semen from a proven bull of high quality; as cows of a herd come into estrous, the semen is thawed and deposited into the female tract manually. The benefits of this procedure are that one bull ejaculate can be used to inseminate several cows and the bull need not undergo the stress of shipping to the service location. As well, semen may be stored and used long after that bull has been retired from breeding service. However, a danger lies in narrowing the genetic pool by servicing too many herds with semen from the same bull. With only one offspring produced per individual, the limiting factor is the number of quality cows
available for insemination (Zavy, 1994; Lohuis, 1995). The absolute maximum possible number of quality offspring is equal to the number of cows inseminated, provided that each insemination is successful (Hunter, 1994; Lohuis, 1995) and that embryonic mortality is not taken into account.

To circumvent the problem of limiting offspring, superovulation is now widely used to recover more than one embryo per cow. This involves a hormone drug regimen including stimulation of folliculogenesis using follicle-stimulating hormone, and prostaglandin to induce estrus and ovulation of more than one oocyte. These animals are then artificially inseminated, and embryos transcervically flushed from the uterus at approximately day 7 after insemination (Hasler, 1992; Suzuki, 1993; Greve et al., 1995; Lohuis, 1995). Several embryos may be recovered from one cow, all fertilized with semen from a proven high quality bull. These embryos are cultured for a short time in vitro to assess morphology, and good quality embryos are transferred to recipients (lesser quality cows) for development to term. This procedure effectively produces more than one embryo from proven parents in a breeding cycle, but the cost of superovulation, flushing, short-term culture and transfer is high, and this technique does not yield a predictable or constant supply of embryos because of individual variance of ovulation response to superovulation (de Loos et al., 1991; Armstrong, 1993; Boland and Roche, 1993; Dieleman and Bevers, 1993; Greve et al., 1995; Lohuis, 1995).

Another method that produces a large number of selected offspring is in vitro maturation, fertilization and culture followed by transfer (Hasler, 1992; Lohuis, 1995; Wrathall, 1995). Embryos are cultured until morula or blastocyst stage at which time they
are transferred non-surgically to the uterus of a recipient heifer who has been synchronized to be at the appropriate point in her cycle. It is well established that viability of embryos following transfer is directly related to cell number at the time of transfer (Betteridge and Loskutoff, 1993). Embryos transferred at the morula or blastocyst stage produce a much higher pregnancy rate than embryos between the 2- and 16-cell stages (Loskutoff et al., 1992).

With the exception of natural matings, all new reproductive technologies involving embryo production in vivo involve manipulation of gametes or embryos in vitro. Damage to the embryo during flushing and transfer, or during the transfer of in vitro produced embryos, and lack of synchronization with recipients may account for some of the early embryonic mortality seen with the use of these techniques (Betteridge and Loskutoff, 1993; Behboodi et al., 1995; Wrathall, 1995). In the case of superovulation, damage may be incurred by disrupting the natural synchronous maturation of cumulus and oocyte by stimulation with exogenous hormones (de Loos et al., 1991).

**In Vitro:**

The production of large numbers of bovine embryos in vitro for scientific study is widely practised today, but survival of these embryos, and the difficulty and cost of culture hamper this practice at the commercial level. The in vitro production of embryos consists of 3 main steps: 1) oocyte maturation; 2) fertilization; and 3) embryo culture. One of the most important factors involved in the production of good quality embryos with high viability is to start with a highly selected group of oocytes. Those oocytes that
are totally cumulus surrounded show a higher proportion of maturation over those oocyte populations with discontinuous or sparse cumulus vestments (Zhang et al., 1995). High rates of metaphase II, which is a feature used to indicate successful oocyte maturation, are readily attainable in vitro. Maturation is required for successful fertilization and embryo development, and has been achieved with simple medium without serum supplementation (Rose and Bavister, 1992). The level of fertilization of matured oocytes in vitro is high, and approximately 60 to 85% of all bovine oocytes fertilized cleave to the 2-cell stage (Lonergan, 1994). The major loss of in vitro produced embryos occurs during subsequent development to the blastocyst stage. Only 30-50% of all fertilized oocytes will develop to morulae and blastocysts in culture (Pollard et al., 1989; Shamsuddin et al., 1992; Kato and Iritani, 1993; Lonergan, 1994); many of the arrested embryos do so at the 8-cell stage, corresponding with failure of the activation of the embryonic genome and possibly influenced by suboptimal culture conditions.

Several factors have been implicated in affecting the survival of embryos produced in vitro including the availability of growth factors, ions, energy substrates, amino acids, vitamins and minerals. These substances may be present in vivo in the bovine oviduct but not available in vitro, resulting in blocking of in vitro development (Lonergan, 1994). One method used to try to optimize culture conditions is to co-culture embryos with bovine oviduct epithelial cells (BOEC), granulosa cells (cumulus cells), or other somatic cell types from the adult. The underlying assumption is that any substances that are produced by these cells in vivo will also be produced in vitro to supplement the culture medium, or the co-culture cells may actively remove some kind of inhibitory factor
present in vitro (Xu et al., 1992A; Kato and Iritani, 1993; Lonergan, 1994; Harvey et al., 1995). When in vitro produced embryos are co-cultured with BOEC, embryo survival does increase significantly over that of embryos produced in minimal essential media (Wiemer et al., 1991; Xu et al., 1992A; Kato and Iritani, 1993). Increased maturation and fertilization levels were observed when oocytes were allowed to mature and fertilize without removal of cumulus cells (Zhang et al., 1995). Also, the age of somatic cell monolayers, either granulosa or BOEC, is correlated with embryo development: the older the co-culture cells the poorer the embryo development (Weimer et al., 1991). It is thought that the presence of co-culture cells, either as monolayers or as suspended cells, may facilitate sperm binding, capacitation and thus enhance fertilization (Zhang et al., 1995).

A common practice is to culture bovine embryos in "non-defined" medium which contains serum. While increasing the proportion of 2-cell embryos surviving to form blastocysts, it initially will inhibit cleavage of presumptive zygotes to the 2-cell stage (Keskintepe and Brackett, 1996). Keskintepe and Brackett (1996) have successfully cultured bovine embryos from presumptive zygote to blastocyst in completely defined medium and suggest that serum is not required for embryo development. As well, Rose and Bavister (1992) have shown that serum is not required for successful maturation, fertilization and culture of embryos to the blastocyst stage. Studies by Wiemer et al. (1991) have shown that cultures with serum not subjected to heat-inactivation supports oocyte maturation and embryogenesis better than cultures containing heat-inactivated serum and cultures without serum. Serum is predominantly uncharacterized and contains
a large variety of materials, including growth factors, hormones, waste products and energy substrates, any of which may or may not be beneficial or inhibitory to embryo development.

**Preattachment/Preimplantation Embryo Development:**

**Morphological Changes:**

During the very early stages of mammalian development, from fertilization to compaction, there are no obvious morphological changes except the cleavages which produce a loosely associated ball of round blastomeres that are apparently identical. These cells are smaller than the oocyte and result in an increased cell number with no change in overall embryo size, effectively increasing the surface area to volume ratio of the embryo (Watson, 1992). With each cellular cleavage new membrane is inserted at the cleavage furrow edges (Izquierdo et al., 1980). Early cleavages are relatively synchronous, but as development continues to compaction these cleavages become asynchronous (Watson et al., 1992A; Lonergan, 1994).

Compaction is the first major morphological change to occur during early mammalian embryo development. Compaction is the flattening of blastomeres to maximize cell contact, and is the first change in the epithelialization process which ends one to two cleavages later with the formation of tight and adherent junctions between outer cells of the embryo and gap junctions between all cells (Kimber et al., 1982; Vestweber et al., 1987; Sheth et al., 1997). Compaction and epithelialization are required for establishment of the two cell populations that differentiate into trophectoderm and
inner cell mass (ICM) lineages (Houliston et al., 1989; Pratt, 1989; Reima, 1990; Watson, 1992; Collins et al., 1995). In a compacted morula there is: 1) a flattened, polarized population of cells with one free (apical) surface, blastomeres contacting all other sides and tight and adherent junctions which will ultimately become the trophectoderm of the blastocyst; and 2) blastomeres surrounded by other blastomeres and lacking tight junctions, which will form the ICM of the blastocyst. It is thought that the manner and order in which blastomeres cleave ultimately determines which will become ICM and trophectoderm cells (Kelly et al., 1978; Ziomek and Johnson, 1981; Balakier and Pedersen, 1982; Kimber et al., 1982; Surani and Barton, 1984; Johnson et al., 1986; Pedersen et al., 1986; Garbutt et al., 1987; Reima, 1990; Fleming and Hay, 1991; Watson, 1992). Blastomeres from 2-, 4- and 8-cell murine embryos that were labelled by injection of horseradish peroxidase contributed evenly to both ICM and trophectoderm lineages, while cells from post-compaction 16-cell and early morula stage murine embryos contributed mainly to one lineage or the other (Balakier and Pedersen, 1986). In mice, even at the late morula stage, it was found that some outer cells contributed to ICM probably by migration of cells from one population to the other. By the blastocyst stage, cells are fate restricted by location (Balakier and Pedersen, 1982; Johnson et al., 1986; Pedersen et al., 1986). Similar studies have not been performed in the cow, but it is assumed that the process is similar in this species as the events leading up to this period of development are similar.

The process of compaction initiates the establishment of interblastomeric tight and gap junctions and polarized cells capable of active transport (Reima, 1990; Larue et al.,
1994; Riethmacher et al., 1995). In mice it has been shown that polarization, initial adhesive contacts and flattening can be accomplished with maternal protein stores alone, but, maintenance of this state and construction of full epithelial junctions involves the de novo production of proteins from the embryonic genome, in particular ZO-1 (Fleming and Hay, 1991; Javed et al., 1993; Sheth et al., 1997) and uvomorulin (E-cadherin) (Pratt, 1989; Reima, 1990; Javed et al., 1993; Larue et al., 1994; Sheth et al., 1997). Without functional tight junctions and zygotic gene expression, the embryo will not form a blastocyst (Fleming and Hay, 1991; Larue et al., 1994; Riethmacher et al., 1995; for review see Watson, 1992A; Sheth et al., 1997; Betts et al., 1997).

Following compaction the next morphological change is the formation of a blastocoelic cavity and the blastocyst. Fluid is moved into the space between blastomeres of the morula as a result of active transport, producing a fluid-filled blastocoel and effectively separating the ICM from all but one segment of overlying trophectoderm (Ziomek and Johnson, 1981; Kidder and McLachlin, 1985; Watson et al., 1992A). In cattle, this process is driven by the activity of a Na⁺,K⁺ ATPase pump basolaterally, and in mice it has been demonstrated that a Na⁺,H⁺ exchanger and Na⁺, glucose co-transporter located apically are also involved in drawing fluid by osmotic transport into the blastocoel where it is trapped from escaping by the tight junctions between outer blastomeres established at compaction (Kidder and McLachlin, 1985; Watson et al., 1992A; MacPhee et al., 1994; Betts et al., 1998).

The final morphological events of preattachment development are hatching of the bovine embryo from the zona pellucida followed by a period of rapid elongation.
Hatching involves possibly an enzymatic digestion of the zona pellucida in vivo, and, in vitro, an increase in the overall size of the blastocyst through swelling of the blastocoel causing rupture of the zona pellucida (Massip and Mulnard, 1980; Massip et al., 1982; Pedersen and Burdsal, 1994). Once hatched, the embryo elongates and attaches to the uterine lining (Pedersen and Burdsal, 1994).

**In Vivo vs In Vitro Embryo Development:**

Differences between embryos produced in vitro or in vivo, either through natural matings or through artificial insemination, raise questions about how well the in vitro culture system mimics the in vivo system. Bovine embryos produced in vitro have been successfully transferred and yielded live offspring, but these embryos were selected on the basis of good morphology. The survival of larger groups of unselected embryos is unknown, but is hypothesized to be lower than that of selected embryos (Shamsuddin et al., 1992; Lonergan, 1994; Farin and Farin, 1995).

Superovulation may force the ovulation of oocytes which were destined to degenerate within the ovary, possibly due to some kind of abnormality (Betteridge and Loskutoff, 1993; Assey et al., 1994; Greve et al., 1995). Changes in the nucleolar maturation oocytes of normal and superovulated animals were found by Greve et al. (1995). Normally the nucleolus vacuolates just prior to ovulation, probably in the final production of rRNA before germinal vesicle breakdown arrest at MII. However, in superovulated oocytes there is quite often no nucleolar vacuolation which may be indicative of failure of rRNA synthesis to resume prior to the forced maturation and
ovulation. A high proportion of these oocytes subsequently fail to reach MII and block at metaphase I of meiosis (Assey et al., 1994; Greve et al., 1995). It has also been shown that in vitro produced embryos which are transferred and develop to term result in heavier birth weight and larger sized offspring than embryos derived in vivo (Behboodi et al., 1995).

Bovine embryo development to blastocyst in vivo spans approximately 6 to 7 days from fertilization, with the 2-cell stage at 23-31 hours post insemination (hpi), the 4-cell stage at 36-50 hpi and the 8-cell stage at about 56-64 hpi (Lonergan, 1994). Cleavage to 16-cell is at 85 hpi and development of the morula is at around 120 hpi with cavitation occurring shortly thereafter, around 140 hpi. Development in vitro requires 24 to 48 hours longer than in vivo, with blastocysts appearing at about 170 hpi. The major difference in timing occurs during development after the 8-cell stage (Grisart et al., 1994; Lonergan, 1994). Grisart et al. (1994) suggest that this developmental lag of approximately 24 hours of in vitro embryos may be due to longer cell cycle duration found in in vitro produced embryos, and this may then cause embryonic transcription to appear to begin earlier, at a lower cell number.

In addition, there are differences in cell number between in vitro and in vivo produced blastocysts. Embryos produced in vitro show a lower number of cells at the blastocyst stage than those in vivo (Iwasaki et al., 1990; Xu et al., 1992A; Lonergan, 1994; Keskinintep and Brackett, 1996). Embryos produced in vitro and transferred give rise to offspring that may be heavier in birth weight with longer gestation periods than
embryos from natural matings (Farin and Farin, 1995), although this has been a matter of controversy (King et al., 1985).

At compaction, in vitro produced bovine embryos show a lesser degree of compaction as compared to in vivo embryos. Iwasaki et al. (1990) found that cell-cell contacts between cells of the ICM of in vitro embryos were not as tight as those found in in vivo embryos, and in vitro embryos tended to undergo asynchronous compaction. Shamsuddin et al. (1992) also found that in vitro produced embryos have shorter and less numerous junctional complexes, a higher degree of cytoplasmic vacuolation and more numerous phagosomes. These results hint at possible differences in calcium ion concentration, microtubule structure and enzyme activity in cultured embryos. In a study by Plante and King (1994) there were no major structural differences found between in vivo and in vitro produced embryos at the light and electron microscopic level, except that at early cleavage stages, blastomerens of in vivo embryos were rounder than those in vitro produced embryos, and a slight time delay was observed in in vitro development compared to that in vivo.

Maternally Controlled Early Development:

During oogenesis, large amounts of mRNA and protein are produced and stored in the oocyte for use during late oogenesis and the cleavage period of development (Dworkin and Dworkin-Rastl, 1990; Nothias et al., 1996). There is maternal contribution of large amounts of histone, tubulin, actin, and desmocollin, as well as smaller amounts of other mRNAs and proteins such as uhomorulin. In mice, mRNAs are translated and
rapidly degraded after fertilization to low levels in the 2-cell embryo. Losses of maternal mRNA up to 70% have been reported in mice between fertilization and the 2-cell embryo stage (Flach et al., 1982; Kidder and McLachlin, 1985; Schultz, 1986; Crosby et al., 1988; Jarrel et al., 1991; Nothias et al., 1996). As maternal mRNA decreases, there is a decrease in protein production until after the maternal-zygotic transition (MZT) when first mRNA content and then protein production increase once again due to de novo synthesis from the embryonic genome (Flach et al., 1982; Kidder and McLachlin, 1985). Between the 1-cell and 8-cell bovine embryo stages, translation drops by as much as 68% (Jarrell et al., 1991), then increases again at the 16-cell stage as protein is produced from embryonic mRNA synthesized from the embryonic genome (Frei et al., 1989; Jarrell et al., 1991; Via et al., 1996).

Little is known about how stored message is stabilized in the mammalian oocyte and subsequently used and degraded during cleavage (Bachvarova, 1981; Renard et al., 1994). It has been found that some specific transcripts in murine embryos have been adenylated and then deadenylated for storage during oocyte maturation (Dworkin and Dworkin-Rastl, 1990). After fertilization, and prior to translation, they are re-adenylated with poly(A) tail length determining translation and longevity. Deadenylation generally appears to lead to degradation. However, most mRNAs are stored highly adenylated in the murine oocyte, with the poly(A) tail added during oogenesis (Wickens, 1990). They are actively translated, deadenylated and subsequently degraded by the late 2-cell stage. In the cow, the period of development controlled by maternally-derived products is longer,
up to the 8- to 16-cell stages. Products from the embryonic genome begin to replace maternal stores and control at this point (Telford et al., 1990).

Onset of Embryonic Transcription:

In mammals, successful initiation of transcription from the embryonic genome is required for further development. In mice, this has been described as the "maternal-zygotic transition" or the MZT. This term however, is inaccurate for the bovine embryo, as this initiation does not occur at the zygote stage of development, but occurs later at the 8- to 16-cell stage. For this thesis, this initiation of embryonic transcription will be described by the term "OET" or the "onset of embryonic transcription". The major burst of embryonic transcription occurs at the 2-cell stage in the mouse (Schultz, 1986; Telford et al., 1990; Schultz, 1993), the 8- to 16-cell stage in the cow (Telford et al., 1990; Barnes and First, 1991), the 4-cell stage in the pig (Telford et al., 1990; Jarrell et al., 1991) and humans (Telford et al., 1990) and between the 64- and to 128-cell stages in the rabbit (Telford et al., 1990). The OET is characterized by decrease in maternally derived mRNA, onset of sensitivity to transcriptional inhibitors such as α-amanitin, greatly increased mRNA production from the embryonic genome, change in the protein profile, and appearance of gene products from paternally donated genetic material (Bolton et al., 1984; Crosby et al., 1988; Frei et al., 1989; Telford et al., 1990; Schoenbeck et al., 1992; Schultz, 1993; Nothias et al., 1996). As well, the OET coincides with the time of development when embryos are particularly sensitive to the environment and development will be blocked in suboptimal culture conditions (Schoenbeck et al., 1992; Kato and
Iritani, 1993). In mice, if transcription is continuously blocked before the OET by the use of α-amanitin, development progresses only to the point at which proteins from embryonic transcription might normally be expected to become active (Bolton et al., 1984; Telford et al., 1990; Barnes and First, 1991; Schoenbeck et al., 1992; Schultz, 1993).

The timing of the OET in cattle was determined by radiolabelled uridine incorporation, detection of specific transcripts using RT-PCR, and radiolabelling proteins and subjecting the embryo extracts to two dimensional gel electrophoresis to examine the relative amounts of protein present between stages of embryos (Frei et al., 1989; Jarrell et al., 1991; Viuff et al., 1996). Low levels of active transcription occur as early as the 2- and 4-cell stages in the bovine embryo as shown by the incorporation of radiolabelled uridine into de novo transcripts (Barnes and First, 1991; Plante et al., 1994; Viuff et al., 1996) and the production of rRNAs (King et al., 1988). There is a larger burst of transcription at the 8-cell stage which marks a more generalized activation of the embryonic genome. In mice it is now known that the OET proceeds in two phases: the first occurs in late 1-cell embryos and is typified by a low level of transcription and the production of heat-shock proteins. This transcription was measured by extracting mRNA and amplifying the transcripts for heat-shock genes (Christians et al., 1995) and by two-dimensional gel electrophoresis of radio-pulsed proteins (Bensaude et al., 1983). This is followed at the 2-cell stage by the major burst of transcription when many different genes are transcribed (Clegg and Piko, 1982; Flach et al., 1982; Bolton et al., 1984; Schultz, 1986; Thompson et al., 1995; Nothias et al., 1996).
The process of compaction requires proteins derived from transcription and translation of embryonic genes, as this process, as well as further embryo development, is prevented by the use of transcriptional inhibitors such as α-amanitin (Kidder and McLachlin, 1985; Watson, 1992). It is not known which transcripts are required for further cleavage and initiation of compaction. Synthesis of uvomorulin is required for maintenance of compaction and blastocoel formation in mice. Compaction is initiated by maternal stores of uvomorulin mRNA and protein, but embryonic expression of uvomorulin is required for maintenance of the compacted state and further development (Vestweber et al., 1987). Cavitation also depends on embryonic transcripts. The transcripts for the α subunit of Na⁺,K⁺ ATPase are present throughout bovine and murine development (MacPhee et al., 1994; Betts et al., 1998), but those of the β subunit of Na⁺,K⁺ ATPase are transcribed one cell cycle before the protein is required (Kidder and McLachlin, 1985; Watson, 1992; MacPhee et al., 1994; Betts et al., 1997). Both the α and β subunits are required for the production of functional protein so control of the transcription and translation of the β subunit may control the timing of cavitation.

Expression patterns for other genes, in particular growth factors and their receptors, have been studied in bovine embryos (for review see Watson, 1992 and Watson et al., 1996). In general, appearance of certain gene transcripts does not correlate well with morphogenesis. Table I lists a few of the examples of maternally derived and embryonic genome derived mRNAs, and differences that exist in their expression between the mouse and the cow. There are fewer genes in cattle transcribed exclusively from the embryonic genome, perhaps due to a longer period of time from fertilization to the major burst of transcription at the OET.
Table I: Some mRNA species expressed in early murine and bovine embryos, indicating whether the transcripts are maternally derived (M) or embryonic genome derived (E).

<table>
<thead>
<tr>
<th>Messenger RNA</th>
<th>Murine</th>
<th>Bovine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast growth factor</td>
<td>E (^1)</td>
<td>M+E (^3)</td>
</tr>
<tr>
<td>Transforming growth factor α</td>
<td>M+E (^2)</td>
<td>M+E (^3)</td>
</tr>
<tr>
<td>Transforming growth factor β</td>
<td>M+E (^1)</td>
<td>M+E (^3)</td>
</tr>
<tr>
<td>Platelet derived growth factor A</td>
<td>E (^1)</td>
<td>M+E (^3)</td>
</tr>
<tr>
<td>Insulin-like growth factor I</td>
<td>M+E (^2)</td>
<td>M+E (^3)</td>
</tr>
<tr>
<td>Insulin-like growth factor II</td>
<td>E (^1)</td>
<td>M+E (^3)</td>
</tr>
<tr>
<td>Insulin receptor</td>
<td>E (^3)</td>
<td>M+E (^3)</td>
</tr>
<tr>
<td>Insulin-like growth factor I receptor</td>
<td>E (^3)</td>
<td>M+E (^3)</td>
</tr>
<tr>
<td>ZO-1 (zona occludens protein-1)</td>
<td>E (^5)</td>
<td>E (^4)</td>
</tr>
<tr>
<td>Epidermal growth factor receptor</td>
<td>E (^3)</td>
<td>- (^3)</td>
</tr>
<tr>
<td>Na(^+),K(^+) ATPase subunit β1</td>
<td>E (^8)</td>
<td>E (^4)</td>
</tr>
<tr>
<td>Embryonic alkaline phosphatase</td>
<td>E (^7)</td>
<td>NP</td>
</tr>
<tr>
<td>Bovine trophoblast protein</td>
<td>NP</td>
<td>E (^4)</td>
</tr>
<tr>
<td>Uvomorulin (E-Cadherin)</td>
<td>M+E (^6)</td>
<td>M+E (^4)</td>
</tr>
</tbody>
</table>

\(^1\)Rappolee et al., 1990  
\(^2\)Doherty et al., 1994  
\(^3\)Watson et al., 1992B  
\(^4\)Watson et al., 1996  
\(^5\)Sheth et al., 1997  
\(^6\)Takeichi, 1988  
\(^7\)Hahnel et al., 1990  
\(^8\)Kidder, 1993  
NP=not present
It is of interest that translation in the early post-OET mammalian embryo does not seem to be coupled to transcription, with mRNAs detected at the OET being poorly translated until subsequent cleavage has occurred (Nothias et al., 1996). It is not clear that the products of all genes are used immediately post-OET, and Kidder (1993) has suggested that post-transcriptional modifications are important in preimplantation/preattachment development. Among those molecules required for successful embryogenesis are ZO-1, uvomorulin, histones, and the $\beta$ subunit of Na$^+$.K$^+$ ATPase. There are undoubtably many more required materials that are as yet unknown.

Control of the Timing of Development:

Debate exists about the control of events leading to the initiation of embryonic gene expression and compaction. Three models have been proposed. The first model is based on the concept of a "zygotic clock" mechanism which predicts embryonic gene expression and compaction occur at a particular time post-fertilization. This is defined by the OET occurring regardless of treatment with inhibitors of DNA replication and mitotic division such as aphidicolin and nocodazole. In the second model it is cleavage that triggers these changes so that no matter how many hours post-fertilization have elapsed, the events will not happen if DNA replication or cleavage is prevented. In the third model it is the environment which affects genetic and morphological changes, although this model is not favoured since a constant in vitro culture environment will support early embryogenesis.
The zygotic clock hypothesis is favoured by Wiekowski et al. (1991), Schultz (1993) and Nothias et al. (1995 and 1996), who suggest that the clock is the major regulator of translation as well as transcription in mice and that primary transcriptional regulation is through the action of histone acetylases. Their research suggests that the clock mechanism works through regulation of chromatin structure. In turn, changes in chromatin structure are known to affect transcriptional status in eukaryotic cells. For example, DNA that is not bound by histones is more transcriptionally active than DNA that is bound, and DNA bound by protamines in particular is more transcriptionally silent than histone-bound DNA (Groudine and Conkin, 1985). The regular packaging of DNA with histones (winding of 146 base pairs of DNA around a core of eight histone proteins) can act as a transcriptional regulator depending on the positioning of the nucleosome along the DNA helix, resulting in a repression of transcription (ie, if binding sites for transcription factors are concealed) or an enhancement of transcription (ie, if activating regulatory segments are brought into proximity) (Felsenfeld, 1992; Thompson et al., 1995; Steger and Workman, 1996; Beato and Eisfeld, 1997).

The most recent zygotic clock hypothesis is that initiation of embryonic transcription is regulated by enzymatic changes which affect chromatin structure, including methylation/demethylation and acetylation/deacetylation. Under-methylation has been found to be localized to areas of transcriptionally active DNA (Groudine and Conkin, 1985). It is thought that methylation enhances histone (or protamine) binding to DNA, so that demethylation, or under-methylation, would result in release of DNA from histones (or protamines), making it accessible to transcriptional or regulatory factors.
Hyperacetylation of histones is highly correlated with increased transcriptional activity (Schultz and Worrad, 1995; Beato and Eisfeld, 1997). In the early 1-cell mouse embryo, histones are hypoacetylated causing increased binding of histone H1 to DNA, and preventing access of transcription factors to the chromatin (Nothias et al., 1995; Schultz and Worrad, 1995; Thompson et al., 1995). Beginning in G2 of the 1-cell embryo there is progressive acetylation of histones. There is evidence from antibody localization and acetylation inhibition studies, that acetylation is part of, and perhaps the first event in, the transition to the early embryonic transcriptional pattern (Nothias, 1995; Schultz and Worrad, 1995; Thompson et al., 1995).

Translational control both before and after the OET in mice is postulated to be controlled by the zygotic clock, but control mechanisms for this process are unknown and are felt to be distinct from those regulating transcription (Nothias et al., 1995). Translational control, especially before the OET in cattle, is thought to involve post-translational modification of factors either required for, or repressive to, translation (Nothias et al., 1995). These modifications could include such changes as glycosylation/deglycosylation and phosphorylation/dephosphorylation of proteins, examples of which occur with proteins required in cell cycle regulation such as the cyclins and mitogen activated protein kinases (Dworkin and Dworkin-Rastl, 1990).

**Inhibition of Embryo Development:**

During early embryo development a complex series of communications must occur between the cytoplasm and nucleus, although it is unknown exactly what these signals are
and how they are manifest. Transfer of nuclei from early embryos (Hasler, 1992) or even primary cell cultures (Campbell et al., 1996) into enucleated oocytes can produce an embryo. This technique, albeit at very low efficiency at this point, allows for the transfer of a normal or manipulated genome from a high quality individual into enucleated oocytes collected from another. This requires synchronizing recipient ooplasm and nucleus so that inconsistencies in communication between the nucleus and ooplasm do not occur (Surani et al., 1980; Tanaka et al., 1995A and 1995B; Samake and Smith, 1996B). One way to achieve synchrony is to incubate embryos in an inhibitor which effectively arrests development for a brief period of time with minimal effect on subsequent development. This treatment is applied to recipient ooplasm and the nucleus-donating embryo so that both are at the same state when transfer occurs. To date, the widest used synchronizing agents for nuclear transfer are nocodazole and aphidicolin.

Nocodazole is a microtubule inhibitor which acts by binding free tubulin monomers and preventing their association into microtubules (Maro and Pickering, 1984; Otaegui et al., 1994). This, in turn, prevents separation of chromosomes from the metaphase plate by disrupting the spindle, and prevents cytokinesis (Tanaka et al., 1995A). Upon removal of nocodazole, embryos cleave immediately (Otaegui et al., 1994; Tanaka et al., 1995A and 1995B; Samake and Smith, 1996A and 1996B). Transfer of embryos after nocodazole treatment has resulted in live offspring (Otaegui et al., 1994). When used at low doses for a maximum period of 12 hours, nocodazole successfully synchronizes blastomeres within an embryo with a minimum of cell death in bovine (Tanaka et al., 1995A and 1995B; Samake and Smith, 1996A and 1996B) and in murine
(Otaegui et al., 1994) embryos. Although nocodazole treated embryos have resulted in viable offspring after transfer, the effects of nocodazole on gene expression, morphogenesis and survival rates have not been fully studied. Another microtubule inhibitor, taxol, has a similar action of inhibiting cytokinesis and chromosome separation in cells, but, instead of depolymerising microtubules, it causes the rapid production of large stabilized microtubule arrays within the cell (Rowinsky et al., 1990; Woods et al., 1995; Darby et al., 1996; Parekh and Simpkins, 1997). Although it has not been used to synchronize mammalian blastomeres, taxol is widely used as an anti-cancer drug where it affects rapidly dividing cells by rapidly polymerizing microtubules, preventing chromosome separation and cytokinesis in a manner opposite to nocodazole (Rowinsky et al., 1990; Parekh and Simpkins, 1997).

Aphidicolin and α-amanitin are two inhibitors routinely used to assess genetic control of embryogenesis. Aphidicolin inhibits DNA synthesis, effectively arresting the nuclear cycle by preventing completion of S phase (Spadari et al., 1982; Petzoldt, 1984; Samake and Smith, 1996A). Treatment of mouse embryos prior to the onset of embryonic transcription does not block the production of transcripts from the embryonic genome (Schultz, 1993), but it does result in transcripts being produced at lower levels than normal (Petzoldt, 1984). The treatment of embryos with α-amanitin (as discussed previously) results in the cessation of transcription from the embryonic genome. If treated before embryonic transcription has begun, embryo development will continue until materials from the embryonic genome are required, but not beyond. If treated after the embryonic genome becomes active, embryogenesis will continue until stores of mRNA and proteins required for embryogenesis are exhausted.
The Alkaline Phosphatases:

The alkaline phosphatases (APs) comprise a family of membrane bound enzymes found across diverse species including bacteria, mammals, reptiles, fungi, plants and amphibians (McComb et al., 1979; Harris, 1982; Millan, 1988; Millan, 1990). The wide distribution of this family of isozymes suggests at least one significant biological function, but their role in vivo remains unclear (Harris, 1982; Narisawa et al., 1997). In vitro the APs are known to remove phosphates from phosphorylated molecules at alkaline pH between 8 and 10.5, hinting at possible in vivo roles in cell-cell signalling, transmembrane signalling, shuttling, and mineralization. Two distinct forms of mammalian APs exist: tissue non-specific AP (TNAP) and tissue-specific APs (TSAPs). The TSAPs may be farther subdivided depending on temperature stability, pH optimum, inhibitor sensitivities and expression patterns into forms with names such as human placental (PLAP), intestinal (IAP), mouse embryonic (EAP) and human germ cell (GCAP) APs.

Structure:

Most of the information about the biochemical structure of AP has been derived from the study of the Escherichia coli enzyme (Millan, 1988; Akiyama and Ito, 1993). Each functional *E. coli* AP isozyme consists of a homodimer of 2 units of approximately 40 to 45 kDa (Fishman, 1974; Kim and Wyckoff, 1989) localized in the periplasmic space between the cell wall and cytoplasmic membrane (McComb et al., 1979). Each mature enzyme has three metal binding sites: two sites involve binding of zinc ions to histidine residues while a third site involves binding of a magnesium ion between lysine and
aspartate residues (Millan, 1990; Dealwis et al., 1995). Each subunit contains two disulfide bridges which appear to be necessary for enzyme activity by providing structural integrity to ion binding sites (Akiyama and Ito, 1993; Sone et al., 1997). Preliminary reports predict that mammalian and insectoid APs have similar structure and basic biochemical properties to bacterial AP (McComb et al., 1979; Kim and Wyckoff, 1989; Millan, 1990; Butterworth, 1994), with the same basic α/β conformation and central conserved β sheet. In addition there are highly variable loops and insertions not present in the E. coli enzyme (Weissig et al., 1993). In all APs of species studied to date, the presence of Zn$^{2+}$ and Mg$^{2+}$ are required for enzyme activity, but the concentrations required for optimal activity varies between species and isoforms (McComb et al., 1979; Kim and Wyckoff, 1989). The APs derived from shrimp, silkworm and grasshopper are also slightly larger than the bacterial AP, ranging from 54 to 56 kDa (Chang et al., 1993). Many mammalian APs are highly glycosylated, and thus range in size from 60 to 90 kDa (Micanovic et al., 1988; Dairiki et al., 1989). In the rat, the glycosylated forms appear to be between 75 and 90 kDa, and the deglycosylated forms are 52 kDa (Nair et al., 1987). Also in contrast with E. coli AP, mammalian AP isoforms are cell membrane anchored at the C-terminus by a glycosylphosphatidylinositol (GPI) anchor, which can be cleaved by membrane bound phospholipase C or circulating serum phospholipase D, resulting in circulating AP in the blood in some disease states (McComb et al., 1979; Harris, 1982; Low and Saltiel, 1988; Micanovic et al., 1988; Dairiki et al., 1989; Harris, 1989; Kim and Wyckoff, 1989; Millan, 1990). It has been suggested by polyacrylamide gel electrophoresis experiments performed by Hamilton et al. (1989) that human APs may
be present as tetramers while membrane bound and as a mixture of dimers and tetramers when in fluids and secretions.

The APs have been extensively studied in the human and mouse in regards to heat stability, chemical inhibitor susceptibility, and electrophoretic mobility (Goldstein et al., 1980; Harris, 1982; Merchant-Larios et al., 1985; Lepire and Ziomek, 1989). The L-forms of several amino acids, including homoarginine, phenylalanine, tryptophan and leucine provide uncompetitive inhibition of APs, whereas the D- forms do not. Since the bacterial AP is not susceptible to inhibition by compounds such as L-homoarginine, L-phenylalanine and levamisole, inhibitor-sensitivity in mammals is probably conferred by the presence of an extra polypeptide loop when compared to E. coli AP (Buttenworth, 1994). Tissue non-specific AP is heat labile and highly susceptible to L-homoarginine inhibition in humans and mice (Harris, 1982; Lepire and Ziomek, 1989; Narisawa et al., 1992). Tissue non-specific AP from mouse, cow and human have all been found to be highly susceptible to levamisole inhibition (Fishman, 1974; Harris, 1989; Bingham et al., 1992). The TSAPs in human and mice are more heat-stable, requiring temperatures above 80°C to cause inactivity, and are readily inhibited by L-phenylalanine (Nair et al., 1987; Lepire and Ziomek, 1989; Narisawa et al., 1992).

**Human:**

Four distinct APs exist in the human: one TNAP and the TSAPs, which are IAP (intestinal), PLAP (placental) and GCAP (germ-cell; Goldstein et al., 1980; Harris, 1982; Garattini et al., 1985; Terao and Mintz, 1987; Knoll et al., 1988; Millan, 1988; Weiss et
al., 1988; Harris, 1989; Manes et al., 1990). The TNAP form is expressed at low levels in most human tissues, with liver, bone and kidney expressing higher levels than other tissues (Harris, 1989). Intestinal AP is expressed in the intestinal epithelium on the brush border (Lewis and Rutan, 1991). Placental AP is expressed in the late first to early second trimester of pregnancy, as well in the lung and the cervix in much lower amounts (Harris, 1989). Germ-cell AP is found in testis and in small amounts in thymus (Harris, 1989). As well, in the second and third trimester of pregnancy, three APs (IAP, TNAP and PLAP) are present in the amniotic fluid (Sembaj et al., 1995).

The genes encoding human PLAP, IAP and GCAP are localized on chromosome 2 (Henthorn et al., 1988; Harris, 1989), while TNAP is localized on chromosome 1 (Terao and Mintz, 1987). All the human TSAP isozymes are more than 85% identical at the protein level (Manes et al., 1990). Human TNAP is 57% identical to IAP at the amino acid level (Weiss et al., 1988; Hahnel et al., 1990).

Membrane-bound AP may be released by cleavage of the GPI anchor by phospholipase C or by circulating serum phospholipase D (Micanovic et al., 1988; Tsonis et al., 1988). In disease states, such as liver damage and cancer, large amounts of AP are released from cells resulting in a measurable increase in circulating APs not normally detected in clinical analyses. In cancer states, there is release of AP isozymes due to ectopic or over-expression of TSAP genes with protein being released into the circulation (Millan, 1988; Harris, 1989; Griffiths, 1992; Millan, 1992). The "Regan" AP isozyme has been found to be overexpressed PLAP in lung, gastrointestinal, ovarian and uterine malignant tumours (Griffiths, 1992). Similarly, the "Nagao" isozyme describes GCAP
bearing tumours, most commonly seminomas (Millan and Manes, 1988). The "Kasahara" isozyme refers to IAP found to be ectopically expressed in certain hepatomas (Harris, 1989).

**Mouse:**

In mouse there are at least three AP isozymes expressed, including TNAP found most conspicuously in placenta, liver, and kidney (Terao and Mintz, 1987; Johansson et al., 1993), IAP in the intestine and EAP (embryonic AP) in the preimplantation embryo and spermatocytes in mid- to late-prophase (Hahnel et al., 1990; Narisawa et al., 1992; MacGregor et al., 1995). There is also a mouse AP pseudogene which is thought to not code for a functional protein (Schurr et al., 1989; Manes et al., 1990). During embryo development, there is a change from EAP to TNAP and IAP expression (Merchant-Larios et al., 1985; Hahnel et al., 1990; MacGregor et al., 1995). Embryonic AP mRNA has been found in 2-cell embryos through to blastocyst (Hahnel et al., 1990; Narisawa et al., 1992). No AP protein or mRNA expression was detectable in oocytes and presumptive zygotes (Johnson et al., 1977; Mulnard and Huygens, 1978), leading to the conclusion that AP is expressed early from the zygotic genome. Alkaline phosphatase activity has been identified on the plasma membrane of adjacent blastomeres beginning at the late 2-cell stage (Mulnard and Huygens, 1978), although others have not detected activity until the late 4-cell or 8-cell stage (Vorbrodt et al., 1977). In the morula and blastocyst stages, some investigators have found AP protein activity to be localized to areas of blastomere-blastomere contact, with activity becoming restricted to the ICM at the late blastocyst
stage (Ishiyama and Izquierdo, 1977; Johnson et al., 1977; Mulnard and Huygens, 1978; Izquierdo et al., 1980; Millan, 1988; MacGregor et al., 1995). Others have described activity as concentrated mainly to apical surfaces of outer cells (Vorbrodt et al., 1977). The mouse TNAP isozyme is not detected until around the time of implantation, and IAP is not detected until late fetal development.

Mouse TNAP and human TNAP are 86% identical at the amino acid level (Terao and Mintz, 1987; Hoshi et al., 1997). Mouse EAP and IAP 75% identical (Hahnel et al., 1990, Manes et al., 1990), and are at least 75% identical (depending on the isozyme) to human TSAPs (Manes et al., 1990). See Table II for a more thorough list of percentage similarities between mouse and human APs.

**Cattle:**

The adult cow has been shown to exhibit a TNAP in liver, bone, kidney, milk and lactating mammary gland (Garattini et al., 1987; Hsu et al., 1987; Bingham et al., 1992), and IAP in the intestine (Weissig et al., 1993). The TNAP isozyme from bovine kidney has been cloned and the amino acid sequence determined by Garattini et al. (1987) and independently by Hsu et al (1987). It has 90% amino acid identity to the human TNAP isozyme. Bovine TNAP is also thought to be membrane attached by a GPI anchor (Garattini et al., 1987).

Presence of AP activity in the bovine follicle has been shown to be dynamic over the estrous cycle, with higher AP activity in follicles in the preovulatory phase and during the first trimester of pregnancy (Henderson and Cupps, 1990). Administration of chorionic
Table II: Percentage similarity between human (h) and mouse (m) alkaline phosphatase isozymes at the amino acid level.

<table>
<thead>
<tr>
<th>mEAP</th>
<th>mIAP</th>
<th>hIAP</th>
<th>hGCAP</th>
<th>hPLAP</th>
<th>hTNAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTNAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>86.3</td>
</tr>
<tr>
<td>mEAP</td>
<td>75.4²</td>
<td>77.4²</td>
<td>75.4²</td>
<td>75.4²</td>
<td></td>
</tr>
<tr>
<td>mIAP</td>
<td>80.3²</td>
<td>76.8²</td>
<td>75.4²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hIAP</td>
<td></td>
<td>86.7²</td>
<td>85.8²</td>
<td>57.0¹</td>
<td></td>
</tr>
<tr>
<td>hGCAP</td>
<td></td>
<td></td>
<td>96.6²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hPLAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>52.0¹</td>
</tr>
</tbody>
</table>

¹Harris, 1982; Weiss et al., 1986; Henthorn et al., 1987
²Manes et al., 1990
³Terao and Mintz, 1987; Hoshi et al., 1997
gonadotropin or leutinizing hormone resulted in increased AP activity suggesting a correlation with AP activity and steroidogenesis (Henderson and Cupps, 1990). In the rat, evidence points to a reorganization in the distribution of AP in the uterus in response to unknown factors present during early pregnancy (Bucci and Murphy, 1995). As AP expression is highly conserved across species it is probable that the bovine pregnant state will be similar to that of the rat. Recent work by McDougall et al. (1998) has found the presence of two additional TSAPs, called TSAP2 and TSAP3, in bovine blastocysts. As well, an IAP form and a putative pseudogene exist in cattle (Garattini et al., 1987; Hsu et al., 1987; Weissig et al., 1993).

Function:

The substrates of APs are unknown in vivo, but, in vitro, AP will function in the dephosphorylation of molecules containing phosphorylated tyrosine, threonine and serine groups (Swarup et al., 1981; Millan, 1990). As AP activity is localized mainly to cell surfaces in organs involved in absorption and transmembrane transport, it is hypothesized that these enzymes may play a role in absorption of metabolites or shuttling of molecules across epithelia (Makiya et al., 1992A). High levels of active AP protein are found in the brush border of the intestine, the maternal-fetal interface in the placenta (Makiya et al., 1992B; Makiya and Stigbrand, 1992), and in the tubules of the kidney, all tissues involved in the absorption or movement of materials across cells (Harris, 1989; Henderson and Cupps, 1990; Johansson et al., 1993). Other hypothesized roles for APs include inorganic phosphate shuttling, vitamin metabolism and shuttling (in particular vitamin B; Rindi et
al., 1995; Narisawa et al., 1997), bone mineralization (Yoon et al., 1989; Zimmerman et al., 1992; Fukayama and Tashjian, 1994), vascularization (Alpaslan et al., 1997), cellular migration (Narisawa et al., 1992; Chang et al., 1993; MacGregor et al., 1995; Kumano et al., 1996), immunoglobulin transport (Makiya and Stigbrand, 1992A; Makiya and Stigbrand, 1992B; Makiya et al., 1992), steroid receptor inactivation or regulation in preovulatory follicles (Henderson and Cupps, 1990), and possibly involvement in phosphorylation/dephosphorylation reactions involved in cell-cell signalling pathways (Swarup et al., 1981; Harris, 1989; Lewis and Rutan, 1991; Narisawa et al., 1992; Hui et al., 1993) or cell-substrate recognition (Narisawa et al., 1992). No elucidated physiological role exists to date for the AP family (McComb et al., 1979; Bingham et al., 1992; Hui et al., 1993; MacGregor et al., 1995). Tissue non-specific AP is thought to function in bone mineralization since osteoblasts are prime expressor cells (Morris et al., 1986; Harris, 1989; Yoon et al., 1989).

Tissue non-specific AP has been used as a marker for migrating primordial germ cells (PGCs) in many mammalian species (Hahnel and Eddy, 1986; Hahnel et al., 1990). It was shown by MacGregor et al. (1995), using TNAP knock-out mice, that although PGCs in the mouse are positive expressors of TNAP, this isozyme is not required for PGC development and migration, nor is TNAP essential for embryonic development to term. Tissue non-specific AP knock-out mice do survive to birth, but shortly thereafter, most die due to apnea, seizures, failure to gain weight, myelophthisic anemia and cranial haemorrhage, all of which are known symptoms of human hypophosphatasia (Narisawa et al., 1997). Interestingly, in these mice mineralization does occur, unlike in human
hypophosphatasia. These afflictions are possibly due to abnormalities in AP expression in spinal nerve roots and neural tube development resulting in defects which remain to be elucidated (Narisawa et al., 1997). These researchers report the survival of one male TNAP knock-out mouse to sexual maturity with the ability to produce offspring, further suggesting that TNAP is not required for the production of functional gametes in the male (Narisawa et al., 1997). There also is an EAP knock-out mouse, which survives embryogenesis and reaches sexual maturity (Narisawa et al., 1997).

The only genetic AP disorder known to date is hypophosphatasia (Muller et al., 1991). This is a congenital, autosomal recessive genetic disease of humans characterized by the lack of, or a marked decrease in, the function of TNAP, apparently causing low or no mineralization of developing bone (Weiss et al., 1988). Different mutations are associated with different degrees of severity but this disease most often results in childhood death (Millan, 1990), with many of the accompanying symptoms listed above for the TNAP knock-out mice. Thus, while studies involving the AP-associated human disease hypophosphatasia and TNAP knockout mice suggest involvement of APs in normal growth and function, they do not enable identification of specific roles for these isozymes. Knockout TNAP mice suffer from a long list of disorders and dysfunctions (Narisawa et al., 1997); the range of these disabilities, and the fact that hypophosphatasia is quite often lethal, strongly suggests that at least TNAP is metabolically important. However, since there is limited survival of TNAP null mice and good survival of EAP null mice, APs may be part of a redundant system. Regardless of their functions, these
genes may be useful for identifying mechanisms of control of gene expression in early embryos.

Other Genetic Markers of Bovine Embryo Development:

Research by Watson et al. (1992 and 1996) and Harvey et al. (1995), has characterized the expression of over 30 genes during preattachment bovine embryo development. In mice, many markers have been found which signal the onset of transcription from the embryonic genome, such as fibroblast growth factor, platelet-derived growth factor A, insulin-like growth factor II and its receptor, ZO-1 and the β1 subunit of Na⁺,K⁺ ATPase (Rappolee et al., 1990; Watson et al., 1992; Kidder, 1993; Doherty et al., 1994). These genes are transcribed actively beginning at the OET, not before, and therefore can be used to assess successful initiation of transcription from the embryonic genome. However, in cattle, a very different pattern emerged. Many transcripts found to be solely embryonic in the mouse are found in the bovine embryo as both stored maternal mRNA and as products of the embryonic genome later in development. For example, many of the growth factors, including fibroblast growth factor, transforming growth factors α and β, platelet derived growth factor A, and insulin-like growth factors I and II and their receptors are all present as mRNA inherited by the oocyte and then mRNA produced by the embryonic genome at the OET (Watson et al., 1992; Watson et al., 1996). Transcripts detected only after the OET include bovine trophoblast protein (involved in maternal recognition of pregnancy), the β1 subunit of Na⁺,K⁺ ATPase, and insulin-like growth factor binding protein 5 (Watson et al., 1996).
Bovine trophoblast protein mRNA is expressed at the blastocyst stage, well after the OET, and as part of signalling of the embryo's presence to the mother in preparation for uterine attachment. The β1 subunit of Na⁺,K⁺ ATPase mRNA was found beginning at the morula stage, in preparation for the assembly of functional Na⁺,K⁺ ATPase for use in blastocoel formation. This transcript is found after the OET and therefore can be used as a marker for preparation for cavitation but not for the OET itself or compaction. Similarly, insulin-like growth factor binding protein 5 transcripts were not detected until the blastocyst stage.

The number of mRNAs in the bovine embryo produced from the embryonic genome appear to be much lower than in the murine system. Transcription from the embryonic genome is known from autoradiography to occur as early as the late 2- and 4-cell preattachment bovine embryo (Barnes and First, 1991; Plante et al., 1994; Vioff et al., 1996). However, those known embryonic transcripts discussed above are only detectable well after the OET would have taken place, thus leaving researchers still with no transcripts to mark the onset of transcription at the 4- and 8-cell stages of development. Alkaline phosphatase in the mouse preimplantation embryo is present only as embryonically produced transcripts. In cows there is more than one AP isozyme expressed in blastocysts. The hypothesis driving this thesis is that at least one bovine AP isozyme will be only embryonic in origin and detectable at the beginning of embryonic transcription.
RATIONALE

The purpose of this thesis is to characterize AP expression at both the mRNA and protein levels in the preattachment bovine embryo, and determine if expression can be used to mark onset of embryonic transcription and provide an indirect measure of embryo health.

To date, the APs have been most extensively studied in adult tissues in mice and humans. Bovine tissues are not well characterized, and recently two new bovine AP isozymes have been discovered. Chapter 1 investigates AP mRNA and protein expression in adult bovine tissues for comparison to known expression patterns in mice and humans, and to lay the foundation for embryo studies. Among mammals, only the murine embryo has been studied for AP expression, and controversy exists with respect to AP protein localization in murine embryos. Chapter 2 investigates bovine AP expression during preattachment embryo development at mRNA and protein levels. While the detection of mRNA by RT-PCR distinguishes between AP isozymes, it does not show whether functional protein is produced. The assays of AP activity, while highly sensitive, quick and relatively simple to employ, do not allow for identification of individual isozymes. This limitation was found also in the mouse, and circumvented using heat incubation and inhibitory chemicals to inactivate the different isozymes. This approach was taken in Chapter 3 to determine which bovine isozymes are expressed as active protein in tissues and embryos.

An ongoing debate in embryology exists as to whether bovine embryo culture in simple media mimics in vivo embryo development. With use of new reproductive
technologies becoming more widespread and increasingly requiring in vitro manipulation, the impact of in vitro procedures on embryo health and development must be investigated. In Chapter 4, embryos produced in vivo were examined for AP isozyme expression to determine if the culture system affects expression of AP mRNA. This is important because even subtle changes in mRNA expression could have drastic effects on morphology and development resulting in lower embryo viability.

In the cow, there is more than one AP isozyme found at the blastocyst stage. The hypothesis driving this thesis is that, in bovine preattachment embryos, at least one AP isozyme will be expressed from the embryonic genome only, and be detectable at the OET. This expression then could be used as an indirect measure of embryo health by marking the onset of embryonic genome transcription, a period in development that is required for subsequent development and embryo survival.
CHAPTER 1

ALKALINE PHOSPHATASE ISOZYME
EXPRESSION IN ADULT BOVINE TISSUES

INTRODUCTION

The alkaline phosphatases (APs) are a small family of enzymes usually localized to the cell-surface and expressed in a highly conserved manner across diverse species (McComb et al., 1979; Swarup et al., 1981; Kim and Wyckoff, 1989; Low and Saltiel, 1988; Millan, 1990). In the mouse, there is an embryonic AP (EAP) form expressed during preimplantation embryo development (Hahnel et al., 1990), then after implantation there is a change to the TNAP form. There also is expression of an intestinal form (IAP) in the intestine. A putative TSAP pseudogene exists in the murine system with a stop codon near the 3' end of the coding portion of the transcript (Manes et al., 1990). The expression of murine isozymes in adult tissues has been well described (McComb and Bowers, 1979; Harris, 1982; Millan, 1990; Hoshi et al., 1997). Adult and tumor AP expression in humans has been extensively studied (Fishman, 1974; Harris, 1982; Garattini et al., 1985; Millan, 1988; Millan and Manes, 1988; Millan, 1992). In bovine tissues, TNAP, IAP and one putative pseudogene have been reported to exist (Garattini et al., 1987; Hsu et al., 1987; Weissig et al., 1993). Recently two additional bovine isozymes have been identified in blastocysts (McDougall et al., 1998), but expression patterns of the bovine isozymes are not well characterized in either adult or embryonic tissues.

This study was undertaken to determine which of the four known bovine AP isozymes are expressed in various adult tissues for comparison to the expression patterns
and isozymes of APs in other species. This will establish a reference for the study of embryonic expression of these four isozymes.

MATERIALS AND METHODS

Total RNA Extraction from Adult Tissues:

Adult bovine liver, kidney, intestine, spleen, brain, thymus, placenta and testis were obtained at slaughter from cattle and flash frozen in liquid nitrogen. Bovine oviduct epithelial cells (BOEC) and cumulus cells were obtained during aspiration of cumulus-oocyte complexes (COCs) from slaughterhouse ovaries. Total RNA was extracted using TRIzol reagent as per the manufacturer’s specifications (see Appendix I for listing of chemicals and suppliers and Appendix II for recipes of solutions). Samples were quantified using a spectrophotometer and immediately reverse transcribed or held at -80°C until required.

Reverse Transcription:

Messenger RNA was reverse transcribed as follows. Total RNA (2-2.5 μg), 5 μl oligo d(T) primer (100 μg/ml) and 2 μl sdH2O were heated to 70°C for 10 minutes, cooled quickly on ice, and then 7.3 μl reverse transcription mix (containing 4 μl First Strand Buffer, 2 μl 0.1 M DTT, 1 μl 10 mM dNTPs and 0.3 μl RNAse inhibitor) was added. Samples were warmed to 46°C for 2 minutes before addition of 1 μl Superscript Reverse Transcriptase II, then incubated for 1 hour at 46°C. After incubation, 30 μl sdH2O was added to each sample and the samples were stored at -20°C until cDNA amplification.
Plasmid Preparation:

Bacteria (E. coli XLI Blue) containing plasmids (pBluescript) with cloned fragments of bovine IAP, TSAP2 and TSAP3 cDNA were available at the start of these experiments (provided by Dr. A. Hahnel, Dept. of Biomedical Sciences, University of Guelph). Bacteria were grown overnight at 37°C in 15 ml LB medium containing 100 µg/ml ampicillin, and plasmids were isolated using an alkaline lysis technique (Ausubel et al., 1994). Bacteria were pelleted by centrifugation at 12,000g for 15 minutes and the pellet lysed by suspension in 100 µl bacterial lysis buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0, 4 mg/ml lysozyme) and incubation at room temperature for 5 minutes. To this mixture, 200 µl 0.2 M NaOH plus 50 µl 20% (w/v) sodium dodecylsulfate was added and samples incubated on ice for 5 minutes, then 150 µl ice cold 3 M potassium acetate, pH 5.6, was added and samples vortexed for 10 seconds. For each 450 µl sample, 0.5 µl 20 mg/ml Proteinase K was added and the mixture incubated for 30 minutes at 37°C. Next, 1 µl DNAse free RNAse A (20 µg/ml) was added and the mixture was incubated at 37°C for at least 30 minutes. Samples were subjected to a standard DNA extraction procedure as follows: to each tube 125 µl 24:1 chloroform:isoamyl alcohol and 125 µl DNA grade phenol were added. Samples were spun in a microcentrifuge (at 12,000g) for 2 minutes and the top phase retained, then another 125 µl 24:1 chloroform:isoamyl alcohol was added and the samples were centrifuged for 2 minutes. Twice the volume of 100% ethanol was added to the retained top phase, followed by vortexing and incubation for 2 minutes at room temperature. The mixture was centrifuged for another 5 minutes and the pellet retained. The precipitate
was washed in 1 ml 70% ethanol, pelleted by centrifugation for 5 minutes, dried under vacuum for 5 minutes and resuspended in 20 µl low TE buffer.

**Polymerase Chain Reaction Amplification and Confirmation:**

**Polymerase Chain Reaction Amplification:**

Copy DNA obtained by reverse transcription was amplified by polymerase chain reaction (PCR) with primer pairs specific for each AP isozyme. Primers were designed from sequences obtained from the literature (IAP: Weissig et al., 1993; TNAP: Garattini et al., 1987) or from previous cloning in Dr. Hahnel’s laboratory (TSAP2 and TSAP3: McDougall et al., 1998). Table III shows primer pairs used to amplify specific AP isozyme cDNAs and control cDNA (actin control primer sequences were generously donated by Dr. A. Wildeman, Dept. Molecular Biology and Genetics, University of Guelph).

All samples for PCR amplification contained 5 µl of cDNA from 20 µl reverse transcription reaction and were amplified using a hot start as follows: 23 µl Mix 1 (containing 5 µl 10X PCR buffer, 1 µl 10 mM dNTPs, 1 µl 50 µM 5’ primer, 16 µl sdH2O) was added to each PCR tube; 5 µl cDNA was added and tubes placed at 80°C for 10 minutes. Then 22 µl Mix 2 (containing 5 µl 15 mM MgCl2, 1 µl 50 mM 3’ primer, 0.5 µl Taq polymerase, 16 µl sdH2O) was added, and samples were amplified for 40 cycles with denaturation at 94°C for 2 minutes, annealing at 57°C for 1 minute and extension at 72°C for 2 minutes. A final extension of 72°C for 10 minutes was performed to ensure full-length copies were produced. Resulting amplified cDNA was separated on a 2% agarose gel containing 0.5 µg/ml ethidium bromide and visualized with UV light.
Table III: Primer sequences used in the polymerase chain reaction amplification of cDNA from bovine tissues and embryos.

<table>
<thead>
<tr>
<th>Sequence to be amplified</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. ( \beta, \gamma ) Actin (left) ( \beta, \gamma ) Actin (right)</td>
<td>5'-ACTGGGCAGACATGGGAGAAGAT-3' ( \text{<strong>} ) 5'-TGCTCGAAGTCGACAGCAGTG-3' ( \text{</strong>} )</td>
</tr>
<tr>
<td>2. TNAP (left) TNAP (right)</td>
<td>5'-ACCGCTGACCACATCCCGTC-3' ( \text{<strong>} ) 5'-CGAGCTGGCTGACAGCTG-3' ( \text{</strong>} )</td>
</tr>
<tr>
<td>3. IAP (left) IAP (right)</td>
<td>5'-GGGTCAAGGGCAACTCAGAAGG-3' ( \text{<strong>} ) 5'-CATTCACACTGGCATCTGGG-3' ( \text{</strong>} )</td>
</tr>
<tr>
<td>4. TSAP2 (left) TSAP2 (right)</td>
<td>5'-GGTCAAGCATTGATGGAAGG-3' ( \text{<strong>} ) 5'-CTCCATTTCTGGTTTATGGC-3' ( \text{</strong>} )</td>
</tr>
<tr>
<td>5. TSAP3 (left) TSAP3 (right)</td>
<td>5'-TGAGGGGAGCTGAATGAGAAGC-3' ( \text{<strong>} ) 5'-TGTCTAGGGGACACTTGCAGT-3' ( \text{</strong>} )</td>
</tr>
</tbody>
</table>

* These primer sequences were obtained from Dr. A. Wildeman, Department of Molecular Biology and Genetics, University of Guelph, Guelph, Ontario, Canada.
1 Garattini et al., 1987
2 Weissig et al., 1993
3 McDougall et al., 1998
Restriction Endonuclease Digestion:

Amplicons from PCR reactions were verified using restriction endonuclease digestion. Each isozyme has a unique nucleotide sequence, and restriction endonucleases were chosen to differentially cleave amplicons depending on the isozyme amplified (Table IV; see Appendix III for AP sequences). Amplicons were recovered from PCR reactions by chloroform:phenol extraction and ethanol precipitation as follows: 100 μl DNA grade phenol and 100 μl 24:1 chloroform:isoamyl alcohol were added to the PCR product and mixed. Samples were then centrifuged for 15 minutes at room temperature and 12,000g in a tabletop microcentrifuge, the top aqueous phase was saved and the DNA precipitated with 1/10 volume 3 M sodium acetate and 2 volumes 100% ethanol. The DNA was allowed to precipitate overnight at -20°C, spun 15 minutes at room temperature, the pellet washed with 500 μl 70% ethanol, spun 15 minutes, and the pellet dried under vacuum for 5 minutes. The pellet was resuspended in 15 μl ddH₂O and subjected to restriction endonuclease digestion according to the manufacturer’s recommendations using 10 units of enzyme per 25 μl reaction. The resulting fragments were separated with a 2% agarose gel and visualized as above.

DNase Treatment:

Primers were specifically designed to span introns in known sequences providing an internal control for genomic DNA contamination. However, to further ensure that there was no contaminating DNA present, a DNase treatment was performed on three replicates of all tissue samples. To 5 μg of RNA, 50 μl of DNase buffer (containing 40
Table IV: Restriction endonucleases and resulting fragment sizes after digestion used to confirm the identity of amplicons from polymerase chain reaction amplification of cDNA from control tissues and embryos.

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>PvuII</th>
<th>HincII</th>
<th>Accl</th>
<th>HindIII</th>
<th>PstI</th>
<th>SmaI</th>
<th>KpnI</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAP</td>
<td>107+264</td>
<td>100+269</td>
<td>369</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TSAP2</td>
<td>109+262</td>
<td>--</td>
<td>104+267</td>
<td>371</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TSAP3</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>12+300</td>
<td>312</td>
<td>87+225</td>
</tr>
</tbody>
</table>

---=not used
mM Tris-HCl pH 7.9, 10 mM NaCl, 6 mM MgCl$_2$, 10 mM CaCl$_2$) and 5 µl RQ1 RNase free DNAse was added and incubated at 37°C for 15 minutes. Samples were extracted with 50 µl TRIS saturated phenol:chloroform as above, and precipitated in 1/5 vol 7.5 M ammonium acetate and 2.5 vol 100% ethanol for at least 30 minutes at -80°C, and then 2 hours at -20°C. The washed pellet was dried for 5 minutes under vacuum, then resuspended in 40 µl sdH$_2$O. Samples were overlaid with mineral oil and incubated for 5 minutes at 99°C to denature any remaining DNAse. The oil was then removed and samples reverse transcribed as described previously.

**Alkaline Phosphatase Enzyme Activity:**

**Paraffin-Embedded Tissue Preparation:**

Tissues were obtained from adult cattle at slaughter, fixed immediately for up to 12 hours in 4% paraformaldehyde, then embedded in paraffin wax using an automated embedder and standard procedure (Ausubel et al., 1994). Sections were cut at room temperature using a microtome at 7 µm thickness, floated on water and dried onto slides overnight at 37°C. Dried sections were dewaxed with three 2 minute soaks in xylenes and rehydrated through a series of 2 minute washes in 100% ethanol (twice), 95% ethanol, 90% ethanol, 70% ethanol, 50% ethanol, and two separate rinses in 3X PBS, then held in 1X PBS. Sections were equilibrated in 0.1 M Tris-HCl pH 10.0 for 10 minutes prior to AP protein activity assay.
**Frozen Tissue Preparation:**

Tissues were obtained from animals at slaughter, placed into OCT Embedding Medium, plunged into liquid nitrogen and stored at -80°C. Tissues were sectioned at 7 μm thickness at -20°C in a cryostat, post-fixed for 20 minutes with 4% paraformaldehyde at room temperature, washed twice in 3X PBS and then held in 1X PBS. Sections were equilibrated in 0.1 M Tris-HCl pH 10.0 for 10 minutes before AP protein activity assay.

**Cell Smear Preparation:**

Bovine oviduct epithelial cells were recovered from oviducts and cumulus cells were recovered by follicle aspiration. These cells were washed three times in PBS+0.1% PVP (PBS+PVP) by allowing cells to settle then removing the supernatant and replacing with fresh PBS+PVP. Settled cells (10 μl) were dotted onto positively charged slides and smeared. Cells were dried onto slides overnight at room temperature, fixed for 20 minutes in 4% paraformaldehyde, rinsed in 1X PBS and equilibrated in 0.1 M Tris-HCl pH 10.0 for 10 minutes before AP protein activity assay.

**Naphthol AS-MX Phosphate Assay:**

Alkaline phosphatase enzyme activity was determined using the azo-dye coupling technique outlined by Ziomek et al. (1990). Briefly, sections were equilibrated for 10 minutes in 0.1 M Tris-HCl pH 10.0. Colour reaction buffer was prepared directly before use from 0.25% alkaline solution Naphthol AS-MX Phosphate (see Appendix II). Sections were placed in the dark at room temperature for 15 minutes for colour reaction,
washed three times with 1X PBS, mounted with Aqua-Polymount, and examined for red fluorescent precipitate using a Leitz-Aristoplan fluorescence microscope (Wild Leitz GmbH, Werzlar, Germany) equipped with a rhodamine filter.

**NBT/BCIP Assay:**

Sections were equilibrated for 10 minutes in 0.1 M Tris-HCl pH 10.0, then equilibrated for another 10 minutes in NBT/BCIP buffer (Appendix II) without NBT/BCIP substrate. This buffer was then replaced with NBT/BCIP buffer containing substrate (Appendix II; 10 ml NBT/BCIP buffer plus 200 μl NBT/BCIP stock solution, made fresh before use). Sections were allowed to stain in the dark for 20 minutes at room temperature, then counterstained with a 5 second wash in 1% methyl green in PBS, rinsed three times in PBS and mounted in Aqua Polymount. Sections were visualized by light microscopy for the presence of blue-purple precipitate to measure AP activity.

**RESULTS**

**Plasmid Controls:**

Primer specificity was tested on plasmid controls before use on tissue samples. Plasmids were linearized, amplified by PCR with specific primer pairs (see Table III), and amplicons subjected to restriction endonuclease digestion. As shown in Figure 1, plasmid controls yielded the expected amplicons, as well as the predicted restriction endonuclease fragment patterns (see Table IV for predicted fragment sizes), confirming that the primers
FIGURE 1: Example of plasmid control, 2% agarose gel stained with ethidium bromide and visualized with UV light. Panel A shows intestinal alkaline phosphatase (IAP) containing plasmid that has been amplified using the polymerase chain reaction with primers specific for IAP (Lane 2) and TSAP3 (Lane 3). Lane 1 contains 100 base pair ladder. Panel B shows fragments obtained after restriction endonuclease digestion of the band from Panel A. Lane 4 is PvuII digested amplicon (264 and 107 bp); Lane 5 is HincII digested amplicon (269 and 100 bp); Lane 6 is Accl digested amplicon (369 bp, no cutting).
amplified the proper sequences and did not cross react with other isozymes. Plasmid controls were verified three times each using three different plasmid preparations.

**Alkaline Phosphatase mRNA Expression:**

Messenger RNA analysis was performed on mRNA from at least three different individuals, in three replicates of each tissue. The presence of actin mRNA was used to confirm success of extraction and reverse transcription reactions. Only samples that were positive for γ actin (277 bases) or β actin (441 bases) were used in subsequent PCR amplification of other isozymes. As this involved non-quantitative extraction and PCR methods, cDNA levels were variable and these results are qualitative, not quantitative.

The presence of TNAP in kidney and liver, and IAP in the adult intestine of cattle has been previously reported (Garattini et al., 1987; Hsu et al., 1987; Weissig et al., 1993). These results were confirmed and extended with the detection of TNAP amplification product from cDNA of bovine cumulus cells, thymus, placenta, brain tissue and BOEC (Figure 2). Bovine TSAP2 cDNA was detected in cDNA samples from cumulus cells, spleen and testis, and TSAP3 cDNA was found in samples from kidney, BOEC, spleen, thymus and testis. Surprisingly, there were no AP transcripts detected in submandibular gland preparations even though AP activity has been reported to be typical of tissues with high transmembrane activity (Harris, 1989). As can be seen in Figure 3, DNAse treatment of samples with amplicons larger than expected (approximately 600 bp) eliminated those amplicons. Primers were designed to span known intron sites conserved
FIGURE 2: Alkaline phosphatase (AP) mRNA expression in adult bovine tissues as determined by RT-PCR and visualized under UV light on a 2% agarose gel stained with ethidium bromide. Panel A=actin primers; B=tissue non-specific AP primers; C=intestinal AP primers; D=tissue specific AP 2 primers; E=tissue specific AP 3 primers. Lane 1=100 bp ladder; 2=cumulus cells; 3=bovine oviduct epithelial cells; 4=thymus; 5=placenta; 6=brain; 7=spleen; 8=testis; 9=submandibular gland.
FIGURE 3: Effect of DNAse treatment on mRNA from placental tissue prior to PCR. Amplicons were separated on a 2% agarose gel stained with ethidium bromide and visualized with UV light. Lane 1=100 bp ladder. Lanes 3 through 6 contain PCR amplicons from cDNA from untreated mRNA; Lanes 8 through 12 contain PCR amplicons from cDNA from DNAse treated mRNA. Lanes 2 and 8=actin primers; 3 and 9=intestinal alkaline phosphatase (AP) primers; 4 and 10=tissue specific AP 2 primers; 5 and 11=tissue specific AP 3 primers; 6 and 12=tissue non-specific AP primers. Note the absence of amplicons of approximately 600 bp in treated samples.
in mice and humans, so if there was DNA contamination present in mRNA preparations products significantly larger than from cDNA would be expected. Treatment of RNA extractions with DNase confirmed that these bands were derived from genomic DNA contamination and not from cDNA.

Alkaline Phosphatase Enzyme Expression:

Tissue sections (kidney, intestine and liver) and cells (BOEC and cumulus cells) subjected to either the azo-dye coupling technique or the NBT/BCIP substrate colour reaction exhibited identical patterns of staining (see Figure 4). Therefore, the majority of tissues were subjected only to the NBT/BCIP technique for AP activity detection as this technique gave sharper localization with reduced background over the azo-dye coupling technique for frozen and paraffin embedded tissues. Paraffin embedding is performed at approximately 65°C. When liver and kidney paraffin embedded sections were compared with frozen sections following staining it was found that frozen sections exhibited surface staining while paraffin embedded sections did not (see Figure 5). This suggested that bovine TNAP, an isozyme transcribed in both liver and kidney, is affected by the embedding process. Therefore further tests of localization were performed only with frozen tissue sections.

Enzyme Localization:

In the bovine liver, AP activity was present on all surfaces of hepatocytes (Figure 6A), as well as on a population of cells thought to be possibly "pit cells" (Ross et al.,
FIGURE 4: Alkaline phosphatase enzyme activity in bovine intestinal epithelium, a comparison of two different protein activity assays, Naphthol AS-MX Phosphate/Fast Red TR Salt and NBT/BCIP.

Panel A: Intestinal epithelium (X40 magnification) incubated with Naphthol AS-MX Phosphate and Fast Red TR Salt which forms a red precipitate visible with light microscopy.

Panel B: Intestinal epithelium (X40 magnification) incubated with Naphthol AS-MX Phosphate and Fast Red TR Salt. Under fluorescence with a rhodamine filter the precipitate fluoresces red.

Panel C: Intestinal epithelium (X40 magnification) incubated with NBT/BCIP, with the resultant precipitate being dark blue and more easily identifiable with less background.
FIGURE 5: Alkaline phosphatase enzyme activity in paraffin embedded and frozen sections. All sections are stained with the NBT/BCIP alkaline phosphatase protein activity assay. Tissues are shown at X40 magnification.

Panel A: Frozen section and paraffin section of liver incubated with NBT/BCIP after sectioning. Note the lack of staining in the paraffin embedded section.

Panel B: Frozen section and paraffin section of kidney incubated with NBT/BCIP after sectioning. Note the lack of staining in the paraffin embedded section.
FIGURE 6: Alkaline phosphatase enzyme activity in various adult bovine tissues frozen in liquid nitrogen in OCT embedding compound and cryosectioned. A=liver; B=kidney cortex; C=small intestine; D=spleen; E=thymus; F=testis; G=bovine oviduct epithelial cells; H=cumulus cells.
Kidney frozen sections (Figure 6B) showed AP activity on the epithelial lining of tubules, mainly of the proximal and distal convoluted tubules and loops of Henle and not in collecting ducts or glomeruli. In the bovine intestine, AP protein activity was predominantly localized to the brush border of the surface epithelium and did not extend into the crypts (see Figure 6C). In the spleen (Figure 6D), and thymus (Figure 6E), the majority of cells were negative for activity. In the spleen, the white pulp was characteristically intensely AP positive while the red pulp was negative. The testis showed a ring staining pattern, with the peritubular cells being positive (in agreement with Narisawa et al., 1992), while all other cells were negative (see Figure 6F). Both BOEC and cumulus cells were intensely AP activity positive over cell surfaces (see Figures 6G and 6H).

**DISCUSSION**

This study aimed to determine isozyme expression patterns of the four AP isozymes in normal adult bovine tissues. Intestinal AP mRNA was found in intestinal tissue as was described by Weissig et al. (1993), and as found in mouse (McComb et al., 1979; Manes et al., 1990; Millan, 1990), and humans (McComb et al., 1979; Henthorn et al., 1988; Harris 1989; Millan, 1990). Tissue non-specific AP was found in various tissues, including the liver and kidney (as described previously by Garattini et al., 1987 and Hsu et al., 1987), cumulus cells, BOEC, thymus, brain and placenta. In humans, the placenta expresses a distinct AP isozyme, PLAP (Millan, 1986; Millan, 1988; Harris, 1989), while in mouse, the placental form was found to be the TNAP isozyme (Terao and
Mintz, 1987; MacGregor et al., 1995). The bovine placenta expresses the TNAP form, like mice. Two additional TSAPs have been detected in adult cattle (McDougall et al., 1998), TSAP2 and TSAP3. Tissues expressing mRNA for these isozymes often also expressed TNAP mRNA, a phenomenon not described in other species. Tissue specific AP2 mRNA was detected in cumulus cells, spleen and testis, and TSAP3 mRNA was found in kidney, BOEC, spleen, thymus and testis. Tissues with high transmembrane activity have characteristically expressed mRNA for at least one AP isozyme, but submandibular gland did not contain mRNA for any of the AP isozymes, even though this tissue is characterized by high transmembrane activity.

The localization of substrate precipitate found in bovine tissues was found to agree with patterns found in mice and humans, with AP localized to the brush border of the intestinal epithelium, the epithelium of kidney tubules and hepatic cords. All these sites of activity are involved in transmembrane transport. Other positive cell types, like BOEC and cumulus cells, may have transmembrane activity, or AP may be involved in some other process in these cells. A complication in cattle is that tissues transcribe mRNAs for more than one AP isozyme, and the protein activity assay does not indicate which isozymes are translated to functional protein. Liver and intestine each exhibit mRNA for only one isozyme, TNAP and IAP respectively, so that it must be these isozymes that are being translated in these tissues. The decrease in AP protein activity upon paraffin embedding also suggests that TNAP and TSAPs may have different temperature sensitivities as do human and murine isozymes. This observation is further investigated in Chapter 3.
The original cloning of bovine TSAP2 and TSAP3 was from blastocysts. It was necessary to explore AP expression in bovine tissues to serve as controls for studies with bovine embryos. These results show that cattle do express several AP isozymes in a tissue-specific manner similar to the human and mouse, that TSAP2 and TSAP3 are not cloning artifacts and may be equivalent to mouse EAP, and that the APs should provide an interesting tool for studying gene expression in the preattachment bovine embryo.
CHAPTER 2
ALKALINE PHOSPHATASE ISOZYME
EXPRESSION IN BOVINE PREATACHMENT EMBRYOS

INTRODUCTION

In the mouse an embryonic AP (EAP) is expressed during preimplantation development (Hahnel et al., 1990) beginning at the time of the OET, but this isozyme does not appear to be required for successful embryo development (Hahnel et al., 1990; Narisawa et al., 1997). There is also a murine TNAP expressed after implantation (MacGregor et al., 1995). During preimplantation mouse embryogenesis, AP protein activity is localized to areas of cell-cell contact between blastomeres, with lack of activity reported on free surfaces of blastomeres (Ishiyama and Izquierdo, 1977; Johnson et al., 1977; Mulnard and Huygens, 1978; Izquierdo et al., 1980). This activity was detected as early as the late 2-cell or early 4-cell stage, but the basolateral localization and early expression were investigated in other studies with conflicting results (Solter et al., 1973; Vorbrodt et al., 1977).

The purpose of this study was to examine which of the four known bovine AP isozymes are expressed during preattachment embryogenesis at both the mRNA and protein levels, to examine the timing of expression, and to localize AP activity and compare it to that of the mouse. During the course of these experiments, it was found that TSAP2 and TSAP3 mRNAs are not constantly transcribed, and that their transcription might occur only during certain parts of the cell cycle. Due to small amounts of RNA present within embryos, large numbers of embryos are required for experiments.
Therefore, synchronizing agents were used to produce a higher proportion of embryos in the same part of the cell cycle. For this study, two cleavage inhibitors which work through interaction with microtubules were used: nocodazole which prevents polymerization, and taxol which prevents depolymerization of microtubules. An additional nuclear cell cycle arrester, aphidicolin, was used to confirm regulation of expression.

**MATERIALS AND METHODS**

**In Vitro Embryo Production:**

**Culture With Serum:**

Preattachment bovine embryos (presumptive zygote, 2-cell, 4-cell, 8-cell, 16-cell, morula, blastocyst, hatched blastocyst) were produced by standard in vitro culture techniques (Xu et al., 1992). All cultures were maintained at 38.5°C in 5% CO₂ in humidified air. Bovine ovaries were obtained at slaughter from local abattoirs and washed twice in PBS. Cumulus-oocyte complexes (COCs) were aspirated from follicles less than 1 cm diameter using an 18-gauge needle attached to a flask and vacuum pump, into modified Ham’s F-10 medium (see Appendix IV for recipes for all in vitro culture media), matured for 22 to 24 hours in in vitro maturation medium (IVM) in groups of 100 per 750 μl IVM, washed twice in HEPES, and placed in modified Tyrodes medium (in vitro fertilization medium, IVF), 100 COCs in 750 μl IVF. Sperm was prepared by thawing two 0.5 ml frozen straws at 38°C for 2 minutes followed by swim-up for 1 hour in Sperm TALP (modified Tyrodes medium; 0.2 ml extended sperm per 1 ml Sperm
TALP) and pelleting by centrifugation. Sperm was added to IVF wells at a concentration of 1 million sperm/ml and incubated with COCs for 18 to 20 hours. Presumptive zygotes were stripped of cumulus cells by gentle vortexing and co-cultured with bovine oviduct epithelial cells (BOEC) in groups of 100 per 750 μl in vitro culture medium (IVC). An additional 100 μl IVC was added on days 4 and 6 after insemination.

Bovine oviduct epithelial cells for co-culture were prepared the day of oocyte aspiration as follows: oviducts were trimmed of connective tissue and rinsed in Ham’s F-10 medium and the epithelium stripped by squeezing the oviduct with fine forceps forcing sheets of epithelial cells from the oviduct into 5 ml IVC medium. Large sheets of cells were broken by aspiration three times through a 22.5-gauge needle and then cultured in 1.5 ml IVC for 24 hours. Overnight, the sheets of cells rolled into cylinders. These cylinders were washed three times with IVC, and 10 to 20 cylinders added to IVC medium for 24 hours before embryo culture to condition the medium (Xu et al., 1992B).

**Culture Without Serum:**

In a preliminary study, embryos were cultured in protein and serum free medium by Ms. Semple (Animal Biotechnology Embryo Laboratory; University of Guelph; Hochi et al., 1996). Intestinal AP mRNA was found in a portion of embryos. To determine if serum-free culture conditions contributed to IAP expression, embryos were produced as above but cultured in IVC which was protein free (TCM-199 supplemented with 0.1% polyvinyl alcohol, 1 mM L-glutamine, and 1 mM sodium pyruvate) but contained BOEC in culture drops (Hochi et al., 1996).
Culture with Cell-Cycle Inhibitors:

To further study the gaps in the expression of TSAP2 and TSAP3 at the early stages of expression, and to synchronize embryos more closely with respect to cell-cycle, embryos were prevented from cleaving from 2-cell to 4-cell for a period of 12 hours. Cell-cycle inhibitors were prepared as a concentrate in DMSO at the following concentrations: Nocodazole at 50 μM; taxol at 5 mg/ml and aphidicolin at 1 mg/ml. These concentrates were diluted to working solutions with IVC medium to 10 μM nocodazole, 5 μg/ml taxol and 4 μg/ml aphidicolin just prior to use.

Embryos were produced in vitro as described above with serum-supplemented medium. As presumptive zygotes cleaved to the 2-cell stage, they were removed from culture, and placed in IVC supplemented with either 10 μM nocodazole, 5 μg/ml taxol or 4 μg/ml aphidicolin. Embryos were incubated in inhibitor for 12 hours at 38.5°C in 5% CO₂ in humidified air, then washed 6 times in HEPES and placed into culture with serum and BOEC until appropriate stage (4-, 8-, 16-cell, morula and blastocyst for nocodazole and taxol treatments) or age (equivalent to early 16-cell and blastocysts stages for aphidicolin treatment) of development. Groups of 20 embryos were washed three times in PBS+PVP and then frozen in 10 μl PBS+PVP for RT-PCR analysis and 3 replicates of 5 embryos each were prepared for AP protein activity assay.

Embryo Selection

Messenger RNA Analysis by Embryo Stage:

Embryos were removed from IVC at the appropriate time and stage of development. Stages included presumptive zygotes (up to 20 hpi), 2-cells (20-40 hpi),
4-cells (40-60 hpi), 8-cells (60-80 hpi), 16-cells (80-110 hpi), morulae (110-160 hpi) and blastocysts (160+ hpi). Hatched blastocysts were collected upon hatching from the zona pellucida, as determined by light microscopy, no later than day 9 post-insemination. Embryos were rinsed three times in phosphate-buffered saline (PBS) containing 0.1% polyvinylpyrrolidone (PBS+PVP), and embryos of like stage and age were pooled in groups of 20 and frozen immediately at -80°C.

Total RNA was extracted from pools of embryos as described in Hahnel et al. (1990). Briefly, frozen pools of embryos were combined with 100 µl embryo extraction buffer, 75 µl RNA grade phenol, 75 µl 24:1 chloroform:isoamyl alcohol and 5 µg E. coli ribosomal RNA. This mixture was vortexed for 1 minute and microcentrifuged at 12,000g for 15 minutes in 250 µl long microcentrifuge tubes. The top aqueous phase was extracted with 100 µl 24:1 chloroform:isoamyl alcohol by inversion for 30 seconds. Samples were again centrifuged for 15 minutes and the mRNA in the aqueous phase precipitated with 1/5 volume of 10 M ammonium acetate and 2.5 volumes 100% ethanol. The RNA was precipitated at -20°C overnight, centrifuged for 20 minutes, the pellet washed in 200 µl 70% ethanol and dried for a maximum of 5 minutes under vacuum. The pellet was resuspended in 4 µl sterile-deionized water (sdH₂O) and immediately reverse transcribed.

**Messenger RNA Analysis by Age:**

For further analysis, embryos were removed from culture every 10 hours and only like age embryos of the same stage pooled into groups of 20. Total RNA was extracted as above and reverse transcribed immediately.
Reverse Transcription:

Messenger RNA extracted from embryos and tissues was reverse transcribed as follows. To each sample of RNA from approximately 20 embryos or 2.5-5 μg total tissue RNA, 5 μl oligo d(T) primer (100 μg/ml) and sdH₂O to 16 μl was added. Samples were heated to 70°C for 10 minutes, cooled quickly on ice, then 7.3 μl reverse transcription mix (containing 4 μl First Strand Buffer, 2 μl 0.1 M DTT, 1 μl 10mM dNTPs and 0.3 μl RNase inhibitor) added. Samples were warmed to 45°C for 2 minutes before addition of 1 μl Superscript Reverse Transcriptase II, then incubated for 1 hour at 46°C. After incubation, 30 μl sdH₂O was added and samples stored at -20°C until cDNA amplification.

Polymerase Chain Reaction Amplification:

Polymerase chain reaction (PCR) amplification was performed on all frozen reverse transcribed samples as per Chapter 1, with actin, TNAP, IAP, TSAP2 and TSAP3 primers. Primers for bovine and mouse hypoxanthine phosphoribosyltransferase (left primer: 5'-GTAATGATCAGTCAACGGGGGAC-3'; right primer: 5'-CCAGCAAGCTTGGCAACCTTAACCA-3') were used for amplification as follows: 35 cycles of 94°C for 45 seconds, 54°C for 45 seconds, and 72°C for 80 seconds.

Alkaline Phosphatase Enzyme Activity Assay:

Naphthol AS-MX Phosphate and Fast Red TR Salt:

Alkaline phosphatase enzyme activity was investigated using the azo-dye coupling technique described by Ziomek et al. (1990). All steps were performed at room temperature. Embryos of each stage (2-, 4-, 8-, 16-cell, morula and blastocyst) were
removed from culture, rinsed three times in PBS+PVP, and fixed for one hour in 4% paraformaldehyde, pH 7.4. After fixing, embryos were rinsed three times in PBS+PVP, incubated for 15 minutes in the dark with 0.25% Naphthol AS-MX Phosphate (alkaline solution in 0.1 M Tris-HCl buffer, pH 10.0) containing 5-10 mg Fast Red TR Salt prepared fresh and filtered through a 0.22 µm filter immediately before use. Stained embryos were rinsed three times in PBS+PVP, mounted on slides using PBS and vaseline to seal the coverslips, and examined immediately with both a fluorescent light microscope (Leitz-Aristoplan equipped with rhodamine filter) and a scanning confocal microscope (Bio-Rad 600 MRD confocal microscope with rhodamine filter).

As a control for the AP protein activity assay, several different pH values were used to ensure that the protein activity seen was not due to activity of an acidic or neutral phosphatase. Tris buffer (0.1 M Tris) was prepared at pH 10, pH 7.4 and pH 5.6. A potassium acetate buffer was also prepared at pH 5.6 and pH 3.6. Embryos were subjected to the same AP protein activity assay described above with these buffers instead of 0.1 M Tris pH 10.0.

RESULTS

Alkaline Phosphatase mRNA Expression:

Although IAP cDNA was originally cloned from bovine blastocysts (McDougall et al., 1998), IAP mRNA was found only occasionally in blastocyst preparations. There was no relationship between culture with or without serum and expression of mRNA for
IAP, as can be seen in the gel in Figure 7. Blastocysts that were positive were from both culture systems, and negative results were found in blastocysts from both culture systems (see Table V).

Bovine TSAP2 was detected at the early 4-cell stage, between 40 and 50 hpi. Between 50 and 60 hpi, no amplification product was detected. Messenger RNA was detected again between 60 and 90 hpi at the 8- and 16-cell stages, but not between 90 and 100 hours at the 16-cell stage, and then detected again from 100 hours to the end of the period of in vitro culture (220 hpi) (see Figures 8 and 9). Bovine TSAP3 was detected first at the 60 hour 8-cell stage, with an "on-off" pattern similar to that of TSAP2. Between 70 and 80 hpi 8-cell stage embryos did not show cDNA amplification product. From 80 hpi to the end of in vitro culture (220 hours) TSAP3 amplification product was detected. Bovine TNAP was consistently detected in expanded and hatching blastocysts beginning at 200 hours post-insemination (hpi). The time-course experiment to map AP transcription was repeated three times. Therefore, there were three separate pools of embryos collected, and the RNA extracted and reverse transcribed resulting in a minimum of 60 embryos being examined for each transcript. In addition, each PCR reaction was repeated at least twice.

PCR-reagent controls showed that there was no contamination of PCR reactions. In samples where no band was seen on the gel from pools of 20 embryos, pools of 100-120 embryos were subjected to the same procedure to confirm the lack of isozyme mRNA. Positive reaction samples were subjected to restriction endonuclease digestion.
FIGURE 7: A 2% agarose gel stained with ethidium bromide and visualized using ultraviolet light of intestinal alkaline phosphatase cDNA amplified by polymerase chain reaction from reverse transcribed mRNA of blastocysts cultured in vitro with or without heat inactivated steer serum. Lane 1=100 bp ladder; Lanes 2, 3, 4 and 5=blastocysts cultured without serum; Lanes 6, 7, 8 and 9=blastocysts cultured with serum; Lane 10=positive control, intestinal cDNA; Lane 11=negative control (no cDNA).
Table V: Intestinal alkaline phosphatase (IAP) mRNA expression: A comparison between in vitro embryos produced with and without serum supplemented culture medium.

<table>
<thead>
<tr>
<th>Culture with serum</th>
<th>Culture without serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAP mRNA +</td>
<td>IAP mRNA -</td>
</tr>
<tr>
<td>n=31</td>
<td>n=91</td>
</tr>
<tr>
<td>total n=122</td>
<td></td>
</tr>
</tbody>
</table>

n=number of individual embryos studied
FIGURE 8: Alkaline phosphatase (AP) isozyme mRNA expression in preattachment bovine embryos as determined by RT-PCR (2% agarose gel stained with ethidium bromide and visualized with UV light). Panel A=actin controls; Panel B=tissue non-specific AP; Panel C=intestinal AP; Panel D=tissue specific AP 2; Panel E=tissue specific AP 3. Lane 1=100 bp ladder; 2=ocytes; 3=2-cell; 4=4-cell; 5=8-cell; 6=16-cell; 7=compact morula; 8=blastocyst; 9=hatched blastocyst; 10=positive PCR controls; 11=negative PCR controls.
FIGURE 9: Alkaline phosphatase (AP) isozyme mRNA expression by time and stage of development of in vitro produced embryos as determined by RT-PCR. Note the "on-off" pattern for tissue specific APs 2 and 3. Gels are 2% agarose stained with ethidium bromide and visualized with ultraviolet light. Panel A: actin positive controls; Panel B: tissue specific AP 2; Panel C: tissue specific AP 3. Lane 1=100 bp ladder; 2=40-50 hour 4-cell embryos; 3=50-60 hour 4-cell embryos; 4=60-70 hour 8-cell embryos; 5=70-80 hour 8-cell embryos; 6=80-90 hour 16-cell embryos; 7=90-100 hour 16-cell embryos; 8=100-110 hour 16-cell embryos; 9=110-120 hour 16-cell embryos.
Alkaline Phosphatase mRNA Expression in Cell-Cycle Arrested Embryos:

Embryo mortality was low in nocodazole and taxol treated embryos. Aphidicolin treated embryos appeared to cleave relatively normally to the morula stage, when many attempted compaction and cavitation, but no embryos formed morphologically healthy looking blastocysts. Nocodazole treated embryos expressed actin and HPRT at all stages of development, but expression of TSAP2 and TSAP3 mRNA was not detected until the blastocyst stage (see Figure 10). Nocodazole appeared to have a specific effect on TSAP2 and TSAP3 transcription, but it was not clear it was through inhibition of the cell cycle. Therefore the effect of other inhibitors were examined. Taxol treated embryos expressed HPRT at all stages, but actin message was not detectable in any samples. Message for TSAP2 and TSAP3 was detected only at the very late blastocyst stage (see Figure 11). Aphidicolin treated embryos expressed actin, HPRT, TSAP2 and TSAP3 mRNA at the equivalent time of development of early 16-cell embryos and blastocysts (see Figure 12). Treatment with aphidicolin resulted in poor embryo morphology and weak but positive cDNA signals after amplification in many samples. Either nocodazole and taxol inhibit the ability to detect specific mRNA, or these transcripts are sensitive to microtubule disruption by increased turnover or decreased transcription, or both. These experiments were performed in three replicates of 20 embryos each from three separate in vitro cultures. Each PCR was repeated at least twice to confirm PCR results.
FIGURE 10: Alkaline phosphatase mRNA expression in embryos treated with 10 μM nocodazole for 12 hours. 1=100 bp ladder; 2=4-cell embryos; 3=8-cell embryos; 4=16-cell embryos; 5=morula stage embryos; 6=blastocysts; 7=positive PCR control; 8=negative PCR control. Panel A=actin controls; Panel B=hypoxanthine phosphoribosyltransferase controls; Panel C=tissue specific alkaline phosphatase 2; Panel D=tissue specific alkaline phosphatase 3.
FIGURE 11: Alkaline phosphatase mRNA expression in embryos treated for 12 hours with 5 μg/ml taxol. 1=100 bp ladder; 2=4-cell embryos; 3=8-cell embryos; 4=16-cell embryos; 5=morula; 6=blastocysts; 7=positive PCR control; 8=negative PCR control. Panel A=actin; Panel B=hypoxanthine phosphoribosyltransferase; Panel C=tissue specific alkaline phosphatase 2; Panel D=tissue specific alkaline phosphatase 3.
FIGURE 12: Alkaline phosphatase mRNA expression in embryos treated for 12 hours with 4 µg/ml aphidicolin. TSAP2=tissue specific alkaline phosphatase 2; TSAP3=tissue specific alkaline phosphatase 3; HPRT=hypoxanthine phosphoribosyltransferase; 1=100 bp ladder; 2=16-cell equivalent; 3=blastocyst equivalent; 4=negative PCR control (no cDNA).
Alkaline Phosphatase Enzyme Activity:

Activity was present in all stages of preattachment bovine embryos (see Figure 13). Cumulus cells were also AP activity positive. Confocal microscopic localization of the precipitate shows activity mainly between blastomeres of 2- and 4-cell embryos. The amount of signal appeared to decrease from the 8- to 16-cell stages of development. At the morula stage (Figure 13F), the precipitate appears to be intracellular, and within only outer blastomeres. Interblastomeric areas of trophoderm cells of blastocysts were strongly positive, and there was no signal associated with the ICM.

Embryos treated for phosphatase activity in buffers of more acidic pH showed little (pH 7.4) or no (pH <7.4) staining (Figure 14). The APs have been shown to exhibit low levels of protein activity at pH 7.4, but not at more acidic pH (Fishman, 1974; McPherson, 1990). Expression at pH 7.4 was more diffuse than at pH 10.0, suggesting the possibility of an active neutral phosphatase since the expression pattern did not coincide with the pattern of APs at pH 10.0.

Alkaline Phosphatase Enzyme Activity in Cell-Cycle Arrested Embryos:

Nocodazole treatment did not affect protein expression significantly, although it appeared that there was less activity at the 16-cell, morula and blastocyst stages. Up to the 8-cell stage, expression mimicked that in control embryos, with protein localized interblastomERICally. Activity decreased in the 16-cell stage and morula, and stayed low in blastocysts (see Figures 15A through 15E).
FIGURE 13: Scanning confocal images of alkaline phosphatase enzyme activity in preattachment bovine embryos to the blastocyst stage after Naphthol AS-MX Phosphate/Fast Red TR Salt activity assay staining. A=unfertilized oocyte; B=2-cell embryo; C=4-cell embryo; D=8-cell embryo; E=16-cell embryo; F=compacted morula; G=trophectoderm of expanded blastocyst; H=section through inner cell mass region of expanded blastocyst. Note the positive activity of cumulus cells in A surrounding the oocyte, and the interblastomeric localization of protein activity in B and C. The pattern in F appears to be intracellular in the outer located blastomeres. The inner cell mass itself was negative but obscured by trophectoderm signal in H. White colour denotes highest protein activity, while red indicates areas of very low activity.
FIGURE 14: Alkaline phosphatase enzyme activity in blastocysts subjected to Naphthol AS-MX Phosphate/Fast Red TR Salt assay at pH 5.6 (Panel A), pH 7.4 (Panel B) and pH 10.0 (Panel C). Lowest activity is seen as red, highest activity is seen as yellow-white.
FIGURE 15: Alkaline phosphatase enzyme activity as visualized with scanning confocal microscopy after Naphthol AS-MX Phosphate/Fast Red TR Salt activity assay in embryos treated for 12 hours with 10 μm nocodazole (A through E) or 5 μg/ml taxol (F through J). White colour denotes areas of high protein activity, yellow is moderate and red is low protein activity. A and F=4-cell embryos; B and G=8-cell embryos; C and H=16-cell embryos; D and I=compact morulae; E and J=trophectoderm layer of expanded blastocysts (note the lack of AP enzyme activity compared with the blastocyst in Figure 13).
Taxol treated embryos displayed a marked decrease in AP protein activity at all stages when compared to control and nocodazole treated embryos (see Figures 15F through 15J). Activity was located diffusely over blastomeres, and not in interblastomeric regions. There was no apparent decrease in signal at the 8-cell stage, and no apparent increase in signal at the morula and blastocyst stages as was found in controls and nocodazole treated embryos.

Embryos that were treated with aphidicolin were not examined for AP protein activity due to poor morphology after culture; most embryos were fragmented, and the zona pellucida was extremely fragile, which resulted in lysis during the staining protocol.

**DISCUSSION**

**Alkaline Phosphatase Transcription:**

This study aimed to determine the expression patterns for isozymes of the AP family in bovine in vitro produced embryos. Messages for four distinct isozymes are present during preattachment embryo development, each with its own characteristic expression pattern.

Bovine TSAP2 and TSAP3 are the earliest AP genes to be transcribed in bovine development. General transcription from the embryonic genome has been detected at very low levels as early as the 2-cell stage by de novo $^3$H-uridine incorporation (Plante et al., 1994; Viuff et al., 1996). There is a major burst of transcription at the 8-16-cell stage that has been shown through α-amanitin sensitivity to be required for continued development (Telford et al., 1990). There is rRNA transcription at the 4-cell stage in
cattle (Bilodeau-Goeseels and Schultz, 1997). Bovine TSAP2 is the only other specific gene known to be transcribed prior to the burst of transcription at the 8-cell stage, and bovine TSAP3 is one of the few specific genes whose transcription is known to coincide with the bovine OET. Thus, these APs may be useful for study of regulation of the OET in cattle.

Both TSAP2 and TSAP3 exhibited a curious "on-off" pattern of early transcription between 40 hpi and 110 hpi in in vitro produced embryos, with gaps of approximately 10 hours where no mRNA was detected. It is possible that these isozymes exhibit cell cycle dependent transcription, a dependency which is either lost, or apparently lost, with accumulation of asynchronously dividing cells. It is also possible that mRNA levels during this time are at, or just above, the level of detection, resulting in seemingly cyclic expression patterns.

Cell cycle synchronizing drugs were used to test the dependence of TSAP2 and TSAP3 transcription on cell cycle. Nocodazole and taxol both successfully inhibited cleavage with a minimum of embryo mortality. However, rather than observing the "on-off" pattern after release, mRNA expression for TSAP2 and TSAP3 was delayed until the blastocyst stage. Protein expression was decreased at the morula and blastocyst stages of nocodazole treated embryos and in taxol treated embryos of all stages. The lack of mRNA expression for TSAP2 and TSAP3 until blastocyst stage could be due to several factors. First, these drugs may affect accessibility of the transcripts to RT enzymes or primers. Second, these inhibitors could destabilize message so that mRNA is degraded at a faster rate. This could be directly, or, more likely, through their effects on normal
microtubule assembly and disassembly. The possibility of faster turnover due to increased translation is unlikely as protein levels did not seem to increase with inhibitor treatment, although protein may be produced and not post-translationally modified to its active form. Third, these inhibitors may act to suppress transcription of these genes. It is notable that not all mRNA species studied were similarly affected. Nocodazole affected TSAP2 and TSAP3, but not actin or HPRT. Taxol affected TSAP2 and TSAP3, as well as actin, but not HPRT. Changes in AP activity patterns corresponded with delayed transcription and loss of maternal protein stores. These results are suggestive of a role for microtubules in TSAP2 and TSAP3 expression separate from their role in the cell cycle. To confirm that any drug which blocks the cell cycle would not have the same effect, embryos were treated with aphidicolin at the same stage. Aphidicolin has no known effect on microtubules, but blocks DNA synthesis. Embryos treated with aphidicolin did not appear to have any disruption in TSAP2 and TSAP3 expression. Schultz (1993) has shown that nocodazole will prevent zygotic genome activation in the mouse while aphidicolin will not. These results, and those of Schultz (1993), suggest that there are embryonic transcripts that are sensitive to microtubule disruption. However, in this study, microtubule inhibitors did not appear to affect development significantly.

The appearance of IAP mRNA expression in a portion of blastocysts, regardless of culture with or without serum, is possibly due to timing of differentiation of endoderm within the embryo. Intestinal AP is expressed exclusively in normal adult bovine intestinal epithelium, an endodermal-derived tissue. It is possible that IAP mRNA is seen only in those blastocysts which have endoderm present. As endoderm is produced after
cavitation, the end point of this experiment would coincide very closely with the onset of endoderm differentiation, so in some samples endodermal cells may exist and be expressing IAP mRNA while in others the endodermal cell population may not yet be established.

Alkaline Phosphatase Enzyme Activity:

Active AP protein was detected using a colour substrate staining technique in which a substrate for AP is digested by active protein and results in a coloured precipitate deposited in the area of active protein. Both substrate assays, NBT/BCIP and Naphthol AS-MX Phosphate/Fast Red TR Salt, gave the same localization, but the background for the latter was higher, especially when visualized with fluorescence microscopy. The NBT/BCIP assay yielded a dark blue/purple precipitate which was easily identified, so that this technique was used for tissues and heat and chemical inactivation experiments described in Chapter 3. For embryos, however, the localization of active AP protein was difficult to determine when using NBT/BCIP because areas between blastomeres were obscured by any surface staining when visualized by light microscopy. For protein localization studies Naphthol AS-MX Phosphate/Fast Red TR Salt was used and visualized using a scanning confocal microscope with an argon laser. The NBT/BCIP colour reaction is widely used in immunohistochemical staining protocols to determine the localization of an AP-conjugated antibody bound to antigen. In this protocol incubation with levamisole is commonly used to block expression of native APs. The procedure used here is based on the same principle that active AP will produce a precipitate from the
NBT/BCIP substrate, only the active AP is native to the tissue and not supplied as an antibody conjugated enzyme.

Alkaline phosphatase protein activity was detected in both immature and mature oocytes. Transcription of AP was found only in immature oocytes in COCs. The presence of bovine TNAP and TSAP2 mRNA in isolated cumulus cells was revealed by RT-PCR. Cumulus cells could not be separated reliably from immature oocytes and therefore it was impossible to determine which isozymes contribute to the positive activity seen in mature COCs. It is well documented that cumulus cells surrounding the oocyte aid in the development and the survival of the oocyte (Zhang et al., 1995), therefore it is possible that cumulus cells may have an effect on the production of AP RNA in the immature oocyte. The maturing oocyte may also transcribe at least one AP isozyme.

These experiments suggest that AP activity observed in oocytes, 2-, 4- and 8-cell embryos is due to active maternally derived protein, with protein turnover or specific OET-related degradation causing decreased signal at the 16-cell stage. Subsequent protein synthesis from translation of either or both TSAP2 and TSAP3 is then responsible for AP activity beginning at the 16-cell and morula stages of development. The protein activity is located intracellularly at the morula stage and only within outer blastomeres. This is possibly protein being transported to the cell surface for insertion into the cell membrane. It appears that either the central blastomeres do not transcribe AP genes, or do not translate AP mRNA. By the blastocyst stage expression of either or both TSAP2 and TSAP3 is localized to the trophectoderm only. These results suggest that expression of TSAP2 and TSAP3 is sensitive to cell position and is part of the first differentiation of cell
types in the bovine embryo. They are good candidates for studies of regulation of expression of AP isozymes both across tissue types and in embryogenesis.
CHAPTER 3
INHIBITION AND HEAT INACTIVATION
OF BOVINE ALKALINE PHOSPHATASE ENZYMES

INTRODUCTION

In bovine oocytes and early cleavage stage embryos, functional AP protein is detected, but not the corresponding mRNA. After the 4-cell stage, there is mRNA for TSAP2 and TSAP3, as well as AP activity. Activity assays do not distinguish between isozymes, and it is possible that not all AP mRNAs are translated or form functional protein. Therefore, a technique that identifies the isozymes responsible for activity was needed for AP expression patterns in preattachment bovine embryos to be fully defined. One of the characteristics of APs examined to date is that they exhibit different sensitivities to temperature and chemical inhibitors. The major difference lies in the behaviour of the TSAPs compared with TNAP. In humans and mice, TNAP is inactivated by temperatures below 60°C and incubation in L-homoarginine (HOM) and levamisole (LEV) (Harris, 1989; Lepire and Ziomek, 1989; Millan, 1990). The human and mouse TSAPs are thermal tolerant, and usually more sensitive to L-phenylalanine (PHE) (Harris, 1989; Lepire and Ziomek, 1989; Millan, 1990). Table VI summarizes inhibitors and temperatures required to inactivate the different known AP isozymes. Individual TSAPs may be distinguished by temperature incubation in the mouse, with EAP requiring 70°C for inactivation while IAP requires 95°C. In humans, GCAP and IAP are not distinguishable using temperature incubation. Chemical inactivation with PHE will distinguish mouse EAP from IAP, but not human TSAPs. Levamisole will distinguish IAP, PLAP and GCAP in the human (Table VI). As well, EDTA has been found to be
Table VI: Temperature (°C) and concentrations (mM) of three alkaline phosphatase (AP) protein inhibitors required to inactivate mouse (m) and human (h) APs. TNAP=tissue non-specific AP; IAP=intestinal AP; PLAP=placental AP; GCAP=germ cell AP; EAP=embryonic AP; LEV=levamisole; PHE=L-phenylalanine; HOM=L-homoarginine.

<table>
<thead>
<tr>
<th>AP isozyme</th>
<th>Temp</th>
<th>LEV</th>
<th>PHE</th>
<th>HOM</th>
</tr>
</thead>
<tbody>
<tr>
<td>mEAP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>95</td>
<td>&gt;5</td>
<td>50</td>
<td>&gt;20</td>
</tr>
<tr>
<td>mIAP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70</td>
<td>&gt;5</td>
<td>20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>mTNAP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60</td>
<td>1</td>
<td>40</td>
<td>&lt;10</td>
</tr>
<tr>
<td>hIAP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60</td>
<td>6.8</td>
<td>0.8</td>
<td>40</td>
</tr>
<tr>
<td>hPLAP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;65</td>
<td>1.7</td>
<td>1.1</td>
<td>&gt;50</td>
</tr>
<tr>
<td>hGCAP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60</td>
<td>2.7</td>
<td>0.8</td>
<td>36</td>
</tr>
<tr>
<td>hTNAP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;56</td>
<td>0.03</td>
<td>31</td>
<td>2.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Lepire and Ziomek, 1989
<sup>b</sup>Harris, 1989
a potent TNAP inhibitor in both mice and humans by chelating divalent ions required for activity (Harris, 1989; Lepire and Ziomek, 1989; Millan 1990).

This study was undertaken to determine if temperature tolerance or chemical inhibitors could be used to separate activity due to the different bovine AP isozymes in adult tissues and preattachment embryos.

**MATERIALS AND METHODS**

**Tissues:**

**Preparation:**

Adult bovine tissues (liver, kidney, intestine, spleen, testis) were recovered at slaughter and flash frozen in OCT embedding compound in liquid nitrogen and stored at -80°C until cryosectioning. Tissues were sectioned at 7 μm thickness using a cryostat at -20°C. Sections were mounted on slides and fixed 20 minutes with 4% paraformaldehyde, washed three times in PBS and held in PBS for treatment.

Cumulus cells and bovine oviduct epithelial cells (BOEC) were recovered from in vitro culture and washed three times in PBS+PVP and smeared on slides. Cells were allowed to dry overnight and fixed for 20 minutes in 4% paraformaldehyde and held in PBS until treated.

**Heat Inactivation:**

Tissue sections were incubated for 1 hour in PBS in an oven (for temperatures less than 80°C) or water bath (for temperatures over 80°C) at temperatures ranging from room
temperature to 99°C in 5°C increments. Slides were removed after one hour, rinsed in PBS, tissue sections equilibrated for 10 minutes in 0.1 M Tris pH 10.0 and subjected to the NBT/BCIP AP activity assay as described previously (see Chapter 1).

**Chemical Inactivation:**

Inhibitors were prepared first as 500 mM stock solutions in PBS, and these were further diluted to working solutions in 0.1 M Tris pH 10.0 (see Appendix V) directly before use. Working inhibitor solutions included levamisole (LEV), L-phenylalanine (PHE), L-homoarginine (HOM), and EDTA each at 50 mM, 25 mM, 10 mM, 5 mM and 1 mM concentrations. Tissue sections on slides were incubated for one hour in 0.1 M Tris pH 10.0 containing an inhibitor. Alkaline phosphatase activity was then determined by incubation for 20 minutes in each inhibitor at the above concentrations in 0.1 M Tris pH 10.0 containing NBT/BCIP substrate (200 µl substrate per 10 mL inhibitor plus buffer) at room temperature in the dark. Sections were rinsed three times in PBS, mounted with Aqua-Polymount, and observed under a light microscope. Scoring of tissue sections was considered positive if there was cell-specific blue precipitate formed, and negative only if there was no precipitate present.

**Embryos:**

**Preparation:**

Embryos were produced by in vitro maturation, fertilization and culture as discussed previously in Chapter 2. Embryos were removed from culture at specific stages
of development (presumptive zygote, 2-cell, 4-cell, 8-cell, 16-cell, morula and blastocyst) and rinsed three times in PBS+PVP, fixed in 4% paraformaldehyde for 1 hour, washed three times in PBS+PVP and held in PBS+PVP until treatment.

**Heat Inactivation:**

Results from tissue controls showed that incubation at 70°C would distinguish TNAP from the TSAPs, so embryos were incubated at this temperature for 1 hour in 200 µl PBS in capped microcentrifuge tubes to prevent evaporation. After 1 hour, embryos were recovered from tubes, washed three times in PBS+PVP and equilibrated for 10 minutes in 0.1 M Tris pH 10.0 and subjected to the NBT/BCIP AP protein activity assay as described previously (see Chapter 2). Embryos were then ranked as positive or negative by presence or absence of blue precipitate as seen by light microscopy.

**Chemical Inactivation:**

Results from adult tissue controls showed that incubation in 50, 25 or 10 mM PHE and HOM and 10, 5 or 1 mM LEV in 0.1 M Tris pH 10.0 for one hour would provide a pattern of inactivation for AP isozymes. Embryos were incubated in inhibitor alone in 0.1 M Tris pH 10.0 for one hour, then for 20 minutes in 0.1 M Tris pH 10.0 plus inhibitor containing NBT/BCIP substrate as described above. Embryos were washed three times in PBS+PVP, mounted on slides and scored as above.
RESULTS

Tissues:

Heat Inactivation:

Each tissue type was examined in three replicates of five tissue sections each from at least three different individuals. Bovine tissues that expressed only TSAP mRNA were found to require higher temperature of incubation to remove all protein activity (see Table VII and Figure 16). Intestinal epithelium remained AP activity positive up to 90°C. Similarly, bovine spleen, which expresses mRNA for TSAP2 and TSAP3, also required incubation for one hour at 90°C to eradicate AP activity. Liver, which is positive for TNAP mRNA only (see Chapter 1), required incubation of at least 65°C to produce negative results. Kidney, BOEC and cumulus cells transcribe a combination of TNAP and TSAPs as determined by RT-PCR (Chapter 1), and thus could express both proteins. These enzymes acted more like TNAP than TSAPs in the heat inactivation assay, requiring incubation at a temperature similar to that of liver for inactivation of AP protein. Samples which transcribed TNAP were heat inactivated between 60°C and 65°C, but any differences did not correlate with second isozyme transcribed.

Chemical Inactivation:

Replications for this study are as described above. Kidney, liver and intestinal tissues were tested for AP protein activity at 50, 25, 10, 5 and 1 mM concentrations of EDTA, LEV, HOM and PHE. All three tissues were rendered AP activity negative by similar concentrations of EDTA, showing that EDTA was not useful as a diagnostic
Table VII: Inhibitor concentrations (mM) and temperature (°C) required to inactivate alkaline phosphatase (AP) enzyme activity in adult bovine tissues with a one hour incubation. BOEC=bovine oviduct epithelial cells; PHE=L-phenylalanine; HOM=L-homoarginine; LEV=levamisole; IAP=intestinal AP; TSAP2=tissue specific AP 2; TSAP3=tissue specific AP 3; TNAP=tissue non-specific AP.

<table>
<thead>
<tr>
<th>Tissue (n)(^d)</th>
<th>mRNA(^a)</th>
<th>Temp</th>
<th>PHE</th>
<th>HOM</th>
<th>LEV</th>
<th>AP(d)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine (45)</td>
<td>IAP</td>
<td>90</td>
<td>50</td>
<td>&gt;50</td>
<td>10</td>
<td>IAP</td>
</tr>
<tr>
<td>Liver (45)</td>
<td>TNAP</td>
<td>65</td>
<td>&gt;50</td>
<td>25</td>
<td>&lt;1</td>
<td>TNAP</td>
</tr>
<tr>
<td>Kidney (45)</td>
<td>TNAP</td>
<td>60</td>
<td>50</td>
<td>25</td>
<td>&lt;1</td>
<td>TNAP, TSAP(^c)</td>
</tr>
<tr>
<td>BOEC (45)</td>
<td>TNAP</td>
<td>65</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>1</td>
<td>TNAP, TSAP(^c)</td>
</tr>
<tr>
<td>BOEC (45)</td>
<td>TSAP3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cumulus (45)</td>
<td>TNAP</td>
<td>60</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>5</td>
<td>TNAP, TSAP(^c)</td>
</tr>
<tr>
<td>Cumulus (45)</td>
<td>TSAP2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen (45)</td>
<td>TSAP2</td>
<td>90</td>
<td>25</td>
<td>50</td>
<td>5</td>
<td>TSAP</td>
</tr>
<tr>
<td>Spleen (45)</td>
<td>TSAP3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)alkaline phosphatase mRNA present as determined in Chapter 1 by RT-PCR
\(^b\)alkaline phosphatase protein determined to be present using these data
\(^c\)values indicate that these tissue APs behave more like liver (TNAP) suggesting that if the TSAP form is present it is in small amounts or the presence of heterodimers may produce differential inhibitor concentration behaviour. Temperature data indicate a TNAP form.
\(^d\)number of sections/slides examined for each treatment
FIGURE 16: The effects of heat incubation of alkaline phosphatase (AP) enzyme activity in intestine and liver as seen with light microscopy (100X magnification) after NBT/BCIP AP activity assay. Panels A, B and C are intestinal epithelium incubated at room temperature, 50°C and 100°C, respectively. Panels D, E and F are liver incubated at 55°C, 60°C and 65°C, respectively. Panels C, E and F were considered negative.
inhibitor for bovine APs. Levamisole appeared to be a potent TNAP inhibitor by effectively blocking all AP activity in liver at 1 mM concentration. However, LEV did not inhibit IAP protein activity until 10 mM concentration, suggesting that LEV might be useful for distinguishing TSAP from TNAP. Kidney, which may express a combination of TNAP and TSAP3, behaved similarly to TNAP with LEV inhibition. Similarly, HOM was useful for distinguishing TNAP (25 mM) from IAP (>50 mM). However, kidney behaved as IAP when incubated with HOM. See Table VII for a summary of concentrations of inhibitors required to inhibit AP protein activity in tissues, and Figure 17 for examples of kidney and intestine incubated with PHE and HOM.

Once a diagnostic range was established for each inhibitor, spleen, BOEC and cumulus cells were also tested. The concentrations used were 10, 5 and 1 mM LEV, and 50, 25 and 10 mM HOM and PHE. Oviduct epithelial cells transcribe the same mRNAs as kidney, TNAP and TSAP3, and presented a similar, although not identical, inhibitor profile as kidney, requiring high levels of PHE and HOM and low levels of LEV for AP inhibition. Cumulus cells, which transcribe mRNA for TNAP and TSAP2, required high concentrations of PHE and HOM, but also relatively high levels of LEV for inhibition of AP protein. Spleen transcribes only TSAP mRNA, TSAP2 and TSAP3 with a profile similar to intestine, requiring high LEV (5 mM) for inhibition, but dissimilar from all other samples with low PHE (25 mM) requirements for inhibition.
FIGURE 17: Inhibition of alkaline phosphatase (AP) enzyme activity by one hour incubation in various concentrations of chemical inhibitors as seen by light microscopy of tissues subjected to the NBT/BCIP AP activity assay. Panels A, B and C are kidney incubated in 10, 25 and 50 mM L-phenylalanine, respectively; Panels D, E and F are kidney incubated in 10, 25, and 50 mM L-homoarginine, respectively; Panels G, H and I are intestine incubated in 10, 25 and 50 mM L-homoarginine, respectively.
Embryos:

Heat Inactivation:

Embryos were incubated at 70°C for 1 hour since it was found with adult tissues that this temperature dependably distinguished TNAP, or a combination of isozymes containing TNAP, from TSAPs. Oocytes and embryos up to and including the 16-cell stage were negative for AP activity when incubated for one hour (Table VIII, Figure 18), while morulae, blastocysts and hatched blastocysts were AP activity positive after one hour of incubation.

Chemical Inactivation:

As found with tissues, the inactivation of AP by chemical inhibitors depended upon the isozyme(s) present, and changed with each stage examined. Oocytes and 2-cell embryos required low concentrations of LEV (<1 mM) to inhibit AP activity and inhibition was accomplished with exceptionally low HOM (<10 mM) and PHE (<10 and 25 mM) concentrations. At the 4- and 8-cell stages, AP activity was inhibited with low PHE (25 mM), moderate HOM (50 mM) and low LEV (<1 mM) when compared to adult tissues. At the 16-cell stage, AP activity was inhibited by low levels of PHE (25 mM), but now required high and moderate levels of HOM (>50 mM) and LEV (5 mM). In the morula, AP inhibition required high concentrations PHE (>50 mM) and HOM (>50 mM), but still moderate LEV (10 mM) levels. By the blastocyst and hatched blastocyst stages there was definite high requirements for all three inhibitors (>50 mM PHE and HOM, >10
Table VIII: Inhibitor concentrations (mM) and temperature (°C) required to inactivate alkaline phosphatase (AP) enzymes in preattachment bovine embryos with a one hour incubation. M=morula; Bl=blastocyst; HBl=hatched blastocysts; PHE=L-phenylalanine; HOM=L-homoarginine; LEV=levamisole; IAP=intestinal AP; TSAP2=tissue specific AP 2; TSAP3=tissue specific AP 3; TNAP=tissue non-specific AP.

<table>
<thead>
<tr>
<th>Stage (n)</th>
<th>mRNA</th>
<th>Temp°</th>
<th>PHE</th>
<th>HOM</th>
<th>LEV</th>
<th>AP(d)^d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocyte (72)</td>
<td>-^a</td>
<td>&lt;70</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;1</td>
<td>TNAP</td>
</tr>
<tr>
<td>2-cell (65)</td>
<td>-^a</td>
<td>&lt;70</td>
<td>25</td>
<td>&lt;10</td>
<td>&lt;1</td>
<td>TNAP</td>
</tr>
<tr>
<td>4-cell (59)</td>
<td>TSAP2</td>
<td>&lt;70</td>
<td>25</td>
<td>50</td>
<td>&lt;1</td>
<td>TNAP</td>
</tr>
<tr>
<td>8-cell (58)</td>
<td>TSAP2 TSAP3</td>
<td>&lt;70</td>
<td>25</td>
<td>50</td>
<td>&lt;1</td>
<td>TNAP</td>
</tr>
<tr>
<td>16-cell (60)</td>
<td>TSAP2 TSAP3</td>
<td>&lt;70</td>
<td>25</td>
<td>&gt;50</td>
<td>5</td>
<td>TNAP TSAP</td>
</tr>
<tr>
<td>M (59)</td>
<td>TSAP2 TSAP3</td>
<td>&gt;70</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>5</td>
<td>TSAP</td>
</tr>
<tr>
<td>Bl (43)</td>
<td>TSAP2 TSAP3</td>
<td>&gt;70</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>10</td>
<td>TSAP</td>
</tr>
<tr>
<td>HBl (50)</td>
<td>TSAP2 TSAP3 TNAP IAP^b</td>
<td>&gt;70</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;10</td>
<td>TSAP</td>
</tr>
</tbody>
</table>

^aThere was no AP mRNA detected in oocytes and 2-cell embryos by RT-PCR
^bIAP mRNA in the hatched blastocyst was sporadic as explained in Chapter 2
^cEmbryos were incubated at 70°C, <70 represents inhibition of AP activity while >70 denotes activity detected after incubation
^dAP isozyme determined to be present based on the data presented
FIGURE 18: The effects of heat incubation of alkaline phosphatase (AP) enzyme activity in preattachment bovine embryos as seen with light microscopy after NBT/BCIP AP activity assay. Panels A and B are blastocysts incubated at room temperature and 70°C, respectively; Panels C and D are 8-cell embryos incubated at room temperature and 70°C, respectively. Note the lack of activity in the 8-cell embryo at 70°C.
mM LEV) to block AP activity, suggesting a predominant TSAP activity (Figure 19). These results are summarized in Table VIII.

**DISCUSSION**

Adult bovine tissues transcribing mRNA for only TSAPs clearly required higher temperature to inhibit AP activity than tissues transcribing TNAP alone or in combination with a TSAP. This corresponds with work reported by Lepire and Ziomek (1989) who found that mouse TSAPs were more thermal tolerant than TNAP; similar results were reported by Harris (1989) for the human. For the mouse and human, it was found that TNAP has a low temperature tolerance, and is inactivated by incubation at approximately 60°C (Harris, 1989; Lepire and Ziomek, 1989). For the mouse, the TSAPs showed a much higher temperature tolerance than TNAP, requiring temperatures up to 95°C before inactivation occurred (Lepire and Ziomek, 1989). Studies with human tissues also showed that TSAPs generally required higher temperatures than TNAP for inactivation, although not nearly to the extent of the murine system (Harris, 1989). Similarly, when applied to bovine tissues expressing only one AP mRNA form, and therefore protein for that isozyme, it was found that individual TSAPs require much higher temperature to render protein inactive than the TNAP isozyme. Intestinal AP required 90°C for inactivation, while TNAP in liver required only 65°C, again showing that the bovine AP family acts more like the murine than the human family of isozymes. Tissue specific AP2 and TSAP3 were never detected in tissues without the presence of another isozyme so the
FIGURE 19: Inhibition of alkaline phosphatase (AP) enzyme activity by one hour of incubation in various concentrations of chemical inhibitors, as seen by light microscopy of preattachment bovine embryos subjected to the NBT/BCIP AP activity assay. Panels A and B are blastocysts incubated in 5 mM levamisole and 50 mM L-phenylalanine, respectively; Panels C and D are 8-cell embryos incubated in 25 mM L-phenylalanine and 1 mM levamisole, respectively.
temperature sensitivities of these individual isozymes in adult tissues are unknown. Kidney and BOEC both expressed mRNA for TSAP3 and TNAP, and required approximately 60°C for inactivation. Cumulus cells, which expressed mRNA for TNAP and TSAP2, also required 60°C for inactivation. Liver, which expresses only TNAP mRNA, and therefore only TNAP protein, required 65°C for inactivation. When examined by light microscopy, tissues with either positive or negative activity did not appear to "fade out" or weaken at any point, which argues against the presence of two homodimer populations. If there were two homodimer populations it would be expected that the TNAP population would be inactivated well before the TSAP population, resulting in a drop of activity at approximately 60°C. This was not observed, strongly suggesting that either the only population present is TNAP, or a set of heterodimers of TNAP and TSAP is present. When spleen was tested for temperature stability, it was found that tissues with only TSAP mRNA (TSAP2 and TSAP3) acted much like tissues expressing a single TSAP (intestine), requiring very high temperature to inactivate the AP protein present. This confirmed that heat inactivation of AP enzyme activity in bovine tissues distinguishes TSAPs from TNAP, as in the murine system.

Inhibition of AP activity with chemical inhibitors LEV, PHE and HOM also distinguished TNAP from IAP. Of the tissues examined in this study, intestine and liver expressed IAP and TNAP respectively. Both of these tissues presented the classical inhibitor pattern found in mouse (Lepire and Ziomek, 1989) and human (Fishman, 1974; Harris, 1989; Millan, 1990), with IAP requiring moderate PHE (50 mM) and high HOM (>50 mM) and LEV (10 mM) concentrations to effectively inhibit activity, while liver
TNAP required high PHE (>50 mM) concentrations and low HOM (25 mM) and LEV (5 mM) concentrations. Liver and intestinal tissues in mice and humans do not present the possibility of the existence of heterodimers between TNAP and TSAPs, whereas several bovine tissues do present the possibility of more than one isozyme mRNA being transcribed and forming functional protein. Heat and chemical inhibitors of AP activity did not allow confident determination of combinations of TNAP and TSAP isozymes in bovine tissues. It is not known if thermal and chemical tolerance of possible heterodimers would differ from that of homodimers. The presence of mRNA for more than one isozyme does not mean that active protein for all isozymes is produced, but tissues with mRNA for more than one isozyme did not behave as tissues expressing only one isozyme. This suggests that these tissues may produce two functional isozymes, either as two distinct homodimer populations, or as a heterodimer population, and that local tissue or environmental factors may also contribute to differences in thermal tolerance between isozymes and combinations of isozymes found in bovine tissues.

Bovine embryos were found to express mRNA for the two TSAP isozymes TSAP2 and TSAP3, but it was not known which isozymes were translated to produce the detected protein. A complicating factor was that AP enzyme activity was detected in immature oocytes, mature oocytes, and all stages of in vitro produced embryos, including presumptive zygotes and 2-cell embryos, before transcription from the embryonic genome begins. This is only possible if AP protein is produced during oogenesis in the ovary. Cumulus cells, which surround the developing oocyte, are thought to aid in oocyte growth and development in the ovary, and may affect protein production in the oocyte during the
period before ovulation. These cells contain mRNA for both TNAP and TSAP2, so it is unknown which isozyme is expressed in the immature and mature oocyte, presumptive zygote and 2-cell embryo, although the protein must be produced in the maternal system. Incubation of embryos at 70°C can distinguish TNAP from TSAPs, as incubation at 60°C inactivates TNAP. Oocytes, 2-cell, 4-cell, 8-cell and 16-cell embryos were all rendered AP activity negative by incubation at 70°C, suggesting the presence of TNAP protein. As well, the concentrations of LEV and HOM required for inactivation of AP enzyme activity also strongly suggested TNAP presence up to at least the 4-cell stage (see Table VIII). At the 4-cell stage TSAP2 mRNA was detectable, but LEV inhibition suggests the presence of TNAP until the 16-cell stage, while HOM and PHE inhibition suggest that there may be presence of TSAP protein beginning at the 4-cell stage and continuing until the 16-cell stage. By the morula and blastocyst stages the chemical inactivation profile for HOM and LEV strongly point to TSAP, with a gradual replacement of TNAP with at least one form of TSAP at the 16-cell and morula stages. However, which TSAP isozyme is present is unknown; IAP cannot be present as transcripts for IAP are not detected in early embryos, TSAP protein is clearly not present at very early embryo stages, and IAP mRNA is not detected until late blastocyst. The PHE values, however, are confusing: although PHE is useful for distinguishing TNAP and TSAPs in human tissues (Table VI), its ability to distinguish TNAP from TSAPs in the mouse is limited. This also appears to be the case with bovine embryos, although it was clear that PHE will inactivate TNAP only at high (>50 mM) concentrations in control tissues. It is possible that TNAP, TSAP2 and TSAP3 proteins are all present in the later stages of
preattachment embryo development, producing a complex population of proteins with differing sensitivities to PHE, or that the TSAP population of embryos acts differently than in control tissues and other species, to PHE, for some unknown reason. However, the majority of the data indicate the presence of TNAP protein which is gradually replaced by TSAP protein before embryo attachment.

The preattachment bovine embryo does not contain detectable polyadenylated mRNA for AP until the 4-cell stage, but AP activity is present on the cell surface of oocytes, presumptive zygotes, 2- and 4-cell embryos (see Chapter 2), providing evidence that a maternally derived protein is present. This early AP activity is lost after 1 hour of incubation at 70°C, showing that this activity involves TNAP. Bovine compact morulae and blastocysts were found to have AP activity after 1 hour of incubation at 70°C suggesting that this activity is due primarily to at least one form of TSAP. However, due to limitations in the technique, the individual TSAPs cannot be distinguished using temperature of inactivation, so it is unclear if TSAP2, or TSAP3, or a combination of the two, are responsible for AP enzyme activity seen at these later stages. These data show that AP protein expression is dynamic over time in preattachment bovine embryos, beginning with maternally derived TNAP that is replaced by at least one type of TSAP protein at the morula stage. This suggested progression generally correlates with observed transcription of TSAP2 and TSAP3 from the embryonic genome. Chemical inhibition of the preattachment embryonic isozymes agrees with control tissue observations that no clear pattern of inhibition with combinations of isozymes is present, and to suggests that other cell processes may affect the inhibition profile as well. There are slight changes in
chemical inhibition profile at each stage of development without observed transcriptional change.

These data suggest that TNAP is the major AP protein found in the oocyte and up to the 8-cell stage in the preattachment bovine embryo. Then, at the 16-cell and morula stages, there is a change to TSAP, either TSAP2 or TSAP3 or both, while TNAP is degraded.
CHAPTER 4

IN VIVO VERSUS IN VITRO ALKALINE PHOSPHATASE
mRNA AND ENZYME EXPRESSION

INTRODUCTION

The cost of research with embryonic tissue has always been high, so that availability of these tissues is low. The development of in vitro culture provides a more cost-effective means to obtain large numbers of embryos for study, as well as to increase the number of offspring from quality animals. However, bovine embryo production in vitro is not capable of yielding more than approximately 30% development to the blastocyst stage from all fertilized oocytes, so that efficiency of this technique is low (Pollard et al., 1989; Shamsuddin et al., 1992; Kato and Iritani, 1993; Lonergan, 1994), with many embryos arresting in development, presumably due to genetic or metabolic defect, intolerance to some component of the culture system, or lack of some essential component in the medium. For these reasons, culture medium is commonly supplemented with serum, resulting in undefined conditions. Attempts to culture bovine embryos in strictly defined medium have met with limited success (Rose and Bavister, 1992; Grisart et al., 1994; Keskintepe and Brackett, 1996).

It is known that in vitro and in vivo produced bovine embryos are morphologically and developmentally distinct. The period of development to the blastocyst stage in vitro is between 8 and 9 days, 24 to 48 hours longer than in vivo (Lonergan, 1994). Cell numbers are lower in vitro at the blastocyst stage than in vivo (Iwasaki et al., 1990; Xu et al., 1992A; Lonergan, 1994; Keskintepe and Brackett, 1996). Embryos produced in
vitro also display weaker interblastomeric junction formation at compaction and undergo asynchronous compaction (Iwasaki et al., 1990). The variety of differences existing between in vitro and in vivo produced embryos suggest that culture systems do not accurately mimic the environment in vivo, which in turn suggests that there may be other differences, including variation in onset of gene expression or protein production. This study was designed to determine whether transcription of AP isozymes and AP protein activity are different in embryos produced in vitro and in vivo.

**MATERIALS AND METHODS**

**Embryo Production:**

**In Vitro:**

Embryos were produced in vitro as previously described (Chapter 2). Briefly, oocytes were recovered from slaughterhouse ovaries, allowed to mature overnight, and fertilized with frozen-thawed semen. Presumptive zygotes were stripped of cumulus by gentle pipetting, and embryos were cultured with BOEC until the appropriate stage of development.

**In Vivo:**

Embryos were produced by superovulation and artificial insemination of *Bos taurus* animals that were either to be euthanized (21 cows) or permanent University of Guelph research animals at Elora Dairy Research Station (7 cows; Elora, Ontario).
Those animals to be euthanized were used to produce embryos between days 2 and 5 post-insemination, corresponding to 4-, 8-, and 16-cell embryos. Animals in the research herd at Elora Diary Research Station were used to produce embryos between day 6 and 8 post-insemination.

For the production of day 2 to 4 embryos, 21 animals were synchronized with intramuscular injections of 2.0 ml of prostaglandin, then observed for heat behaviour 2 and 3 days after injection. Animals were injected intramuscularly with 2.5 ml Follitropin every 12 hours for 4 days, starting 10 days after detection of heat to stimulate follicle growth. On day 12 after heat detection they were also injected with 2.0 ml prostaglandin intramuscularly. Gonadotropin releasing hormone was administered on day 13 after heat, and animals were artificially inseminated that morning and 12 hours later with frozen-thawed semen from a proven bull (GENCOR, Ontario, Canada). Animals were slaughtered on days 2, 3 and 4 after insemination. Intact uteri and ovaries were obtained at slaughter (MGI Meat Packers, Kitchener, Ontario), and transported to the laboratory for embryo recovery. Oviducts and the cranial 2 inches of uterine horns from both sides of the tracts were carefully dissected and all connective tissues removed. Embryos were flushed from oviducts with 10 ml of PBS+PVP injected into the fimbrial end of the oviduct from a 10 ml syringe with a 16 gauge needle. Embryos were separated by stage and washed three times in PBS+PVP until use in either RT-PCR or AP enzyme activity assays.

Embryos of morula and blastocyst stage were produced using the same superovulation protocol. Embryos were recovered with non-surgical flushing from 7 cows
at days 6 and 8 post-insemination using 500 ml PBS supplemented with 1% heat inactivated steer serum. Embryos were separated by stage, washed three times in PBS+PVP and held in PBS+PVP until use in either RT-PCR or AP enzyme activity assays.

**RNA Extraction and RT-PCR:**

Samples for RT-PCR were frozen in 10 µl PBS+PVP in pools of approximately 20 embryos. Total RNA from pools of embryos was extracted and reverse transcribed with oligo d(T) primer as described in Chapter 2, except that 2 µl SeeDNA DNA/RNA co-precipitant was added to the precipitation step. Briefly, after extraction, 2 µl SeeDNA co-precipitant, 1/10 volume 3 M sodium acetate, and 2 volumes 100% ethanol were added, mixed by vortexing, and incubated at room temperature for 2 minutes, then on ice for 10 minutes. Samples were spun at 13,000 rpm for 30 minutes at 4°C, and the pellet washed, then dried on ice for 5 minutes, resuspended, and reverse transcribed as described previously. Samples were amplified using actin, TNAP, IAP, TSAP2 and TSAP3 primers as described in Chapter 2. Actin primers were used as positive RT-PCR controls, and adult bovine tissues were amplified along with embryos as positive PCR controls. Negative controls consisted of amplification without cDNA.

**Restriction Endonuclease Confirmation of PCR Product:**

Positive PCR amplification reactions were subjected to restriction endonuclease digestion as described previously (Chapters 1 and 2) to confirm that the primers were not cross reacting with other isozymes cDNA.
**Alkaline Phosphatase Enzyme Activity Assay:**

Embryos were fixed for one hour in 4% paraformaldehyde, washed three times in PBS+PVP, equilibrated for 10 minutes in 0.1 M Tris pH 10.0, subjected to the Naphthol AS-MX Phosphate/Fast Red TR Salt AP activity assay as described in Chapter 2, and visualized with confocal microscopy.

**RESULTS**

**Alkaline Phosphatase mRNA Expression:**

Embryos produced in vitro for this study reproduced the expression patterns for actin, IAP, TSAP2, TSAP3 and TNAP reported in Chapter 2. Table IX shows total in vivo embryo yields obtained from 28 cows, and division of the embryos for assay. Actin mRNA was detected in 8- and 16-cell embryos, morulae and blastocysts, but not in 4-cell embryos. Only two 4-cell embryos were obtained for RT-PCR, and the amount of mRNA recovered was probably below detectable levels considering the extraction and RT-PCR protocol employed. The 4-cell embryos were not analysed further for mRNA content. There was no amplification product for TNAP and IAP from 8- and 16-cell embryos, morulae and blastocysts. Amplification of TSAP2 was detected only in morulae, while TSAP3 was detected in 16-cell embryos, and more strongly in morula stage embryos. Neither TSAP2 nor TSAP3 were detected in blastocysts. Only three blastocysts were obtained, and the negative results for TSAP2 and TSAP3 may be due to insufficient
Table IX: Embryo yields from 28 superovulated *Bos taurus* animals in the in vivo production of embryos and their allocation to either mRNA isozyme study (mRNA) or to alkaline phosphatase enzyme activity assay (Activity).

<table>
<thead>
<tr>
<th>Stage</th>
<th>mRNA</th>
<th>Activity</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-cell</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4-cell</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>8-cell</td>
<td>26</td>
<td>5</td>
<td>31</td>
</tr>
<tr>
<td>16-cell</td>
<td>21</td>
<td>5</td>
<td>26</td>
</tr>
<tr>
<td>Morula</td>
<td>17</td>
<td>5</td>
<td>22</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>3</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>
starting material for messages less abundant than actin. Unlike the in vitro situation, TSAP2 and TSAP3 were not detected in 8-cell embryos, although there were as many embryos in the in vivo as in vitro derived group (Figure 20). Restriction endonuclease digestion of positive amplification products from morulae confirmed that the amplified isozymes were the expected isozymes (Figure 21). These results are summarized in Table X.

**Alkaline Phosphatase Enzyme Activity:**

Very low AP enzyme activity was seen in 4-, 8- and 16-cell in vivo produced embryos when visualized with confocal microscopy, and low and generalized cell surface staining was seen at the morula stage. The ICM of the blastocyst was negative for activity, while the trophoderm was very positive. Trophoderm exhibited intense activity at regions of blastomere-blastomere contact, as well as very low activity over apical surfaces. Figure 22 shows scanning confocal micrographs of 4-, 8-, 16-cell, morula and blastocyst stages of in vivo produced embryos. The amount of activity throughout in vivo preattachment development appeared much lower than in in vitro produced embryos, and there was very little AP activity at the 4-cell stage, suggesting that there is loss of maternal protein in vivo before the 4-cell stage.
FIGURE 20: Alkaline phosphatase (AP) mRNA expression in in vivo produced embryos as found by RT-PCR (2% agarose gel, stained with ethidium bromide and visualized under ultraviolet light). A=actin controls; B=tissue specific AP 2; C=tissue specific AP 3; D=intestinal AP; E=tissue non-specific AP. Lane 1=100 bp ladder; 2=4-cell embryos (Note that 4-cell embryos were only examined for actin); 3=8-cell embryos; 4=16-cell embryos; 5=morulae; 6=blastocysts; 7=positive PCR control; 8=negative PCR control.
FIGURE 21: Restriction endonuclease digest of alkaline phosphatase (AP) isozyme cDNA found in in vivo produced morulae (2% agarose gel, stained with ethidium bromide and visualized with ultraviolet light). Lane 1=100 bp ladder; Lanes 2, 3 and 4 are tissue specific AP 2 digested with HinfI, PvuII and Accl, respectively; Lanes 5, 6 and 7 are tissue specific AP 3 digested with SmaI, PstI and KpnI, respectively.
Table X: A comparison of alkaline phosphatase (AP) mRNA presence and enzyme activity between in vitro and in vivo produced bovine embryos. IAP=intestinal AP; TSAP2=tissue specific AP 2; TSAP3=tissue specific AP 3; TNAP=tissue non-specific AP.

<table>
<thead>
<tr>
<th>Stage (n)</th>
<th>mRNA</th>
<th>Protein (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IAP</td>
<td>TSAP2</td>
</tr>
<tr>
<td>in vitro</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-cell (300)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4-cell (180)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>8-cell (180)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>16-cell (180)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Morula (180)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Blastocyst (180)</td>
<td>+/ -</td>
<td>+</td>
</tr>
</tbody>
</table>

| in vivo    |      |       |       |       |             |
| 2-cell     | N/A  | N/A  | N/A  | N/A  | N/A         |
| 4-cell (26) | N/A  | N/A  | N/A  | N/A  | + (5)       |
| 8-cell (21) | -    | -    | +    | -    | + (5)       |
| Morula (17) | -    | +    | +    | -    | + (5)       |
| Blastocyst (3) | -   | -    | -    | -    | ++ (5)      |

* Messenger RNA for APs; +=amplicon detected after RT-PCR; -=no amplicon detected after RT-PCR.

b Messenger RNA for IAP was detected in some samples.

c N/A=data not available due to insufficient mRNA recovery.
FIGURE 22: Alkaline phosphatase enzyme activity as seen with scanning confocal microscopy after staining in vivo produced embryos with Naphthol AS-MX Phosphate/Fast Red TR Salt. A=4-cell; B=8-cell; C=16-cell; D=morula; E=blastocyst with focusing through the inner cell mass; F=blastocyst with focusing on trophectoderm.
DISCUSSION

The lack of actin amplicon in in vivo produced 4-cell embryos is not surprising, as only 2 embryos for RT-PCR were recovered from tracts, resulting in too little mRNA for study. It was found that in vivo produced 8-cell embryos were negative for AP transcripts, although there was a sufficient number of embryos for actin cDNA to be detected. Therefore, it is likely that there are no AP transcripts produced before the 16-cell stage in bovine in vivo derived embryos. It is not known if TSAP2 and TSAP3 mRNA persist through blastocyst development in vivo. Only three blastocysts were recovered, and although this sample was positive for actin cDNA amplification, the signal is much weaker than for other positive samples, suggesting that the levels of actin mRNA are just at the detectable level. The AP mRNAs are probably present in lower concentrations, and therefore too low for detection. From in vitro results, it is reasonable to hypothesize that because TSAP2 and TSAP3 were detected in morulae, blastocysts may also be positive for these transcripts when greater numbers are obtained. Most mRNAs from the embryonic genome that have been detected in mice and cattle during embryogenesis persist to the blastocyst stage.

The pattern of AP mRNA expression differs between in vitro and in vivo produced embryos. In the in vitro system (see Chapter 2), mRNAs for TSAP2 and TSAP3 were first detected at the 4- and 8-cell stages respectively. The onset of TSAP3 mRNA expression is delayed for two cell cycles in vivo, from the 4-cell stage to the 16-cell stage, and TSAP2 expression in vivo lags behind in vitro expression by three complete cell cycles (4-cell to morula stage). This suggests that AP transcription, or message stability,
is sensitive to culture conditions, and that TSAP2 and TSAP3 are not required until the blastocyst stage or later.

Only very low levels of AP enzyme activity were detected from 2-, 4-, 8- and 16-cell in vivo produced embryos when compared with in vitro produced embryos of the same stages. The 16-cell stage probably represents the beginning of translation of protein from embryonic transcripts, as the level of AP activity increased steadily to the blastocyst stage. The morula, however, did not exhibit the same intracellular AP staining in in vivo produced embryos as seen with in vitro embryos. This pattern of AP activity also suggests that AP is not required until the blastocyst stage at the earliest, and that activity seen in vitro during earlier cleavage stages is caused by in vitro culture conditions. Determination of the source of AP activity in in vitro produced embryos will require further study, but the presence of deadenylated message or post-translational modification of maternally derived protein are possibilities.

The differences in AP mRNA levels and AP enzyme activity between in vitro and in vivo derived embryos suggest differences with respect to timing of gene expression in the two systems. Grisart et al. (1994) suggest that transcription in in vitro produced embryos may occur sooner than that in embryos in vivo due to a lengthening of the early cell cycles, possibly in response to environment, allowing for a transcriptionally permissive state. The results presented here support this hypothesis.
GENERAL DISCUSSION

The Bovine Species as a Model:

The bovine embryo provides an interesting model for studying early mammalian development for several reasons. First, the period of time for preattachment development is longer than in other animal models such as the mouse, spanning 9 to 10 days in culture. This results in events being separated by longer periods of time, and allows the closer study of these events. The period of time between fertilization and the onset of low levels of embryonic transcription, followed by the major burst of transcription, is longer than in mice, allowing for better distinction between maternally driven events and those initiated and controlled by the embryo itself. Second, cattle are economically important herd animals which unfortunately experience a large percentage of embryo loss due to preattachment embryo death. Elimination of early embryonic death both in vitro and in vivo is important to industry and research. Third, bovine embryos are produced in sufficient numbers for study by relatively simple techniques in vitro. Fourth, in this species it is possible, using the new reproductive technologies, to produce relatively high numbers of in vivo embryos for comparison to in vitro embryos.

In the bovine embryo, relatively few mRNAs produced from the embryonic genome have been identified, but autoradiographic studies confirm that there is a major increase in mRNA production at the 4-, 8- and 16-cell stages (Plante et al., 1994; Viuff et al., 1996). None of the transcripts known to be produced only from the embryonic genome are detected in 4- and 8-cell embryos. Transcripts with later onsets of transcription would be useful for studies of normal progression of embryonic
transcriptions and cell differentiation. Four AP isozymes were identified in adult cattle and blastocysts. Their expression patterns were examined in the in vitro produced preattachment bovine embryo to determine if they could be used as specific markers of steps in the onset of transcription and embryonic gene expression, and to also determine embryo health status, assuming that a normal pattern of gene expression is one indicator of embryo health. Bovine AP expression patterns have not been well studied, and the expression patterns of these genes in adult tissues and preattachment embryos were investigated. These experiments showed that the bovine isozymes are clearly expressed during development, with different isozymes appearing at different stages. However, functional AP protein is present at all stages of embryo development, and attempts to clarify which isozyme is expressed as functional protein were difficult to interpret. Individual isozymes, and combinations of isozymes, did not present consistent behaviour to inhibitors when expressed in various tissues and combinations.

**Messenger RNA Expression:**

In adult cattle, AP expression was found to be similar to that in both humans and mice. Expression of TNAP and IAP was quite consistent over the three species. There is more variability in adult expression patterns of the remaining TSAPs (murine EAP, human PLAP and GCAP and bovine TSAP2 and TSAP3). It is interesting to note that mouse testis tissue expresses EAP (Hahnel et al., 1990; Narisawa et al., 1992) and the adult bovine testis expresses TSAP2 and TSAP3. Furthermore, in the developing murine embryo, EAP is expressed up to implantation (Hahnel et al., 1990), and in the bovine
embryo, mRNA for TSAP2 and TSAP3 are expressed during preattachment development. These expression patterns suggest that murine EAP and bovine TSAP2 and TSAP3 may be homologous in distribution in the mouse and cow.

Intestinal AP was originally cloned from bovine blastocysts, but not all blastocysts expressed IAP mRNA. It is hypothesized that IAP expression would be detectable very shortly after the late blastocyst stage, but embryos of this age are extremely difficult to obtain, and impossible to maintain in culture. Of the tissues examined in the adult cow, only intestine was found to consistently express IAP mRNA; intestine is of endodermal origin. In the late blastocyst the major germ layers are being formed, and it is possible that in a few blastocysts endoderm has differentiated and is producing detectable, albeit low levels of IAP mRNA. The end point of in vitro culture coincides with the timing of development of the major germ layers, and would account for the detection of IAP mRNA in only a few preparations.

As discussed in Chapter 2, TSAP2 and TSAP3 exhibited a cyclic pattern of expression. This cyclic expression does not appear to be cell cycle dependent, but does appear to be reliant on microtubule function for detection. It is possible that the gaps in expression are caused by early rapid turnover of mRNA for these isozymes just after initiation of their transcription. If this pattern were cell cycle dependent it might be expected to continue throughout development, but as development continues the cleavages progress from synchronous to increasingly asynchronous. Thus, any cyclic expression patterns for these genes would be masked. The possibility of using cell cycle synchronization agents to synchronize a population of embryos was investigated and found
to be not feasible because of adverse effects on transcription of the AP isozyme family of genes. Nevertheless, these studies suggested that microtubules play an important role in either stability, storage or detection of AP and actin transcripts. Synchronization agents such as aphidicolin and nocodazole are widely used in the new reproductive technologies, especially in nuclear-cytoplasmic synchronization in nuclear transfer procedures. Treatment with aphidicolin has been found in mice not to affect transcription from the zygotic genome at the 1-cell stage and into the 2-cell stage (Schultz, 1993); this also appears to be the case with the bovine embryo. Treatment of embryos with nocodazole and taxol caused the delay of TSAP2 and TSAP3 mRNA expression until the late blastocyst stage. Either taxol affects the stability of only a certain subset of mRNAs (namely the AP and actin gene transcripts) causing them to be undetectable, or taxol has the ability to affect mRNA levels in embryos by increasing mRNA turnover or decreasing production. One possibility is that taxol somehow interferes with procedures used to detect mRNA, but as HPRT mRNA was readily detectable in the same preparations this is unlikely. Another possibility is that taxol affects mRNA levels through increased turnover or degradation of mRNA, which appears to be much more likely, although the actual means by which this would occur is unknown. Nocodazole also caused delay of TSAP2 and TSAP3 mRNA expression to the late blastocyst stage, but not that of mRNA for actin and HPRT, which suggests that mRNA expression of TSAP2 and TSAP3 genes is somehow affected by the microtubule status of the embryo. Nocodazole has also been shown to delay transcription from the zygotic genome in mice (Schultz, 1993), and treatment with this inhibitor confirms that this is also the case with the bovine embryo.
While taxol is not traditionally used in embryological studies, nocodazole is routinely used to synchronize cells of embryos, and aphidicolin and nocodazole are used to synchronize nucleus, cytoplasm and recipient ooplasm status for nuclear transfer. It seems that although these inhibitors do provide blastomere synchronization with a minimum of cell mortality, they also affect transcription from the genome for at least one subset of mRNAs (the AP mRNAs) and could have drastic consequences for embryo viability by delaying or preventing the expression of transcripts that may be required for embryogenesis.

Tissue specific AP2 and TSAP3 genes are the first identified genes known to be transcribed from the embryonic genome at the 4- and 8-cell stages in the cow to date. Several genes have been characterized to date during both murine (Takeichi, 1988; Hahnel et al., 1990; Rappolee et al., 1990; Kidder, 1993; Doherty et al., 1994; Sheth et al., 1997) and bovine in vitro embryo development (Watson et al 1992B; Watson et al., 1996). In the mouse there are many known zygotically produced transcripts detected at the OET and not before (Table 1). In the bovine embryo it was found that many of the same transcripts were detected as maternally derived mRNAs as well as embryonically produced mRNAs; for example, transcripts for platelet derived growth factor A, insulin-like growth factor II, the insulin-like growth factor I receptor and the insulin receptor were all found to be embryonic transcripts in mice, and maternal and embryonic messages in the cow. The only bovine transcripts known to be produced solely from the embryonic genome have been detected after the major burst of transcription has occurred. These include those encoding bovine trophoblast protein and ZO-1, which are detected at late blastocyst and 16-cell stages respectively (Watson et al., 1996). Tissue specific AP2 and TSAP3
genes are transcribed at the 4-cell and 8-cell stages, respectively, and not at the 2-cell stage or in the oocyte, making it possible to use these two types of transcripts as markers at the mRNA level for the onset of gene expression from the bovine embryonic genome.

Alkaline Phosphatase Enzyme Expression:

High AP enzyme activity has been localized to cell membranes of tissues with high transmembrane transport activity, such as the intestine, kidney and liver (Harris, 1989). In the mouse and human, strong AP protein activity is found on the brush border of intestinal epithelium, as was found in the bovine intestine. As well, localization of protein activity in bovine kidney was the same as for the mouse, with kidney tubules being strongly positive and glomeruli being negative. As well, in the liver, hepatocyte surfaces were strongly positive for AP activity. In the adult bovine reproductive tract, BOEC and cumulus cells were also examined for AP protein activity and both cell types were strongly positive over entire cell membranes. The role of active AP protein is unknown, and while both BOEC and cumulus cells have been implicated in the growth, development and survival of embryos and oocytes respectively, it is not known whether expression of AP on the surfaces of these cell types is involved in cumulus or BOEC interaction with oocytes.

As mentioned previously, several tissues expressed mRNA for more than one AP isozyme. The difficulty lay in determining which isozyme was expressed at the protein level, and not transcribed and then degraded. Tissues such as liver and intestine expressed only one type of AP mRNA: TNAP and IAP, respectively. These mRNA isozymes must
be the isozymes expressed as protein in these tissues. However, in tissues such as kidney, BOEC, cumulus, spleen and testis, in each of which a combination of AP mRNAs is expressed, it is not known which isozymes are expressed as active protein. Several possibilities exist. First, only one mRNA type is translated resulting in a population of homodimer proteins for that isozyme. Second, there could be translation of all mRNA types, resulting in a population of homodimers for both isozymes. Third, there could be translation of all mRNA types, resulting in a heterodimer population of protein. Finally, there could be translation of both mRNA types, resulting in a mixed population of homodimers of both isozymes as well as heterodimers. To determine which isozymes are expressed as active protein, heat and chemical inactivation of active AP enzyme from a variety of adult tissues and preattachment embryos was performed. However, while heat inactivation proved useful for distinguishing TSAPs from TNAP, it could not distinguish combinations of TNAP and TSAPs, nor individual TSAPs from each other. Similarly, inhibitor profiles for tissues presented a complex set of characteristic inhibitor concentrations, which did not appear to be totally dependant on the isozyme present. Instead, inhibitor profiles seemed to differ between tissues with the same isozyme mRNA profile, suggesting that other factors such as tissue type or environment may also affect AP response to inhibitors.

The localization of active AP protein is dynamic over preattachment in vitro bovine embryo development. In the oocyte, the oolemma is positive for activity, as is the presumptive zygote. However, upon cleavage to two cells, the localization shifts to interblastomeric, and remains interblastomeric until the 8-cell stage. At this point there

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appears to be a decrease in the amount of protein present, and loss of high protein activity interblastomerically in 8- and 16-cell embryos. At the morula stage activity appears to be mainly intracellular in blastomeres of the outer layer of the embryo, and then, once again, activity becomes sharply intercellularly localized between cells of the trophectoderm of the blastocyst.

Generally, in in vitro produced embryos it appears that TNAP protein is produced maternally in the oocyte and remains the only active AP form until the 8- to 16-cell stages. Active protein over this period is localized to areas that are interblastomeric until the 8-cell stage, at which stage activity appears to fade between blastomeres, followed by a reduction in total AP protein activity, presumably due to the turnover of maternally derived TNAP. Levels of AP protein activity remain low until the morula stage, when translation of embryonic AP mRNA for either or both TSAP2 or TSAP3 occurs, apparently selectively in outer blastomeres of compacted morulae. Expression then moves interblastomERICally once again, to areas between tropheCTodERM blastomeres. No activity was seen in or on the surface of ICM cells.

In embryos that had been synchronized with nocodazole and taxol there was a drastic change in protein activity levels, which coincided with the change in mRNA expression patterns. Aphidicolin treated embryos could not be examined because of extreme brittleness of the zona pellucida, which resulted in lysis of all embryos in the Tris pH 10.0 buffer. Treatment with nocodazole produced a pattern of AP activity like that of control embryos, but instead of just being localized interblastomerically, AP activity was also found over entire cell membranes to the 8-cell stage. A decrease in enzyme
activity occurred at the 16-cell stage, with a slight increase at morula stage. The level of enzyme activity appeared to be lower in treated morulae and blastocysts than in similar control embryos, and was not only localized to outer blastomeres of compacted morulae. Taxol treated embryos displayed greatly reduced overall AP activity, generally located over entire blastomeres. At the morula stage, AP enzyme activity appeared to be localized only to outer blastomeres, but at extremely low levels, and in blastocysts the trophectoderm was very weakly positive, as was the ICM. As discussed previously, treatment of embryos with nocodazole and taxol delayed TSAP2 and TSAP3 mRNA expression until the late blastocyst stage. These data and the apparent reduction in protein levels at the 16-cell, morula and blastocyst stages agree in that if no mRNA is produced from the embryonic genome, maternally derived TNAP is degraded by the 8-cell stage. If there is no translation of AP mRNA at this point, then protein levels remain low.

It appears that embryos treated with nocodazole and taxol survive through preattachment development relatively well, showing that AP expression, especially at later stages of development, is not required for embryo viability in vitro. The effects of taxol and nocodazole on the microtubule status of an embryo changed AP mRNA expression from the embryonic genome. There was no increase in AP enzyme activity at or directly after the time of treatment with these inhibitors, so that increased turnover of mRNA by translation into protein with subsequent degradation of mRNA is highly unlikely. As there was no increase in enzyme activity, the probability is that any mRNA is degraded directly after production, or there is no transcription of these genes, the latter being in
agreement with observations made by Schultz (1993) in mice where nocodazole delays transcription from the embryonic genome.

Alkaline Phosphatase Expression in vivo vs in vitro:

There is evidence that embryos of mice and cows produced in vitro are not identical to embryos produced in vivo. Although in vitro produced bovine embryos have been transferred and resulted in the birth of live, apparently healthy offspring, reports by Farin and Farin (1995) and Crosier et al. (1998) show that in vitro produced embryos have lower numbers of mitochondria, increased lipid content, decreased cell number at the blastocyst stage, and an increased number of vacuoles. These reports are in agreement with results published by Shamsuddin et al. (1992). In a study by Plante and King (1994), it was found that abnormalities found in in vitro produced embryos were also found in in vivo embryos, suggesting that these differences are not unique to in vitro produced embryos. Conflicting reports also exist as to whether or not in vitro embryos produce offspring of higher birth weight and larger size (Behboodi et al., 1995; Farin and Farin, 1995; King et al., 1985). In this study, the presence of mRNA for each of the AP isozymes was investigated by stage of development to determine if differences at the level of gene expression exist. Embryos produced in vitro and in vivo showed changes in the timing of TSAP2 and TSAP3 mRNA expression suggesting that the culture system does not mimic the in vivo system. The remote possibility exists that the procedure used for superovulation of oocytes in this research could account for the differences in gene expression, but as only one or possibly two embryos can be retrieved from one animal
without superovulation, the study of in vivo produced embryos on such a small scale (without superovulation) is not feasible. Embryos flushed from animals after superovulation have been shown to produce live, healthy offspring with no apparent differences to offspring from normal matings. Gene expression in embryos can be markedly changed by depletion of contents of the culture medium, addition of certain components to the culture system (such as serum with its undefined nature), and stress conditions (for example, expression of the heat shock proteins may be induced by incubating embryos at higher temperatures than normal body temperature). It appears that the culture system used in this study provided a less than optimal environment for embryos, causing a deviation in gene expression from that in those embryos produced in vivo. However, the changes in expression were not large, and did not appear to affect embryogenesis. The pattern of AP mRNA expression also suggests that this family of isozymes remains a good model for the study of embryonic gene expression during early embryo development in vivo. Neither TSAP2 nor TSAP3 mRNA appear to be maternally derived in in vivo produced bovine embryos, as shown by the lack of mRNA detected at the 8-cell stage. Eventually, these transcripts are produced from the embryonic genome differentially, so that mRNA for TSAP3 appears one cell cycle prior to that for TSAP2. In future, these transcripts could be used as an indicator of gene expression at a particular stage of development, such as at the 16-cell stage, to ensure that embryonic transcription is occurring as expected from the embryonic genome at that stage of development.

As the expression of mRNA for TSAP2 and TSAP3 was delayed in vivo compared to that in vitro, it was not surprising that AP protein expression differed between in vivo
and in vitro derived embryos. It was not possible to recover 2-cell embryos or zygotes using the procedures reported in this thesis, so maternal inheritance of protein is not known, but, as in vitro embryos are produced from oocytes recovered by aspiration from slaughterhouse ovaries, the expression of maternal protein is expected to be the same in at least the zygote for in vivo produced embryos. The turnover rate of AP proteins in the cow is unknown, and these studies showed that there were extremely low levels of active AP protein present in in vivo produced 4-, 8- and 16-cell embryos. Only at the morula stage, the stage at which mRNA for TSAP2 becomes apparent and TSAP3 mRNA is present, is active AP protein readily detected. The level of activity, as seen by the intensity of staining, is low, and does not appear to be intracellular. Expression of active protein at the blastocyst stage is the same in in vivo as in in vitro produced embryos, strongly localized to regions of cell-cell contact of the trophectoderm layer only.

These results show that in vivo and in vitro produced embryos have distinct gene expression patterns for the AP family of isozymes. Expression of APs in vitro at earlier stages of development may be indicative of stress exerted on the early stages of embryo development in the culture system and not at later stages, as at morula and blastocyst stages the expression of both mRNA and protein is similar to that of in vivo produced embryos. Grisart et al. (1994) have suggested that in vitro produced embryos exhibit early transcription because of an artificially induced lengthening of early cell cycles in response to the environment. These data support this theory, which would also suggest that gene expression in early embryo development is largely controlled by time post insemination, a hypothesis which has also been suggested by Schultz (1993). Lengthening
of the early cell cycles in vitro would then make the in vitro produced embryo initiate gene expression from the embryonic genome earlier with respect to embryo stage than in vivo. An in vivo produced embryo would then express, for example, TSAP3 mRNA at the 16-cell stage, while an in vitro produced embryo would express TSAP3 mRNA at the 8-cell stage. While the stage of development may be different, the age of development (or time post insemination) would be the same. A complication to this is that the exact moment of fertilization is not known, either in vivo or in vitro, so that the exact time post-insemination cannot be determined, although it may be estimated by stage of development.

As the numbers of embryos recoverable from superovulation protocols is still low and the procedure extremely labour intensive in contrast to in vitro production of embryos, in vivo produced embryos were not examined for individual isozyme determination using heat and chemical inactivation. However, as the expression of APs is not markedly different between in vivo and in vitro produced embryos, it is hypothesized that the active protein seen at the morula stage is probably TSAP3, from mRNA produced one cell cycle before, at the 16-cell stage. Then TSAP2 and TSAP3 would hypothetically contribute to the active AP protein population at the blastocyst stage.

These data show that members of the AP family of isozymes are useful as markers for embryonic genome activation in both in vitro and in vivo produced embryos, and that they could provide a tool for studying effects of in vitro culture and manipulations on gene expression. Successful transfer from maternal to embryonic control of gene expression during development is required for subsequent embryo development. By
examining the expression of these isozymes during embryogenesis, it could be determined whether successful transfer of control had occurred, as seen by the onset of transcription of TSAP2 and TSAP3 genes. Observation of specific isozyme expression patterns by stage would enable the culture conditions to be manipulated so that developmental patterns mimicked those of the in vivo system. Various original procedures in the production of bovine embryos could then be monitored for differences in gene expression using a simple PCR based assay for AP isozyme gene expression. For example, different hormone levels, serum effects, culture media components and stress conditions could be investigated to determine if these factors affect gene expression in bovine embryos produced either in vitro or in vivo. Use of AP gene expression would not provide an absolute measure of embryo viability, but would instead provide insight into possible effects of environmental factors on embryo development.
SUMMARY AND CONCLUSIONS

The research reported in this thesis determined the expression patterns of the AP family of isozymes at both the mRNA and enzyme levels in adult bovine tissues and in preattachment bovine embryos to determine if this family could be used as a marker for early embryonic gene expression. The expression patterns for four AP isozymes in cattle were determined to be similar to corresponding isozymes in the mouse and human, although bovine AP expression patterns tended to resemble those in mice more closely than those in the human. Incubation of tissues displaying AP enzyme activity at different temperatures revealed that, as in the mouse and human, TNAP and TSAP isozymes could be distinguished, with TNAP inactivation occurring at approximately 60°C while TSAPs remained active up to high temperatures approaching 95°C. As well, incubation of bovine tissues in the presence of inhibitors of AP enzyme activity known to be effective for the mouse and human isozymes also distinguished AP isozymes, particularly TNAP from TSAPs. The enzyme populations present in tissues expressing mRNA for more than one isozyme could be a population of heterodimers, as revealed by temperature and chemical inactivation of enzyme activity.

The preattachment bovine embryo expressed mRNA for TSAP2 and TSAP3 at the 4- and 8-cell stages of development, respectively; these are the earliest known embryonically produced transcripts found in bovine embryos to date. Genes of the other two isozymes, TNAP and IAP, are expressed beginning only at the late blastocyst stage. Enzyme expression in the preattachment embryo was found to change over time, beginning with maternally derived TNAP and replaced gradually by TSAP expression by
the morula and blastocyst stages. Alkaline phosphatase enzyme activity is localized to interblastomeric regions from the 2-cell to 8-cell stages and again at the blastocyst stage, where activity is found exclusively between cells of the trophectoderm. At the morula stage, activity appears to be mainly intracellular, with low activity on cell surfaces possibly indicating transport of protein that is to be inserted into the membrane. These experiments also showed that APs appear not to be required for early bovine embryo development, as interruption of transcription of TSAP2 and TSAP3 mRNA and concurrent decrease in active protein levels do not affect embryo development to the blastocyst stage.

The expression patterns of AP differ between in vitro and in vivo produced preattachment embryos, with in vitro produced embryos expressing mRNA for TSAP2 and TSAP3 at the 4- and 8-cell stage, respectively, and in vivo produced embryos expressing TSAP2 and TSAP3 at the morula and 16-cell stages, respectively. These results show that the in vitro culture system used in these experiments does not mimic the environment found in vivo, and results in changes in gene expression during preattachment development. As well, the expression of AP enzyme activity was also changed, with in vivo produced embryos showing strong expression only at the blastocyst stage. The localization of protein, however, was not different between in vivo and in vitro produced bovine embryos.

In conclusion, cattle express the family of AP isozymes in a similar manner to the mouse and human, and preattachment bovine embryos express two isozymes, TSAP2 and TSAP3, from the embryonic genome in a manner that would allow their use in determining successful onset of embryonic genome activity at very early stages of
development. Alkaline phosphatase, while not required for embryo development, could be used as a marker for the onset of early genome activity and an indirect indicator of embryo health, and to aid in the development of new reproductive technologies and culture systems that mimic more exactly the environment in vivo.
LITERATURE CITED


Betts DH, Barcroft LC, Watson AJ. Na/K-ATPase-mediated $^{86}$Rb$^+$ uptake and


Garattini F, Hua JC, Udenfriend S. Cloning and sequencing of bovine kidney alkaline phosphatase cDNA. Gene 1987; 59: 41-46.


Harris H. Multilocus enzyme systems and the evolution of gene expression: The alkaline phosphatases as a model example. The Harvey Lectures 1982; 76: 95-123.


Kimber SJ, Surani MAH, Barton SC. Interactions of blastomeres suggest changes in cell surface adhesiveness during the formation of inner cell mass and trophoderm in the preimplantation mouse embryo. J Embryol exp Morph 1982; 70: 133-152.


MacGregor GR, Zambrowicz BP, Soriano P. Tissue non-specific alkaline phosphatase is expressed in both embryonic and extraembryonic lineages during mouse embryogenesis but is not required for migration of primordial germ cells. Development 1995; 121: 1487-1496.

MacPhee DJ, Barr KJ, De Sousa PA, Todd SDJ, Kidder GM. Regulation of Na\textsuperscript{+},K\textsuperscript{+}-ATPase \textalpha subunit gene expression during mouse preimplantation development. Dev Biol 1994; 162: 259-266.


Otaegui PJ, O’Neill GT, Campbell KHS, Wilmut I. Transfer of nuclei from 8-cell stage mouse embryos following use of nocodazole to control the cell cycle. Mol Reprod Dev 1994; 39: 147-152.


Petzoldt U. Regulation of stage-specific gene expression during early mouse


Surani MAH, Barton SC. Spatial distribution of blastomeres is dependent on cell division order and interactions in mouse morulae. Dev Biol 1984; 103: 335-343.


Wickens M. In the beginning is the end: regulation of poly(A) addition and removal during early development. TIBS 1990; 15: 320-324.

Wieckowski M, Miranda M, DePamphilis ML. Regulation of gene expression in preimplantation mouse embryos: Effects of the zygotic clock and the first


## APPENDIX I
### CHEMICALS, SUPPLIES AND SUPPLIERS

<table>
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<th>Material</th>
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Eosin Y
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Fast Red TR Salt
First Strand Buffer
Follicle Stimulating Hormone (Folltropin-V)
Gentamicin
Glucose
Guanidinium Thiocyanate
Ham’s F-10
Hematoxylin
Heparin (Hepalean)
HEPES
HincII
Hinfl
Hydrochloric Acid
Isoamyl alcohol
KpnI
L-Glutamine
L-Homoarginine
L-Phenylalanine
Levamisole
Lutenizing Hormone
Lysozyme
Magnesium Chloride (for PCR)
Magnesium Chloride (for solutions)
Methanol
Methyl Green

Fisher Scientific, Unionville, Ont, Canada
Sigma Chemical Co., St Louis, MO, USA
Sigma Chemical Co., St Louis, MO, USA
Gibco BRL, Burlington, Ont, Canada
Vetpharm, London, Ont, Canada
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Fisher Scientific, Unionville, Ont, Canada
Gibco BRL, Burlington, Ont, Canada
Fisher Scientific, Unionville, Ont, Canada
Organon Teknica, Toronto, Ont, Canada
Gibco BRL, Burlington, Ont, Canada
New England Biolabs, Mississauga, Ont, Canada
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Gibco BRL, Burlington, Ont, Canada
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Gibco BRL, Burlington, Ont, Canada
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Fisher Scientific, Unionville, Ont, Canada
Allied Chemical, New York, NY, USA
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APPENDIX II

SOLUTIONS

0.25% Alkaline Naphthol AS-MX Phosphate Solution:
0.25 g Naphthol AS-MX Phosphate
100 ml 0.1 M Tris-HCl pH 10.0

2% Agarose Gel:
24.5 ml ddH₂O
0.5 ml 50X TAE Buffer
0.5 g Agarose (Electrophoresis Grade)
1.5 μl 10 mg/ml ethidium bromide

4% Paraformaldehyde:
4 g Paraformaldehyde
100 ml ddH₂O
1 drop 1 M NaOH

Bacterial Lysis Buffer:
50 mM glucose
10 mM EDTA
25 mM Tris pH 8.0
4 mg/ml lysozyme

DNAse Buffer (1 ml):
5 μl 2M NaCl
40 μl 1M Tris pH 8.0 without EDTA
6 μl 1M MgCl₂
100 μl 0.1M CaCl₂
849 μl ddH₂O

Embryo Extraction Buffer (10 ml):
1 ml of 2 M NaCl
2.5 ml of 0.1 M Tris pH 7.4
20 μl of 0.5 M EDTA
6.48 ml ddH₂O

Low TE Buffer:
10 mM Tris pH 8.0
1 mM EDTA
Naphthol AS-MX Phosphate/Fast Red TR Salt (Naphthol Colour Reaction Buffer):
0.2 ml Naphthol AS-MX Phosphate (0.25% alkaline solution pH 10.0)
4.8 ml 0.1 M Tris-HCl pH 10.0
5-10 mg Fast Red TR Salt

NBT/BCIP Colour Reaction Buffer (with substrate):
10 ml NBT/BCIP Buffer (above)
200 μl NBT/BCIP stock solution

NBT/BCIP Buffer (without substrate):
0.1 M Tris pH 9.5
0.05 M MgCl₂
0.1 M NaCl
100 ml sdH₂O

PBS (10X stock)
80 g NaCl
2 g KCl
11.5 g Na₂HPO₄ 7H₂O
2 g KH₂PO₄
1000 ml sdH₂O

PBS (1X working solution):
1 ml 10X PBS stock
9 ml sdH₂O

PBS+PVP (100ml, 0.1%):
100 ml PBS
0.1 g PVP

PCR Buffer (10X):
1 ml of 1 M Tris pH 8.3
2.5 ml of 2 M KCl
10 mg of 1 mg/ml bovine serum albumin
to 10 ml sdH₂O

Potassium Acetate Buffer pH 3.6:
A = 11.55 ml glacial acetic acid
    1000 ml sdH₂O
B = 19.6 g potassium acetate
    1000 ml sdH₂O
for pH 3.6: 46.3 ml solution A
            3.7 ml solution B
            50 ml sdH₂O
Potassium Acetate Buffer pH 5.0:
A = 11.55 ml glacial acetic acid
    1000 ml sdH₂O
B = 19.6 g potassium acetate
    1000 ml sdH₂O
for pH 5.0: 14.8 ml solution A
            35.2 ml solution B
            50 ml sdH₂O

Reverse Transcription Buffer:
4 µl First Strand Buffer
2 µl 0.1 M DTT
1 µl 10 mM dNTPs
0.3 µl RNAse Inhibitor

TAE (50X Stock solution):
242 g Tris base
57.1 ml glacial acetic acid
37.2 g Na₂EDTA.2H₂O
sdH₂O to 1 liter

TAE (1X running buffer):
1 ml 50X Stock
49 ml sdH₂O
0.5 µg/ml ethidium bromide

TRIS saturated phenol:chloroform (500 µl):
10 µl TRIS pH 8.0 without EDTA
990 µl sdH₂O
Mix together and take 500 µl of this
Add 500 µl RNA grade phenol
Take 250 µl of bottom, organic phase
Add 250 µl 24:1 chloroform:isoamyl alcohol
APPENDIX III

BOVINE ALKALINE PHOSPHATASE SEQUENCES, PRIMERS AND RESTRICTION ENDONUCLEASE SITES

Restriction Endonuclease Sites:

<table>
<thead>
<tr>
<th>Restriction Endonuclease Sites:</th>
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</thead>
<tbody>
<tr>
<td>AccI: 5' GT</td>
</tr>
<tr>
<td>5' GT</td>
</tr>
<tr>
<td>HincII: 5' GTC</td>
</tr>
<tr>
<td>5' GTT</td>
</tr>
<tr>
<td>HinfI:  5' G</td>
</tr>
<tr>
<td>KpnI:  5' GGTAC</td>
</tr>
<tr>
<td>PstI:  5' CTGCA</td>
</tr>
<tr>
<td>PvuII: 5' CAG</td>
</tr>
<tr>
<td>Smal:  5' CCC</td>
</tr>
</tbody>
</table>

Primer sites are in green, restriction endonuclease sites are in red.

Intestinal Alkaline Phosphatase:

ACCTGTGTGGGGTCAAGGGCACAATGAGAACCATTGGTGAATGCGCAAGCCCGCCCCGCTACA
left primer

ACCAGTGCAAACGACACCTGAGGAGTGTCATCTGTGATGAAACGGGCGCAAGAAAG

CAGGGAAAGTCCGTGGGAGTGGTGACCAACCACCAGGGTGAGATGCATGGCCTTCCTCCCAGCAGCCGG

CCTACGCACACGATGCAAACCCAACTGGTACTCGACACGCCACTGCCTGATGACAC

AGATGAATGGCTGCCAGGACATCGGCACACAGCTGCTCAACAACATGGATATTTGACGTGA

~PvuII~HincII

TCCTGGGTGGGAGGCCGAAAATATCATGTTCTCTGTGGGACCCCAACACCTGAATACCCAG

ATGATGCCAGTGTGAAATGGAGTCCGGGAAGCAGAAACCTGGTGCAAGCATGGCAGG
right primer

CCAAGCAACGGGGAACCGTACGTGAAAGATGACACGACAGGGAAGGGAAGGCGACATGGCAGG

ACTCCAGTGTAAACACACTTCATGGGCTCTTTTGAGCCGGCGAGACTGAAATGATTGTC

AGCAAGACACACAAAGGACCCGACACTGCAGAAATGACAGAGGTGGCCCTGCGAGCTG

TAAGCAGGAACCCAGGGGCTCTCTACCTCTTTTGTGGAGAGGAGGCGGCAATTGACACGGTC

ACCATTGACAAAAGCTTATATATTGCACTGACGAGGCGGGGTATTTGTTGACAAATGCCATCG

CCAAGGGCTACTAGTCACCTGACACTGCAACGCTGATCTTGATCTCGACAGGACTCG

CTCAGTCTCTCTCTTTTGTGGCTATACACTGGCAGTGGGACCTCCATCTTTTTGGCTGCGAC

CCAGCAAGGCCATTAGACAGCAAGTCTCTACACTCCATCTCTCATGAGAGGCGGCAAGGCT
ATGCGCTTGGCGGGGGCTCAGGCGAGGAAGAGGACCCCTCGT
ACCAGCAGCAGCGGCCTGCTGGCCCGAGGCTAGCGAGACCCACAGGGGGCGAGGTGGCGG
TTCCGCGCGCCGGCCCCGGCAGCGCAGCCTGGTGCACGGCGTCAGGAGGAGGACCTTCGT
CGACATCATGGCCTTGGGCTGGCTGGGAACCTACACGGACTTGCCAACTGACGCCC
CCACCAACCAGCAGCATCCCAGACGCCCAGCAGAGACGGGCCACCTTGGGAGCCAGCCG
CGCTGCTGGCTGGGCGATGCTGCTGCTGCTGGCTCCATTGTACTAA

Tissue Specific Alkaline Phosphatase 2:
ACCTGTGTGGGTCAAAGACCCGTATGAAGGTGTATGAAAGTCAGGGCCGAAAATTTA
left primer
ATCAATGTAACACAGACATATGGGAAACGCTACTGACATGGGAAACCCGAGAAAAG
CAGGGAAAGTCAGGGTGGAGTGTGACACACACCAGCAGTGCAGGCTCCTCCAGGGCGG
CCTACGCGACAGCCTTAAACCTGTATTCTGATTGCTAATATGGCCTGGCGAGGCA
AGAGGGAGGGTGGGGTCAAGACCCGTATGAAGGTGTATGAAAGTCAGGGCCGAAAATTTA
PvuII AccI
tcctgggtgaggccgcaatatatactgattttccgagggaaaaaccgaccctctgaataccag
GCAATACAAACAGAATGGGATTCGGAAGGACAAACGCAACGTCTGGTGCAGGAGTGGCAT
right primer
CAGAGCAACCAGGAGCCGCTCATGGTGGAAACGCACCGCGCGCTCCTTCAGGGCGGCAATG
ACTCCAGGTGAAACACACCTCCTGCGCCCTCTTTGGACCCAGGAGATATGACCTATGACCTAT
GACATCCACGGCGAGCACATCAAGGCACTATCTTGAGGGAGATGACGGAGTCAGGGCGG
CGTGTGCTGAGCAGAAACCCCTCCGGGCTTTCTCCTTCTGCTGGAGGGAGGCGCCGCAATTG
CAGGGCGCTACCTGGAAGCATAGCTTATGCCTGCGGACTGACGGAGGCGGCTGATGTTGCAAAAT
GCCATCGCCAAGGCTAGGCCGACTCACCAGTGAAAGCAGCAGACACTGACCCTTGTCAGCAACC
GACCACTCCACGTCTTACTTGGTGCTACCCCACTTGCCTGGAGACCCTCATTTTGGG
CTGCGCCGATGCGAAAAAGCTGGCAAGTCTATACACCCACCTTCTGACGAGGACACCCCG
CCGGGCAACCGCTGCTGCTACGACCTGATGTGAAATGGAAAGGAGCAGCATGGAC
GCCAGTACAGCAGCAATCCCGAGCTGCCTATGGCAGACCACGGCAGGGCCGTGAGGAC
CTTCGGCGCGCATGTTCATTGGCCTTGCGCTGCTGGGACACCTTTGCTGGGACACCCCGCCT
Tissue Specific Alkaline Phosphatase 3:

ACCTGTGTTGGGCTCAAGACCAACATGAGGACCATTTGTTGTAAGTGCAAGCCGCCCGGCTTTG
ACCAGTGCACACGACACGTGGGAATGAGGTCACGTCTGTGATAAACCGGCGAACGAAGAAAG
CAGGGAAGTCAGTTGGGAGTTGGTGGACCAACCAACAGGGTGCCAGATGCACTCCCCAGCAGG
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AGCTGCTCGACGTGCCCTAGACAGCGAGACCCCAAGCCCGGTGAGGACGAGTGCCAGTATGGCAC
^PstI right primer
GAGGCCGGTCGAGCAACCTTGATGGCAATGGAAAGGAGAGCCAGGACCACGACCAGGCACTGAC
TGCCCTTTGCTGCTGCTGAGCCCTACACACCAGACTGCAACCCTCAACCTCAACCCATAGTG
GTCCCTCGACACCGCACCACCAGCGTCTGGCCCATCCTCAGTGCACGTGGGGCGG
CCCTGCTCTGCTGGTGGTCACGCCCTCCTGCCGCTGCTCGGCACAGGCGGGTCTCTCCTCC
CGCCCGCCACCTCTGAGGACCAAAACACCCCTGGAAAGCTGAACCCCTACTTCTGTGGAG
AGCCACAGTCTCTCGAGGCCCACACCTCACTGAGCTATTTGAGGAGGCTGCCACA
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TTCAGCAGCCCCCTTCTAGGAACCCAGCGGTATCTTTACAGAGAGACAGCGACACAGAGGA
GAGGAGACTTTGTCCAGGTCTCAGCTGCTGTGAGGTTGGCCCCGGTGCTGCCCCTGGGAGATC
CCAGGAGCAGCGGGAGAGCTGGGGGTGGGGACAGAGGCCCCGCCCTCCTGGGAGGGAGGA
AGCAGCCCTCAAATAAAAAGCTGTCCAAGTGAAAAA

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APPENDIX IV

CULTURE MEDIA FOR IN VITRO BOVINE EMBRYO PRODUCTION

Oocyte Collection Medium:
9.8 g (1 package) Ham's F-10 powder
10.0 ml HEPES
20.0 ml heat inactivated steer serum
10.0 ml penicillin/streptomycin solution
0.2 ml heparin (Hepalean)
1.2 g NaHCO$_3$
1000 ml Milli-Q filtered H$_2$O
pH to 7.35-7.40

In Vitro Maturation Medium:
200 ml TCM-199 (Earles) with 25 mM HEPES
55 mg sodium pyruvate
29.2 mg L-glutamine
1.2 ml penicillin/streptomycin solution
10-14 ml Milli-Q filtered H$_2$O
Osmolarity 280-290 mOsm
22 ml 10% (v/v) steer serum

In Vitro Culture Medium:
100 ml TCM-199 (Earles)
55 mg sodium pyruvate
14.6 ml L-glutamine
0.6 ml penicillin/streptomycin solution
6-8 ml Milli-Q filtered H$_2$O
Osmolarity 280-290 mOsm
1 ml 35% bovine serum albumin
12 ml 10% steer serum

Modified Tyrodes Medium (Primary Stock Solution):
2.28 M NaCl
158 mM KCl
250 mM NaHCO$_3$
35 mM NaH$_2$PO$_4$·H$_2$O
200 mM CaCl$_2$·2H$_2$O
100 mM MgCl$_2$·6H$_2$O
**TALPs (for 100 ml each):**

<table>
<thead>
<tr>
<th></th>
<th>Sperm TALP</th>
<th>HEPES TALP</th>
<th>In vitro Fertilization TALP</th>
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<tr>
<td>NaCl</td>
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<tr>
<td>KCl</td>
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<tr>
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<td>1.0</td>
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<tr>
<td>Na-lactate</td>
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<tr>
<td>HEPES</td>
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<td>1.0</td>
<td>--</td>
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<tr>
<td>CaCl$_2$ 2H$_2$O</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
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<tr>
<td>MgCl$_2$ 6H$_2$O</td>
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<td>Milli-Q H$_2$O</td>
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<td>80.31</td>
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<td>NaHCO$_3$</td>
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<td>10.0</td>
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<tr>
<td>Na-pyruvate</td>
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<td>0.1</td>
<td>0.1</td>
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<tr>
<td>Gentamicin</td>
<td>0.2</td>
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<td>0.1</td>
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<tr>
<td>35% (v/v) BSA</td>
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<td>0.87</td>
<td>1.8</td>
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<td>Osmolarity</td>
<td>295-305</td>
<td>280-290</td>
<td>290-305</td>
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<td>pH</td>
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</table>
APPENDIX V

ALKALINE PHOSPHATASE PROTEIN INHIBITOR SOLUTIONS

NBT/BCIP Buffer (without substrate):
- 5 ml 2 M Tris pH 10.0
- 5 ml 1 M MgCl₂
- 2 ml 5 M NaCl
- 88 ml sdH₂O

500 mM Stock Solutions:

- **Levamisole**: 0.2408 g levamisole
  2 ml PBS

- **L-phenylalanine**: 0.1652 g L-phenylalanine
  1 ml PBS
  dropwise 1 M NaOH until into solution
  to 2 ml PBS

- **L-homoarginine**: 0.1047 g L-homoarginine
  2 ml PBS

- **EDTA**: 0.37224 g EDTA
  1 ml PBS
  dropwise 1 M NaOH until into solution
  to 2 ml PBS

Working Solutions:

- **50 mM working solution**: 1 ml 500 mM stock inhibitor solution
  9 ml NBT/BCIP buffer (without substrate)

- **25 mM working solution**: 500 µl 500 mM stock inhibitor solution
  9.5 ml NBT/BCIP buffer (without substrate)

- **10 mM working solution**: 200 µl 500 mM stock inhibitor solution
  9.8 ml NBT/BCIP buffer (without substrate)

- **5 mM working solution**: 100 µl 500 mM stock inhibitor solution
  9.9 ml NBT/BCIP buffer (without substrate)

- **1 mM working solution**: 20 µl 500 mM stock inhibitor solution
  9.980 ml NBT/BCIP buffer (without substrate)

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