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DISSERTATION

IDENTIFICATION OF GENES WITH GREATER mRNA CONTENT IN 17.5-DAY THAN 15.5-DAY BOVINE EMBRYOS

Submitted by

Michelle D. Glover

Department of Physiology

In partial fulfillment of the requirements

for the Degree of Doctorate of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 1999

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COLORADO STATE UNIVERSITY

MAY 20, 1999

WE HEREBY RECOMMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY MICHELLE D. GLOVER ENTITLED IDENTIFICATION OF GENES WITH GREATER mRNA CONTENT IN 17.5-DAY THAN 15.5-DAY BOVINE EMBRYOS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTORATE OF PHILOSOPHY.

Committee on Graduate Work

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ABSTRACT OF DISSERTATION

IDENTIFICATION OF GENES WITH GREATER mRNA CONTENT IN 17.5-DAY THAN 15.5-DAY BOVINE EMBRYOS

Considerable embryonic loss occurs between days 15 and 18 of gestation in cattle (estrus=day 0), when several critical cellular and molecular events occur. To gain insight into these events, the mRNA differential display technique was used to identify transcripts present in greater abundance in 17.5-day compared to 15.5-day bovine embryos. Eight unique cDNA fragments were identified.

Four of the eight candidate clones were confirmed by Northern blot analysis to be up-regulated between days 15.5 and 17.5 of pregnancy. These four cDNA fragments were subcloned and sequenced. It was revealed that nucleotide sequences for three genes had known homologs; one cDNA fragment was homologous to human and rat allograft inflammatory factor-1 (AIF-1), the second was homologous to human LERK-5 cDNA, and the third gene was bovine IFN-τ. One cDNA fragment did not match sequence identities to previously reported genes.

A gene fragment encoding bovine AIF-1 and the unidentified gene were selected for the following studies. To determine if elevated gene expression continues beyond day 17.5 of pregnancy, Northern blot analysis was conducted using total RNA isolated from five embryos collected from days 17.5 to 36 of gestation. In addition, Northern blot

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analysis was used to determine whether these genes were expressed in several adult bovine tissues. The mRNA encoding AIF-1 was present in developing placenta through day 36 of gestation, and abundant levels were observed in adult bovine spleen and lung. The novel cDNA fragment hybridized only to total RNA isolated from 17.5-day embryos; hybridization of the probe was not evident in adult tissues.

A cDNA library constructed from 25-day bovine embryos was used to isolate additional nucleotide sequence for bovine AIF-1 and the novel cDNA fragment. The cDNA clone encoding AIF-1 had 88% and 84% sequence identity to human and rat AIF-1; its open reading frame encoded a protein that was 82% and 78% homologous to the amino acid sequences for human and rat AIF-1. Additional sequence information obtained for the novel cDNA fragment was not homologous to previously reported genes.

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CHAPTER I

REVIEW OF LITERATURE

INTRODUCTION

Commercial market sales of beef and dairy products comprise approximately 25% of total cash receipts for all farm commodities in the United States including corn, soybeans, wheat, vegetables, fruits, and all livestock products. In 1995, it was estimated that 103 million cattle were present on farms in the United States valued at approximately 63 billion dollars (US Bureau of the Census, 1996). Reproductive failure is one the most economically devastating factors to cattle producers, resulting in financial losses estimated to be as high as 1.4 billion dollars nearly two decades ago (Gerrits et al., 1979). There has been little progress in reducing infertility since this report was published. According to the U.S. Dept. of Agriculture, the number of cattle in the United States has not changed significantly since 1980; therefore, estimated financial loss due to reproductive failure probably continues to be at least a billion dollars annually.

Several studies have been conducted to characterize the percentage of pregnancies ending in failure. Calving rates to a single insemination for dairy cows are 45-55% (Mawhinney and Roche, 1978) and ~60% for beef cattle (Roche et al., 1977; Diskin and Sreenan, 1980; Roche et al., 1981). Diskin and Sreenan (1980) reported comparable calving rates, although percentages varied with age, climate, and physiological factors.

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Fertility loss is influenced by many factors including detrimental effects of a short estrous cycle preceding insemination (Erb et al., 1958), high temperature and humidity (Stott and Wiersma, 1973), irregular hormone secretion patterns (Erb et al., 1976), and poor nutrition (Francos et al., 1977). Furthermore, others (Tanabe and Casida, 1949; Laing, 1949; Casida, 1953; Tanabe and Almquist, 1953; Hawk et al., 1955) have reported 40-50% prenatal mortality in animals with histories of infertility ('repeat-breeders'). Some infertility in cattle also is accounted for by the presence of chromosomal abnormalities. These aberrations account for approximately 7.5% of reproductive failure (Wilmut et al., 1986), and may be inherited or arise during meiosis, fertilization or early cleavage stages (King, 1985). Since fertility in cattle is affected by a variety of factors, it is important to note that most of the studies cited in this review were conducted under good management conditions.

When discussing reproductive loss, it is necessary to recognize that the birth of a calf relies upon successful completion of a series of important biological events, beginning with fertilization in the oviduct of the recently ovulated oocyte. Fertilization rates have been investigated in heifers (Kidder et al., 1954; Beardon et al., 1956; Henricks et al., 1971; Wishart and Young, 1974; Spitzer et al., 1978; Maurer and Chenault, 1983) and cows (Ayalon, 1969; Boyd et al., 1969; Ayalon, 1972; Maurer and Chenault, 1983) by examining the morphology of ova or embryos within 8 days after breeding before embryos hatch from the zona pellucida. Fertilization rate is estimated by determining the number of cleaved ova as a proportion of all fertilized and unfertilized ova recovered. Fertilization rate may be nearly 100% in first service heifers; embryonic death accounts

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for nearly all reproductive loss (Ayalon, 1978). Fertilization failure occurs in approximately 10-15% of cows with normal breeding histories (Boyd et al., 1969), but this rate increases to approximately 40% in repeat-breeders (Tanabe and Almquist, 1953).

After fertilization, the newly formed zygote will continue developing as it travels from the oviduct to the uterus. Between days 6 and 8 post-ovulation, the embryo enters a pre-attachment phase; the morula stage embryo forms a blastocyst that will continue to expand until hatching from the zona pellucida. Subsequently, the bovine embryo will elongate and secrete INFτ, a peptide responsible for maternal recognition of pregnancy (Godkin et al., 1982). On approximately day 18 of gestation, the embryo will adhere to the uterine endometrium, a process necessary for successful placentation (Wathes and Wooding, 1980). If fertilization rates account for approximately 25% of reproductive failure, the remaining 75% of failure can be attributed to early embryonic mortality during the period when these critical events occur (Sreenan and Diskin, 1986). Several investigations have been conducted using direct slaughter methods to determine the time of early embryonic mortality. The reported time of loss varies among studies, but in fertile groups of cattle, most embryonic mortality occurs between days 8 and 18 (Ayalon, 1978; Diskin and Sreenan, 1980; Roche et al., 1981). More recently, in a symposium devoted entirely to reproductive failure, Sreenan and Diskin (1986) narrow this margin, reporting that most embryonic loss occurs between days 15 and 18 of gestation. In infertile animals, embryonic mortality may occur earlier than in fertile cows. Ayalon (1978) reported that most embryonic death in repeat breeders occurs around day 7 after service, when the morula is developing into a blastocyst; however Hawk et al. (1955)

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estimated that 52% of embryos perished between days 16 and 34 of gestation, with the majority of loss occuring prior to day 25 of pregnancy.

A better understanding of the factors affecting embryonic survival is necessary if methods to reduce early embryonic mortality are to be developed. Understanding the intimate dialog that occurs between the developing embryo and maternal environment between days 15 and 18 is essential. This dialog involves secretion of various autocrine, paracrine and endocrine factors by the embryo and endometrium. Morphological changes that occur in embryonic trophoblast and uterine endometrium during this period have been well documented (Wathes and Wooding, 1980), but cellular and molecular changes that occur at this time are practically unstudied.

FERTILIZATION

The presence of a healthy embryo is dependent upon the successful union of male and female gametes. At the time of fertilization, a mature spermatozoon travels through the oviduct to fuse with a mature oocyte recently ovulated from the ovary. Enzymes, consisting mostly of hyaluronidase, are present in the bull sperm acrosome and disperse the cumulus oophorus surrounding the oocyte (Bedford, 1982). After penetration through the cumulus, the sperm plasma membrane covering the acrosome contacts the glycoprotein coat of the oocyte, the zona pellucida, and attaches to receptor sites. The zona pellucida is formed by three major glycoproteins: ZP1, ZP2, and ZP3 (Hasegawa et al., 1991). ZP3 functions as a sperm surface receptor and induces the acrosome reaction. Proteins present on the sperm head including glycosyl transferase, proteinases, and

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glycosidases may bind with ZP3 in a lock and key mechanism (Wasserman, 1990). Once bound, the sperm undergoes the acrosome reaction. The release of hydrolytic enzymes and sperm motility (Yanagimachi, 1981) act synergistically allowing the sperm to pass through the zona pellucida. After a sperm has entered the perivitelline space, a change in the zona occurs preventing additional sperm from penetrating the ovum (Hafez, 1993). Microvilli present on the vitelline membrane and membranes covering the equatorial segment of the sperm fuse, activating the oocyte to complete meiosis and extrude a second polar body. Chromatin in the sperm head decondenses and eventually both male and female pronuclei form (Hafez, 1993). In the cow, individual pronuclei migrate to the center of the ovum; pronuclei break down, and maternal and paternal chromosomes mix during the first cell division. Completion of this process yields a two-cell embryo (Betteridge and Fléchon, 1988).

EARLY EMBRYONIC DEVELOPMENT

Fertilization occurs in the ampullary-isthmic junction of the oviduct on day 1 (estrus=day 0) of pregnancy in cattle, and the embryo travels through the oviduct during the first 4 days following fertilization (Hamilton and Laing, 1946). Embryonic loss is minimal during this time (Ayalon, 1978) although critical developmental events occur including cell differentiation and activation of the embryonic genome. The zygote undergoes the first mitotic cell division in which each blastomere receives a full complement of chromosomes approximately 24 to 28 hours after fertilization (Thibault, 1966). The resulting blastomeres are totipotent, so each is fully capable of giving rise to

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an intact fetus. The cells remain totipotent until the eight-cell stage in sheep embryos (Papaioannou and Ebert, 1986) and the 4-cell stage in cattle (Willadsen, 1989). After this time, the blastomeres appear to differentiate depending on their stage in the cell cycle (Fehilly and Willadsen, 1986) and position in the embryo (Denker, 1981).

As observed *in vivo* in mice and *in vitro* in cattle, cleavage occurs asynchronously beginning with the second cell division (Barlow et al., 1972; Massip et al., 1983; Chisholm et al., 1985). Some blastomeres divide earlier and more rapidly than others, forming cells that are small in size and contribute to the inner layer of the embryo (Ziomek and Johnson, 1981; Massip et al., 1983). This inner layer eventually forms the inner cell mass which gives rise to the embryo (Surani and Barton, 1984; Garbutt et al., 1987). Earlier dividing cells also are more likely to initiate blastocoele formation in mice (Garbutt et al., 1987). Later dividing cells are larger in size and tend to contribute to the trophoblast, which eventually forms the chorion (Massip et al., 1983; Surani and Barton, 1984; Garbutt et al., 1987).

Another molecular event that occurs during embryonic development is activation of transcriptional activity from the embryonic genome. Early embryos are mostly controlled by macromolecules present in the oocyte including rRNA, mRNA, tRNA, and ribosomes that are produced during oogenesis. In cattle, these molecules control development until the 8-cell stage, at which time transcriptional activity is initiated from the embryonic genome (Telford et al., 1990). An interesting phenomenon occurs when culturing bovine embryos *in vitro*; at the 8 to 16-cell stage of development, embryos may become “blocked” and cease to develop (Camous et al., 1984; Gandolfi and Moor, 1987).

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This phenomenon is coincident with activation of the embryonic genome and may demonstrate the embryo's sensitivity to certain culture conditions during this period. This block can be eliminated by co-culturing embryos with other tissues including trophoblastic vesicles or oviductal epithelial cells (Camous et al., 1984; Eyestone et al., 1987; Gandolfi and Moor, 1987). This indicates that various factors secreted by the oviduct stimulate embryonic growth and development to the blastocyst stage. For example, platelet-derived growth factor, secreted by oviductal cells, stimulates development during the fourth cell cycle (Larson et al., 1992; Thibodeaux et al., 1993), and addition of an antibody to platelet-derived growth factor partially delays embryonic development (Thibodeaux et al., 1993). Although the microenvironment of the oviduct is important for development, it is not essential since early stage embryos continue to develop in culture in the presence of tissues other than oviductal epithelial cells.

The zygote reaches the uterus by day 5 and consists of 16 cells encased in the zona pellucida, which holds the blastomeres together. At the 16 to 32-cell stage in the cow, boundaries between the cells become obscure as the embryo undergoes the process of compaction (Hamilton and Laing, 1946; Barnes et al., 1987). Several ultrastructural events occur during compaction which result in increased intercellular communication and eventual blastocoele formation. During compaction, blastomeres lose their spherical shape and flatten on each other (Betteridge and Fléchon, 1988); outer cells of the compacting embryo become polarized with ultrastructural differences between outer and inner poles (Sutherland et al., 1990). In the mouse, these differences include microvillous investment, ability to bind fluorescent dyes, and distribution of alkaline phosphatase, 5'

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nucleotidase, and proteins associated with the cytoskeleton, such as actin and vinculin (Ziomek and Johnson, 1981; Lois and Izquierdo, 1984; Ziomek and Lepire, 1984). In mice and cattle, the lateral surfaces of the outer polarized cells do not have microvilli, but do form gap and tight junctions (Ducibella, 1977; Linares and Ploen, 1981). Gap junction formation between blastomeres is postulated to increase cell to cell communication, regulating development (Solter and Knowles, 1975). The intercellular communication system provided by gap junctions in the embryo is demonstrated by electrical coupling and transfer of low molecular weight dyes between the blastomeres (Lo and Gilula, 1979; Goodall and Johnson, 1982). The protein subunits that form gap junctions in the morula have been characterized and appear to be similar in the mouse, cow, and rat (Hertzberg and Skibbens, 1984). Gap junctions not only form between the outer cells of the compacting morula, but also the inner polygonal cells (Betteridge and Fléchon, 1988). There is unequal distribution of gap junctions between the blastomeres in the mouse (Caveney, 1985); more gap junctions are present among the inner polygonal cells and the outer polarized cells than between the two layers. It has been speculated that these uneven zones of distribution may influence embryonic development (Caveney, 1985).

Apical tight junctions appear between outer compacted cells of the morula on days 6 to 8 of pregancy (Hamilton and Laing, 1946; Massip et al., 1981; Barnes et al., 1987). Tight junctions form an ion and antibody barrier that facilitates the accumulation of fluid within a central cavity, termed the blastocoele (Ducibella, 1977). This cavity is created and maintained by the presence of Na^+/K^+ pumps within the cell layers that

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transport ions across the membranes into the center of the compacted morula (Benos and Biggers, 1981). Water then flows into the embryo due to osmotic pressure causing the blastocoele to increase in size. Formation of the blastocoelic cavity marks the transition from the morula to blastocyst stage embryo. Early blastocysts contain approximately 100 cells (Mannaerts, 1986; Picard et al., 1986) that continue to divide mitotically as the blastocyst expands, thinning the zona pellucida. Newly expanded blastocysts contain approximately 160 cells (Mannaerts, 1986).

Bovine blastocysts hatch or escape from the zona pellucida between days 9 to 11 of gestation. The zona pellucida is torn as the embryo expands and contracts (Betteridge et al., 1980). Expansion is caused by cellular hyperplasia and fluid accumulation in the blastocoele. Also, enzymes such as plasmin may facilitate zona lysis (Coates and Menino, 1994). By day 11, spherical embryos measure approximately 375 µm in diameter and contain approximately 1000 cells, of which less than 25% contribute to the inner cell mass (Renard et al., 1978).

PERI-IMPLANTATION EMBRYONIC DEVELOPMENT

Approximately 65-70% of embryonic loss in fertile cattle occurs during the peri-implantation period (Ayalon, 1978; Diskin and Sreenan, 1980; Roche et al., 1981). Embryonic growth and survival rely on communication between the uterus and embryo. This close dialogue becomes evident during the peri-implantation period when several critical molecular and cellular events occur. These events include: 1) embryonic elongation; 2) secretion of bovine IFNτ, a peptide responsible for maternal recognition of

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pregnancy; and 3) adherence of the embryo to the uterine endometrium during the process of implantation.

Uterine-Embryo Communication and Embryonic Elongation

Bovine embryos undergo a logarithmic elongation phase after shedding the zona pellucida between days 9 to 11 of gestation. Bovine blastocysts are transformed from a 3 mm sphere on day 13 to a 25 cm long filamentous vesicle on day 17 of pregnancy (Hafez, 1993). Keller and Seidel (1996) demonstrated that day 13 embryos undergo a 36-fold increase in surface area and 30-fold increase in length by day 15. Cellular hyperplasia of the trophectoderm and extraembryonic endoderm continues until day 21 in cattle as the blastocyst invades the contralateral uterine horn (Leiser, 1975; Hafez, 1993).

Onset and rate of elongation are extremely variable among embryos (Boyd et al., 1969), and appear to rely on factors secreted by the uterus. In sheep, uterine factors that affect embryonic elongation have been demonstrated, but not characterized (Fléchon et al., 1986). Several observations suggest that factors secreted by the uterus affect embryonic growth. For example, embryonic elongation is not seen *in vitro* despite expansion of cultured blastocysts (Wintenberger-Torrès, 1956). Furthermore, when Garrett et al. (1988) administered progesterone injections (100 mg/day) to pregnant cows from days 1 to 4 post-estrus, embryos recovered on day 14 of gestation were longer than controls. Progesterone treatment also stimulated a variety of endometrial proteins, prostaglandin $F_{2\alpha}$ secretion, and corpus luteum regression in cyclic cows (Garrett et al., 1988). More recently, Nephew et al. (1994) administered human chorionic gonadotropin

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(hCG) (100 IU), progesterone (12 mg), or vehicle on 11.5 days of pregnancy (estrus=day 0) in ewes. On day 13 of pregnancy, reproductive tracts were collected, and blastocysts were flushed from the uterine horns. Day-13 blastocysts from ewes treated with hCG were longer and had higher protein and interferon-τ concentrations than blastocysts from vehicle-treated ewes. Unlike observations made earlier by Garrett et al. (1988), progesterone treatment did not affect embryonic growth. Furthermore, hCG treatment increased pregnancy rates in ewes, whereas progesterone did not, possibly indicating that hCG treatments influenced embryonic growth and uterine secretions resulting in increased pregnancy rates. Differences in the results between these studies may be due to species differences, dosage differences, or the time at which progesterone was administered.

The ability of the uterus to regulate embryonic growth has been demonstrated using embryo transfer experiments in sheep and cattle. For example, embryonic development is stimulated when a delayed embryo is transferred to an advanced uterus (Lawson and Cahill, 1983), and retarded in the converse situation (Wilmut and Sales, 1981; Ashworth and Bazer, 1989). Albihn et al. (1991) demonstrated that embryos transferred asynchronously +3 days or -3 days to recipient heifers were accelerated or retarded respectively when compared to synchronously transferred embryos. Although two days of asynchrony is tolerated between embryo and recipient, pregnancy rates decline more when an advanced embryo is transferred to a delayed recipient uterus than the converse (Hasler et al., 1987).

Not only is embryo growth affected by uterine secretions, the uterus is affected by

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the residing embryo. Ashworth and Bazer (1989) investigated the pattern of protein secretion from the endometrium of ewes on day 8 of pregnancy that received a day 6 embryo on day 4 post-estrus. Secretion of several proteins was stimulated in recipient ewes pregnant with advanced embryos. Proteins secreted by a day 8 uterus pregnant with a day 10 embryo closely resemble the protein secretion pattern of control ewes at day 10 of pregnancy, indicating that advanced embryos have the ability to alter the uterine environment of recipient cows. Progesterone levels also affect uterine environment. Geisert et al. (1991) observed that recipient cows treated with exogenous progesterone from 1 to 5 days post-estrus and receiving a day 8 embryo on day 5 maintained their pregnancy, indicating that progesterone stimulated appropriate uterine development. These studies vividly demonstrate the ability of the uterus and embryo to communicate during the peri-implantation period of pregnancy, and potential for hCG and progesterone to influence pregnancy rates.

Uterine-Embryo Communication and Maternal Recognition of Pregnancy

After ovulation, the ovarian follicle is transformed into the corpus luteum, which is responsible for secretion of progesterone. Progesterone stimulates glandular epithelium of the uterine endometrium to provide an environment capable of nourishing and sustaining a conceptus (Bazer and First, 1983). When a female is not pregnant, the corpus luteum regresses, allowing another estrous cycle to occur; regression of the corpus luteum is termed ‘luteolysis’. Prevention of luteolysis and maintenance of progesterone secretion in pregnant animals are vital to sustaining pregnancy.

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Removing the uterus from ruminants can prevent corpus luteum regression for nearly the entire length of gestation (Bazer et al., 1986); this indicates that a factor inducing luteolysis is secreted from the uterus. In ruminants, this luteolytic factor is prostaglandin $F_{2\alpha}$, which is released from the uterus in a pulsatile fashion, inducing corpus luteal regression (McCracken et al., 1972; Bazer et al., 1986).

Progesterone performs two important functions during the estrous cycle; it not only prepares the uterine endometrium for the establishment of pregnancy, but also is essential for the production of $PGF_{2\alpha}$ and luteolysis. Progesterone secretion during the early and mid-luteal phase of the estrous cycle increases phospholipid stores (Boshier et al., 1987) and prostaglandin synthase activity involved in the conversion of arachidonic acid to $PGF_{2\alpha}$ (Eggleston et al., 1990). Estrogen and progesterone also regulate oxytocin receptor synthesis in the endometrium; the presence of these receptors in the uterus is essential for the production of $PGF_{2\alpha}$ (McCracken et al., 1984). Exposure of the uterus to progesterone during diestrus suppresses expression of the oxytocin receptor gene. Continuous progesterone exposure elicits a negative feedback loop and causes down-regulation of progesterone receptors in the endometrium. Reduction of progesterone receptors allows for increased synthesis of estrogen receptors, increased estrogen sensitivity, and enhanced oxytocin receptor production in the uterine endometrium (Spencer et al., 1995).

Oxytocin is secreted in a pulsatile fashion from large luteal cells (Wathes and Denning Kendall., 1992) and the posterior pituitary (Hooper et al., 1986). Approximately 95% of $PGF_{2\alpha}$ pulses correspond to pulses of oxytocin (Hooper et al., 1986). Oxytocin

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binds to oxytocin receptors present in the uterine endometrium causing an immediate increase in phospholipase C activity and $PGF_{2\alpha}$ production (Flint et al., 1986; Silvia and Homanics, 1988; Mirando et al., 1990; Mirando et al., 1993; Burns et al., 1996). $PGF_{2\alpha}$ enters the uterine venous drainage and is transferred to the ovarian artery via a countercurrent exchange system (Bazer et al., 1997). Upon entering the ovary, $PGF_{2\alpha}$ binds to receptors present on luteal cells, terminating the secretion of progesterone and initiating cell death (Bazer et al., 1997). There are several theories whereby the $PGF_{2\alpha}$ induces luteolysis; these include: 1) decrease in luteal blood flow; 2) activation of protein kinase C; 3) influx of high levels of calcium; and/or 4) activation of a cytotoxic cascade (Niswender and Nett, 1994).

Corpus luteum function is maintained during early pregnancy, indicating that the presence of the conceptus inhibits luteolysis. This period of recognition, termed ‘maternal recognition of pregnancy’ (Short, 1969), occurs on approximately day 12 in sheep and day 16 to 17 in cattle. It has been demonstrated using embryo transfer experiments that embryos transferred synchronously prior to day 12 result in the maintenance of pregnancy in sheep (Moor and Rowson, 1966; Moor, 1968). Furthermore, aqueous extracts from day-14 to 15 conceptuses infused into the uterine luteum extend luteal function in non-pregnant ewes, identifying the elongated blastocyst as the possible source of the antiluteolytic substance (Rowson and Moor, 1967). These results led to the partial identification and purification of a protein called trophoblastin (Martal et al., 1979) which was sensitive to heat and proteases, and secreted in minimal amounts after day 20 from sheep conceptuses.

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Godkin et al. (1982) completed the final purification of a protein secreted by the ovine conceptus that matched previously described characteristics of the antiluteolysin. This protein was the major product secreted from day-13 to 21 ovine embryos (Godkin et al., 1982), the major translation product of mRNA isolated from day-16 sheep conceptuses (Hansen et al., 1985; Anthony et al., 1988), and consisted of approximately 3 to 4 isoforms having a molecular weight of approximately 19,000 (Godkin et al., 1982; Imakawa et al., 1987). When injected into the uterine lumen of non-pregnant ewes, the protein, later called ovine trophoblast protein-1 (oTP-1), dampened pulsatile peaks of $PGF_{2\alpha}$ from the uterus and prolonged the lifespan of the corpus luteum (Godkin et al., 1984; Vallet et al., 1988).

A secretory product immunologically related to oTP-1 has been identified in cattle; this protein is called bovine trophoblast protein-1 (bTP-1) (Imakawa et al., 1989). Bovine trophoblast protein-1 also has at least 4 isoforms ranging in molecular weight between 22,000 and 24,000. Translation studies demonstrate that the major difference between oTP-1 and bTP-1 is the presence of an asparagine-linked carbohydrate molecule on bTP-1.

Molecular cloning of cDNA for oTP-1 (Imakawa et al., 1987; Stewart et al., 1989; Charlier et al., 1989; Klemann et al., 1990a) and bTP-1 (Imakawa et al., 1989; Stewart et al., 1990; Hansen et al., 1991) revealed that coding regions for bTP-1 have 90%, 85%, and 63% identity to cDNAs for oTP-1, bovine interferon ω, and bovine interferon α respectively (Imakawa et al., 1989). Amino acid sequence homology shows that bTP-1 is 80% homologous to oTP-1, 72% to IFN-ω, and 50% to bIFN-α. IFN-α, IFN-β, and IFN-

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ω belong to the Type I interferon family because they are probably derived from the same ancestral gene and are clustered on the same chromosome (DeMaeyer and DeMaeyer-Guignard, 1988). Bovine trophoblast protein-1 and oTP-1 are functional interferons having antiviral activity similar to other Type I interferons (Pontzer et al., 1988; Roberts et al., 1989; Klemann et al., 1990b; Martal et al., 1990); they also compete with IFN-α for binding to Type I IFN receptors (Stewart et al., 1987; Hansen et al., 1989; Knickerbocker and Niswender, 1989). Trophoblast interferons have little similarity to Type II or γ interferons. Because oTP-1 and bTP-1 are secreted from the trophoblast, have high sequence homology, and respond poorly to viral stimulation, Roberts et al. (1992) suggested the trophoblast interferons be given the nomenclature interferon tau (IFNτ).

Trophoblast interferons have been studied to determine the onset, rate, and site of expression. Ovine IFNτ has been detected in day-10 embryos, although high rates of oIFNτ are not secreted until approximately day 13 of gestation (Godkin et al., 1982; Hansen et al., 1985). Once induced, synthesis of oIFNτ increases over three orders of magnitude between day 12 and day 15 to 16 of pregnancy, the time of peak production. A single 16-day conceptus cultured *in vitro* may secrete up to 100 μg of oIFNτ during a 24 hour period (Ashworth and Bazer, 1989). Furthermore, similar patterns were observed for oIFNτ mRNA content using *in situ* hybridization techniques, confirming the data on protein production. Farin et al.(1989, 1990) observed that oIFNτ and bIFNτ mRNAs are synthesized in the trophectoderm, the outer epithelial layer of the conceptus. The amount of oIFNτ mRNA per cell increases significantly on approximately day 13, when spherical

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blastocysts begin to elongate into a filamentous form. Parallel changes occur in the cow, although peak bIFNτ production occurs between days 16 to 19 of pregnancy (Bartol et al., 1985). It has been speculated that rapid embryonic growth and increased IFNτ synthesis are coincident in order to provide an adequate amount of IFNτ, thus preventing pulsatile secretion of $PGF_{2\alpha}$ and luteolysis. On day 17 in cattle, the period of maternal recognition of pregnancy, embryos vary greatly in size ranging from 15 mm to 250 mm in length (Thatcher et al., 1994). It has been theorized that embryos shorter than 150 mm in length were potentially less viable since they occupied less than one-third of the uterine horn. Factors regulating embryonic growth and IFNτ production are critical to the ultimate survivability of conceptuses. At this time, these factors are mostly unknown.

Uterine-Embryo Communication and Implantation

The survivability of mammalian embryos relies upon successful implantation of the embryonic trophoblast into the uterine endometrium. The interface between the fetus and maternal tissues eventually results in formation of the placenta, a temporary organ responsible for protection and nourishment of the fetus until it is capable of surviving outside the uterine environment (King, 1993). The extent of invasiveness of the trophectoderm into the endometrium is dependent on the species. Rodents and humans have highly invasive blastocysts that penetrate through the uterine epithelium, deep into the uterine stroma (Perry, 1981). This type of hemochorial placentation results in maternal blood bathing the fetal chorion.

In many ungulates, including the cow, implantation is superficial and noninvasive

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when compared to other mammalian species (King et al., 1982). The cow exhibits a synepitheliochorial type of placentation, a mixture of maternofetal hybrid syncytium with cellular uterine epithelium apposed to fetal cellular chorion (Wooding and Morgan, 1993). Although this type of placentation consists of little more than close apposition of the trophoblastic and uterine epithelial layers, the term 'implantation' is still applied for ease of discussion. Since integrity of the embryonic and maternal cell layers is retained, a dramatic increase in surface area between the fetus and mother is necessary for adequate gas and nutrient exchange. Bovine conceptuses rapidly elongate from spherical to tubular vesicles proliferating to nearly fill the entire length of the uterus by day 21 in cattle (Leiser, 1975). Also, formation of interdigitating fetal and uterine microvilli increase surface area tremendously to facilitate nutrient and gas exchange (King, 1993).

Embryonic trophectoderm consists of two cell types prior to day 18 of pregnancy, binucleate and uninucleate trophoblast cells. Binucleate cells, appearing on days 14 to 15 of gestation, are larger than uninucleate trophoblast cells and constitute 15-22% of the total trophoblast through the final week of pregnancy (Wooding, 1983). Binucleate cells are formed when uninucleate trophoblast cells undergo nuclear division that is not followed by cytokinesis (Wimsatt, 1951). As early binucleate cells mature, they become rounded, increase in size, and develop characteristic cytoplasmic granules (Hoffman and Wooding, 1993).

On day 18 of gestation in the cow, the embryo and uterine epithelium come into close proximity. At this time, cells comprising the uterine epithelium become more regularly columnar, and a significant portion are found to have two nuclei, forming a

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uterine syncytium (Wathes and Wooding, 1980). Microvillar interdigitation between the embryonic trophoblast and maternal endometrium is present by day 19 of pregnancy. The adherence process is gradual, beginning at the embryonic disc and spreading progressively towards the vesicle ends (King, 1993). During this time, mature binucleate cells migrate from the chorion, through the trophectodermal tight junctions, and fuse with uterine columnar cells forming fetomaternal hybrid trinucleate cells (Wooding and Morgan, 1993). The granules present in the fetal binucleate cell pass into the hybrid trinucleate cell and are released by exocytosis to the maternal tissues and eventually maternal circulatory system. Mitosis does not occur once binucleate cells fuse with a single uterine epithelial cell or with existing uterine syncytium; therefore, new granulated binucleate cells are continuously escaping through trophectodermal tight junctions, fusing with uterine epithelial cells, releasing their granules, and degenerating (Hoffman and Wooding, 1993).

This unique system functions in delivering a variety of hormones from the fetus to the maternal circulation throughout gestation. Placental lactogen has been immunolocalized to fetal binucleate cell secretory granules in the cow (Flint et al., 1979; Duello et al., 1986; Wooding and Beckers, 1987), along with three other binucleate and giant cell markers: 1) bovine pregnancy-associated glycoprotein (Zoli et al., 1992); the molecule recognized by the monoclonal antibody to the SBU-3 antigen (Beckers et al., 1988); and a bovine chorionic gonadotropin-like protein (Morgan et al., 1989). Placental lactogens are members of the prolactin/growth hormone gene family and have the ability to interact with prolactin and growth hormone receptors (Talamantes, 1975; Kelly et al.,

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1976). The action of these hormones is speculative, although they potentially target the fetus, mother, and uteroplacental unit; these hormones may be involved in fetal and/or placental growth, modulation of maternal energy metabolism, growth of the mammary epithelium, maintenance of steroidogenesis by both the placenta and corpus luteum, control of osmotic balance in the fetus, development of fetal immune competence, and modulation of the maternal immune system (Schuler et al., 1993). Furthermore, it has been demonstrated that fetal binucleate cells are capable of synthesizing and delivering progesterone to the maternal circulation (Ullman and Reimers, 1989).

Between days 20 to 28 of gestation, trinucleate cells continue to expand by fusion with binucleate cells, forming syncytial plaques (Wooding and Morgan, 1993). Many of the original uterine columnar cells degenerate and are phagocytosed by uninucleate chorion cells (Wathes and Wooding, 1980). Residual uninucleate uterine epithelial cells divide to form fat cuboidal cells that continue to proliferate eliminating the syncytial plaques by days 40 to 50 of pregnancy (Wooding and Morgan, 1993). This produces a layer of uterine cuboidal cells with a scalloping basement membrane, structurally unique from the original uterine columnar epithelium. Embryonic binucleate cells are continually produced throughout pregnancy and fuse with uterine epithelial cells; once binucleate cell granules are released, the hybrid trinucleate cells degenerate and are phagocytosed by the chorion (Wathes and Wooding, 1980).

CONCLUSION

Significant economic loss in the beef and dairy industry is due to early embryonic

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mortality. It is estimated that approximately 40-45% of pregnancies are prematurely terminated in reproductively normal cattle, and nearly 65-70% of this loss occurs between days 15-18 of gestation.

Several critical cellular and molecular events occur during the peri-implantation period of development including embryonic elongation, secretion of INFτ, and attachment of the embryonic trophectoderm to the uterine epithelium. A better understanding of events affecting embryo survivability could be valuable in reducing early embryonic death. Morphological changes that occur during this time have been documented; identifying changes in gene expression would seem an appropriate next step.

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CHAPTER II

IDENTIFICATION OF GENES WITH GREATER mRNA CONTENT IN 17.5-DAY THAN 15.5-DAY BOVINE EMBRYOS

INTRODUCTION

Embryonic wastage results in substantial economic loss to the cattle industry. This loss was estimated to be as high as 1.4 billion dollars nearly two decades ago (Gerrits et al., 1979) and likely is much higher today. Failure to achieve term pregnancy is estimated to be approximately 40-45% in beef and dairy cows that have received a single insemination (Roche et al., 1977; Mawhinney and Roche, 1978). Fertilization failure typically accounts for 25% of this reproductive loss in ruminants while the remaining 75% of failure is attributed to early embryonic mortality (Boyd et al., 1969; Sreenan and Diskin, 1986). The majority of early embryonic wastage occurs between days 15 and 18 of pregnancy (estrus=0) (Sreenan and Diskin, 1986), when several critical events occur including maternal recognition of pregnancy and attachment of the trophoblast to the endometrium. Changes in gene expression likely are related to these developmental events. Although morphological changes that occur at this time have been well documented (Wathes and Wooding, 1980; Guillomot and Guay, 1982), cellular and molecular changes remain practically unstudied.

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Studying developmentally regulated gene expression in preimplantation and peri-implantation embryos has been problematic due to the paucity of tissue. To better understand changes in gene expression in bovine embryos between day 15.5 and 17.5 of gestation (estrus=0), we used the RT-PCR based technique developed by Liang and Pardee (1992), mRNA differential display (Figure 1). This technique not only allowed detection of changes in expression of known factors present in peri-implantation embryos, but also unknown potential mediators of significant events during this period of development.

MATERIALS AND METHODS

Embryo Collection

Cows were artificially inseminated 12 hours post-estrus (day 0 = estrus), and conceptuses were collected on days 15.5 and 17.5 of pregnancy. Cows were confined in a cattle chute and given an epidural block of 2% procaine. A sterile 24 gauge Foley catheter was inserted through the cervix into the uterine body, and the balloon was inflated immediately cranial the cervix to ensure that the catheter remained in place. Modified Dulbecco's phosphate-buffered saline supplemented with 0.1% BSA was flushed through the uterus according to the nonsurgical procedure developed by Elsden et al. (1976). Upon recovery, embryos were measured, rinsed through 0.1% BSA in sterile modified Dulbecco's phophate-buffered saline and frozen in liquid nitrogen. To minimize potential confusion from the substantial size variation among elongating embryos, only 15.5-day bovine embryos ranging in length from 2 to 6 cm were used.

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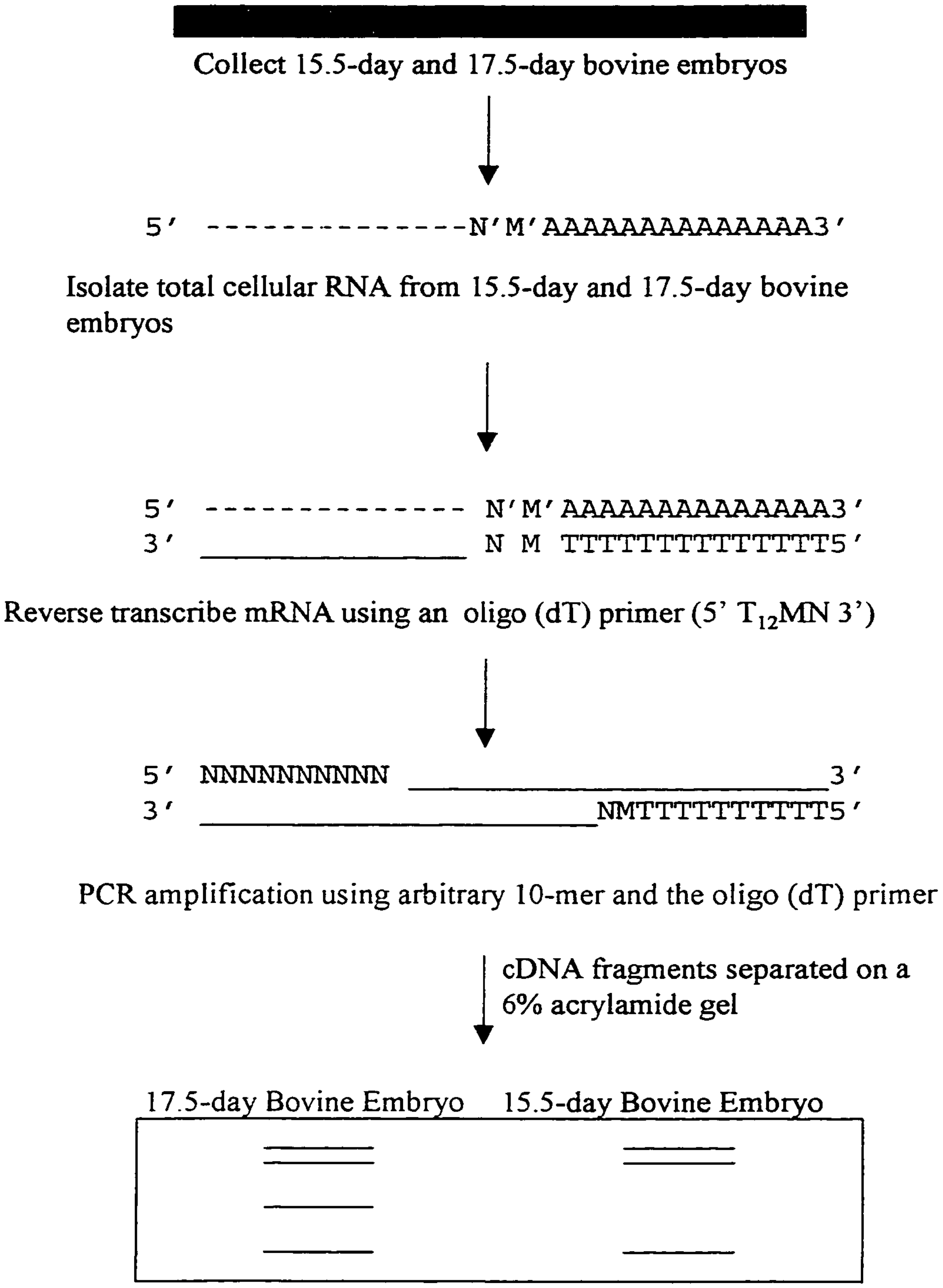

Fig. 1 Schematic of mRNA differential display technique. The diagram of the gel represents cDNA fragments generated using a single primer set from a 17.5-day and 15.5-day bovine embryo. The red fragment illustrates a single amplicon present in 17.5-day embryos, but not 15.5-day embryos. This cDNA fragment is isolated, reamplified, and subcloned. Dashed lines are RNA; solid lines are DNA; M indicates A, C, or G. N can be A, C, G, or T.

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Messenger RNA Differential Display Technique

Total RNA Isolation

Total RNA was isolated from two 17.5-day and two 15.5-day bovine embryos using a modified protocol developed by Chomczynski and Sacchi (1987); the modifications are indicated below. Unless otherwise stated, all solutions were prepared with 0.1% diethyl pyrocarbonate. A stock denaturing solution was prepared by dissolving 250 g guanidinium thiocyanate in 293 ml water, 17.6 ml of 0.75 M sodium citrate, and 26.4 ml of 10% sarcosyl in water. Lysis buffer was prepared from the stock, 1 ml for 17.5-day embryos and 0.5 ml for 15.5-day embryos, by adding 0.1 M 2-mercaptoethanol and 25 μg poly(C) (Boehringer Mannheim, Indianapolis, IN). Lysis buffer was added to each vial containing a frozen embryo, and lysate was sheared through a 22 g needle, transferred to a 1.5 ml microcentrifuge tube, and extracted using 1/10 volume 2 M sodium acetate (pH 4.0), 1 volume phenol (pH 4.3), and 1/5 volume chloroform-isoamyl alcohol 49:1. After centrifugation (Eppendorf Centrifuge, model 5415C, Westbury, NY) at 16,000 x g for 15 min at 4°C, the aqueous phase was transferred to a clean microcentrifuge tube, and RNA was precipitated overnight at -20°C by adding an equal volume of isopropyl alcohol. RNA was collected by centrifugation at 16,000 x g for 15 min at 4°C, and the pellet was rinsed briefly with 500 μl of 70% ethanol. After removing the supernatant, the pellet was dried for 1 min in a Savant SpeedVac^{R} and resuspended in 23 μl diethyl pyrocarbonate-treated water. To remove contaminating DNA, samples were treated for 30 min at 37°C with 3 U RQ1 RNase-Free DNase (Promega, Madison, WI) in the recommended buffer containing 1 mM DTT, 40

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mM Tris-HCl (pH 8.0), 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$, 20 U RNAsin and water, bringing the final volume to 40 µl. Following DNase treatment, 200 µl of diethyl pyrocarbonate-treated water were added to each sample, and RNA was extracted with sodium acetate, phenol, and chloroform-isoamyl alcohol as previously described. The aqueous phase was transferred to a clean microcentrifuge tube and precipitated overnight with an equal volume of isopropyl alcohol. Total RNA was collected and rinsed with 70% ethanol as described previously, resuspended with 20 µl diethyl pyrocarbonate-treated water, and the concentration of RNA was calculated by removing an aliquot and determining the A_{260}. RNA was diluted to 0.25 µg/µl, aliquoted (0.5 µg) into 0.5 ml Microcentrifuge tubes and stored at -70°C.

Reverse Transcription

Total RNA (0.5µg) and oligo (dT) primer 5'-T_{11}MN-3' (where M represents A, C, or G, and N represents all four nucleotides) (20 µM) were combined, and the volume was brought to 12.4 µl with diethyl pyrocarbonate-treated water. Total RNA was reverse transcribed using one of twelve oligo (dT)+2 base pair primers; each of the 12 5'-T_{11}MN-3' primers was used in separate reactions. Samples were incubated for 10 min at 70°C and cooled to 45°C. The remaining components were combined in a mastermixture, prewarmed to 45°C, and added to the samples yielding a final volume of 20 µl and final amounts of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml BSA, 10 mM dithiothreitol, 40 U RNAsin (Promega, Madison, WI), 0.2 mM of each dNTP, and 200 U Superscript RT II enzyme (Gibco BRL, Gathersburg, MD). Samples were

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incubated at 45°C for 1 hr, 95°C for 5 min, and cooled to 4°C.

Polymerase Chain Reaction

Condensate was collected by centrifugation at 16,000 x g for 30 sec. For each 20 μl PCR mixture, 2 μl of each RT reaction were added to 18 μl of PCR solution to obtain the final amount of 1x Vent polymerase buffer (New England Biolabs, Beverly, MA), 2 μM of each dNTP, 5 μCi of (^{35}S)-deoxy-ATP (1000 Ci/mmol; Amersham, Cleveland, OH), 0.5 U Vent exo-polymerase (New England Biolabs, Beverly, MA), and 0.5 μM of 1 arbitrary decamer. Five unique arbitrary decamers were used in separate PCR reactions per sample. The sample was overlaid with 40 μl mineral oil and subjected to 40 cycles in a model 9600 thermocycler (Perkin Elmer/Cetus, Norwalk, CT) using the following parameters: 94°C for 30 sec, 42°C for 60 sec, and 72°C for 30 sec. Radiolabeled PCR product (3 μl) was combined with 3 μl stop solution (USB Sequenase^{R} Version 2.0 T7 DNA Polymerase, Cleveland, OH), boiled for 2 min to denature the DNA, placed on ice, and separated on a 6% polyacrylamide sequencing gel. The gel was exposed to Kodak X-Omat film for 12 hr, and fragments that were consistently differentially expressed between the two embryonic stages were identified. If differentially expressed fragments were present, the gel was exposed to Kodak X-Omat film for an additional 12 hr. A razor blade was used to remove the bands present in greater abundance in 17.5-day compared to 15.5-day embryos from the Kodak X-Omat film. Once removed, the x-ray was realigned with the acylamide gel, and DNA fragments were excised with a razor blade using the x-ray film as a template. Fragments were placed in 100 μl sterile water in 0.5

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ml microcentrifuge tubes, and the acrylamide gel was reexposed to Kodak X-Omat film for 12 hr to guarantee that the correct band was excised. Tubes containing the cDNA fragments were boiled for 15 min and centrifuged for 5 min at 16,000 x g to pellet debris. The supernatant was transferred to a 0.5 ml microcentrifuge tube; 8 μl of 3 M potassium acetate, 2.5 μl of glycogen (20 mg/ml), and 300 μl of 100% isopropyl alcohol were added, and samples were incubated at -20°C overnight to precipitate the DNA. DNA was collected by centrifugation, rinsed in 70% ethanol, and dried briefly as previously described. Samples were resuspended in 10 μl water.

Reamplification of PCR Products

To reamplify the differentially expressed product, DNA (5 μl) was added to 95 μl of PCR solution to obtain a final amount of 2.5 μM of the respective oligo (dT) primer, 1x Vent polymerase buffer, 20 μM of each dNTP, 1.25 U Vent exo-polymerase, and 2.5 μM of the respective decamer; no (^{35}S)-deoxy-ATP was present. Samples were overlaid with mineral oil, and the same PCR parameters were used as described previously except the last cycle was followed by a 72°C extension time for 10 min.

Southern Blot Analysis

Southern blot analysis was used to identify cDNA fragments that were unique and are not represented in multiple differential display analyses. After reamplification, DNA from each PCR reaction (50 μl) was separated on a 2% agarose gel, denatured in 0.5 M NaOH/1.5 M NaCl for 30 min, neutralized in 0.5 M Tris (pH 7.5)/1.5 M NaCl for 30 min,

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and transferred overnight by capillary action to nylon membrane (Schleicher and Schuell, Keene, NH). The membrane was cross-linked at 1200 Joules using a CL-1000 Ultraviolet Crosslinker (UVP, Upland, CA) and prehybridized for 2 hrs at 42°C in 10 ml of solution containing 50% formamide, 5x Denhardt's solution, 5x SSC, 0.5% SDS, 5 µg poly (A) RNA, and 1.5 mg boiled salmon sperm DNA. DNA (25 ng) from each PCR reaction was radiolabeled with (^{32}P)-deoxy-CTP (3000Ci/mmol; ICN, Costa Mesa, CA) using Random Primers DNA Labeling System (Gibco/BRL, Gaithersburg, MD). Following incubation, 1×10^7 cpm of probe was denatured in 0.3 M NaOH for 5 min, neutralized with 0.05 M Tris (pH 7.2)/0.3 M HCl, and immediately added to the Southern Blot. After hybridizing overnight at 42°C, the membrane was rinsed for 20 min at 42°C in 2x SSC/0.1% SDS, 15 min at 65°C in 1x SSC/0.1% SDS, and 10 min at 65°C in 0.1x SSC/0.1% SDS to remove nonspecifically bound probe. Then, the membrane was exposed to Kodak X-Omat film for approximately 1 hr. If a single band was present on the Southern blot, the corresponding cDNA fragment was used to probe the following Northern blots. If multiple bands were present, the largest cDNA fragment was identified and used in the following Northern blots analyses.

Northern Blot Procedure Used to Verify Differential Expression Between 15.5-day and 17.5-day Bovine Embryos

Embryo Collection

Northern blot analysis was conducted with a new set of embryos to verify an increase in gene expression between days 15.5 and 17.5 of pregnancy as detected by

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differential display. Due to the relatively small amount of tissue present on day 15.5 of pregnancy, cows were superovulated with follicle stimulating hormone (FSH-P) (Schering), and embryos were collected on day 15 of pregnancy. Cows were superovulated according to the following regimen (days post estrus):

Day 10 p.m.	6 mg FSH i.m.
Day 11 a.m.	6 mg FSH i.m.
Day 11 p.m.	4 mg FSH i.m.
Day 12 a.m.	4 mg FSH i.m.
Day 12 p.m.	2 mg FSH i.m.
Day 13 a.m.	2 mg FSH i.m. 25 mg prostaglandin $F_{2\alpha}$ i.m.
Day 13 p.m.	2 mg FSH i.m. 12.5 mg prostaglandin $F_{2\alpha}$ i.m.
Day 14 a.m.	2 mg FSH i.m.

Cows usually show estrous behavior in the morning of the fifteenth day using this regimen. Embryos were collected on day 17.5 of pregnancy from cattle that were not superovulated, and all embryos collected were rinsed and frozen as described in the differential display technique section above.

RNA Isolation

Total RNA from six 15-day embryos ranging in size from 2 to 6 cm and two 17.5-day embryos were isolated according to the protocol developed by Chomczynski and Sacchi (1987), described previously with additional modifications. Lysis buffer was prepared as described previously from the denaturing stock solution and added to embryos, 1 ml for 17.5-day embryos and 0.5 ml for 15.5-day embryos. Lysate was sheared through a 22 g needle, transferred to a 1.5 ml microcentrifuge tube, and phenol-

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chloroform-isoamyl alcohol extracted as described previously. The aqueous phase containing the RNA was transferred to a clean microcentrifuge tube after centrifugation, and RNA was precipitated overnight. RNA was collected by centrifugation, and the pellet was rinsed briefly with 500 µl of 70% ethanol. After drying the pellet, RNA was resuspended in 300 µl lysis buffer, and transferred to 1.5 ml microcentrifuge tubes. An equal volume of isopropanol was added, and RNA was again precipitated overnight at -20°C. RNA was collected and rinsed with 70% ethanol as described previously, resuspended with 50 µl diethyl pyrocarbonate-treated water, and the concentration was determined using spectrophotmetry (A_{260}=40 ug RNA). RNA was stored at -70°C until further use.

Northern Blot Procedures

After the final ethanol precipitation, samples were resuspended in 1 mM EDTA and heated to 70°C for 15 min. Sample buffer (19 µl) containing 660 µl formamide, 130 µl 10x MOPS, and 210 µl formaldehyde (37%) was added to each sample, and samples were incubated for 5 min at 70°C. Following incubation, loading buffer containing glycerol and bromophenol blue was added to each sample, and RNA was separated on a 0.66 M formaldehyde/1% Seakem GTG agarose denaturing gel. Gels were rinsed 4x for 5 min in water and transferred by capillary action to nylon membranes overnight. Membranes were cross-linked at 1200 Joules as described earlier for the Southern blot procedures.

Differentially expressed fragments chosen from the Southern blots were

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radiolabeled with (^{32}P)-deoxy-CTP (3000Ci/mmol, ICN) using the Random primers DNA labeling system as described for Southern blots, and used to probe the Northern blot. Prehybridization and hybridization of probe to membranes were described in the above Southern blot section. After hybridization, membranes were rinsed for 30 min at 42°C in 2x SSC/0.1% SDS, 30 min at 50°C in 0.5x SSC/0.1% SDS, and 15 min at 50°C in 0.1x SSC/0.1% SDS. Membranes were exposed to Kodak X-Omat film for 24-72 hr, and exposed to a phosphoimaging screen (Molecular Dynamics, Sunnyvale, CA) for 8 and 16 hr. Resulting images were used to quantify differences in steady state mRNA content between day-15 and day-17.5 bovine embryos.

Complementary DNA fragments encoding mRNAs demonstrated to be differentially expressed between embryonic stages were further cloned and sequenced. After probing with the differentially expressed fragments, membranes were stripped by soaking in boiling 0.1x SSC/0.1% SDS for 20 min. The metabolic gene, IB15 cyclofilin (Danielson et al., 1988), was radiolabeled and hybridized to the membranes to confirm equivalent amounts of RNA were loaded into each lane; bands were quantified by phosphoimaging.

Cloning and Sequencing

Four differentially displayed fragments identified from the previous experiments were reamplified using their respective primers and cloned using the Invitrogen TA cloning kit according to the manufacturer's instructions. A modified mini alkaline-lysis/PEG precipitation procedure (Perkin-Elmer Corporation, Norwalk, CT) was used to

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prepare plasmid DNA for sequencing. Plasmid DNA containing the differentially expressed fragment was sent to Macromolecular Resources at Colorado State University to be sequenced. The sequences were compared to known mammalian sequences using the BLAST algorithm (Altschul et al., 1990). From the Northern blot and sequence data, two differentially displayed fragments, allograft inflammatory factor-1 (AIF-1) and fragment 7, were used to complete the following experiments.

Embryonic Gene Expression During Early Pregnancy

Embryo Collection

Bovine embryos were collected on days 17.5, 30, 33, 35, and 36 of pregnancy. Day-17.5 embryos were collected, rinsed, and frozen according to procedures described previously. Later stage embryos were collected according to the following nonsurgical procedure developed in our laboratory. A metal tube 40 cm in length with an outer diameter of 7 mm and an inner diameter of 6 mm, and a stainless steel cervical expander measuring 48.5 cm in length with a diameter of 6 mm tapering to 3 mm were buffed to remove all rough edges and sterilized by autoclaving. After sterilization, the expander was placed inside the metal rod so that the expander tip was exposed. Pregnant cows were given an epidural block of 5 ml of 2% procaine, and their vulva was thoroughly cleaned. Expander and rod were gently threaded through the cervix; once inside the internal os, the expander was slowly removed leaving the hollow rod in place. Tygon^R tubing S-50-HL (60 cm) with an inner diameter of 0.63 cm was placed over the metal tube, and a 60 ml luer-lock syringe filled with modified Dulbecco's PBS plus 0.4% BSA

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was anchored to the opposite end of the Tygon tubing. The entire contents in the syringe were expelled into the uterus, and the uterus was gently massaged to dislodge the embryo. While applying gentle pressure, uterine contents were aspirated into the syringe. This procedure was repeated until the embryo was recovered. Embryos were rinsed and extra-embryonic membranes were frozen as described previously.

RNA Isolation and Northern Blots

These procedures were described previously in the Northern blot section; a few exceptions are described here. After isolating RNA from individual embryos, total RNA (25 µg) was separated by gel electrophoresis and capillary transferred to nylon membranes. The partial cDNA for AIF-1 was radiolabeled as described previously and used to probe the membrane. After rinsing away excess probe, the membrane was exposed to Kodak X-Omat film for 72 hr, and further exposed to phosphoimaging analysis for 24 and 48 hr to quantify the level of expression among embryos. After collecting data for AIF-1, the membrane was stripped as described previously. This procedure was repeated using radiolabeled DNA fragment 7 to probe the membrane. The membrane was exposed to Kodak X-Omat film for 96 hr, but for fragment 7, not exposed for phosphoimaging analysis.

To provide an internal control, membranes were stripped and hybridized to a radiolabeled cDNA fragment encoding the 28S ribosomal subunit (Ambion, Austin, TX) (Hillis and Dixon, 1991). Membranes then were rinsed to remove excess probe, exposed to Kodak X-Omat film for 20 min, and further exposed to phosphoimaging analysis for 5

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and 15 min to quantify the steady state content of 28S ribosomal subunit cDNA.

To verify information obtained using the 28S ribosomal subunit as an internal control, membranes were stripped and hybridized to radiolabeled cDNA encoding human γ-actin (Gunning et al., 1983). Membranes were rinsed, exposed to Kodak X-Omat film for 6 hr, and further exposed to phosphoimaging analysis for 30 min and 1.5 hr to quantify the steady state content of γ-actin mRNA.

Northern Blots to Determine Differences in Gene Expression Among Various Adult Tissues

Tissue Collection, RNA Isolation, and Northern Blot Procedures

Adult tissues including brain, heart, kidney, liver, lung, anterior pituitary, skeletal muscle, and spleen were collected immediately from a slaughtered heifer, dissected into pieces (~1 gm) and frozen in liquid nitrogen.

Total RNA was isolated as described above with a few modifications. Lysis buffer was prepared from denaturing solution stock as previously noted. Lysis buffer (5 ml) was added to a 50 ml conical tube, and 0.5 g tissue was added to each tube. Tissue was homogenized (Polytron, Brinkman Instruments, Westbury, NY), transferred to a 10 ml Falcon tube, and extracted using 1/10 volume 2 M sodium acetate (pH 4.0), 1 volume phenol (pH 4.3), and 1/5 volume chloroform-isoamyl alcohol 49:1. After centrifugation at 16,000 x g for 15 min at 4°C (Beckman, Accuspin FR$^{(TM)}$, Fullerton, CA), the aqueous phase was transferred to a clean Falcon tube, and RNA was precipitated overnight at -20°C by adding an equal volume of isopropyl alcohol. RNA was collected by

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centrifugation at 16,000 x g for 15 min at 4°C, and the pellet rinsed briefly with 5 ml of 70% ethanol. After removing the supernatant, the pellet was dried for 1 min in a Savant SpeedVac^R, resuspended in 300 μl of denaturing solution, and transferred to a 1.5 ml microcentrifuge tube. The remaining procedures were as described in the Northern blot sections above.

Cloning of Allograft Inflammatory Factor and Fragment 7 cDNA from a 25-day Bovine Embryo cDNA Library

The 3' partial cDNA fragment for AIF-1 and differential display fragment 7 were used to screen a bacteriophage lambda, Uni-ZAP cDNA library prepared from 25-day bovine embryos (Xie et al., 1995). Approximately 400,000 plaques were screened for AIF-1 and ~800,000 plaques were screened for fragment 7 according to the manufacturer's instructions. Positive clones were isolated, and pBluescript phagemids were excised by the *in vivo* excision protocol using ExAssist helper phage and *E. coli* SOLR strain (Stratagene, LaJoya, CA). Rescued plasmids were restriction digested with Xho 1 and Eco R1, separated by gel electrophoresis, and hybridized to the original probe to verify their identity. Plasmids were prepared and sequenced by Macromolecular Resources using T3 and T7 primers, two synthesized specific primers for AIF-1, and six synthesized specific primers for cDNA fragment 7. Nucleotide and amino acid sequence searches were performed using the BLAST algorithm, and motif searches were generated using the prosite database.

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RESULTS

Differential Display and Southern Blot Analysis

Differential display was used to identify genes that encode mRNAs that increase in abundance in 17.5-day relative to 15.5-day bovine embryos. Total RNA was isolated from embryo samples, reverse transcribed using one of twelve oligo (dT) primers (5'-T_{11}MN-3'), and PCR-amplified using the respective anchored primer and one of five decamers resulting in 60 different primer combinations per sample. The RNA from two 15.5-day and two 17.5-day bovine embryos were reverse transcribed and amplified using 60 primer combinations resulting in 240 PCR reactions. Complementary DNA profiles generated using the same primer set in each embryo were compared side-by-side. Each of the 60 differential displays yielded 150-200 bands per lane; if each band represents a unique mRNA, we would have examined approximately 50% of the estimated 10,000-20,000 mRNA species present. As indicated in Table 1, some mRNAs are represented more than once, so the number of unique mRNAs examined was lower. Although it is difficult to determine the number of mRNA species evaluated, Liang and Pardee (1992) estimated that ~25% of the total mRNA population could be examined using 60 different primer combinations.

Fifteen amplicons (designated bands 1-15) putatively present in larger amounts in 17.5-day than 15.5-day embryos were excised, reamplified, and used as radiolabeled probes in subsequent Southern and Northern blot analyses. All amplicons ranged from 240 to 600 bp in length, and with the exception of cDNA fragment 10, yielded a single

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Table 1. Summary of cDNA fragments identified by differential display

Fragment Number	Northern analysis confirmation	Approximate mRNA size, kb	Avg. relative increase in transcription	Sequence homologies
1	Confirmed	0.8	4.6-fold	Human and rat AIF-1
2	Confirmed	5.0 4.5 2.0	11-fold 6.6-fold ?	Human LERK-5
3	Not confirmed			
4	Not determined			
5	Not confirmed			
6, 8, 10, 11, 12, 14	Confirmed	1.6	2.5-fold	Bovine interferon-τ
7, 9, 15	Confirmed	1.8	10.6-fold	None found
13	Not confirmed			

Fragment numbers (1-15) are designated randomly. Fragments with shared sequence homologies by Southern blot are shown in the same row. Complementary DNA fragments shown in bold italics were radiolabeled and used in Northern blot analysis to confirm differential expression; the results confirmed the original display (confirmed), did not confirm the original display (not confirmed), or this method lacked sensitivity to produce a notable band (not determined). The mRNA transcript size determined by Northern blot analysis is shown, as is the average increase in mRNA content in 17.5-day relative to 15.5-day bovine embryos.

band when separated by gel electrophoresis on a 1% agarose gel. Fig. 2 illustrates differential displays obtained with four separate primer combinations. Four representative cDNA fragments are shown that are present in greater abundance in 17.5-day embryos (lanes 3 and 4) compared to 15.5-day embryos (lanes 1 and 2).

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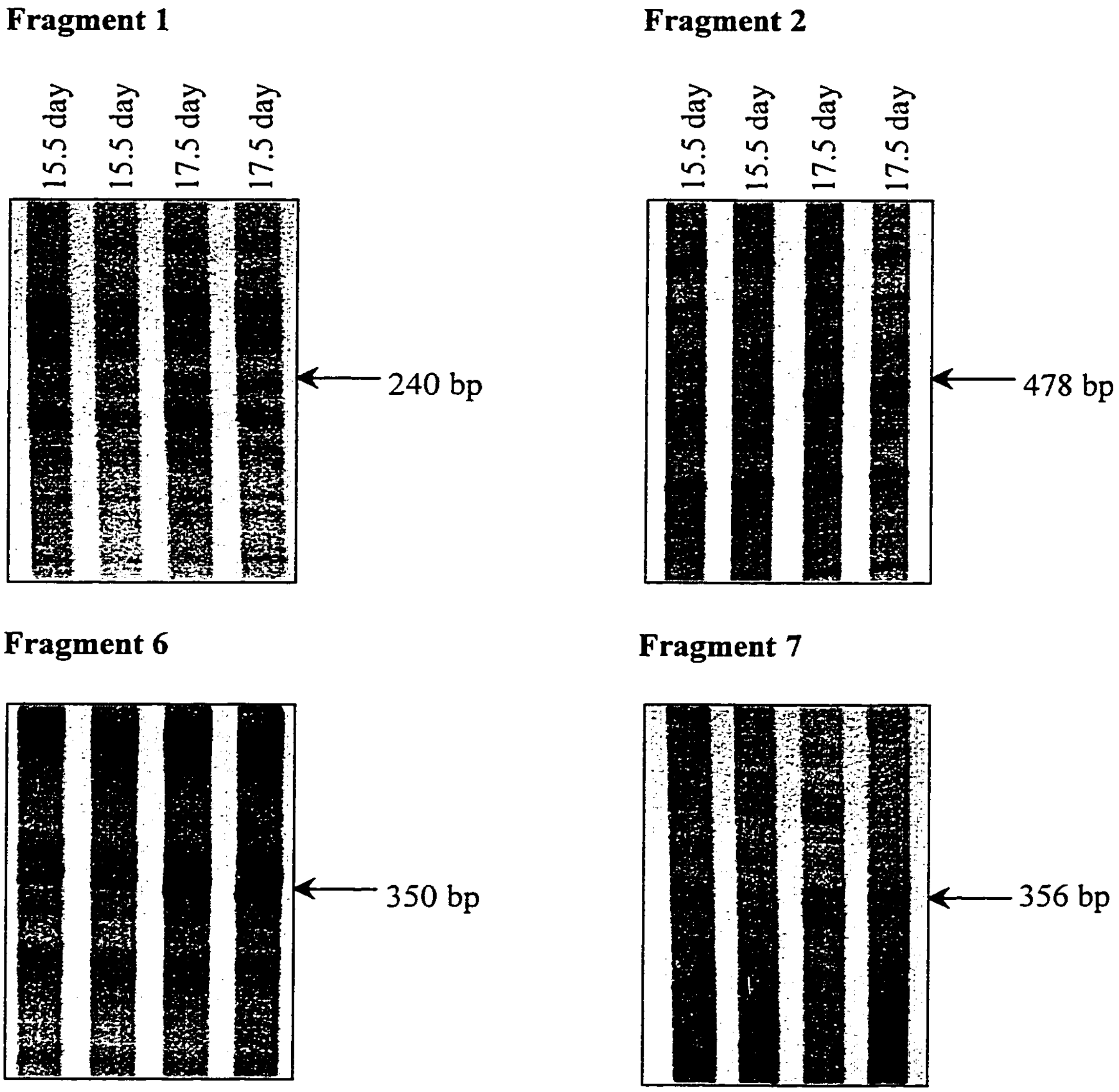

Fig. 2 Representative band patterns from differential display analysis showing PCR fragments generated from up-regulated genes expressed in 17.5-day more abundantly than 15.5-day bovine embryos. Total RNA was isolated from bovine embryos collected on 17.5 and 15.5 days gestation and subjected to differential display analysis. X-ray films exposed to denaturing 6% polyacrylamide gels demonstrating band patterns generated from four different primer sets are shown. Arrows indicate a higher band intensity in 17.5-day (lanes 3 and 4) than in 15.5-day (lanes 1 and 2) embryos. Primer combinations used to generate each pattern are: fragment 1, 5'-T_{11}CA-3' and 5'-TGGAGATCTG-3'; fragment 2, 5'-T_{11}CC-3' and 5'-TGGAGATCTG-3'; fragment 6, 5'-T_{11}GG-3' and 5'-TGGAGATCTG-3'; and fragment 7, 5'-T_{11}GG-3' and 5'-GTACTGAACC-3'. The approximate size of PCR fragments is indicated next to the arrows and estimated using DNA size standard ϕX174/HaeIII.

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The 15 cDNA fragments identified by differential display analysis are in Table 1. Differentially expressed amplicons were reamplified by PCR using the appropriate primer set. The resulting reactions were used in two ways: 1) amplified cDNA fragments were separated on a 1% agarose gel and transferred to nylon membrane; 2) the same cDNA fragments were radiolabeled and used as molecular probes to identify unique amplicons on the Southern blot. There were several cDNA fragments that appeared to be identical, although they were PCR amplified using different primer sets.

Complementary DNA fragments represented by a single band on the Southern blot (bands 1, 2, 3, 4, 5 and 13) were chosen and used in the following Northern blot analyses. In samples where several cDNA fragments appear to represent the same up-regulated gene, the largest amplicon was chosen and used in subsequent Northern blot analyses (bands 6 and 7).

Northern Blot Analysis with Differentially Displayed cDNA Fragments and Nucleotide Sequencing Analysis

To confirm that the mRNA concentration of differentially displayed cDNA fragments was greater in 17.5-day compared to 15.5-day bovine embryos, Northern blot analysis was conducted using two individual 17.5-day embryos collected from unsuperovulated cows, and a pool of six 15-day bovine embryos collected from superovulated cows. The pool of 15-day embryos was needed to obtain adequate amounts of total RNA to conduct Northern blot analyses. Northern blots were screened using ^{32}P-labeled cDNA fragments (bands 1, 2, 3, 4, 5, 6, 7 and 13) from PCR mixes. Of the eight cDNA fragments that appeared to be differentially expressed, four (bands 1, 2, 6

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and 7) had a higher mRNA concentration in 17.5-day than 15-day embryos; equal concentration was noted for three fragments, and no signal was detected for one cDNA fragment.

A single band ~800 bp in length was detected in all samples (Fig. 3) when using fragment 1 as a probe. Expression of this transcript was 4.3-fold and 4.9-fold greater in individual 17.5-day embryos than the 15-day pool. Three distinct mRNA transcripts were noted for fragment 2, a major mRNA species ~5000 bp in length, and two minor transcripts ~4500 bp and ~2000 bp in length (Fig. 3). The major transcript was present on the Northern blot in 11.3-fold and 10.6-fold more abundance in 17.5-day than 15-day embryos; the ~4500 bp transcript was 4.2-fold and 9-fold more abundant in 17.5-day embryos compared to the 15-day pool. Intensity of the smallest band (~2000 bp) was not measured due to a break in the agarose gel that separated the hybridization band. Fragment 6 yielded a single mRNA transcript ~1600 bp in length, and relative content was 2.5-fold more in 17.5-day than 15-day embryos (Fig. 3). Finally, a single transcript of ~1800 bp in length was shown for fragment 7; it was 9.4-fold and 11.7-fold more abundant in 17.5-day embryos than the 15-day embryo pool (Fig. 3). RNA loading was normalized among all samples using cyclofilin cDNA.

The four differentially expressed cDNA fragments were subcloned into the plasmid pCRII vector; inserts were excised, radiolabeled using (^{32}P)-deoxy-CTP, and used to reprobe the Northern blots containing total RNA isolated from both stages of embryos to verify that the correct fragment had been cloned. In all cases, equivalent results were obtained from Northern blots probed with fragments generated by PCR or

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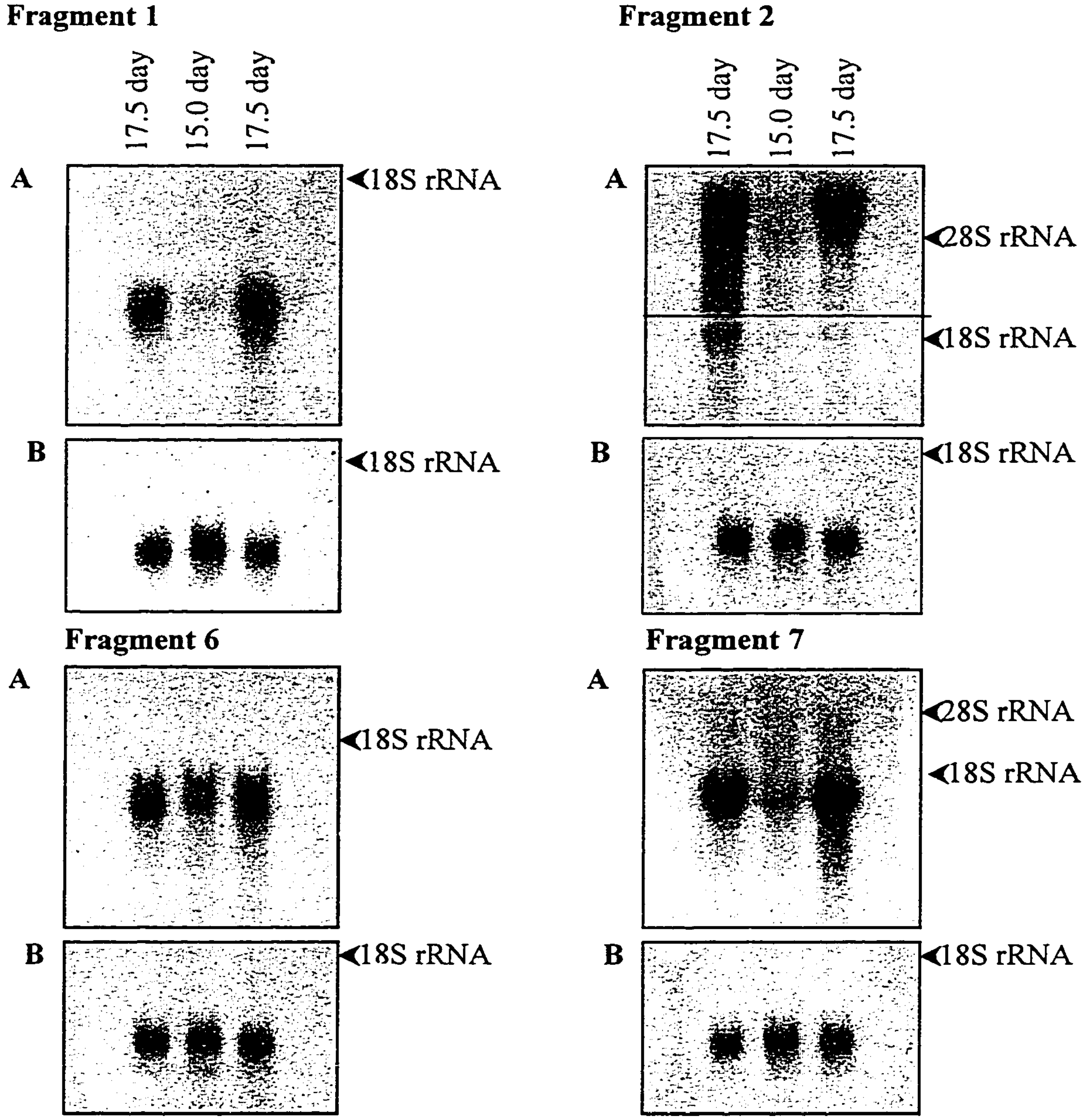

Fig. 3 Northern blot analyses demonstrating increased mRNA content for differential display bands 1, 2, 6 and 7. Total RNA (20μg per lane) was isolated from two 17.5-day and a pool of 15-day bovine embryos. A) Hybridization patterns demonstrating increased mRNA content in 17.5-day relative to 15.5-day bovine embryos are indicated for each differentially expressed cDNA fragment. Messenger RNA content was transcriptionally up-regulated 4.6-fold, up to 11-fold, 2.5-fold and 10.6-fold for cDNA fragments 1, 2, 6 and 7. The line drawn through the illustration of fragment 2 indicates a space where the gel was broken. B) Cyclofilin was used to normalize for loading differences among embryos. 28S and 18S ribosomal RNA are indicated as size markers.

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subcloned inserts. The four differentially expressed cDNA fragments were sequenced by Macromolecular Resources at Colorado State University, and results were compared to known mammalian sequences using BLAST, basic local alignment tool. Three cDNA fragments had significant sequence identity to a known entity (bands 1, 2 and 6), and one cDNA fragment demonstrated no sequence homology (band 7) to previously reported genes. The nucleotide sequence for cDNA fragment 1 was found to be 87% identical to bases 175-422 of human allograft inflammatory factor-1 (GenBank accession no. U19713), and 84% identical to bases 236-483 of rat allograft inflammatory factor-1 (GenBank accession no. U17919). Complementary DNA fragment 2 had 79% sequence identity to bases 2662-2853 of human LERK-5 mRNA (GenBank accession no. U81262) (Fig. 4). Complementary DNA fragment 6 had 97% sequence identity to the 3'-end of bovine interferon−τ (bIFNτ) (GenBank accession no. M31557), an abundant and well characterized gene product responsible for the maternal recognition of pregnancy. Two cDNA fragments (bands 2 and 6) were not pursued in the following experiments. Complementary DNA fragment 2 had large multiple mRNA transcripts that may increase the difficulty in obtaining additional nucleotide sequence information, and bIFNτ (band 6) has been well characterized in cattle. In the following experiments, we used cDNA fragment 1 (allograft inflammatory factor-1) and cDNA fragment 7.

Northern Blot Analysis Identifying Continued Presence of cDNA Fragments Through 36-day of Pregnancy

Single conceptuses were collected from cows on days 17.5, 30, 33, 35, 36 of

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TTTTTTTTTTTTTTCC **AATTGGAC**T**GCTACTTCTGGAAAAA**C**GACAT**T**A**C 50
CAAATTGGACA**GCTACTTCTGGAAAAA**C**AACAT**C**A**T

TCCAAAAAT**AACAATAATG**G**GAGCAAA**CA**CAAAAATAACCAAGTCCTC**T**G** 100
TCCAAAAAG**AACAATAATG**A**GAGCAAA**TG**CAAAAATAACCAAGTCCTC**C**G**

AAGGCATG**TCACG**TCACCA**TAGACTAGGAAGT**G**C**A**AGCCCCA**A**A**T**A**C**CAG** 150
AAGGCATC**TCACG**GAACCG**TAGACTAGGAAGT**A**C**G**AGCCCCA**C**A**G**A**G**CAG**

GAAATCAC**TGT**TCACT**CATC**GTG**TTT**T**CAACAATGA**G**AAGATGT**CCCCCC 200
GAAGCCGA**TGT**GACTG**CATC**ATA**TAT**T**TAACAATGA**C**AAGATGT**T**CC**GGC

C**TTTA**GCTTCTGTGCTGTGTTTTCCTTCCTCATGGCTGAAGTGTTCGCT 250
G**TTTA**

AGAATCCACCGGGGGACGTCCAGGGGGCTTCAGCTGAAAGTTCACCTGAC 300
CCCTTCCTTCCTCGCTCCCCCCTCCCCTCGCCCCTTCCTGCTGGGAAAAG 350
AAGCGAGTAAACAGGAAACCTACTTTTTATGTGCTATGCAAAATAGACAT 400
CTTTAACATAGTCCCGTTACTATGGTAACACTTTGCTTTCTGAATTGG*GG* 450
AAAAAAAAAAAA 462

Fig 4. Nucleotide sequence for bovine LERK-5 cDNA. The nucleotide sequence for the cDNA fragment encoding bovine LERK-5 is shown above and nucleotide numbering is on the right. The nucleotide sequence for human LERK-5 cDNA is indicated below. Consensus nucleotides are in bold type. PCR primers annealed to locations indicated in italics.

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pregnancy to determine the presence of bovine allograft inflammatory factor-1 (bAIF-1) and cDNA fragment 7 in developing placenta. Total RNA was isolated from extraembryonic membranes of individual embryos and separated by gel electrophoresis; fragments 1 and 7 were radiolabeled and used to probe the resulting Northern blots. Gene expression was normalized using two radiolabeled cDNAs: γ-actin cDNA and 28S ribosomal cDNA. Bovine AIF-1 was present as a single mRNA transcript of ~800 bp in all samples (Fig. 5). Expression of this gene, normalized to γ-actin mRNA, was 10-fold, 2.5-fold, 6.3-fold, and 7.6-fold greater in older embryos than mRNA levels present in the 17.5-day bovine embryo. Expression was 3.5-fold, 1.1-fold, 2.6-fold, and 2.4-fold greater than levels present in the 17.5-day bovine embryo when using 28S ribosomal RNA to normalize for loading differences. It may be that γ-actin is more valid because that large amounts of 28S rRNA present on the membrane may preclude binding of the 28S rRNA probe in a linear range.

Fragment 7 also was used to probe the Northern blot (Fig. 5). A single band of ~1600 bp was identified only in the RNA obtained from the 17.5-day embryo; no bands were identified in samples from 30, 33, 35, and 36-day bovine embryos.

Northern Blot Analyses Identifying the Presence of Differentially Expressed Genes in Bovine Adult Tissues

Total RNA was isolated from bovine brain, heart, kidney, liver, lung, anterior pituitary, skeletal muscle, and spleen to determine if the differentially expressed genes are present in adult tissue. Northern blots were probed with ^{32}P-radiolabeled bAIF-1 and

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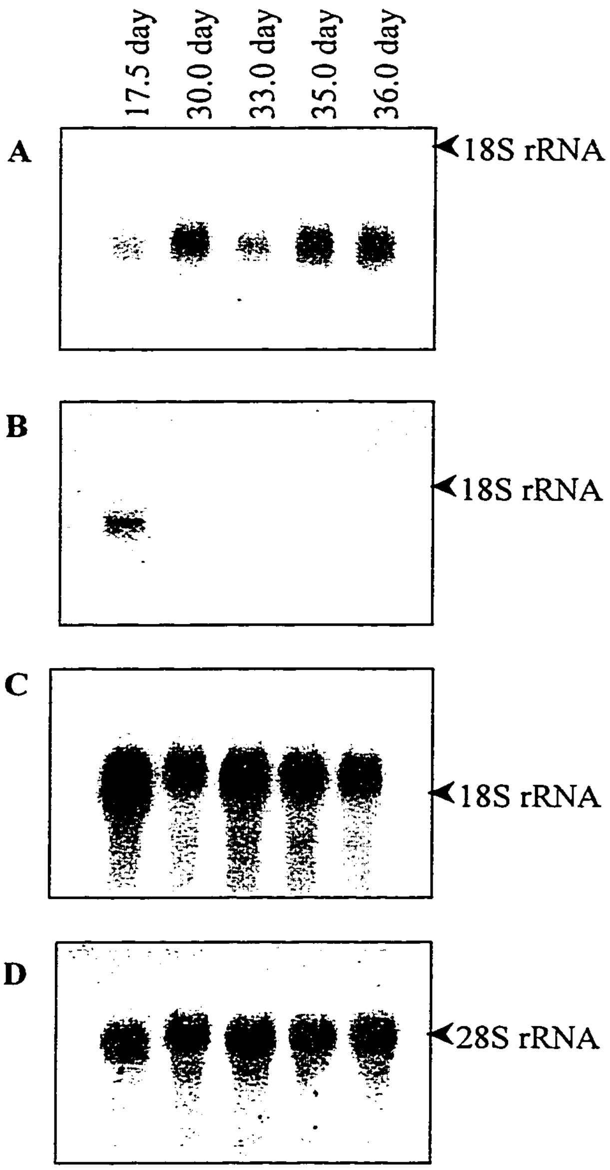

Fig. 5 Northern blot analyses of bovine embryos obtained between days 17 and 36 of pregnancy. Total RNA (20 μg per lane) was isolated from embryos obtained on days 17, 30, 33, 35, and 36 of pregnancy. A) Hybridization patterns demonstrating the continued presence of allograft inflammatory factor-1 through day 36 of pregnancy. A single mRNA transcript of ~800 bp was present in all lanes. B) Hybridization patterns were generated using ^{32}P-labeled cDNA fragment 7. A single band of ~1800 bp was present in 17-day embryos; no bands were visibly present during the remainder of gestation. C) Human gamma-actin was used to normalize for loading differences among embryos. D) 28S ribosomal RNA probe was used to confirm results obtained using human gamma-actin mRNA. 18S and 28S ribosomal RNA are indicted as size markers.

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cDNA fragment 7, and message levels were normalized to 28S ribosomal RNA. When probed with bAIF-1, a single mRNA transcript of ~800 bp was identified in lung and spleen at levels 10-fold and 2.4-fold greater than in the 17.5-day bovine embryo; hybridization bands were barely detectable in brain, kidney, and liver (Fig. 6).

A single mRNA transcript of ~1600 bp in length was identified in the 17.5-day bovine embryo when the Northern blot was probed with ^{32}P-radiolabeled cDNA fragment 7. A slightly visible hybridization band was present in kidney, and no bands were present in remaining adult tissues (Fig. 6).

cDNA Library Screening and Nucleotide Sequences of Differentially Expressed cDNA Fragments 1 and 7

To determine the full-length nucleotide sequence of these genes, a 25-day bovine embryo cDNA library kindly provided by Dr. RM Roberts was screened. Four independent clones were obtained using ^{32}P-radiolabeled AIF-1; the nucleotide sequence for the longest clone (688 bp insert) is shown in Fig. 7A. With the exception of a 1 bp mismatch, the 3' terminal region of the clone was identical to cDNA fragment 1 obtained from the original differential display analysis. The full-length nucleotide sequence had a presumed open reading frame of 441 bp from the possible ATG start site to a TGA stop codon, with a deduced protein sequence of 147 amino acid residues. The cDNA clone also contained a 143 bp 3' untranslated region that included a typical polyadenylation signal (Proudfoot and Brownlee, 1976) 17-22 bases upstream from the poly (A) tail. The full-length sequence was compared to known nucleotide sequences using the BLAST

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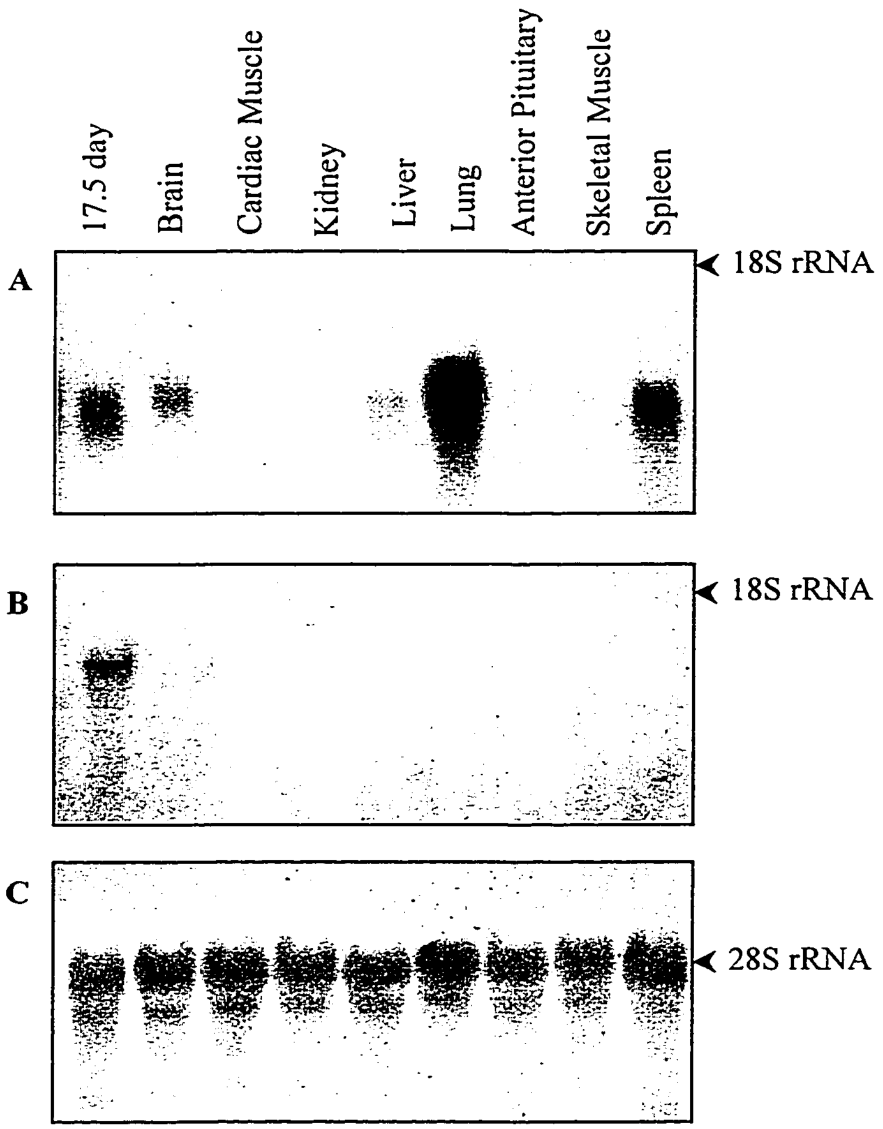

Fig. 6 Representative autoradiograms of AIF-1 mRNA and Fragment 7 mRNA in 17-day bovine embryo and adult bovine tissues. Total RNA (20 μg per lane) was isolated from one 17.5-day bovine embryo and adult bovine organs. A) Northern blot hybridized to ^{32}P-radiolabeled allograft inflammatory factor-1 cDNA. A single band of ~800 bp is present abundantly in bovine embryo, lung, and spleen. Also, bands are barely detectable in brain, kidney, and liver. B) A single mRNA transcript of ~1800 bp is visible in the 17.5-day bovine embryo sample probed with ^{32}P-radiolabeled cDNA fragment 7. With the exception of a marginally visible band present in kidney, no hybrization bands are present in adult tissues. C) 28S ribosomal RNA was used to normalize for loading differences among all samples. 18S or 28S ribosomal RNA are indicated as size markers.

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A

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GGCAGGCCTCCAGGAAGCTGATGGGAGAGGAGAGCTGGCAGACAGAGAAGGCACCCAGTC   60
TGTGTTGTTTCCTCCAACATCTCAACCAGCACCTGCTGAGCTATGAGCGAAACTAGGGAT  120
                                           M  S  E  T  R  D
TTACAGGGAGGAAAAGCCTTTGGGCTGCGGAAAGCCCAGCAGGAAGAGAGAATCAATGAA  180
 L  Q  G  G  K  A  F  G  L  R  K  A  Q  Q  E  E  R  I  N  E
ATCAACCAGCAATTCCTGGATGATCCCAAATACAGCAGTGATGAGGACCTGCCCTCCAAA  240
 I  N  Q  Q  F  L  D  D  P  K  Y  S  S  D  E  D  L  P  S  K
CTGGAAGCCTTCAAGAAGAAATACTAGGAGTTTGACCTGAATGAAGATGGAGGTATCGAT  300
 L  E  A  F  K  K  K  Y  M  E  F  D  L  N  E  D  G  G  I  D
ATCATGTCCCTGAAGCGAATGATGGAGAAACTTGGGGTTCCCAAGACCCACCTGGAGCTA  360
 I  M  S  L  K  R  M  M  E  K  L  G  V  P  K  T  H  L  E  L
AAGAAATTAATCATGGAGGTATCCAGTGGCCCTGGGGAGACTTTCAGCTACTCTGACTTT  420
 K  K  L  I  M  E  V  S  S  G  P  G  E  T  F  S  Y  S  D  F
CTCAAGATGATGTTGGGCAAGAGATCTGCCATTCTAAAAATGATCCTGATGTATGAAGAG  480
 L  K  M  M  L  G  K  R  S  A  I  L  K  M  I  L  M  Y  E  E
AAAGCAAGAGAACAGGAGAAGCCAACAGGTCTCCCAGCCAAGAAAGCTATCTCTGAGTTG  540
 K  A  R  E  Q  E  K  P  T  G  L  P  A  K  K  A  I  S  E  L
CCCTGATTTGTGGTGAGGGAGGTGTGGTGGAATTGAAGGGGCTTCTAATGACTCCAACAT  600
 P  .
AGAAAAAGAAGACAAAATTGTGAACCAGAGCCAGACTAACTTAAATAAATTATCCTCCTT  660
CAGATGAAAAAAAAAAAAAAAAAAAAA                                   687
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B

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BOVINE AIF-1   ---..E.... ........R. ......IN.. .Q........ ..........    47
HUMAN AIF-1    .......... .......... .......... .......... ..........    50
RAT AIF-1      ......SK.. .......... ........G. ..H....... ......Q...    50

Consensus      AACMSQTRDL QGGKAFGLLK AQQEERLDEI NKQFLDDPKY SSDEDLPSKL    50

BOVINE AIF-1   ....K..... ...ED.G... ......M... .......... ...M.....P    97
HUMAN AIF-1    .G..E..... .......... .......... .......... ...G......   100
RAT AIF-1      ....T..... .......... .......... .......... ...R......   100

Consensus      EAFK.KYMEF DLNGNGDIDI MSLKRMLEKL GVPKTHLELK KLI.EVSSGS   100

BOVINE AIF-1   .......... K......... .......... ...Q.....L ..........   147
HUMAN AIF-1    ......P... .......... .......... ...K...... ..........   150
RAT AIF-1      E......... .......... .R........ NK.HQ..... ..........   150

Consensus      GETFSYSDFL RMMLGKRSAI LKMILMYEEK ARE.EKPTGP PAKKAISELP   150
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Fig. 7 Nucleotide and peptide sequences for bovine AIF-1. A) The nucleotide sequence encoding bAIF-1 cDNA is shown on top and nucleotide numbering is on the right. The deduced amino acid sequence is indicated below, and the putative polyadenylation signal sequence is underlined. The PCR primers annealed to sequences indicated in bold. B) Comparison of amino acid sequences from bovine, human, and rat AIF-1. Mismatches among the sequences are noted. The consensus sequence is shown on the bottom. A '.' indicates a mismatch among all sequences. The amino acid is shown if present in two of the three sequences.

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computer program; 88% and 84% sequence homology was identified to human and rat allograft inflammatory factor-1 (GenBank accession no. U19713 and U17919) at the nucleotide level. The deduced protein is 16.8 kD; a search of Swiss-Prot and TREMBL databases using BLAST indicated that deduced amino acid sequence was 82% homologous to the amino acid sequence for human allograft inflammatory factor-1 (GenBank accession no. P55008) (Fig. 7B). Several potential amino acid motifs were determined using Prosite including a single PKC phosphorylation site, 5 casein kinase II phosphorylation sites, 2 N-myristoylation sites, and 1 amidation site.

Differential display fragment 7 also was ^{32}P-radiolabeled and used to screen the 25-day bovine embryo cDNA library. Four independent clones were identified; the nucleotide sequence for the longest clone (1703 bp insert) is shown in Fig. 8. Three shorter clones were identified from the 25-day bovine embryo library, p7 (718 bp insert), p1 (446 bp insert), and p10 (400 bp insert). The three shorter clones confirmed the sequence obtained from the longest clone. With the exception of a single mismatch, the 3' terminal regions of the clones were identical to cDNA fragment 7 obtained from the original differential display analysis. A probable polyadenylation signal exists 13-18 bases upstream from the poly(A) tail, although it contains a single base pair transition from an adenine to a thymidine residue (ATTAAA). The full-length sequence was compared to known nucleotide sequences using the BLAST computer program; no significant homologies were found to previously published gene sequences.

The full-length nucleotide sequence had a presumed open reading frame of 377 bp from the possible ATG start site to a TAG stop codon, with a deduced protein sequence

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CGAGTTTTTTTTTTTTTTTTTTTTTTTTTTCCTTCCCGCTGCCCCGGAGTTCTGGTTTCC 60
AGAATCACTTGGCGAATAACCAGCAGGTGGGCAGATGTCTTCTCCCCCGCTGGATCGACG 120
CGGTAGGTCGGTTCAGTGCATCATAGTGAGCAGTTAAGGTTCCATCTTCCGGCTGCTCCC 180
GGGCGGTGCTACCAGGGAAAAGGGACGAGTCCGAGAATGAAAGACTACTAGGCGGGAGAG 240
CTTGCCTGACCATTCACCTGGGGCAGTGAGCCTGGCACCCACTCACCGCGGGGCCCTCCA 300
GCCGGATCCAACCACACTTGGAGCTCCGGGCTACTGTCGCCGCTGCGCCAGGAAATCGGA 360
GGCGCCAGGGGAGACCAGCCGCAGCAATCGGCTTAGCTGGACTGCAGCGATTTCGGCTGG 420
CACACCTTTCAGAAAGAAGCCCGACCTCTCCAAGACCGCACAGAGGAAAGCCGCGCCCCG 480
ACTTCCAGATTAACCCAAGCGCACGCGCTCCCGACTTTGCCCGGAAAGAGAGGTGCGGAG 540
CCACGGGGTCCAGGTGAGCAGCACAAGCCGCTGGCCTYTGCCCCCAGCCCGCAGCCCGCT 600
CGCGAGAGGCTGGGGCAGGGTCGGGTGGACCCGAGAGCAGCTGCTGCCGCCGAGGCCCTT 660

CCCCGCGCCCCGCGCCCAGCCCCCCTCGGCCGGCCTGCCATGGCCGACGGCAGCGGCACC 720
M A D G S G T
GGCAGCTCGGGCTCCTGGTGGAATTCGCTAACGAACAGCAGGAAGAAAAACAAGGAGGCC 780

G S S G S W W N S L T N S R K K N K E A

ACCGGGGGTGCGCAGCCGCCAGCCCAGCCTGCCCCCGGGGAACCCGCGCCGCCCGTCCAG 840

T G G A Q P P A Q P A P G E P A P P V Q

GACTGGACGAGCAGTTCCCGGGAGAATCAGCACCCCAGTCTCCTCGGGGGCGCCGGCGAG 900

D W T S S S R E N Q H P S L L G G A G E

CCCCACAAGCTAGACAAGTTGGGCGGGGAAAAATCGGGCAACAGCCGCCGAAATTTGAAG 960

P H K L D K L G G E K S G N S R R N L K

ATCTCGCGCTCAGGCCGTTTTAAGGAGAAGAGAAAAGTGCGCGCCACTCTGCTCCCCGAG 1020

I S R S G R F K E K R K V R A T L L P E

GGAGTCAGGTCCTCGGAGGAAGCGATCTTCCCTGGTGACCCCCACGACGACAAGCAATAG 1080

G V R S S E E A I F P G D P H D D K Q .

CCCGAGAGCTCCAGGACTCTCTTCCCATTACACCTCCTCAGCCCCCAGTTCTAACCCCGA 1140
GAATTCTGACCCTCCCTGTACGTGGAGTCTCATCCCACCAAATTCAGAATATTCGCACCA 1200
GGCCTCCAGATTTTATTGTTCTGGAAACTGAAGTGACAATTGAGTGTTCCGGTAGTTCAC 1260
GACTCAAGGATGCTTTAAATATTTATTCGTGGTAAGAGAAGATACCACAGAGGAACCTGG 1320
TATAA**TTTTTTTTTTTTTTCC**GAAAAACTTCCGTGGCTTTTGCATCCCCCATGGAAATGA 1380
GAAGGGTACTTCTCTAAGAGACCTTAACTCAACTGAAACTATGCAGCCTTCCAACAAGGT 1440
TGGCATGCTCTAATTCTGGACTTGCTATTGGGAAGCAAATCCCAGTGCTATCTTTAATGA 1500
GAAATAAAAGAAATGCACTTTGGGAGTATTTCTTCAGGCTTATACCTTAAGTGAAGGGAT 1560
GGCAGAGAGCAGGAATCATTCTTAAGTAGAAACGTTTTAAACAAGGCAAGATTGCTTTTA 1620
ATGTTGACTTGCTAAACTGTACTGCCTATTTTAGCGAGGAACTATTAAAACTGTTGCACT 1680
GTAAAAAAAAAAAAAAAAAAAAA 1703

Fig. 8 Nucleotide sequence for the unidentified gene. The nucleotide sequence is shown on top, and the deduced amino acid sequence is indicated below. Nucleotide numbering is shown on the right, and the putative polyadenylation signal sequence is underlined. The PCR primers annealed to sequences indicated in bold.

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of 126 amino acid residues. The cDNA clone contained a 625 bp 3' untranslated region. The molecular weight of the putative protein was calculated to be 13.5 kD, and a search of protein databases using BLAST indicated that the deduced amino acid sequence was not homologous to previously known amino acid sequences.

DISCUSSION

Significant embryonic loss in beef and dairy cattle occurs between days 15 to 18 of gestation (Roche et al., 1977; Mawhinney and Roche, 1978; Sreenan and Diskin, 1986). During this period, several critical events occur that are necessary for the maintenance of pregnancy. These include embryonic elongation, maternal recognition of pregnancy, and movement of the embryonic trophectoderm into close apposition to the maternal endometrium, resulting in the eventual formation of the placenta. The complex processes involved in the successful completion of these events are dependent on adequate embryo-maternal communication. Several researchers have studied specific molecules involved in this intricate communication system including: 1) the effect of growth factors on embryo elongation (Watson et al., 1992; Keller and Seidel, 1996); 2) the role of bovine interferon-τ during the maternal recognition of pregnancy (Short, 1969); and 3) the presence of placental lactogens secreted from fetal binucleate cell secretory granules after the bovine embryo has attached to the endometrium (Flint et al., 1979; Duello et al., 1986; Wooding and Beckers, 1987).

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mRNA Differential Display Technique

To obtain a broader understanding of the molecular changes that occur in embryos between days 15.5 and 17.5 of gestation, mRNA differential display was used to identify mRNA species present in greater abundance on 17.5 days relative to 15.5 days of pregnancy. We found several advantages using mRNA differential display compared to traditional subtraction library techniques. mRNA differential display aided in generating mRNA profiles for 15.5-day and 17.5-day embryos that could be compared, and differentially expressed cDNA fragments could be easily identified, reamplied, subcloned, and sequenced; furthermore, cDNA fragments were used for Southern and Northern blot analyses. mRNA differential display also provided an extremely sensitive technique that required little tissue, a valuable tool when working with embryos. We were able to detect a previously unidentified gene using this technique, as well as genes that have been characterized but not identified during embryonic development.

There were also disadvantages using mRNA differential display compared to traditional subtraction library techniques. Optimization of reverse transcriptases, PCR polymerases, amplification times and temperatures were essential to produce satisfactory differential displays. Several bands were detected using different primers sets that represented the same gene, requiring additional Southern blot and Northern blot analyses to verify the presence of a unique differentially expressed cDNA fragment (Table 2). The 3' untranslated region of the gene is often recovered making sequence identification more difficult, and performing PCR reactions in duplicate using several different primer combinations makes the process laborious and time-consuming. Also, the abundance of

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Table 2. Summary of primers used to generate cDNA fragments encoding bIFN- τ

Fragment Number	Oligo (dT) + 2 bp primer (5' T_{11}MN)	Decamer	Approx Size (bp)	Number of times bIFN-τ was detected
6	5' T_{11}AT	5' TGGAGATCTG	360	1
8	5' T_{11}AC	5' TGGAGATCTG	360	1
10	5' T_{11}AG	5' TGGAGATCTG	600	1
11,12	5' T_{11}GC	5' TGGAGATCTG	360,350	2
14	5' T_{11}GG	5' TGGAGATCTG	360	1

Six cDNA fragments detected by differential display encoded for bIFN-τ. Five different oligo (dT) + 2 bp primers were used to reverse transcribe and amplify bIFN-τ . A single decamer was used to PCR amplify each bIFNτ cDNA fragment. With the exception of cDNA fragment 10, all cDNA fragments were approximately the same length.

bIFNτ mRNA during these stages of pregnancy may overwhelm the system, reducing the number of rare mRNAs detected.

In this study, three cDNA fragments that shared sequence identities with known genes and one novel gene were isolated. Complementary DNA fragment 1 was homologous to human and rat allograft inflammatory factor-1 (AIF-1). Complementary DNA fragment 2 was homologous to human LERK-5, and cDNA fragment 6 was bovine IFNτ. Complementary DNA fragment 7 did not share sequence homology with previously identified genes.

Bovine Allograft Inflammatory Factor-1

The nucleotide sequence for cDNA fragment 1 shared 88% and 84% sequence

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homology to human and rat allograft inflammatory factor-1 (AIF-1). AIF-1 originally was identified in rats that developed arteriosclerotic lesions during chronic rejection of cardiac allografts (Utans et al., 1995). During the initial stages of cardiac rejection, monocytes/macrophages expressing rAIF-1 accumulate in the vessels. Thirty to 75 days post-transplantation, both vascular smooth muscle cells and macrophages accumulate in the vessels. The smooth muscle cells migrate into the intima in response to the secretion of many cytokines and growth factors present during the inflammation response. Finally, smooth muscle cells secrete extracellular matrix, soluble growth and chemotactic factors, resulting in dramatic cellular proliferation and vessel occlusion (Cramer et al., 1992; Adams et al., 1993).

A 4.5-fold increase in bovine AIF-1 mRNA content was demonstrated in 17.5-day relative to 15-day bovine embryos; bAIF-1 mRNA expression continued through day-36 of pregnancy. Although a slight decrease in expression is seen on day 33 of pregnancy, this is probably due to variation in expression from embryo to embryo. Similar to cardiac transplantation, an allograft is forming between fetal and maternal tissues during implantation as bAIF-1 transcription is increasing; this may indicate an important role of bAIF-1 during pregnancy. At this time, uterine macrophages are abundant in rodents, women, and pigs (Tachi and Tachi, 1989; Bischof et al., 1995). It has been suggested that there are several potential macrophage functions in reproduction including tissue remodeling events and a pivotal role in coordinating the local immune response to the fetus during pregnancy (Bischof et al., 1995). Although, the presence of bAIF-1 mRNA may be from activated macrophages present in the embryonic samples, there is no

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evidence that macrophages are present in preimplantation and peri-implantation embryos. We also do not know whether uterine macrophages express bAIF-1 mRNA. If bAIF-1 mRNA detection is from activated macrophages in 15.5-day and 17.5-day bovine embryos, we must conclude that uterine macrophages expressing bAIF-1 mRNA either infiltrated into the embryonic trophoblast or contaminated the embryonic tissue preparations. For example, it is possible that activated macrophages expressing bAIF-1 mRNA contaminated the day-17 to 36 embryonic samples as they were stripped from the uterine epithelium.

Preliminary evidence using in situ hybridization indicated that bAIF-1 mRNA was expressed from bovine trophoblast cells on 17.5 days of gestation (unpublished observations). Rat AIF-1 mRNA expression and antigen also are found in the differentiating germ cells of the rat testis, suggesting a distinct role in spermatogenesis not related to inflammation (Utans et al., 1995). As in spermatogenesis, bAIF-1 may have a unique role in embryonic cell differentiation.

Whether expression of bAIF-1 mRNA is from uterine macrophages or embryonic trophoblast, it may be stimulated by abundant levels of bIFNτ present during early pregnancy. Rat AIF-1 mRNA was up-regulated six-fold *in vitro* from rat macrophages after stimulation with interferon-γ (INFγ) (Utans et al., 1995). IFNγ, a cytokine secreted by activated T lymphocytes and natural killer cells, is a potent activator of macrophages, affecting many macrophage properties and functions (Williams et al., 1993). Although IFNτ and IFNγ are in separate classes of interferons and bind to different receptors, the downstream effects are often similar. Both Type I and Type II interferons may control

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the regulation of bAIF-1 mRNA content.

Human AIF-1 mRNA content was the highest in cells of lymphoid origin, in particular, human spleen, peripheral blood lymphocytes, and thymus. Less hAIF-1 mRNA was detected in liver, lung, and placenta (Autieri, 1996). Abundant levels of bAIF-1 mRNA also were detected in bovine spleen. Unlike the human, the highest bAIF-1 mRNA content was in the lung; this may be due to the environmental conditions experienced by that cow.

cDNA sequences for AIF-1 have been identified in mouse, rat, human, and cow. The deduced amino acid sequences are nearly homologous, and sequences in the mouse, rat and human contain a single conserved EF-hand helix-loop motif with a conserved calcium-binding loop (Utans et al., 1995; Autieri, 1996). The EF-hand calcium binding motif is present in several proteins that bind intracellular calcium; this superfamily includes troponin and calmodulin (Heinzmann and Hunziker, 1991). These proteins are thought to have a role in calcium activation, calcium buffering, and structural stabilization (Fromherz S and Szent-Gyorgyi, 1995; Renner et al., 1993). Two or more EF-hand domains are required for calcium binding by the EF-hand family of proteins (Heinzmann and Hunziker, 1991); therefore, AIF-1 may not function in calcium binding. The bovine cDNA sequence lacks the conserved EF-hand motif and calcium binding loop demonstrated in rat and human AIF-1 cDNA sequences.

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Bovine LERK-5

Complementary DNA fragment 2 was isolated from a single mRNA differential display that was amplified using a single primer set. Sequence information revealed that this cDNA fragment was PCR amplified from the oligo (dT) plus 2 bp primer anchored at both the 5' and 3' positions. Fragment 2 was radiolabeled and used to probe Northern blots. Three distinct mRNA transcripts were observed; the major transcript was ~5000 bp in length and present in ~11-fold higher abundance in 17.5-day relative to 15-day embryos. This fragment shared 79% sequence identity to bases 2662-2853 of human LERK-5 mRNA (GenBank accession no. U81262). Human LERK-5 is a ligand of the eph-related receptor tyrosine kinase family. Receptor tyrosine kinases (RTKs) have been grouped into families based on structural and functional similarities (Fantl et al. 1993; van der Geer and Hunter, 1994). The eph-like branch of RTKs is the largest family including more than 28 members present in several vertebrate species (Hirai et al., 1987; Tuzi and Gullick, 1994; van der Geer and Hunter, 1994; Hanks and Hunter, 1995). Although little is known about the function of specific RTKs, expression of eph-like RTKs during embryogenesis suggests a role in development (Pandey et al., 1995; Tessier-Lavigne, 1995). Also, overexpression of eph-like RTKs in tumor-derived cell lines implicate these receptors in oncogenesis (Hirai et al., 1987; Boyd et al., 1992). Recently, several ligands have been identified that exhibit extensive cross-binding to different members of the eph-like RTKs (Beckmann et al., 1994; Kozlosky et al., 1995), and have been termed ligands of the eph-like receptor kinases or LERKs.

Complementary DNA clones have been isolated for human and murine LERK-5.

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Cerretti et al. (1995) isolated a clone encoding human LERK-5 that was 1777 bp in length with a potential open reading frame encoding a 333 amino acid peptide. Furthermore, a 2611 bp cDNA for murine LERK-5 was isolated from an 11.5-day mouse embryo library; it had an open reading frame of 336 amino acids that shared 97% sequence identity to human LERK-5 (Cerretti et al., 1995). There is relatively little amino acid sequence identity among LERK proteins. LERK-5 peptide sequence is most similar to LERK-2 (59% amino acid identity), the only other ligand in this family that is a transmembrane protein (Davis et al., 1994; Kozlosky et al., 1995).

Cerretti et al. (1995) used LERK-5 riboprobes to examine Northern blots of fetal and adult human tissues. A single mRNA transcript ~5000 bp in length was observed in fetal tissues including heart, lung, kidney, and brain. Lung was the only adult tissue that had abundant levels of LERK-5 mRNA. As in humans, an mRNA transcript ~5000 bp in length was present in 17.5-day bovine embryos, and preliminary evidence suggests that it was present primarily in adult bovine lung. bLERK-5 mRNA content in adult and fetal bovine tissues should be examined more closely.

Bovine Interferon-tau

Complementary DNA fragment 6 encoded bIFNτ, a protein secreted from the embryonic trophectoderm and responsible for the maternal recognition of pregnancy in cattle (Bazer et al, 1991; Roberts et al., 1992). During luteolysis, prostaglandin $F_{2\alpha}$, a luteolytic agent common to most mammals, is released from the endometrium in a pulsatile manner causing the corpus luteum to regress (Geisert et al., 1992). Bovine

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conceptuses produce bIFNτ, a molecule that extends the lifespan of the corpus luteum, thus stabilizing the uterine environment and allowing for continued growth of the embryo and its membranes (Roberts et al. 1993). Although maximal secretion of bIFNτ occurs between days 16 to 19 of pregnancy (Bartol et al., 1985; Farin et al., 1989), bIFNτ mRNA has been detected as early as day 12 of gestation (Farin et al., 1989). Secretion continues through at least day 38 of pregnancy (Bartol et al., 1985; Godkin et al., 1988).

Bovine IFNτ mRNA content increased ~2.5-fold in 17.5-day relative to 15-day bovine embryos. It was surprising to find that bIFNτ mRNA was detected using six different primer combinations (table 2). Five oligo (dT) plus 2 bp primers were used to reverse transcribe and PCR amplify the cDNA products. A single decamer was used for PCR amplication that annealed specifically to the template ~330 bp upstream from the 3' end of the bIFNτ cDNA (Stewart et al., 1990). After separating the reamplified products by gel electrophoresis on an agarose gel, a single band of approximately ~350 bp in length was present in all samples except cDNA fragment 10. Fragment 10 separated into 2 cDNA fragments approximately 600 bp and 350 bp in length. On the Southern blot, a single hybridization band was visible at ~350 bp in all samples. A hybridization band was not present at 600 bp for cDNA fragment 10.

There are several plausible explanations for these results. Bovine IFNτ cDNA or the oligo (dT) plus 2 bp primer may have contaminated the PCR reaction components, thus explaining the presence of a hybridization band at ~350 bp, but not 600 bp for cDNA. fragment 10. Also, there are several repeated adenine nucleotides dispersed throughout the 3' end of bIFNτ mRNA. Degenerate binding may have occurred between the oligo

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(dT) plus 2 bp primer and the mRNA transcript at the low annealing temperatures used in mRNA differential display analysis. This degeneracy likely would increase the possibility of detecting bIFNτ repeatedly using several primer combinations. IFNτ is the major translation product of mRNA isolated from day-16 ovine (Hansen et al., 1985; Anthony et al., 1988) and bovine conceptuses (Farin et al., 1990; Cross and Roberts, 1991). The abundance of mRNA encoding bINFτ may increase the probability that degenerate hybridization will occur between the transcript and primer. Also, IFNτ is a gene family; it is possible that different oligo (dT) plus 2 bp primers annealed to different members of the INFτ family.

A Novel Gene Product

Complementary DNA fragment 7 encoded a previously unidentified gene with an mRNA transcript ~1800 bp in length that increased ~11-fold in 17.5-day relative to 15.5-day bovine embryos. Fragment 7 mRNA content declined by 30 days of gestation, and was not detected in bovine adult tissues, indicating that this gene may have an important developmental role. A single cDNA clone 1703 bp in length with a potential open reading frame of 377 bp encoding 126 amino acid residues was isolated from 25-day embryos. Neither the nucleotide sequence nor amino acid sequence demonstrated significant sequence homology to any previously known genes.

Again, degenerate binding occurred between the oligo (dT) plus 2 bp primer and the template (Table 3). In this case, the oligo (dT) plus 2 bp primer annealed to the novel

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cDNA at both the 5’ and 3’ positions; the decamer was used, but it did not function effectively as a primer.

Table 3. Summary of primers used to generate the novel cDNA fragments.

Fragment Number	**Oligo (dT) + 2 bp primer (5’ T_{11}MN)**	**Approx. size (bp)**	**Number of times fragment was detected**
7	5’ T_{11}GC	350	1
9	5’ T_{11}GC	350	1
15	5’ T_{11}AC	350	1

Three cDNA fragments detected by differential display analysis encoded the unknown gene product. This fragment was amplified in the 5’ and 3’ direction with the oligo (dT) + 2 bp primer indicating degeneracy between the primer and the template.

The results obtained for cDNA fragments encoding bLERK-5, bIFNτ, and the novel gene product indicate that the anchoring scheme suggested by Liang and Pardee (1992) did not work consistently in this study. Increasing the annealing temperature during PCR amplification probably would decrease recurrent isolation of the same cDNA fragment from multiple differential display analyses, but this also could result in failure to detect important differentially expressed genes. Although the anchoring scheme did not work consistently in this study, mRNA differential display analysis still was effective in detecting several changes in gene expression between embryonic stages.

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Conclusion

mRNA differential display analysis is an elegant technique that resulted in detecting genes that may encode factors potentially involved in the dynamic processes associated with early bovine pregnancy. In this study, we identified four genes encoding mRNAs with increased content in 17.5-day relative to 15.5-day bovine embryos. The importance of bIFNτ in the maternal recognition of pregnancy has been well documented. Its detection provides a powerful demonstration as to our ability to identify important molecular mediators during pregnancy using mRNA differential display. Although the roles of bAIF-1, bLERK-5, and the unknown gene during pregnancy have not been elucidated, the increased expression of these genes during this time indicates that they may have an important role during pregnancy. We can speculate that bAIF-1 may be important in tissue remodeling or fetal-maternal interaction during implantation, and LERK-5 may mediate receptor-binding signal transduction in some fetal tissues. This work provides a solid foundation for the future characterization of these genes and their role during peri-implantation development.

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CHAPTER III

GENERAL SUMMARY AND CONCLUSION

Early embryonic mortality is costly to cattle producers. According to Sreenan and Diskin (1986), the majority of loss occurs between day 15 and 18 of gestation. Several critical physiological events occur during this time including: 1) rapid embryonic growth and elongation; 2) maternal recognition of pregnancy; and 3) attachment of the embryonic trophoblast to the maternal endometrium. Although the morphological changes that occur during the periimplantation period of pregnancy have been well studied, the molecular factors involved in these events are not well understood. We employed mRNA differential display analysis to detect genes that are up-regulated in 17.5-day compared to 15.5-day bovine embryos.

In the past, it has been difficult to study changes in gene expression in preimplantation and periimplantation conceptuses due to the paucity of biological material. mRNA differential display analysis is a sensitive tool that requires minimal amounts of tissue. We have used mRNA differential display analysis to isolate potential mediators involved in the complex multicellular processes that occur during periimplantation pregnancy. Sixty different primer combinations were used to identify 15 potentially differentially expressed cDNA fragments. Northern blot analyses using total RNA isolated from 15-day and 17.5-day bovine embryos were conducted to verify differential expression. Of the 15 cDNA fragments, eight were unique and four were verified to have increased mRNA content on 17.5 days relative to 15 days of pregnancy.

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Three cDNA fragments were from known genes: bAIF-1, bIFNτ, and bLERK-5. One cDNA fragment shared no sequence identity to previously identified genes.

Bovine AIF-1 mRNA content increased ~4.5-fold in 17.5-day relative to 15-day bovine embryos; expression continued through day 36 in bovine embryos and was present in term placenta from rats and humans (Utans et al., 1995; Autierei, 1996). Preliminary *in situ* hybridization results suggest that bAIF-1 is expressed in bovine trophectoderm. AIF-1 was detected in adult bovine lung and lymphatic tissue; it also is found in rat and human spleen. A 25-day bovine embryo library was screened using the cDNA fragment of bAIF-1 as a radiolabeled probe. Four independent clones were obtained; the longest clone was 688 bp in length with a presumed open reading frame of 441 bp. The nucleotide sequence shared 88% and 84% sequence identity to human and rat AIF-1 nucleotide sequences. The deduced amino acid sequence for bAIF-1 was 82% homologous to the amino acid sequence for human AIF-1.

AIF-1 originally was identified in activated macrophages that were present in occluded vessels of rats and human experiencing chronic rejection of cardiac allografts (Utans et al., 1995). Rat AIF-1 also was found in the seminiferous tubules in the testis. We speculate that bAIF-1 may have an important role in tissue remodeling or embryo-maternal interactions involving immunological mechanisms like those occurring during cardiac transplantation.

Bovine IFNτ, identified by mRNA differential display analysis, was present in ~2.5-fold greater abundance in 17.5-day relative to 15-day bovine embryos. Interferon-τ is a well-characterized gene that encodes a protein responsible for the maternal recognition of pregnancy in cattle, sheep, and goats. Secretion of bIFNτ from

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preimplantation and periimplantation embryos results in protection of corpora lutea from luteolysis by modifying pulsatile secretions of $PGF_{2\alpha}$, the luteolytic agent produced in many mammalian species. Identification of bIFNτ indicated that genes, which have an important role during periimplantation pregnancy, could be identified using mRNA differential display analysis, but also would decrease the sensitivity for detecting other differentially expressed genes.

Bovine IFNτ was identified in six differential displays using five unique primer combinations. Although the arbitrary 10-mer was the same in each reaction, the oligo (dT) plus 2 bp primer differed. Also, bLERK-5 and the novel gene product were PCR amplified from the oligo (dT) plus 2 bp primer in both the 5' and 3' directions. These results indicate that the oligo (dT) plus 2 bp primer annealed in a degenerate fashion to the cDNA template. Increasing the annealing temperature during PCR likely would decrease repeated isolation of the same cDNA fragment from multiple differential display analyses.

Three transcripts were present on Northern blot analyses with 17.5-day and 15-day embryonic RNA probed with radiolabeled bLERK-5 cDNA. The major transcript was ~5000 bp in length and increased ~10-fold in abundance in 17.5-day and 15-day embryonic RNA probed with radiolabeled bLERK-5 cDNA. The major transcript was ~5000 bp in length and increased ~10-fold in abundance in 17.5-day compared to 15-day bovine embryos. LERK-5 encodes a protein ligand that promiscuously binds to members of the eph-related receptor tyrosine kinase family (Cerretti et al., 1995). LERK-5 is predicted to be a transmembrane protein that appears to be developmentally regulated in humans, binding to human adult kidney and liver and to fetal heart, lung, kidney, and

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brain. Preliminary evidence also suggests that bLERK-5 in adult cattle is expressed solely in kidney and lung. Because multiple transcripts were demonstrated on Northern blots containing 15-day and 17.5-day bovine embryo RNA, this differentially expressed cDNA fragment was not used to screen a 25-day bovine embryo cDNA library or in Northern blot analyses of bovine fetal and adult tissues.

Lastly, a gene that has not previously been identified was isolated by mRNA differential display analysis. The mRNA encoded by the unknown gene was ~1800 bp in length and increased ~10-fold in abundance in 17.5-day relative to 15-day bovine embryos. This differentially expressed cDNA fragment was used to probe Northern blots containing total RNA isolated from a variety of adult tissues and embryonic trophectodermal tissues for days 17.5, 30, 33, 35, and 36 of gestation. A single band of ~1800 bp in length was present in 17.5-day embryonic samples; all other samples lacked a hybridization band. Furthermore, a 25-day bovine embryo cDNA library was screened using this differentially expressed fragment as a probe. Four individual clones were isolated, the longest being 1703 bp in length. The nucleotide sequence had a potential open reading frame of 377 bp encoding a 126 amino acid peptide. No sequence homologies were found when comparing the nucleotide and amino acid sequences of the unknown gene to sequences previously reported in GenBank or Prosite.

Although we do not understand the function of bAIF-1, bLERK-5, and the unknown gene during periimplantation pregnancy, up-regulation of these genes during this time is suggestive of an important role during this period. These studies have provided a solid foundation to continue characterization of these potential molecular mediators.

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