OVINE UTERINE MILK PROTEINS:
STRUCTURE, BIOSYNTHESIS, AND FUNCTION

By

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This thesis is dedicated to my parents, Edwin Bruce Ing and Mary Hughes Ing, and my husband, William Harold Cox, who have always given me their total support.
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ABBREVIATIONS

beta-estradiol, E$_2$
base pairs, bp
complementary DNA, cDNA
concanavalin A, ConA
corpus luteum, CL
epidermal growth factor, EGF
equilibrium constant, K$_m$
follicle stimulating hormone, FSH
isoelectric point, pI
luteinizing hormone, LH
messenger RNA, mRNA
molecular mass, m
plaque forming units, pfu's
plasminogen activator, PA
progesterone, P$_4$
relative molecular weight, M$_r$
sodium dodecyl sulfate, SDS
SDS-polyacrylamide gel electrophoresis, SDS-PAGE
standard saline citrate (150 mM NaCl, 15 mM sodium citrate), 1X SSC
tissue PA, tPA
two dimensional polyacrylamide gel electrophoresis, 2D-PAGE
ultraviolet, UV
urokinase, uPA
uterine milk, UTM
uterine milk proteins, UTMP
volume/volume, v/v
weight/volume, w/v
The uterine milk proteins (UTMP) are a pair of structurally related basic glycoproteins, distinguishable only by their relative mobilities during electrophoresis on 7.5% (w/v) polyacrylamide gels. They have apparent Mr's = 57,000 and 55,000 and are the major proteins secreted by ovine endometrium under the influence of progesterone. RNA from late pregnant ewe endometrium was isolated for use in in vitro translation assays and for complementary DNA (cDNA) synthesis. Translation experiments, initially with total cellular RNA and subsequently with RNA selected by hybridization with a specific cDNA, demonstrated the production of an Mr = 47,000 protein that was precipitated with antiserum to the UTMP. With microsomal membranes in the translation assay, an intermediate in UTMP biosynthesis of Mr = 57,000 was produced and protected from post-translational proteolytic degradation.

Interesting features of the UTMP mRNA sequence, which was 1352 bases long and contained a 1287 base open reading frame, were two strong start codons and a repeat of 21 bases, six bases apart, that result in a
repeat of seven amino acids. The inferred amino acid sequence agrees well with the N-terminal amino acid sequence of both forms of the UTMP, amino acid composition of the UTMP, and the molecular size of the UTMP precursor obtained from in vitro translation studies. The inferred UTMP amino acid sequence clearly placed the UTMP in the serpin superfamily of protease inhibitors, although no inhibitory activity towards a range of serine proteases was demonstrated.

The progesterone induction of the UTMP was studied in groups of ovariectomized ewes given progesterone for 0, 2, 6, 14 or 30 days. Western blot analyses of proteins of uterine flushes and endometrial explant culture tissue and medium, endometrial RNA analyses on dot and Northern blots, and immunocytochemistry, performed on samples from each ewe, demonstrated the presence of low levels of UTMP mRNA and UTMP protein in endometrial cells after 6 days of progesterone therapy, and increasing levels of UTMP production and secretion after 14 days. The induction of the UTMP was shown on the cellular level by immunocytochemistry to progress from small amounts present in the supranuclear region of epithelial cells in deep and middle depth regions of uterine glands in the day 6 progesterone treatment group to large amounts of the UTMP detected in epithelial cells spread throughout the length of the glands in later groups. Production of the UTMP was also identified in the uteri of intact sheep at day 16 of the estrous cycle and during early pregnancy (days 14 to 22), and in the uterus of the pregnant cow. The UTMP provide a good model of a progesterone responsive secretory protein in a mammal whose synthesis is increased gradually over a period of weeks.
CHAPTER 1
LITERATURE REVIEW

Introduction

The influence of the sheep (Ovis aries) in the world today is profound, as they supply materials that are used universally and bear no cultural taboos. In 1983, the U.S.S.R. led the world in numbers of sheep, with 140 million head (Campbell and Lasley, 1985). Sheep are the basis of a multibillion dollar industry because the sale of wool, meat, and lanolin are all important commodities, with wool alone accounting for an income of $7.8 billion for the world's farmers in 1982. In third world countries, as in ancient times, a family may depend upon sheep for meat, milk, and clothing in exchange for only rough forage and protection.

Because sheep are important animals to people all over the world, their reproduction is worthy of study. Although the sheep was one of the first species to be domesticated, very little control is exercised over them in husbandry today. One reason that shepherds choose a policy of nonintervention in sheep reproduction is that not enough is known about the process to be able to enhance either the number of pregnancies per annum ewe fecundity. Deciding the ram:ewe ratio in the herd may be the only decision the shepherd makes in the fall concerning breeding efficacy in his herd. It is important to ensure that an adequate number of rams is present to detect estrus and breed the ewes during the season of their sexual activity when days are decreasing in length. Ewes that
do not conceive or lose pregnancy early are usually detected only by their failure to produce offspring in the spring. Rebreeding or culling the nonpregnant ewes in the fall would decrease losses for the sheep producer. Pregnancy testing with any of the available techniques, including radiography, ultrasound, serum progesterone assays or vaginal biopsies, is time consuming and expensive and, therefore, impractical for the producers (Jainudeen and Hafez, 1980c). In addition to ensuring that all ewes are pregnant, the sheep producer could increase his profit if all ewes carried multiple lambs. Ewes can easily deliver and care for twins and triplets. Many ewes have multiple ovulations during their estrous cycle, and in some breeds, such as the Boorolla, ewes may consistently have greater than four ovulations per cycle (Davis et al., 1982). After the events of ovulation and conception occur, 20 to 40% of sheep embryos are lost in early pregnancy (Jainudeen and Hafez, 1980b). The uterus may be hostile to embryo survival, as is apparent with asynchronous embryo transfers (Moore et al., 1983). The uterus, however, may also contribute to the development of large lambs that have better chance of survival and greater growth rates (Bell, 1984). It is possible, with increased understanding of the uterine environment and the influence of hormones on it, that husbandry practices may improve sheep fecundity. Basic research elucidating the causes of infertility, early embryonic mortality, and low birth weights may implicate the uterine milieu as important factors. Hormonal therapy and gene transfer therapy to manipulate endogenous hormone levels may be realistic solutions to these problems. Improving our understanding of ovine reproduction may result in increased flock fecundity for the producer and ultimately benefit the consumer.
Replicative Physiology of the Ewe

Information contained in the following brief review of ovine reproductive physiology is provided as background for the experiments and discussions within this thesis. Structure and function of both the ovary and uterus are emphasized. The reproductive system of the female sheep has several aspects that appear to be unique to the species. Physical and behavioral characteristics have, over the years, served to isolate the sheep as one reproductive type or species. Although many species face similar problems in propagation different adaptations have evolved to surpass reproductive obstacles and are worthy of biological investigation. The synopsis will focus on the sheep's reproductive strategy and the limits of our understanding of the processes involved.

Reproductive Tract of the Ewe

Anatomy and physiology of the uterus and oviduct

The ewe has two ovaries that are connected to the uterus by extensions of the tips of the uterine horns, called the oviducts. The ewe has a bipartite uterus which, when nongravid, has two horns (10 to 12 cm long) which join in a short body (1 to 2 cm long; Hafez, 1980). A short cartilaginous cervix forms the aperture between the uterine body and the vagina. At mating, sperm are deposited by the ram at the cervical opening in the vagina.

The uterus undergoes the most dramatic changes of any maternal organ during pregnancy, increasing greatly in its dimensions and biological activity. This is reflected in the increase in blood supply to the uterus, from 2% in the nonpregnant ewe to 20% in the pregnant animal (Jainudeen and Hafez, 1980a). The uterus is a hollow organ that
is composed of three layers of tissues. From outside to inside, they are the perimetrium, the myometrium, and the endometrium. The perimetrium is a serosal layer continuous with the peritoneum and provides the thin outer covering of the uterus. The myometrium is composed of longitudinal muscle layers outside and circular muscle layers inside. The myometrium physically supports the weight of the fetus in the mother and provides expulsive force of labor during parturition.

Anatomy and physiology of the endometrium

The inner lining of the uterus is the endometrium. The ewe resembles other ruminants in having endometrium with two distinct areas. Caruncular areas of endometrium are raised and firm. Caruncles, numbering from 88 to 96 per uterus (Hafez, 1980) are richly endowed with vascular components. During pregnancy, caruncular areas form intimate contact with areas of the fetal membranes called cotyledons. These focal areas of caruncular-cotyledonary contact are termed "placentomes." During middle and late pregnancy, maternal blood perfuses the caruncles and fetal blood circulates through the cotyledons. These areas serve to exchange maternal nutrients for fetal waste products between circulatory systems. The intercaruncular endometrium is a hormonally responsive tissue. In response to progesterone, the thin areas of endometrium between caruncles thicken with the growth of the uterine glands and vascular components. The glands open into the uterine lumen and their epithelia are continuous. Epithelium of deep endometrial glands is primarily composed of secretory cells. Ciliated cells, however, predominate in the necks of the glands beneath the surface epithelium. Under progesterone influence, secretory
cells of glandular epithelium show evidence of active secretion: increased cell height, increased Golgi and rough endoplasmic reticulum membrane systems, and areas of homogeneous material (Davies and Wimsatt, 1966). Two proteins secreted by these cells will be the subject of this thesis. Additional cellular components of the endometrium include macrophages, leukocytes, and melanotic pigment cells. In general, the hormonal action of $E_2$ on intercaruncular endometrium is associated with increased cellular water, protein, and RNA content in the tissue (Miller et al., 1976). In the ewe, $P_4$ also increases cellular protein synthesis and RNA in intercaruncular endometrium (Miller, 1976; Miller and Moore, 1983). $P_4$, with or without $E_2$, increases the proportion of synthesized protein in sheep endometrium that is secreted (Salamonsen et al., 1985).

Physiological States of Reproduction in the Ewe

The mature ewe has three physiological states of reproduction in which she is either having estrous cycles, is pregnant or is anestrous. Sheep are seasonal breeders, being reproductively active during the part of the year in which daylength is shortening. Nonpregnant ewes undergo estrous cycles lasting 17 days (range 16.3 to 17.5 days; Robinson, 1951) from August through December in the temperate northern hemisphere. If bred, they may carry one to three lambs to term in 145 to 149 days. Ewes that are not pregnant and not having estrous cycles are termed "anestrous" and resume cyclic activity with the onset of the season of shortening daylength.

The cyclic ewe

Ovarian structures and hormones. The ewe undergoing estrous cycles has many structures on her ovaries. Follicles develop and regress in
three waves during each cycle. Follicular development is influenced by two pituitary products: follicle stimulating hormone (FSH) and luteinizing hormone (LH). While most follicles regress, a few reach maturity and release an ovum during ovulation. LH influence is involved in the subsequent development of the ruptured follicle into a corpus luteum (CL). The CL grows and produces the steroid hormone P_4. The CL regresses during the process of luteolysis. The events described above, from developing follicle to regressing CL, span two estrous cycles. In each cycle, follicles and CL's develop concurrently. A complex interplay of hormones governs the process and will not be discussed in detail here. Major reproductive hormones from mammalian ovaries are the steroids, progesterone from the CL, and estrogen from the follicles. The primary estrogenic substance in plasma is E_2. The serum level profiles of the two steroid hormones will be described in relation to events of the estrous cycle in the ewe.

Estrus, or the period of sexual receptivity, lasts 24 to 48 hours for the ewe. At day 0 or estrus, blood P_4 levels are minimal (0.3 ng/ml) but E_2 levels have peaked at 20 to 30 pg/ml just 0 to 8 hours prior to the behavioral event (Pant, 1972). Large tertiary follicles, the source of the E_2, ovulate 12 hours prior to the termination of estrus. Metestrus, from days 1 to 3, is characterized by baseline P_4 and E_2 levels in the blood as the ruptured follicle develops into a CL. Diestrus follows, from days 2 to 14 (Erb, 1977). During diestrus, the CL gains the capacity to produce P_4, and blood levels rise from below 0.4 ng/ml on days 0 through 4 to maximal levels of 1.5 to 2.5 ng/ml between days 4 and 9 (Thorburn et al., 1969). Peaks of E_2 occur with the waves of follicular growth at days 3 and 8. P_4 levels in the
blood remain high until luteal regression occurs from days 14 to 16, at which time \( P_4 \) levels in plasma drop to less than 1 ng/ml. Proestrus, from days 15 to 0, encompasses the time of luteolysis and follicular maturation with the preovulatory surge of \( E_2 \) already described.

**Endometrium of the cyclic ewe.** The morphology of intercaruncular endometrium changes during the estrous cycle, primarily in response to \( P_4 \) and \( E_2 \) levels in plasma. During proestrus and early estrus, the endometrium is edematous and highly vascularized (Hafez, 1974). At this time of great \( E_2 \) influence, endometrial glands are highly coiled and epithelial cells are proliferating (Restall, 1966). Enzymes responsive to increased \( E_2 \), localized by histochemistry in endometrial epithelium, include succinic dehydrogenase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, and isocitrate dehydrogenase (Zamiri and Blackshaw, 1977). During metestrus and diestrus, while \( P_4 \) is the predominant steroid hormone, luminal and glandular epithelium is of reduced height (Restall, 1966) and acid phosphatase (Zamiri and Blackshaw, 1977) and alkaline phosphatase (Murdoch, 1971; Hafez and White, 1968) activities are induced. \( P_4 \) also appears to induce the formation of lipid droplets in luminal and upper glandular epithelial cells (Brinsfield and Hawk, 1973).

Hormones act by binding specific receptors and triggering subsequent events via their effects on the transcription of various genes. Therefore, the study of sex steroid receptors may be important in understanding steroid influence in the uterus. \( E_2 \) receptors in the intercaruncular endometrium peak in number at days 0 to 3 of the estrous cycle (Koligan and Stormshak, 1977). They appear to be induced by \( E_2 \), but inhibited by \( P_4 \) (Leavitt, et al. 1985). \( P_4 \) receptors decline in
intercaruncular endometrium to be at lowest levels at day 14 of the cycle. In sheep endometrium, E₂ influence enhances P₄ receptor numbers (Zelinski and Stormshak, 1981), while P₄ depresses its own receptor levels (Miller et al., 1979). The action of E₂ on human breast cancer cells was shown to increase P₄ receptor synthesis and mRNA levels without affecting P₄ receptor degradation (Nardulli et al., 1988), and similar controls may exist in sheep endometrium.

The pregnant ewe

Fertilization and maternal recognition of pregnancy. If a ewe is bred, sperm may fertilize the ovum in the oviduct about 24 to 36 h after the start of estrus (Hafez, 1980). The zygote descends the oviduct between days 2 and 4 of pregnancy, to enter the uterus. The embryo hatches, or sheds its zona pellucida, between days 5 and 6. The embryo elongates and starts to form defined tissue layers and rudimentary organs while in intimate contact with only the secretions of the maternal uterus. On day 15 of pregnancy, the outer embryonic membrane, trophectoderm, forms the initial attachments with the uterine caruncles (Wimsatt, 1950). Maternal recognition of pregnancy in the ewe occurs between days 12 and 13, before the apposition of embryo and uterus, and appears to depend on soluble factors of embryonic-maternal communication. In the ewe, maternal recognition of pregnancy is believed to act by the embryo's production and release of ovine trophoblast protein-1 (Hansen et al., 1985a), whose cDNA was recently cloned and sequenced (Imakawa et al., 1987). Subsequently, the discovery was made that ovine trophoblast protein-1 was a functional ovine interferon. This signal, produced by the embryo from days 12 to 22 of pregnancy, acts locally in an antiluteolytic manner to preserve
the CL. The CL produces progesterone necessary to induce maternal receptivity for the embryo, affecting morphology, metabolism, and secretion in the uterus. Follicles cease to mature, the CL enjoys a prolonged life, and the estrous cycle is interrupted by pregnancy.

**Placentation.** Placentation begins at the time of apposition (day 15). Areas of embryonic membranes contact and attach to maternal caruncles. At this time, endometrial tissue appears to be active in protein secretion, with cells containing well developed rough endoplasmic reticulum (Lawn et al., 1969). There is interdigitation of tissues at the sites of apposition. The caruncular areas go on to develop sulci as the apposed chorion develops folds and, later, villi. The interdigitation of tissues within placentomes greatly increases the surface area for nutrient and waste exchange between mother and fetus. There is no loss of tissue layers between maternal and fetal blood circulation. Thus, the sheep's placentation is considered to be "cotyledonary" and "epitheliochorial", the least invasive type of placentation and the one typical of ungulates.

From day 16 to 18 of pregnancy, trophoblast tissues appose noncaruncular endometrium and invade the necks of uterine glands (Lawn et al., 1969). The chorionic membrane of the early embryonic placenta, which is opposite intercaruncular endometrium, is devoid of villi. By day 22 of pregnancy, areas opposite uterine glands on the intercotyledonary placenta develop thickenings. These are areolae which, by day 30, develop papillae that invade the uterine glands. Around day 100 of pregnancy, the areolae are approximately 3 mm in diameter, but due to placental growth and fetal movement, may not directly overlie the uterine glands. Wimsatt (1950) believes that the
structural changes of the fetal areolae are related to the role of the maternal endometrial glands. The gland-areola units may function as unidirectional transfer points for uptake of uterine secretions by the conceptus.

Epithelium of the intercaruncular endometrium is eroded between days 20 and 65 of gestation. The sheep shows greater destruction of maternal tissue than other ruminants (Wimsatt, 1950). The caruncular epithelium at this time is replaced by a syncytium. The intercaruncular epithelium is mainly apparent in the glands. At day 40 of pregnancy, amorphous material, probably secreted by the intercaruncular endometrium, is present between interplacentomal membranes (Lawn et al., 1969). After day 65, the intercaruncular epithelium regrows.

The endometrial glands appear to be of two types (Davies and Wimsatt, 1966). One, opposite areolae, is composed of ciliated cells and contains secretions; the other, not found opposite areolae, have non-ciliated cells laden with lipid droplets. The glands increase up to four times in length and ten times in width by cellular proliferation. They do not, however, increase in number or branching, as occurs in the pig. In late gestation, intercaruncular endometrium contains increased glandular and vascular components. The glandular epithelial cells are of the simple, columnar type, but are hypertrophied, with large nuclei and many microvilli in apical and lateral cell membranes.

Protein secretion by the endometrium. Intercaruncular endometrium is an active secretory tissue during pregnancy. Miller et al. (1983) found a 25-fold increase in protein secretion by ovine endometrium when they compared in vitro incorporation of radiolabeled leucine per ug DNA between samples taken at day 0 and day 84 of gestation. As early as day
11 of pregnancy, there is an increase in protein synthesis by intercaruncular endometrium as compared to that of the ewe at day 11 of the estrous cycle (Findlay et al., 1982). RNA levels in the intercaruncular endometrium tissue also increase in the pregnant ewe. E$_2$ receptors are present in the endometrium in low levels during pregnancy, while P$_4$ receptor levels remain high. The enzymes beta-glucuronidase (Murdoch and O'Shea, 1977), acid phosphatase (Findlay et al., 1981) and alkaline phosphatase (Guillemot et al., 1981) increase during pregnancy possibly as a result of progesterone influence; these enzymes have been postulated (on very tenuous grounds) to be involved in the process of implantation.

**Hormones during pregnancy.** Progesterone is the predominant steroid hormone of pregnancy, and the placenta is the major source of P$_4$ in the ewe after day 50 of pregnancy and can provide sufficient P$_4$ necessary for maintenance of pregnancy in animals ovariectomized after that time. The ability of the placenta to provide all necessary P$_4$ does not occur in the goat, cow, and sow where the CL continues to be a the primary source of progesterone throughout pregnancy (Flint and Ricketts, 1979). Sheep-goat chimeras have been developed in sheep, but require that the fetal placenta be of sheep origin, possibly because of its high rates of P$_4$ production (Meinecke-Tillmann and Meinecke, 1984; Fehilly et al., 1984). Peripheral plasma levels of P$_4$ rise from 2 to 3 ng/ml before day 50 to peak at 10 to 17 ng/ml at about day 120 of gestation (Bassett et al., 1969). Systemic P$_4$ levels decline gradually until day 140, at which time they drop rapidly to be 1 to 3 ng/ml at parturition. Peripheral plasma E$_2$ levels are undetectable until just prior to parturition. At lambing, E$_2$ peaks to 110 to 400 pg/ml in
systemic blood. Other hormones are produced during pregnancy, but these will not be discussed here as they have not been shown to influence uterine glands and their secretions.

**Fetal development.** The embryo begins to form major organ systems at the beginning of fetal development, between days 34 and 40 of gestation. The fetus grows more rapidly as gestation continues. Birth weights for lambs range from 1.8 to 7.6 kg (Barcroft, 1945), dependent on the numbers of fetuses and the nutritional status of the dam, as well as the genetic potential of both. In the case of multiple births, there appears to be competition in the uterus between fetuses for the limited number of uterine caruncles, as the number of placentomes in each placenta relates directly to the weight of the fetus (Mellor and Murray, 1981). Poor nutrition for the ewe in late pregnancy may also decrease fetal birth weight and adversely affect the growth spurt of the prenatal lamb (Robinson, 1977).

**Parturition and uterine involution.** After 145 to 149 days of gestation, the process of parturition occurs and the lamb is born. This is regulated by a complex pattern of several hormones and is coordinated with the onset of lactation in the ewe. Beginning after parturition is the process of involution of the postpartum uterus. During involution, the intercaruncular luminal epithelium appears to remain intact while there is a rapid loss of glandular epithelium and uterine weight (O'Shea and Wright, 1984). Reepithelialization of caruncles is the slowest process of involution, reaching completion about four weeks after parturition. Ovulations without estrous behavior (called "silent heats") have been reported during uterine involution; however, normal estrus activity does not resume until four weeks post-partum. The ewe
may enter an anestrous period before resuming cyclic activity in the appropriate season. Two breeds of sheep, Merino and Dorset Horn, have estrous cycles for an extended season and can conceive and produce two lamb crops per year without artificial hormonal intervention (Robinson, 1951).

The anestrous ewe

Non-cyclic and nonpregnant ewes are anestrous. In this phase of the reproductive cycle, the ovaries have few distinguishable features except for small follicles. \( P_4 \) and \( E_2 \) levels are at baseline levels \( (P_4 = 0.11 \text{ ng/ml}; \text{Thorburn et al.}, 1969) \). The uterus is small and contracted. Endometrium is thin, with minimal glandular development in the intercaruncular areas. Luminal and glandular epithelial cells are short, without signs of active secretion.

The Study of Uterine Secretory Proteins

Components of Uterine Secretions

Since the time of Aristotle (384-322 B.C.) there has been interest in the secretions of the uterus (Roberts and Bazer, 1980). This function is thought to make the uterus a suitable environment for the succession of events, including conception, preimplantation embryonic growth, implantation and placental development, and fetal development, all crucial to pregnancy. Much emphasis was put on the secretions of the uterus prior to conceptus implantation. During this period, the fluid within the uterus has the most intimate association with the conceptus of any maternally-derived material. As such, the conceptus is believed to rely completely upon the uterine fluid for its nutritional needs. For this reason, uterine secretions have been referred to as "histiotroph", "uterine milk", and "culture medium" (Roberts and Bazer,
The placenta in large domestic animal species has no loss of tissue layers between embryonic and maternal blood supplies. Therefore, these species may be more dependent on endometrial secretions for nutrition after placentation than other species such as man, whose hemochorial placenta allows fetal tissue to be in direct contact with maternal blood (Roberts and Bazer, 1988). The ungulate placenta, for example, does not allow the transfer of immunoglobulins from maternal blood to the fetus as man's does. The secretion of materials from glands and their subsequent uptake by the areolae of the fetal placenta are a part of an alternative embryotrophic route.

There are many components of uterine secretions, including water, electrolytes, sugars, minerals, vitamins, prostaglandins and proteins (Bazer and First, 1983). Proteins secreted by the endometrium are discussed in the short review provided here. Few such proteins have been purified and well characterized, and even some of these remain mysterious as to their function in the uterus. Biochemical characteristics and proposed physiological functions of secreted uterine proteins are discussed with an emphasis on those of farm animals.

Methods Used in the Study of Proteins of Uterine Fluid

Studies of uterine fluid components usually involve collection of materials by flushing intact uteri either surgically or nonsurgically. This method is routinely employed in collecting samples from animals during their estrous cycle, early pregnancy, or pseudopregnancy (Basha et al., 1980a), or from ovariectomized animals given exogenous steroids. As the duration of progesterone influence lengthens in ovariectomized animals under hormonal therapy and pseudopregnant animals, uterine fluid accumulates and can be directly aspirated from
the uterine lumen. Uterine fluid can be harvested also from one uterine horn of animals made unilaterally pregnant by surgical restriction of their conceptuses to their other horn (Bazer et al., 1979). Another useful technique involves the synthesis of uterine proteins by endometrial explants in in vitro culture and the harvest of secreted products in the culture medium (Basha et al., 1980b).

Any of the above techniques preclude the possibility of direct collection of conceptus-derived materials in samples but may allow serum contamination by blood or by tissue transudation. In fact, uterine flushings of animals with nonpregnant uteri have two dimensional SDS-polyacrylamide gel patterns similar to those of serum. Hormones control uterine secretory activity as well as uterine blood flow and vascular permeability. At the time of implantation, vascular permeability increases to provide a flux of plasma components into the uterus along with uterine secretions (Keys et al., 1986). To distinguish serum proteins from uterine secretory proteins, some investigators compare levels of activities of proteins within uterine secretions to those in plasma and consider elevated levels within the uterus as proof of uterine origin. This conclusion may not be accurate because of assumptions of uterine fluid volume and because serum may contain enzyme inhibitors that mask the presence of the enzymes it contains. The use of radiolabelled precursors in in vitro culture of endometrium allows identification of radiolabelled products in culture medium as a more viable proof of endometrial origin and release of proteins. Immunocytochemistry using antisera against uterine proteins can detect the presence of specific proteins within uterine cells where they are produced or taken up by tissue. The development of nucleic acid probes
specific for uterine proteins allows levels of their mRNA to be measured in homogenized uterine tissue by Northern blotting analysis. With nucleic acid probes, very low levels of mRNA within individual cells can be detected by the very sensitive technique of in situ hybridization. Thus, the earliest production of uterine proteins specific to pregnancy may be described at the level of the cell.

Factors Affecting the Synthesis of Uterine Secretory Proteins

Steroid hormones

The synthesis of uterine proteins is affected by many factors. The best characterized molecules affecting the protein synthesis of the uterus are the steroid hormones, $P_4$ and $E_2$. One of the best studied steroid-induced uterine proteins is uteroglobin of the rabbit. Although its function is unknown, it is the major protein produced by the endometrium under $P_4$ influence and is presumed to be taken up by the rabbit blastocyst (Beier, 1980). In the investigation of the hormonal regulation of uteroglobin, $P_4$ was found to (1) be an endometrial cell mitogen, (2) increase transcriptional activity in endometrium, and (3) specifically increase uteroglobin gene transcription (Shen et al., 1983). Prolonged $P_4$ influence, however, was found to decrease the last two effects. Low levels of $E_2$ were found to potentiate the action of $P_4$. The ovariectomized rabbit has a two day lag after $P_4$ administration until uteroglobin transcription is induced, while the intact doe does not. The lag may be explained by the endometrium of the ovariectomized rabbit being incompletely differentiated and requiring time under steroid influence to develop complete glandular capacity.

In the farm animal, $P_4$ and $E_2$ act synergistically to induce maximal uterine production of secreted proteins. In gilts given exogenous
steroids for 11 days after ovariectomy at day 4 of the cycle, the amount of protein in uterine flushes varied with the amount of P_4 (Knight et al., 1974b). Low levels of exogenous E_2 appeared to increase total uterine secretory protein, but large doses were inhibitory. In the ovariectomized ewe given P_4 or the unilaterally pregnant ewe, uterine fluid and secretory protein accumulated in small amounts at day 30. By day 136 of pregnancy, uterine fluid containing secreted protein may be present in very large amounts (1.1 liter 16 g protein; Moffatt et al., 1987b). The action of P_4 appears to be required on a long term basis for full induction of uterine secretory capacity. In vitro studies of endometrial explants from treated ewes showed that P_4 treatment of ewes resulted in increased synthesis of secretory proteins. Endometrial explants from ewes treated with the estrogen estrone released unlabeled proteins at higher rates than controls, suggesting that exudation of previously made proteins was occurring.

In addition to hormone levels and their duration, the sequence of hormonal signals appears to affect protein synthesis of the uterus. Prepubertal gilts treated with exogenous steroids for 15 days produced uterine proteins characteristic of adult animals but in lesser quantities than observed in mature gilts under the same treatment regimen (Murray and Grifo, 1976). This reduction of uterine secretory capacity may explain why a prepubertal gilt cannot maintain pregnancies even when treated with exogenous steroids. Schedules of steroid injections designed to simulate the steroid levels of the cyclic animal were given to ovariectomized ewes used as recipients in embryo transfer experiments (Moore et al., 1983). For normal embryo development a minimal steroid schedule requirement was found to include sequential
simulation of luteal phase P_4, estrus E_2, and luteal P_4. The synchrony of the embryo transfer was also necessary for embryo development, as it is in intact recipients. The pregnancy failures of the prepubertal gilts may have been due to the lack of an appropriate schedule of both E_2 and P_4. There appear to be several events necessary to result in full secretory capability of the uterus.

The effects of E_2 and P_4, separately and in combination, on the enzymes in intercaruncular endometrium of ovariectomized ewes have been determined (Murdoch and White, 1968). P_4 increased the measured activities of acid and alkaline phosphatases, glucose-6-phosphate dehydrogenase, glutamic-oxaloacetic transferase and succinic dehydrogenase while amylase activity was decreased. E_2 significantly affected only the activity of acid phosphatase, in a negative manner. However, these housekeeping genes probably constitute only a minor portion of the total protein synthesized and relate to the growth of the endometrium. The influence of P_4 appears to induce the synthesis and secretion of uterine proteins by the endometrium that are different from those found in uterine flushes of animals early in the estrous cycle. The pattern of proteins produced by the uterus in response to pregnancy or progesterone varies greatly between farm animal species (ewe: Bazer et al., 1979, pig: Basha et al., 1980a, cow: Bartol et al., 1985, mare: Zavy et al. 1982b). Specific proteins secreted by the uterus are discussed in following sections.

**Conceptus products**

In the pregnant animal, conceptus products affect the synthesis and secretion of some uterine proteins. Endometrial explants from gravid and nongravid horns of unilaterally pregnant gilts cultured with
radioactive precursors showed a similar pattern of secreted proteins from both horns (Basha et al., 1980b). However, the explants from the horn containing the conceptuses secreted significantly more protein than that from the unoccupied horn. When epithelial cells from endometrium of day 13 pregnant and nonpregnant ewes were incubated with medium from cultured day 15 blastocysts (Salamonsen et al., 1986), in both cases the conditioned medium enhanced the secretion of uterine proteins. However, the effect was greater in epithelial cells from the pregnant endometrium. The proteins secreted appeared similar to those induced by P4 and E2. A purified conceptus product which is the major protein of the sheep embryonic trophoblast, ovine trophoblast protein-1, has been shown to enhance the secretion of at least 11 endometrial proteins when added in culture to endometrial explants from cyclic or early pregnant ewes (Vallet et al., 1987). Ovine trophoblast protein-1 was shown to bind receptors with high affinity in endometrial preparations from cyclic and anestrous ewes (Godkin et al., 1984). As the signal for maternal recognition of pregnancy in the ewe, ovine trophoblast protein-1 is believed to act locally on the uterus, possibly exerting its effects through changes in protein synthesis. Some of these appear to be secreted. Thus, the ovine embryo within the uterus communicates to help bring about the dramatic changes in the mother associated with pregnancy.

Functions of Uterine Proteins

Introduction

Many functions must be performed by proteins in uterine secretions. Proteins in histiotroph may provide the amino acids building blocks necessary for conceptus development and growth (Roberts
et al., 1984b). They may act more specifically to transport limiting or insoluble nutrients from the uterus into the conceptus. There is evidence that uteroferrin, a secreted uterine protein, is taken up by the embryonic placenta at the areolae, where it enters fetal circulation, and is found ultimately within the allantoic fluid, where it may be held as a nutrient reserve (Roberts et al., 1986b).

In addition to having roles in fetal nutrition, proteins in uterine fluid may also regulate dramatic tissue remodeling that occurs in association with pregnancy. Both conceptus and uterus grow and change shape, as well as cooperatively form a new organ, the placenta, with tissues derived from both sources. Enzymes such as proteases are implicated in destruction of old tissue form and enhancement of cell migration. Other enzymes help rebuild tissue in new form by forming new cell contacts and adhesive molecules. Proteases and glycosidases may alter surface components of the embryo or endometrium and facilitate implantation. Proteolytic processes are strictly controlled by the presence of specific enzyme inhibitors which are also proteinaceous (Mullins and Rohrlich, 1983).

Proteins are involved in communication between embryonic and maternal systems that must exist to direct the complex series of events involved in establishment and maintenance of pregnancy. The sheep embryo releases a protein, ovine trophoblast protein-1 that prevents luteolysis within the mother. It is likely that proteins released by the uterus have effects on the development of the embryo. Growth factors, identified in uterine fluid, have been implicated in promotion of embryonic growth. The uterus supports the growth of embryos when both are at the same physiological day of pregnancy. Embryos do not
survive in the oviduct (Bazer, 1975) or in the asynchronous uterus. The failure of the embryos to survive may be due to the lack of a uterine protein necessary to their development. Alternatively, the embryos may die because of the presence of a protein that is inhibitory to their growth or for reasons not related to secreted proteins. The latter possibility is supported by the survival of embryos in vitro during the same stages of pregnancy.

In addition to supplying nutrients and allowing for conceptus growth and development, the uterus must also tolerate the conceptus, which is a foreign allograft within the mother. Immunosuppressive proteins in uterine secretions are believed to promote tolerance of the conceptus by the maternal immune system.

**Effect of Indirectly Reducing or Increasing Uterine Secretory Proteins on Pregnancy**

Studies to determine if the presence of normal amounts of secreted uterine proteins affects pregnancy have been done with pregnant gilts in two ways: (1) passive immunization to reduce the amount of secreted uterine proteins and (2) exogenous steroid therapy to increase them. Three miniature gilts were passively immunized on days 5, 7, 9, 11, and 13 of pregnancy with rabbit antiserum to porcine P4-induced uterine secretions (Daniel, 1972). One gilt farrowed normally, but the two others did not farrow and did not return to estrus for over 172 days. Control gilts given pig serum had normal gestations. A more extensive study employed antiserum to a major P4-induced protein of the porcine uterus, utroferrin, to immunize pregnant gilts on days 7, 9, 11, 13, and 15 of pregnancy, as well as a second group of gilts on days 34, 36, 38, 40, and 42 (Chen and Bazer, 1973). The early immunization time period corresponded to the start of utroferrin synthesis, and the
later, to a period of high production and uptake by the fetus. The gilts immunized in early pregnancy were hysterectomized on day 30 of gestation and had reduced placental lengths and protein concentrations in allantoic fluids within their litters. The gilts in the late immunization group, hysterectomized at day 50, had reduced placental and fetal sizes in their litters. In a study of increased uterine secretions, P\textsuperscript{4} (3.3 mg or 1.1 mg) and E\textsubscript{2} (0.55 ug) were administered daily to pregnant gilts (Knight et al., 1974a). Gilts that had received the higher P\textsuperscript{4} dose, shown to increase secreted uterine proteins in nonpregnant gilts (Knight et al., 1974b), had longer placentae, and greater allantoic fluid volumes and wet empty uterine weights at hysterectomy on day 40 of gestation than gilts on the lower P\textsuperscript{4} dosage. Though many unforeseen factors may be affected by these treatments, these in vivo studies suggest that secreted uterine proteins enhance size of placentae and fetal components, in early and late pregnancy, and probably affect survivability of conceptuses.

**Specific Proteins of the Uterus**

**Proteins with roles in fetal nutrition**

**Uteroferrin.** The uterine secretions of the pig have been investigated more completely than those of any other species. The most well characterized protein secreted by the porcine uterus is uteroferrin, a glycoprotein that contains two Fe atoms per molecule and has a relative molecular weight (M\textsubscript{r}) of 35,000 and an isoelectric point (pI) of greater than 9.5 (Roberts and Bazer, 1980). Uteroferrin is the major secretory product from endometrium of gilts which are pregnant or pseudopregnant and comprises 10 to 25% of the secreted protein of the uterus. Uteroferrin production is induced by P\textsuperscript{4} in ovariectomized gilts.
and appears to have maximal secretion when low levels of E$_2$ is present, while high levels of E$_2$ are inhibitory. In the pregnant gilt uteroferrin can first be detected at day 10 of pregnancy. The uterus shows maximal synthesis of uteroferrin at about day 60 of pregnancy when approximately 2 g per day are synthesized and secreted. This pig protein has acid phosphatase activity towards p-nitrophenyl phosphate (pH optimum = 4.9, K$_m$ = 2.2 mM; Roberts and Bazer, 1984) and other substrates. Uteroferrin is an acid phosphatase that is resistant to tartrate, a compound which is an inhibitor of other isozymes.

There are at least three forms of uteroferrin: native uteroferrin, reduced uteroferrin, and high molecular weight uteroferrin (Roberts et al., 1987). Native uteroferrin is purple with an absorption maximum at around 545 nm, due to its two FeIII ions. In the presence of mild reductants (L-ascorbate or beta-mercaptoethanol) uteroferrin is converted to a pink form, with an absorption maximum at about 508 nm. This shift in absorption maximum is associated with the reduction of one FeIII to FeII. Pink uteroferrin has greatly enhanced acid phosphatase activity compared to native, purple uteroferrin. A pink high molecular weight form of uteroferrin (M$_r$ = 80,000) is also detectable in porcine uterine secretions and allantoic fluid. It is believed to be a heterodimer containing uteroferrin (Baumbach et al., 1986) and a second protein in a one to one complex. The large pink molecule is stable at neutral pH and has an acid phosphatase activity comparable to that of reduced uteroferrin (Roberts et al., 1987). The uteroferrin within this high molecular weight complex is noncovalently bound to a second associated protein; the two are easily dissociated by low pH or strong reducing conditions.
The uteroferrin-associated protein is basic, \( P_4 \)-induced, and has three immunologically related forms of \( M_r = 40,000, 46,000 \) and 50,000 (Baumbach et al., 1986). The uteroferrin-associated protein immunolocalizes to endometrial glandular epithelium, as was uteroferrin. Endometrial RNA from day 45 pregnant gilts was used in an in vitro translation assay followed by immunoprecipitation to identify a single product relating to uteroferrin-associated protein. This translated species of \( M_r = 45,000 \) is believed to undergo post-translational processing, including glycosylation and proteolysis, to yield the three mature forms of the uteroferrin-associated protein.

Proteins similar in enzymatic and physical properties to uteroferrin have been identified in uterine secretions of cows (Dixon and Gibbons, 1979; Ketcham et al., 1985) and mares (McDowell et al., 1982). In neither case, however, are the proteins as predominant as they are in the pig. The uteroferrin-like protein of the mare has been partially characterized; it is less stable than uteroferrin and rapidly loses color and acid phosphatase activity. Uteroferrin is a member of a large family of tartrate-resistant acid phosphatases that possess similar amino acid sequences (Hunt et al., 1987) and cDNA sequences (Ketcham et al., 1988). One closely related molecule is found in spleen, placenta and bone. The latter, probably originating from osteoclasts, is increased in serum of patients with diseases of bone resorption. The spleen molecule is elevated in the serum of patients with Gaucher's disease and hairy cell leukemia (Ketcham et al., 1985).

The complementary DNA (cDNA) for uteroferrin has been cloned. Endometrial mRNA from a pregnant gilt was isolated and used to make cDNA and to generate a lambda bacteriophage gt11 expression library (Simmen
et al., 1988a). Polyclonal antiserum for uteroferrin was raised in rabbits and used to identify clones containing uteroferrin sequences. DNA from two clones bound antibody that specifically reacted with uteroferrin on Western blots. This "epitope selection" reconfirmed that the clones contained sequences encoding uteroferrin antigenic determinants. DNA sequencing of three short cDNA clones corresponded with previously determined sequence of uteroferrin peptides. Radiolabeled DNA probes from positive clones were used as probes for Northern blots of porcine RNA from uterus, liver and mammary tissues. A single RNA species of 1.7 kilobases in length was identified in uterine tissue only. From in vitro translation of porcine uterine mRNA, a single protein of $M_r = 31,000$ was immunoprecipitated. Uteroferrin mRNA was at peak levels in the last half of pregnancy. This result contrasted with the peak synthesis of uteroferrin which occurred at around day 60 of pregnancy (Basha et al., 1980a). Synthesis has been shown to decline sharply after day 75 in explant cultures, and to be present in reduced amounts after day 60 to 75 in uterine extracts and fetal allantoic fluid (Simmen et al., 1988a). Therefore, the control of uteroferrin synthesis appears to exist at the posttranscriptional level in the uterus of the pig during late pregnancy.

The glycosylation and phosphorylation of uteroferrin appear to be tantalizingly similar to those of the UTMP, the subjects of this thesis. Uteroferrin produced by endometrial explants in vitro is larger than uteroferrin found in uterine secretions or uteroferrin deglycosylated by endoglycosidase H ($M_r = 37,000; 35,000; and 33,000; respectively; Roberts et al., 1986a). The differential glycosylation of uteroferrin appears to account for its different sizes. Newly
synthesized uteroferrin appears to carry two carbohydrate chains while native uteroferrin carries only one, possibly as a result of "trimming" by glycosidases within the uterus. Uteroferrin produced by endometrial explants in vitro carries a mannose-6-phosphate residue, which is an intracellular marker for lysosomal enzymes. Uteroferrin is a good substrate for UDP-N-acetyl glucosamine 1-phosphate transferase (Couso et al., 1986), an enzyme that transfers a phosphate and N-acetyl glucosamine group to a mannose residue on the carbohydrate chains of lysosomal enzymes after recognition of specific protein structure determinants (Lang et al., 1984). A phosphodiesterase removes the outer residue, thus uncovering the mannose-6-phosphate residue on lysosomal enzymes. However, uteroferrin produced in explant culture retains the N-acetylglucosamine residue covering the mannose-6-phosphate which may account for its extracellular delivery during its P₄-induced overproduction (Baumbach et al., 1984). The massive production of uteroferrin may overload the capacity of the phosphodiesterase and other components of the cellular machinery to target the product to an intracellular location, thus making uteroferrin a hypersecreted lysosomal enzyme. Only 3% of the mature uteroferrin molecules found in the uterus, however, carry a mannose-6-phosphate residue. This loss may be a result of the action of glycosidases within the uterine fluid.

Despite uteroferrin's relation to intracellular acid phosphatases and lysosomal enzymes, the function of uteroferrin is believed to be delivery of iron to the conceptus. Iron is a limiting nutrient involved in growth of piglets. Uteroferrin has been shown to enter the fetal placenta at the areolae and then pass to fetal circulation (Roberts et al., 1986b). Uteroferrin, labeled with radioactive iron and injected
into the umbilical vein, transfers iron rapidly to fetal liver (the site of erythropoiesis). Much of the radiolabeled iron is finally recovered in hemoglobin (Buhi et al., 1982). The carbohydrate chains of uteroferrin have been shown to be responsible for the uptake of the glycoprotein by reticuloendothelial cells of the fetal liver (Saunders et al., 1985). Uteroferrin is cleared by the fetal liver and kidney, but is also found in allantoic fluid, where it donates iron to fetal transferrin which can in turn recirculate to the liver. Uteroferrin in uterine secretions may also have antibacterial action by binding iron and restricting its bioavailability to microbes. However, this is unlikely because secreted uteroferrin is saturated with iron.

It has been proposed that uteroferrin is a lysosomal acid phosphatase, but under the influence of P₄, is recruited to be produced in great excess of previous levels and be secreted. In the uterine secretions, uteroferrin's delivery of iron to the conceptuses is probably vital to the survival of the offspring and therefore to the success of reproduction of the pig (Roberts and Bazer, 1984).

**Lactotransferrin.** Lactotransferrin is the major product of the mouse uterus under E₂ influence and is a member of the transferrin family of iron-binding proteins (Pentecost and Teng, 1987). Murine lactotransferrin is a glycoprotein with Mr = 70,000 and a pI = 9.5 and, like uteroferrin, may serve as an iron reservoir. Lactotransferrin may serve as a bacteriostatic agent within the uterus of the mouse by binding iron and limiting the availability of iron as a nutrient. Lactotransferrin has also been identified by immunocytochemistry in human secretory phase endometrium (Tourville et al., 1970) and in P₄-induced bovine uterine secretions (Dixon and Gibbons, 1979).
Retinol-binding protein. A member of the family of retinol-binding proteins has been identified as a progesterone responsive component of porcine uterine secretions. This retinol-binding protein ($M_r = 20,000$; J. Clawitter and R. M. Roberts, unpublished results) is distinct from cellular and serum retinol-binding proteins previously identified (Adams et al., 1981). Retinol or vitamin A, necessary for reproductive function and fetal development, is a fat-soluble vitamin and is probably transported to the conceptus through the aqueous uterine fluid by the retinol-binding protein, as iron is by uteroferrin.

Enzymes and inhibitors

Proteases. Proteolytic enzymes have been identified in uterine secretions of pigs. These $P_4$-induced enzymes include leucine aminopeptidase, and cathepsin B(1), D, and E$_2$ activities (R. M. Roberts et al., 1976). The proteolytic activities are not reflected in serum and, therefore, are believed to be of uterine origin. Leucine aminopeptidase, like uteroferrin, is found to accumulate in allantoic fluid. Leucine aminopeptidase, also known as leucine naphthylamidase, is localized to the surface epithelium of the pig uterus as a membrane-bound enzyme. A soluble form of leucine aminopeptidase, possibly released by proteolytic activity on the endometrial membranes, has been purified and has a $M_r = 480,000$, $K_m = 0.14$ mM, and pH optimum of approximately 7 (Mancarella et al., 1981). Similar aminopeptidases have been identified in the uterus around the time of implantation. However their physiological roles are unclear.

Cathepsins are generally regarded as lysosomal enzymes. Their activities within tissues are related to intracellular protein degradation. In assays done at acidic pH, cathepsin D activity was
found to be increased in uterine flushes of ovariectomized gilts given P₄ as compared to those of control animals (Hansen et al., 1985b). However, activity of cathepsin D was undetectable at pH greater than 7, and probably isn't active in utero. Cathepsin D production specifically increases in amount during implantation and postpartum uterine involution in the rat uterus (Elangovan and Moulton, 1980).

In the rat given E₂, a plasma membrane bound-activator of a soluble arginine esterase proenzyme is induced (Jazin et al., 1988). Other proteases identified in the rat uterus include a trypsin-like activity stimulated by E₂ (Katz et al., 1976) and a latent collagenase, activatable by proteolysis but inhibited by P₄ (Tyree et al., 1980).

A chymotrypsin-like enzyme is proposed to be involved in lysis of the zona pellucida of mice (Hoversland and Weitlauf, 1981). Unhatched mouse embryos transferred to ovariectomized mice underwent zona lysis only if both P₄ and E₂ were administered, treatment which induced an activity similar to that of chymotrypsin within the uterine fluid.

Plasminogen activator (PA) is a protease found in uterine flushings of pigs and other species that specifically cleaves the zymogen plasminogen to produce the active, nonspecific protease plasmin. PA has been implicated in normal processes such as tissue remodeling and in disease processes such as tumor spreading (Mullins and Rohrlich, 1983). There are two types of PA: "tissue PA" (tPA) which requires fibrin for full activity, and "urokinase" (uPA) which has no fibrin requirement. Antibodies to these types of PA do not cross react, and the two proteins are clearly distinct. PA activity in uterine flushes of the pig is high under E₂ influence. The rat uterus under E₂ influence also increases its total PA activity, its overall size, and
total fluid volume (Kneifel et al., 1982). It is hypothesized that PA may have a role in tissue remodeling involved in uterine growth. Proteases in uterine secretions may also have a role in implantation because administration of protease inhibitors in utero has been shown to inhibit the ability of blastocyststo implant in rabbits and mice (Denker, 1980; Dabich and Andary, 1974).

Protease inhibitors. The pig conceptus actively secretes PA and, when placed in ectopic sites, invades maternal tissues (Samuel, 1971). The porcine uterus is resistant to such embryonic invasiveness and suffers no erosion of tissue layers during placentation. The uterine surface and upper glandular epithelium secrete a plasmin inhibitor (Fazleabas et al., 1985) during the time of implantation when the embryos secrete PA (Mullins et al., 1980; Fazleabas et al., 1983). This inhibitor also interacts with trypsin and to a lesser extent chymotrypsin and falls into the class of Kunitz inhibitors (Roberts et al., 1987). It is ineffective against elastase, pepsin, papain and collagenase. There are four isoforms of the trypsin/plasmin inhibitor that are related immunologically. Limited N-terminal amino acid sequence of one form has shown it to have strong sequence homology with aprotinin, a \( M_r = 6500 \) trypsin inhibitor found in bovine lung, pancreas and other tissues (Fritz et al., 1979). The porcine inhibitor has been shown by immunocytochemistry to coat the external surface of the trophoblast and is thought to limit proteolysis locally (Fazleabas et al., 1982). The trypsin/plasmin inhibitor may also act generally to prevent proteolytic degradation of proteins of the uterine fluids that may be vital to the embryo.
Protease inhibitors have been identified as products of the endometrium of species other than the pig. In the human, endometrium releases both tPA and uPA into explant culture medium under the influence of E₂ (Casslen et al., 1986). Estrogen influence also enhances the release of a PA-I from human endometrium which forms complexes with both tPA and uPA and has an M_r = 50,000. An inhibitor of PA has been identified as a secretory product from the uterus of the skunk under P^ influence (Fazleabas et al., 1984). This PA inhibitor (M_r = 70,000) was a major protein component of the uterine fluid during embryonic diapause or delayed implantation and throughout gestation. Specific roles of proteases and their inhibitors in uterine secretions have not been established.

**Glycosidases.** Glycosidases have been shown to have increased activities in uterine fluids of many species during pregnancy. Progesterone-induced secretion of several lysosomal enzymes was studied in the gilt, ewe, and the mare (Hansen et al., 1985b). All activities studied (acid phosphatase, arylsulphatase, lysozyme, beta-N-acetylglucosaminidase, alpha-galactosidase, beta-galactosidase, beta-glucosidase, beta-glucuronidase, and alpha-mannosidase) were enhanced in uterine flushes of ovariectomized gilts given P^ as compared to those given the corn oil vehicle, but to widely varying extents. Activities, measured at pH = 7.0, were found to be low since this is well above the acid pH optima of the enzymes. Beta-N-acetylglucosaminidase from pig uterus (M_r = 82,000 to 89,000) was partially purified and was found to resemble lysosomal beta-hexosaminidase in substrate specificity and pH optimum (4.4). Radiolabeled beta-N-acetylglucosaminidase could be immunoprecipitated from endometrial explant culture medium, showing that
it was synthesized in the uterus. Lysozyme, also found in human cervical secretions under E2 control, is an antibacterial enzyme that digests bacterial cell walls. Total lysozyme activity rises in pig uterine flushings with P4 therapy but specific activity does not. Therefore, it is unclear whether lysozyme is specifically induced by P4 (Roberts et al., 1987).

Beta-N-acetylglucosaminidase was increased in uterine flushings of ovariectomized mares and ewes given P4 when compared to levels in uterine flushings of control animals given only the drug vehicle, corn oil. In the sheep, alpha-L-fucosidase, beta-N-galactosidase, beta-N-acetylglactosaminidase, and beta-N-acetylglucosaminidase levels in uterine fluids were all elevated compared to serum levels (C. P. Roberts et al., 1976). At day 15, pregnant ewes had significantly greater activities for all of the glycosidases mentioned above in their uterine fluid than nonpregnant animals did. Uterine secretions from cows during early pregnancy had markedly increased activities for beta-N-acetylglucosaminidase, beta-N-acetylglactosaminidase, and alpha-fucosidase when compared to those in serum (Roberts and Parker, 1974).

Glucosephosphate isomerase activity, which interconverts glucose-6-phosphate and fructose-6-phosphate, is increased in uterine flushings of early pregnant gilts and pony mares (Zavy et al., 1982a). In ovariectomized gilts given E2, glucosephosphate isomerase activity is also increased. Glucosephosphate isomerase is an example of an intracellular enzyme that may be carried into the uterine lumen with secretion of other molecules during E2 influence.

In the pregnant mare, localized areas of endometrium are highly glycosylated immediately prior to implantation (Whyte and Allen, 1985)
and may serve as target sites for embryonic attachment. It is possible that glycosidases and proteases act in the uterine fluids of many species to alter surface interactions between conceptus and uterus.

**DNA polymerases.** RNA- and DNA-dependent polymerases are proteins which are induced by the synergistic action of P₄ and E₂ during pregnancy in rabbit endometrium (Chilton and Daniel, 1978). The induced DNA-dependent polymerase is identical to the nuclear protein and is of interest for its role in gene activation.

The RNA-dependent polymerase has viral and cellular characteristics and may be associated with viral particles. Viral particles have been identified in intercellular spaces of pregnant mouse endometrium and budding from basal membranes of secretory endometrial cells and from embryos. The RNA-dependent polymerase could provide an intrinsic reverse transcriptase activity that might amplify or modify genes during cellular differentiation. Expression of viral and proviral RNA has been demonstrated in pregnant mouse endometrium (Fowler et al., 1977; Saviolakis et al., 1982). The regulation and importance of these viral-related proteins during mammalian pregnancy is not understood, but may involve gene induction.

**Immunosuppressive proteins**

The conceptus is a hemiallograft within the uterus of the mother and differs from the mother in that half of its major histocompatibility complex genes that are from the father. It is not understood why the maternal immune system does not destroy the conceptus by the cytopathic process of graft rejection. Three hypotheses for maternal immunotolerance of the fetus were proposed by Medawar in 1953: the fetus is anatomically separate from the mother, the fetus is
antigenically immature, or the maternal immune system is inert (see Warner et al., 1988). The zona pellucida of the embryo does appear to act as a barrier to immune recognition by the mother. However, after hatching and implantation the fetal and maternal tissues appear to be in intimate contact. The second hypothesis is not correct because the maternal immune system recognizes and reacts to paternal antigens carried by the fetus, as demonstrated by circulating antibodies to paternal antigens during pregnancy (Rodger and Drake, 1987). The hypothesis of the incompetence of maternal immune system is incorrect because females typically withstand immune challenges very well during pregnancy. However, the conceptus is not attacked and destroyed by the maternal immune system. Local immune regulation may be responsible for tolerance of the fetal allograft in the uterus. Proteins released by the embryo such as ovine trophoblast protein-1, an interferon of the sheep, have been implicated as having roles in such immunoregulation (Imakawa et al., 1987).

Uterine secretory proteins have been assigned roles involving immunosuppression in several species. Uterine secretions were induced by P₄ therapy in ovariectomized ewes in which skin autografts and allografts had been placed in utero (Hansen et al., 1986). The sheep were given 0, 50 or 200 mg of P₄ per day for 30 days at which time they were hysterectomized. All autografts were healthy on histological examination, while allografts in ewes without P₄ had been resorbed. In P₄-treated animals, allografts were present but necrotic. Thus, the ovine uterus under the influence of P₄ appeared to be capable of mounting an immune response to destroy a tissue allograft but, under the influence of P₄ the graft destruction was slightly attenuated. Uterine
secretions of P4-treated animals in the graft study demonstrated greater immunosuppressive activity than controls in an assay involving the suppression of phytohemagglutinin stimulation of lymphocytes. In cyclic and pregnant ewes, uterine flushings collected on days 4, 9 and 14 also had nondialysable components that inhibited lymphocyte blastogenesis induced by phytohemagglutinin (Segerson, 1981). At day 14 of pregnancy, uterine flushings had the most immunosuppressive material of the samples analyzed. The immunosuppressive character of crude and partially purified uterine flushes from day 14 pregnant ewes was studied further with an assay involving interleukin-2 dependent T-lymphocyte activation (Segerson, 1988). The uterine flushes contained proteins of $M_r = 248,000, 70,000$, and $14,000$ that suppressed interleukin-2 activation of T-lymphocyte blastogenesis. A large protein ($M_r$ greater than 660,000) secreted by the ovine uterus during late pregnancy was found to inhibit blastogenesis of lymphocytes in phytohemagglutinin and mixed lymphocyte reactions (Hansen et al., 1987b).

In the uterine fluid of the gilt, a protein of $M_r = 15,000$ was identified and shown to suppress the response of maternal T-lymphocytes to the mitogen phytohemagglutinin (Murray et al., 1978). This activity was shown to be elevated in uterine fluids of pregnant animals when compared to those of nonpregnant animals. In the cow, similar activity was identified in two fractions of partially purified material of $M_r$ greater than 219,000 and $M_r = 14,000$ (Segerson et al., 1986). A human placental protein (PP14), found in uterine fluids of pregnant women, appears to have immunosuppressive activity in an assay involving the
mixed lymphocyte stimulation of mitogenesis (Bolton et al., 1987). PP14 is related to alpha-2 microglobulin and is found in seminal fluid, where it is believed to act as a local immunosuppressant.

The molecular events responsible for the immunosuppression remain to be elucidated. The complex role of the immune system in pregnancy requires further information to be understood. Tolerance of the fetal graft is another example of special communication and synchrony between mother and conceptus that is required during pregnancy and may be partially mediated by secreted uterine proteins. It is not known whether the immunosuppression is a result of the secreted proteins coating the conceptus and masking its foreign antigenic determinants, or by more specific effects on components of the maternal immune system.

**Growth factors**

Because of the rapid proliferation of both maternal and conceptus tissues during pregnancy, attempts were made to define cell growth stimulating factors in uterine fluid of pregnant animals. Uterine flushes from day 10 and 12 cyclic and pregnant sows contain a mitogenic activity that is capable of stimulating DNA synthesis of fibroblast and epithelial cell lines as well as that of porcine uterine stromal cells in primary culture (Simmen et al., 1988b). The partially purified growth factor is an extremely stable polypeptide of $M_r = 4,800$. The mitogen is not detected in uterine cytosol or in serum from sows at day 12 of pregnancy. The growth factor from porcine uterine fluids appears to be distinct from epidermal growth factor (EGF) in antibody and receptor binding studies and in stimulation of growth in an epithelial cell line that EGF inhibits. The tissue origin of the porcine growth factor has not been determined. A growth factor from pregnant sheep
uteri shows no mitogenic effect on rat fibroblasts but promotes growth of cell lines derived from E₂-responsive tumors (Ikeda and Sirbasku, 1984). This mammary-uterine-pituitary tumor cell growth factor has $M_r = 4,200$ and is active at nanomolar concentrations. Other polypeptide growth factors including EGF (Gonzalez et al., 1984), colony-stimulating factors (Pollard et al., 1987), and transforming growth factors (Nickell et al., 1983) have been isolated from uteri and placentae of various species. The uterine growth factors may have autocrine activity in promoting growth of the uterus in response to steroids or pregnancy. In addition, they may support conceptus growth and be a part of communication between mother and conceptus.

**Uterine Milk Proteins of the Ewe**

**Prevalence and Progesterone-induction**

The subjects of this dissertation are the major proteins produced and secreted by sheep endometrium under the influence of P₄, the uterine milk proteins (UTMP; Bazer et al., 1979). In ewes made unilaterally pregnant as previously described, the nongravid, surgically-ligated horn accumulates uterine secretions as a thick, creamy fluid called uterine milk (UTM). The volume and protein content of the UTM increases from 8 mls and 19 mg, respectively at day 30 of gestation to 750 mls and 17 g at day 144 (Moffatt et al., 1987b). Ovariectomized ewes treated with corn oil, estrone (5 µg/day), P₄ (50 mg/day), or both estrone and P₄ for 30 days accumulate UTM in their uteri, although two out of three ewes in the first two treatment groups did not possess endogenous secretory fluids. This daily dose of P₄ has been shown to be adequate for pregnancy maintenance in ovariectomized ewes (Alexander and Williams, 1966). Although uterine fluid volumes and protein content are not
significantly different for the treatment groups, explant cultures of endometrium from P-treated ewes release more newly synthesized protein into the medium than those from estrone-treated ewes.

The UTMP are the major protein components identified by SDS-PAGE in pregnant ewe UTM, in culture media from endometrial explants of day 30 to 144 pregnant ewes, and in culture media from endometrial explants of ovariectomized ewes given P₄ for 30 or more days (Moffatt et al. 1987a). Endometrium of ovariectomized ewes given corn oil or estrone did not secrete the UTMP in vivo or in vitro. Therefore the production of the UTMP appeared to be progesterone-dependent. Antiserum to purified UTMP coupled to bovine thyroglobulin was raised in roosters and used in immunocytochemical analysis of sheep uteri from days 60, 90, 120 and 140 of gestation that were fixed in Bouin's solution and embedded in paraffin. An avidin-biotin immunoperoxidase staining procedure was used on sectioned material. Sections developed with dilute chicken antiserum to the UTMP showed immunostaining that was mainly localized to apical borders of glandular and luminal epithelial cells of the endometrium. Some antigen was detected in basal and lateral epithelial cell borders and in luminal contents of uterine glands. Control sections developed with preimmune rooster serum or antiserum preadsorbed against the UTMP showed no staining at the dilutions used for antiserum. Immunostaining was not observed in other tissues of the uterus or in cyclic ovine uteri. The UTMP appeared to be specifically produced in the uterine glands of the intercaruncular endometrium of ewes.

To see if the UTMP gain access to the fetus, fetal membranes and tissues from day 120 and 140 of gestation were subjected to immunocytochemistry (Moffatt et al., 1987b). Only the intercotyledonary
chorioallantois showed specific immunostaining, along the surface of the fetal mesenchyme. Fetal amnion, kidney, liver, small intestine, thyroid and thymus did not show immunostaining. In a separate study, rabbit antiserum to the UTMP was used to immunostain protein samples from late pregnant ewes on Western blots. The UTMP were detected in samples of allantoic and amniotic fluids and maternal plasma (Newton et al., 1988). It appeared that the UTMP were taken up by the fetal placenta and accumulated in allantoic fluid. The UTMP were present in some samples of maternal sheep serum in small amounts.

Purification and Characterization

Purification

The UTMP were purified from UTM by two column chromatography steps (Moffatt et al., 1987a). After dialysis, basic proteins in UTM were bound on a carboxymethylcellulose column and eluted in a high salt buffer. Gel filtration chromatography of the eluate on Sephacryl S-200 yielded a protein peak containing UTMP that appeared to be free of contaminants by SDS-PAGE analysis. Cream-colored clots found in UTM were dissolved with strong protein denaturants and run on SDS-PAGE to show that the clots were also primarily composed of the UTMP.

Protein structure

The UTMP are a pair of basic polypeptides of $M_r = 57,000$ and 55,000, as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE; Moffatt et al., 1987a). With equilibrium sedimentation analysis, the UTMP have a weight-average $M_r = 50,700 \pm 4200$. Amino acid analysis of purified UTMP showed that
they contain high levels of leucine and lysine, little tyrosine and no tryptophan (Hansen et al., 1987a). The lack of tryptophan accounts for UTMP's low absorption at 280 nm when compared to that of most proteins.

Hansen et al. (1987a) showed that the UTMP are related to each other by peptide mapping and N-terminal amino acid sequencing. Two dimensional peptide mapping was done on individual species of radioiodinated UTMP after they were separated by preparative SDS-PAGE. The two samples were reduced, alkylated, and digested with chymotrypsin. "Fingerprints" of radiolabelled peptides were generated in the first dimension by electrophoresis and in the second dimension by thin layer chromatography. Fluorographs showed very similar peptides generated from each of the UTMP. N-terminal acid amino sequencing was done on samples containing both UTMP (Hansen et al., 1987a). Determinations on pooled UTMP at separate facilities agreed on a single sequence for the first 20 amino acid residues. The initial residues had some background signals, but only one strong primary signal was obtained in each of the following cycles. Although it's possible that one species had a blocked N-terminus and gave no signal, this experiment was interpreted to mean that the two species of UTMP have identical N-terminal structures.

Biosynthesis of the UTMP was studied with endometrial explants from day 140 pregnant ewes that were cultured with [3H]-leucine (Hansen et al., 1987a). In pulse-chase and continuous labeling experiments, a precursor of $M_r = 54,000$ was detected in immunoprecipitated material from homogenized endometrium after 20 min. of incubation with the radiolabel. This initial product was "chased" into the higher $M_r$ forms after 30 minutes of incubation without the label. In cultures with continuous presence of radiolabel, all three $M_r$ forms of UTMP were
apparent in immunoprecipitated material from the explant tissue. It appeared that both mature forms arose from a single precursor, whose $M_r$ was smaller than that of either of the mature forms, probably due to incomplete processing of its oligosaccharide chains.

**Glycosylation**

The UTMP are glycoproteins and contain approximately 5.6% carbohydrate by weight (Hansen et al., 1987a). The carbohydrate of the UTMP consists of 2.8% neutral sugars, 2.5% amino sugars and 0.3% sialic acid by weight. The high content of amino sugars in UTMP carbohydrate analysis suggested that the oligosaccharide chains were at least partially processed from high mannose type chains to complex types. Both species of the UTMP stain with periodic acid-Schiff reagent, a test which is specific for carbohydrate. Uterine fluid proteins from a day 120 pregnant ewe were separated on 2-D PAGE (Hansen et al., 1987a). The gel was incubated with radiolabeled concanavalin A (Con-A), a lectin that specifically binds to N-linked oligosaccharide chains containing alpha-mannosyl residues. Autoradiography showed that several proteins, including both of the UTMP, bound Con-A. A competing ligand for Con-A, alpha-methyl-D-mannoside, eliminated the binding. The conclusion was that both UTMP $M_r$ forms carried N-linked oligosaccharide chains.

How the two $M_r$ species of the UTMP (57,000 and 55,000) differ is unknown. The only known method of distinguishing them is by SDS-PAGE on 5 or 7.5% (w/v) acrylamide gels. It is possible that the species of UTMP differ in their $M_r$ on SDS-PAGE because of differences in the extent to which they are glycosylated. Therefore, the synthesis of the glycoproteins was investigated. Endometrial explants from day 140
pregnant ewes were cultured with D-[3H]-glucosamine (Hansen et al., 1987a). Secreted proteins in the culture medium were analyzed by separation in the first dimension by nonequilibrium pH gradient electrophoresis and in the second by SDS-PAGE (2-D PAGE). Fluorography of the gel revealed that both UTMP species were radiolabeled. The two UTMP species that had incorporated D-[3H]-glucosamine were separated by preparative SDS-PAGE and digested with Pronase to yield radiolabeled glycopeptides. The glycopeptides were separated by gel filtration on a Bio-Gel P-4 column. The elution profiles of radioactivity and their positions relative to a standard were very similar for both samples. Thus, the glycosylation of the two UTMP species appears to be qualitatively, if not quantitatively, similar. Similar endometrial explants were cultured with [3H]-leucine and tunicamycin, which inhibits the addition of N-linked oligosaccharide chains to nascent proteins (Hansen et al., 1987a). Proteins in the medium were separated by SDS-PAGE. Fluorography of the gel showed radiolabeled proteins of approximate Mr = 54,000 and 52,000. Because complete inhibition of glycosylation by tunicamycin is seldom obtained in explant cultures, one explanation of the results is that the molecule of Mr = 54,000 was partially glycosylated while the species of Mr = 52,000 totally lacked oligosaccharide chains. The results of the study of the glycosylation of the UTMP was consistent with the two Mr forms carrying one or two N-linked oligosaccharide chains. The smaller species (Mr = 55,000) was believed to carry one oligosaccharide chain while the Mr = 57,000 form of UTMP had two.
Phosphorylation

Endometrial explants from late pregnant ewes were cultured in the presence of $H_3^{32}PO_4$ produced radiolabeled UTMP (Hansen et al., 1987a). In the presence of tunicamycin, $[^{32}P]$-UTMP was not produced, indicating that the phosphate was present on the carbohydrate portion of the molecule.

The phosphorylation of the UTMP was investigated to see if it resembled that of uteroferrin, which carries the lysosomal enzyme marker mannose-6-phosphate on its oligosaccharide chain. Purified $[^{32}P]$-UTMP from explant culture medium was hydrolysed in conditions that spare the glycosidic bonds of mannose-6-phosphate residues (Hansen et al., 1987a). The components of the hydrolyzed material were separated by descending paper chromatography. Two major peaks of radioactivity were observed, with each containing about half of the total radioactivity in the sample. A sharp peak of radioactivity comigrated with mannose-6-phosphate standards. The other peak was a broad band of radioactivity which comigrated with inorganic phosphate. The UTMP appeared to carry a phosphorylated residue similar to mannose-6-phosphate. The radiolabel that comigrated with inorganic phosphate may have arisen from other phosphorylated residues that were less stable toward hydrolytic conditions or from mannose-6-phosphate residues that are cleaved by a contaminating or endogenous phosphatase activity in the UTMP preparation. Such activities have been problems in studies of the mannose-6-phosphate residues of thyroglobulin (Consiglio et al., 1987).

Function

Because mannose-6-phosphate was known as an intracellular marker for lysosomal enzymes, the UTMP were tested for several such activities
including those of beta-N-acetylglucosaminidase, alpha-galactosidase, beta-galactosidase, beta-glucosidase, beta-glucuronidase, alpha-mannosidase, arylsulphatase, cathepsin B and cathepsin D (Hansen et al., 1987a). The UTMP were also tested for activity on the nonspecific substrate casein in an agar plate at pH 3.0, 4.9 and 7.2. No activity was identified for the UTMP in any of the tests.

Both uteroferrin and the UTMP are secreted by uteri of large animals in great quantities in response to P₄. Whether the UTMP mannose-6-phosphate is masked by a covering residue, similar to that of uteroferrin's, is unknown. The covering residue is proposed to be responsible for the extracellular deposition of uteroferrin during periods of its heavy production by the endometrium of the pig. A similar situation may exist in the case of the UTMP and ovine endometrium.

The function of the UTMP is unknown. They may act merely as a source of amino acids for the conceptus, as nonspecific carrier proteins for other molecules, or as regulators of osmotic conditions within the uterus. The UTMP, by virtue of the large scale of their induction, however, appear to be a good model for the investigation of the action of progesterone.

Present Study of the UTMP

Purpose

The study of the UTMP was undertaken to answer several questions. How do the two UTMP species differ? Do they arise from more than one gene? What are the sizes of the UTMP with and without post-translational modifications? Do they have cleaved signal peptides as most secreted proteins do? What is the nucleic acid sequence of the
UTMP mRNA? What is the amino acid sequence of the UTMP inferred from
the nucleic acid sequence? Are the UTMP sequences related to known
sequences for other proteins? What is the schedule of induction of the
UTMP by P₄? How early in pregnancy can UTMP production be detected?
Are the UTMP present in the ovine uterus during the estrous cycle, in
the liver during pregnancy, or in uteri of other farm animal species
during pregnancy?

Experimental Design

The mRNA of the UTMP was cloned to determine the primary structure
of the peptide portion of the UTMP by inference from the sequence of the
cDNA and to develop nucleic acid probes to be used in its detection.
Endometrial RNA was isolated and used in in vitro translation systems
and in cDNA library construction. A lambda gt11 library was screened
with UTMP antiserum and a 350 base pair insert relating to the UTMP was
identified. This clone was used as a probe for screening a second cDNA
library in lambda gt10. A positive clone containing an almost full
length cDNA insert for UTMP mRNA was subcloned into M13mpl8 filamentous
phage and pUC 13 plasmid for sequencing by the dideoxy method. Sequence
data were compared to other known sequences with the Microgenie computer
program. A study of the P₄ induction of UTMP was accomplished with 18
ovariectomized ewes. Half of the ewes received a priming dose of E₂.
At day 0 of the study, P₄ implants were placed in all but four ewes.
The four day 0 ewes were subjected to blood collection for P₄ analysis,
uterine flushing, and hysterectomy. At day 2, samples from two ewes
were collected and at days 6, 14 and 30, samples from four ewes were
collected. Tissues were fixed for immunocytochemistry, cultured as
explants, and homogenized for RNA isolation. Western blots of uterine
flush, explant tissue and culture medium samples were developed with UTMP antiserum. RNA dot blots and Northern blots were probed with the radiolabeled 350 base pair insert. Samples from the uteri of cyclic ewes, early pregnant ewes, and pregnant cows were also analyzed for the presence of UTMP production.

Goals

In this way, an attempt was made to describe the biochemical structure and function of the UTMP, as well as the events surrounding their induction by P₄. The UTMP project was undertaken with the hope of understanding the mechanism underlying the large scale production of these secreted proteins in the ovine uterus during pregnancy, and the reason for their presence.
CHAPTER 2
THE UTMP ARE MEMBERS OF THE SERPIN SUPERFAMILY
OF SERINE PROTEASE INHIBITORS

Introduction

The secretions of the uterus that occur in response to a pregnancy or to long term progesterone administration have been named "uterine milk" or "histiotroph", and have been postulated to comprise a complex embryo culture medium because of their proposed role in nutrition of the developing conceptus (Roberts and Bazer, 1980). Specific functions of the individual proteins of uterine milk, however, are incompletely understood, but may include direct nutrition of the conceptus, transport of limiting nutrients to the embryo, osmotic support of the feto-placental unit, growth control, inhibition of proteolytic activities released by the embryo or mother, and immunosuppression of the local maternal immune system (reviewed in Roberts and Bazer, 1988). These proteins are assumed to have a role in the success of reproduction.

The proteins of uterine secretions have been examined qualitatively but with few exceptions are poorly characterized. The most extensively investigated protein secreted by the endometrium of a farm animal is uteroferrin, found in the pig. Uteroferrin is a purple protein with acid phosphatase activity and functions in iron transport to pig conceptuses (Buhi et al., 1982; Roberts and Bazer, 1988). Interestingly, uteroferrin, a secreted glycoprotein, carries the lysosomal enzyme marker, mannose-6-phosphate, in masked form (Baumbach et al., 1984). Though uteroferrin shows many characteristics of a
lysosomal enzyme, it is secreted in quantities that may exceed 1 g per day around mid-pregnancy (Basha et al., 1979). The primary structure of uteroferrin has been determined from a combination of amino acid sequencing (Hunt et al., 1987) and cDNA sequencing (Ketcham et al., 1988). By using complementary DNA (cDNA) probes, uteroferrin messenger RNA (mRNA) levels have been shown to be maximal from mid-pregnancy to term at 114 days (Simmen et al., 1988). Uteroferrin has also been identified in the uterus of the mare and cow, but not the ewe.

Uterine milk of the ewe contains primarily two progesterone-induced proteins, called "uterine milk proteins" (UTMP), and up to 15 g of the UTMP can be readily purified from one uterine horn of a sheep in late pregnancy when the fetus has been surgically restricted to the contralateral horn (Moffatt et al., 1987b). The UTMP are a pair of basic glycoproteins that are indistinguishable by peptide mapping but differ in mobilities (Mr = 57,000 and 55,000) on SDS-PAGE (Moffatt et al., 1987a). The UTMP are similar to uteroferrin in that they are uterine secretory proteins that are synthesized under the influence of progesterone (Moffatt et al., 1987b) and appear to possess the mannose-6-phosphate recognition marker (Hansen et al., 1987a). At present, no function has been found for the UTMP but the magnitude of their production in the ewe in response to progesterone may make them good models for the study of steroid hormone action in a higher mammal and suggests that they may have an important function in pregnancy. In the present study, we describe the biosynthesis and molecular cloning of the UTMP, and show that they belong to the serpin superfamily of serine protease inhibitors.
Experimental Procedures

Materials

High molecular weight standards for electrophoresis, protein-A Sepharose, RNase A, trypsin, alpha-chymotrypsin (type VII), pancreatic elastase (type III), and plasmin were obtained from Sigma (St. Louis, MO). Reticulocyte lysate, lambda gt10 and gt11 and host E. coli strains, ProtoBlot kit, T4 DNA ligase and EcoR I were purchased from Promega (Madison, WI). Alkaline phosphatase-linked goat anti-rabbit antibody, oligo dT primer, RNase H, EcoR I linkers, and lambda packaging extracts were obtained through Boehringer Mannheim (Indianapolis, IN). EcoRI methylase, S-adenosyl methionine, and DNA polymerase I were from New England Biolabs (Beverly, MA), reverse transcriptase from Seikagaku (St. Petersburg, FL), and oligo dT-cellulose and polyuridine from Pharmacia (Piscataway, NJ). All radioisotopes were purchased from New England Nuclear (Boston, MA). The M13 cloning and Sequenase kits were obtained through U.S. Biochemicals (Cleveland, OH). Kits for translation with canine pancreatic microsomes and nick translation were purchased from Amersham (Arlington Heights, IL). Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer at the University of Missouri Oligonucleotide Facility.

Purification of the UTMP and N-terminal Amino Acid Sequencing

The UTMP were purified by sequential cation exchange and gel filtration chromatography as previously described (Moffatt et al., 1987a). The UTMP preparation was judged free of contaminating proteins when analyzed by Coomassie blue staining of SDS-PAGE (Roberts et al., 1984a). For amino acid sequencing, the two forms of the UTMP were separated on SDS-PAGE and electroblotted onto a polyvinylidene
difluoride membrane (Matsudaira, 1987). A strip of the membrane was stained with Coomassie Blue to localize the exact positions of the UTMP. The adjacent individual bands were cut from the membrane and subjected to limited N-terminal sequencing with an Applied Biosystems gas phase sequencer (#470A) with an on-line PTH amino acid analyzer.

**RNA Isolation**

Endometrium was collected from uteri of ewes after hysterectomy at day 60 or 100 of gestation (Moffatt et al., 1987a). RNA was extracted from tissue homogenized in guanidine buffer by ethanol precipitation followed by cesium chloride density centrifugation (Chirgwin et al., 1979). This total cellular RNA was further purified twice on oligo dT-cellulose chromatography to generate poly(A)^+ RNA (Aviv and Leder, 1972).

**In Vitro Translation of the UTMP**

Total cellular RNA (3 µg) was translated in a rabbit reticulocyte lysate system with L-[³⁵S]-methionine (10 uCi) in 15 µl reaction mixtures (Pelham and Jackson, 1976). Positive and negative controls were brome mosaic virus RNA (0.25 µg) and no RNA, respectively. After translation for 1 hour at 30° C, products were analyzed by SDS-PAGE. Fluorographs were developed in contact with high speed intensifying screens (DuPont) on Kodak XAR film. Immunocomplexes were collected on protein A-Sepharose from duplicate samples (Velan, 1987) with immune or non-immune rabbit serum, in the presence or absence of 30 ng/ml of UTMP. Immunoprecipitated samples were then analyzed by SDS-PAGE and fluorography.

Poly(A)^+ RNA was translated in the presence of canine pancreatic microsomal membranes with the reticulocyte lysate assay according to the
manufacturer's protocol (1 hour; 37° C) in order to determine if processing events of translocation, signal peptide cleavage, and glycosylation of the UTMP peptide would occur. As a control, poly(A)+ RNA was translated in the absence of microsomes. Poly(A)+ RNA was translated with microsomes in the presence of 0.1% (v/v) Nikkol (Nikko Chemical Corp., Tokyo) to restrict processing events leading to glycosylation. To identify translocated proteins, membranes were stabilized after translation in 1.5 mM CaCl₂ and proteins not inside microsomes were degraded with 0.3 mg of each of trypsin and chymotrypsin (1 hour; 4° C). Controls in the presence of the detergents Nikkol or 1% (v/v) Triton X-100 were performed. Translated proteins were subjected to SDS-PAGE and fluorography.

A cDNA clone, pUTMP, containing the sequence from base 1111 to 1352 on Fig. 2-1, was used to select UTMP mRNA from total cellular RNA for translation (Velan, 1987). Plasmid DNA from the clone, prepared by the alkaline lysis procedure (Maniatis et al., 1982), was bound to nitrocellulose filters which were then incubated in a solution containing 300 ug of total cellular RNA and 5 ug/ml polyuridine. After filters were washed, RNA was eluted, extracted with 50:50 (v/v) phenol:chloroform, and precipitated with ethanol. It was resuspended in 3 ul water, translated in vitro, and products of translation subjected to analysis by SDS-PAGE with or without immunoprecipitation by immune or non-immune serum. Immunoprecipitation was performed on duplicate translation samples as previously described.

Construction and Screening of cDNA Libraries

Complementary DNA was synthesized from 1 ug of poly(A)+ RNA in presence of oligo dT primer, by use of reverse transcriptase and RNase H
(Polites and Marotti, 1986). The cDNA was methylated, ligated to radiolabeled EcoRI linkers, digested with EcoRI, and size selected on a Bio-Gel A-50 (Bio-Rad, Richmond, CA) column (Hyunh et al., 1985). After ligation to lambda gt11 arms, DNA was packaged and plated with E. coli strain Y1088 cells. Analysis of the cDNA library showed that 87.5% of plaque forming units (pfu's) were recombinants and that 3.9 x 10^5 recombinants had been generated from 1 ug of RNA. The library, after amplification in Y1088 cells, was screened in Y1090 cells. Immunodetection was performed with rabbit anti-UTMP serum at 1:250 (v/v) dilution with alkaline phosphatase-linked goat anti-rabbit serum and the ProtoBlot system of color development. On 1 ul dot blots of pure protein, 1 ng or less of UTMP could be specifically detected by using these methods. From about 500 pfu's, two positive clones, "U-1" and "U-2", were detected and were plaque purified with rescreening.

A second library was constructed in lambda gt10 with some modifications: the cDNA reactions were scaled up to start with 5 ug of poly(A)^+ RNA; second strand synthesis was made continuous with the first by the addition of RNase H and more reverse transcriptase in another volume of buffer; and blunt ending was done with DNA polymerase I. This cDNA was ligated to lambda gt10 arms, packaged, and plated with E. coli C600 hfl cells (Hyunh et al., 1985). The library contained 20,000 recombinants. The insert from the lambda gt10 library U-2 clone from the first library was radiolabeled by nick translation (Rigby et al., 1977) and 1000 recombinants from the lambda gt10 library were screened. Filters were baked at 80° C for 2 hours, then prehybridized in a buffer of 50% (v/v) formamide, 5X SSC, 5X Denhardt's solution (Maniatis et al., 1982), 10 mM sodium phosphate, pH 6.5 and 0.05% (w/v)
sodium dodecyl sulfate containing 400 ug/ml heat-denatured herring sperm DNA and 250 ug/ml polyuridine (4 hours; 42° C). The pUTMP probe was substituted in hybridization buffer for the herring sperm DNA in which the filters were incubated (overnight; 42° C). The filters were washed in hybridization buffer without DNA or RNA four times (10 minutes; 42° C). Autoradiographs were prepared with Kodak XRP film and enhancing screens (2 days; -70° C). Twelve positive clones were identified and purified.

Subcloning and DNA Sequencing

Lambda DNA from the gtll clones was prepared as plate lysates and purified by DEAE-cellulose chromatography, phenol extraction and ethanol precipitation (Reddy et al., 1988). DNA was digested with EcoRI and run on a horizontal 1% (w/v) agarose gel (Maniatis et al., 1982). The larger cDNA insert was obtained from U-2 and contained approximately 350 base pairs. It was purified by electroelution and subcloned into the EcoR I site of the plasmid pUC13 (Davis et al., 1986). The "pUTMP" plasmid DNA was prepared by the alkaline lysis procedure (Maniatis et al., 1982), and purified by preparative 1% (w/v) agarose horizontal gel electrophoresis, followed by extraction from the gel (Geneclean, Bioscience 101, La Jolla, CA). Plasmid was denatured with 0.2 M NaOH containing 2 mM trisodium EDTA. After the solution was neutralized, DNA was precipitated with ethanol. At resuspension, 30 ng of M13 reverse primer was added and allowed to anneal (1h; 37° C). DNA sequencing with dideoxynucleotide chain terminators (Sanger et al., 1977) was accomplished with modified T7 DNA polymerase (Sequenase) and [alpha-35S]-thio-dATP by following the Sequenase kit protocol.
Reactions were run on 6% (w/v) acrylamide sequencing gels. Urea was removed from the gels and gels were dried and exposed to Kodak XAR (1 day).

Inserts from positive clones of the lambda gt10 library were subcloned into the EcoR I sites of pUC13 and bacteriophage M13 mpl8 (M13 cloning kit protocol). Purified plasmid and M13 preparations of clones containing the U-5 cDNA insert (bases 95 to 1352 in Fig. 2-1) were sequenced with Sequenase, as described above, with vector- and insert-specific synthetic oligonucleotide as primers.

Epitope Selection

Epitope selection was performed by the method of Salzer et al. (1987) after separating components of clonal plaques of pUTMP on a preparative SDS-PAGE and electroblotting to beta-galactosidase fusion protein onto nitrocellulose (Saul and Yeganeh, 1986). A strip of the blot was developed with antiserum and ProtoBlot to identify the position of fusion protein migration. The area with the fusion protein was cut from the blot and incubated with rabbit antiserum (2 days; 4° C). The nitrocellulose strip was washed and antibody was eluted in 150 mM glycine, pH 2.3 and 200 mM NaCl (5 minutes; room temperature). This eluate was immediately neutralized with 1 M Tris, pH 9.0. Western blots from SDS-PAGE of UTMP and molecular weight markers were incubated with the selected antibody or with non-immune rabbit serum and color developed (Salzer et al., 1987).

RNA Sequencing

Since the sequences of the cDNA clones lacked the 5' end of UTMP mRNA (Fig. 2-4), RNA sequencing reactions were performed with 1 ug of poly(A)* RNA with reverse transcriptase, [alpha-35S]-thio-dATP,
dideoxynucleotide mixes (Earl et al., 1987) and a primer complementary to bases 159 to 180 of Fig. 2-1. RNA sequencing was also performed with a primer complementary to bases 1203 to 1223 (Fig. 2-1) to confirm the presence of repeated nucleotide sequences of 21 bases that were thought to have arisen as a cloning artifact. Gel and autoradiograph development were as for DNA sequencing.

**Sequence Analysis**

The Microgenie program (Beckman Instruments, Palo Alto, CA) was used to search for nucleotide and inferred amino acid sequences in the Genbank Genetic Sequence Data Bank (BBN Laboratories Inc., Cambridge, MA) and National Biomedical Research Foundation Protein Sequence Database (Georgetown University, Washington, D.C.), respectively, that were similar to those of the UTMP. The inferred amino acid sequence of the UTMP was aligned with individual sequences by using Microgenie programs and these were compiled by eye with the help of other serpin sequence alignments (Suzuki et al., 1987; Ye et al., 1987). Protein structure, amino acid analysis, and molecular mass calculations were also performed with Microgenie software.

**Protease Inhibitor Assay**

The UTMP were tested for their ability to inhibit proteolysis in the Azocoll protease assay (CalBiochem). Proteases tested were trypsin and chymotrypsin (40 ug in 20 ul), collagenase (CLS III, Worthington, 40 ug in 20 ul), elastase (4 ug in 2 ul), plasmin (80 ug in 20 ul), and thrombin (Parke-Davis, 400 ug in 20 ul). Amounts of individual proteases used were chosen in test reactions so that substrate digestion was similar in extent. Proteases were incubated with 10 ul of water, UTMP (10 mg/ml) or bovine serum albumin (10 mg/ml) for 5 minutes at 37°
C. After addition of 1 ml Azocoll solution reactions were incubated (15 minutes; 37° C) and the dyed peptides released from the substrate were measured at A_520 against substrate solution alone. Molar ratios of proteases to the UTMP ranged from approximately 6 for thrombin, 2 for plasmin, 1 for trypsin and chymotrypsin, and 0.1 for collagenase and elastase.

Results

N-terminal Amino Acid Sequence of Individual UTMP

N-terminal amino acid sequencing was performed on individual UTMP species to determine whether the two forms were dissimilar and to confirm amino acid sequence for analysis of DNA sequencing results. The first twenty-nine amino acid residues were identified in the N-terminus of both species of the 57,000 and 55,000 Mr forms of the UTMP separately. The sequences were identical to each other (Fig. 2-1) and differed from the inferred amino acid sequence at only two residues, Lys_{24} = Arg and His_{28} = Leu.

In Vitro Translation of the UTMP

In vitro translation of endometrial RNA from pregnant ewes produced two major proteins of Mr = 47,000 and 55,000 on fluorographs (Fig. 1, bands "a" and "b", respectively). Both were precipitated with immune serum in the presence of bovine serum albumin. With UTMP in the immunoprecipitation reaction, the protein band "a" was selectively reduced in intensity. The nonimmune rabbit serum control showed nonspecific precipitation of only small amounts of both "a" and "b" bands.

Hybrid selected RNA translated one predominant product at Mr = 47,000 (Fig. 2-2, band "a"). Because of the presence of a major rabbit
reticulocyte protein band seen with Coomassie Blue staining at the same gel position, the translation product appears to be split into two bands. A band of $M_r = 55,000$ was also present in immunoprecipitated material and as a minor component from hybrid select translation (Fig. 2-2, band "b"). However, immunoprecipitation in the presence of UTMP did not eliminate band "b", so it is possible that it precipitated nonspecifically. No bands were evident from immunoprecipitation with non-immune serum.

In the presence of microsomes, poly(A)$^+$ RNA from pregnant ewe endometrium translated three proteins of $M_r$'s 47,000, 55,000, and 57,000 (Fig. 2-3, bands "a", "b", and "c", respectively). The "c" band apparently increased in intensity as the "b" band diminished when microsomes were added to translation assays (Fig. 2). The "c" product was protected from digestion by trypsin and chymotrypsin by translocation into the microsomes. Trypsin and chymotrypsin completely degraded translated proteins in the presence of detergents. All three bands decreased in intensity in translation reactions with Nikkol, but "c" was most affected. Nikkol selectively disrupts the glycosylation function of microsomes (Walter and Blobel, 1983).

Cloning and Sequencing Strategy for the UTMP

The pUTMP clone, identified by immunoscreening the lambda gt11 library, contained a cDNA of 350 base pairs, as determined agarose gel electrophoresis. DNA sequencing showed that the insert contained 240 base pairs, in addition to a poly(A) tail of undetermined length. To prove that the clone contained sequences specific to the UTMP, epitope selection was performed with fusion protein and immune rabbit serum. Antibody selected by the fusion protein specifically bound the UTMP on a
Fig. 2-1. The nucleotide sequence derived for the UTMP mRNA and the inferred N-terminal and inferred amino acid sequences of the protein.

The nucleotide sequence appears above the inferred amino acid sequence of the UTMP precursor. Nucleotide sequence is numbered above, with negative numbers preceding the first in-frame start codon. The amino acids encoded in the open reading frame are indicated in the single letter code under the center of their codons. N-terminal amino acid sequence agreed with the inferred amino acid sequence in region marked by bold underlining. The N-terminal sequence did not agree with the inferred sequence at two positions (which are indicated by amino acid residues under the inferred ones). Amino acids are numbered to the right of the sequence, beginning at the start of the N-terminal sequence. The two AUG start codons, the UAG stop codon and the AAUAAA polyadenylation signal are lightly underlined. Potential N-linked glycosylation sites (N-X-S/T) are indicated with an asterisk. Two repeats of 21 bases, encoding repeats of seven amino acids, are indicated by a broken line above the sequence.
Fig. 2-2. In vitro translation of the mRNA from pregnant sheep endometrium in a rabbit reticulocyte lysate system.

This figure shows fluorographs of the 7.5% (w/v) SDS-PAGE gels used to analyze the cell free translation products. Proteins in lanes 1 through 4 were translated from total cellular RNA, while those in lanes 5 through 8 were from hybrid selected RNA. Reactions were subjected to immunoprecipitation with immune serum and bovine serum albumin (lanes 2 and 6), nonimmune serum and bovine serum albumin (lanes 3 and 7), and immune serum and UTMP (lanes 4 and 8). Distances of migration of molecular weight standards on the gel are indicated at left.
Fig. 2-3. In vitro translation of the UTMP in the presence of microsomal membranes.

Poly(A)$^+$ RNA from pregnant ovine endometrium was used to translate proteins in vitro as in Fig. 2-2. Negative (-) and positive (+) controls are shown. Lane 1 shows products of translation without microsomes. Microsomes are included in the translation reactions shown in lanes 2 through 6. Products in lanes 3 and 4 were translated in the presence of Nikkol. Protease treatment was performed after translation in reactions in lanes 4 through 6, and the proteolysis of the last reaction was carried out in the presence of Triton X-100. A key to reaction components, below the lanes, uses abbreviations: microsomes, M; Nikkol, N; proteases, P; and Triton-X 100, T.
Western blot (results not shown). Normal rabbit serum controls bound several proteins, but not the UTMP.

The lambda gt10 cDNA library, screened with the radiolabeled cDNA insert from pUTMP, yielded a clone with an almost full-length cDNA for the UTMP of 1260 base pairs, "U-5" (Fig. 2-4). The U-5 coding strand was successfully subcloned into M13 mp18 and sequenced. The U-5 insert was also subcloned into pUC13 and the anticoding strand was sequenced. The U-5 clone was sequenced in both directions except for unidirectional sequencing of 23 bases at the 3' end (Fig. 2-4). The U-5 sequence contained the entire pUTMP sequence and therefore the entire 3' end of the mRNA. The pUTMP sequence differed from the U-5 sequence at the pUTMP 5' end (nucleotide 1111 was C instead of G), one in the middle (nucleotide 1197 was G instead of T), and at the 3' end (ACC was present following nucleotide 1352 and just prior to the poly(A) tail). Only the first change affected the inferred amino acid sequence.

The 5' end of the U-5 cDNA terminated within the coding region at a position corresponding to the seventh amino acid from the N-terminus of the UTMP. To obtain complete sequence of the 5' end of the UTMP mRNA, dideoxy sequencing of poly(A)+ RNA was performed. This allowed the sequence from base 159 to -48 to be determined (Fig. 2-1). The sequence conflicted with the U-5 sequence at three bases at the 5' end of the latter. The amino acids inferred from the mRNA sequence at conflict points with U-5 sequence agree with N-terminal amino acid sequence results, and the nucleotide and inferred amino acid sequences at these points reported in Fig. 2-1 are from the mRNA. The mRNA sequence stopped abruptly at base -48 of the sequence reported. The nucleotide sequence determined for the UTMP mRNA contained exactly 1400 bases with
Fig. 2-4. Sequencing strategy for the UTMP mRNA.

The sequences of two cDNA clones, pUTMP and U-5, were merged together with oligonucleotide-primed sequence of UTMP mRNA to determine the complete nucleotide sequence reported in Fig. 2-1. In it, the bold line indicates protein-coding region and the thin line, noncoding regions. (E)'s denote EcoR I linker sites used in cloning. Horizontal arrows below the sequence cartoon denote DNA sequencing (______) and mRNA sequencing (_ . _ . _). Sequencing reactions were primed with specific synthetic oligonucleotide primers (closed circles) or with vector specific primers (vertical bars). A scale in bases is below.
The polyadenylation signal AAUAAA at bases 1334 to 1339 was identified in both pUTMP and U-5 clones. Two in-frame start codons were identified, making the putative signal sequence either 25 or 20 amino acids long. A repeated sequence of 21 bases, resulting in a repeat of seven amino acids, was found in U-5 and was confirmed by RNA sequencing (Fig. 2-1). The proposed sequence for the UTMP mRNA was determined bidirectionally for 88% of the bases.

Analysis of the Inferred Amino Acid Sequence for the UTMP

The nucleotide sequence for the UTMP mRNA sequence encodes an open reading frame for 429 amino acids from Met_25 to Glu_404 (Fig. 2-1). The amino acid composition of the inferred sequence for UTMP (Glu_1 to Glu_404) agrees with that determined empirically for the UTMP (Table 1). The composition study from direct analysis predicted a larger protein (m = 50,887 Da) than the inferred sequence did (m = 46,339 Da), but was based on the assumption that the UTMP contain two Tyr residues while the inferred sequence clearly demonstrates only one. If carbohydrate accounts for 5.6% of the UTMP by weight as reported (Hansen et al., 1987a), the glycosylated protein was predicted to have m = 49,088 Da, which compares to m = 53,700 Da predicted previously. The molecular masses predicted by Microgenie for the UTMP precursors, starting at Met_25 or Met_20 are 49,222 and 48,554 Da, respectively, and are consistent with the sizes of the unprocessed UTMP peptides from in vitro translation of M_r = 47,000 and 55,000. Two possible sites for N-linked glycosylation of the UTMP were identified in the inferred amino acid sequence at Asn_197 and Asn_243 (Fig. 2-1).
Table 1. Amino acid compositions inferred from UTMP nucleotide sequence and determined by direct analysis.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Inferred Sequence</th>
<th>Direct Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>27 (6.7)</td>
<td>31 (6.9)</td>
</tr>
<tr>
<td>Arg</td>
<td>10 (2.5)</td>
<td>13 (2.9)</td>
</tr>
<tr>
<td>Asx</td>
<td>39 (9.7)</td>
<td>42 (9.4)</td>
</tr>
<tr>
<td>Half-Cys</td>
<td>2 (0.5)</td>
<td>4 (1.0)</td>
</tr>
<tr>
<td>Glx</td>
<td>47 (11.6)</td>
<td>49 (11.0)</td>
</tr>
<tr>
<td>Gly</td>
<td>6 (1.5)</td>
<td>8 (1.9)</td>
</tr>
<tr>
<td>His</td>
<td>22 (5.4)</td>
<td>24 (5.5)</td>
</tr>
<tr>
<td>Ile</td>
<td>25 (6.2)</td>
<td>25 (5.6)</td>
</tr>
<tr>
<td>Leu</td>
<td>59 (14.6)</td>
<td>68 (15.3)</td>
</tr>
<tr>
<td>Lys</td>
<td>45 (11.1)</td>
<td>44 (10.0)</td>
</tr>
<tr>
<td>Met</td>
<td>14 (3.5)</td>
<td>16 (3.5)</td>
</tr>
<tr>
<td>Phe</td>
<td>22 (5.4)</td>
<td>25 (5.7)</td>
</tr>
<tr>
<td>Pro</td>
<td>18 (4.5)</td>
<td>19 (4.3)</td>
</tr>
<tr>
<td>Ser</td>
<td>17 (4.2)</td>
<td>14 (3.1)</td>
</tr>
<tr>
<td>Thr</td>
<td>29 (7.2)</td>
<td>37 (8.4)</td>
</tr>
<tr>
<td>Trp</td>
<td>0 (0.0)</td>
<td>0 (0.1)</td>
</tr>
<tr>
<td>Tyr</td>
<td>1 (0.2)</td>
<td>2 (0.5)</td>
</tr>
<tr>
<td>Val</td>
<td>21 (5.2)</td>
<td>23 (5.1)</td>
</tr>
</tbody>
</table>

\[ m \] 46,339 Da 50,887 Da

Values presented are residues per molecule, followed by molar percents.

Molecular masses (m) were calculated from residues per molecule.

\[ a \] Deduced from the nucleotide sequence from cDNA and mRNA.

\[ b \] Data from Hansen et al. (1987)
Alignment of Amino Acid Sequences of the UTMP with Those of Serpins.

The amino acid sequences of the serpins alpha-1 antichymotrypsin, alpha-1 antitrypsin and ovalbumin were identified as being similar to the inferred amino acid sequence of the UTMP by Microgenie analysis. Nucleotide sequence similarity to that for the UTMP was extensive for the above and also for protein C inhibitor, another member of the serpin superfamily. The amino acid sequences of these serpins and antithrombin III were aligned to the UTMP sequence (Fig. 2-5). Predicted secondary protein structure of the serpins determined by x-ray crystallography of alpha-1 antitrypsin (Loebermann et al., 1984), indicated below the sequences in Fig. 2-5, agrees well with that shown above from predictions of the Microgenie program for the UTMP sequence using the algorithm of Carnier et al. (1978). One of the repeats of seven amino acids, from Lys304 to Ile310, is not aligned to residues in any of the other sequences. Regions with the fewest matching residues appear at the extreme N-terminus and at a C-terminal region aligning with the reactive centers of the serpins.

Protease Inhibition Assays

The UTMP had no significant inhibitory activity on the activities of the proteases tested when compared to proteolysis without UTMP or in the presence of an equivalent amount of bovine serum albumin.

Discussion

The two mature secreted forms of the UTMP are distinguishable only by SDS-PAGE ($M_r = 57,000$ and $55,000$). Previous amino acid sequence analysis of UTMP preparations containing both forms yielded single
Fig. 2-5. Alignment of UTMP amino acid sequence with those of selected serpins.

The inferred sequence of ovine UTMP (o-UTMP) precursor was aligned to similar sequences of other members of the serpin superfamily. The o-UTMP sequence is numbered above from the N-terminus of the mature protein (Fig. 2-1). Human alpha-1 antichymotrypsin (h-alACT), baboon antitrypsin (b-alAT), chicken ovalubmin (c-Ovalb), and human antithrombin III (ATIII) sequences were obtained from the National Biomedical Research Foundation Protein Sequence Database. The sequence for human protein C inhibitor (h-PCI) is from Suzuki et al. (1987). Dashes indicate open spacing to optimize similarity in alignments. Lower case letters denote amino acids of signal sequences that are cleaved prior to protein maturation. The slash before the ATIII sequence indicates that some of its N-terminal amino acids are not shown. Residues of o-UTMP are enclosed in boxes with identical ones in other sequences at the same position. Secondary protein structures predicted from o-UTMP are indicated above the sequence, while the structures from x-ray diffraction analysis of alpha-1 antitrypsin (Loebermann et al., 1984) are shown below (alpha-helix = solid line, beta-sheet = broken line, reactive site = arrow).
signals at each cycle, so that the two were assumed to have common N-terminal sequence (Hansen et al., 1987a) and to be closely related molecules. However, the possibility existed that one of the proteins had a blocked end. Analysis of the N-terminal amino acid sequences of the individual UTMP forms, presented here, generated identical results to Hansen et al. (1987a) and prove that the two do indeed share common N-terminal structure. This sequence also matches the amino acid sequence deduced from the UTMP cDNA in all but two positions, thus confirming that the nucleotide sequence presented in Fig. 2-1 represents the UTMP through their N-terminal regions. This N-terminal sequence also matches the previous sequence determinations of 20 amino acids from mixed preparations of UTMP species except that Ala$_{27}$ (Fig. 2-1) was a Ser.

Since the two forms of the UTMP appeared to have similar peptide structure by N-terminal amino acid sequence and peptide mapping analyses (Hansen et al., 1987a), one mRNA and one peptide precursor were predicted for the UTMP. The UTMP appeared to have a single mRNA precursor of approximately 1600 bases when RNA was analyzed on Northern blots and compared to double-stranded DNA standards (Ing and Roberts, manuscript in preparation). In vitro translation of total cellular RNA from pregnant endometrium and of RNA that hybridized to pUTMP DNA, however, produced two proteins with relative $M_r$s of 55,000 and 47,000 that precipitated with antiserum to the UTMP. The nucleotide sequence determined for the UTMP contains two strong start signals, a rare feature in eukaryotic mRNAs (Shaper et al., 1988). Consensus sequences flanking start codons, identified by mutagenesis and surveys of known sequences indicate that a purine, three nucleotides upstream of the A of
the AUG codon, is the most important feature of a functional start codon (Kozak, 1986). Both potential start codons of the UTMP sequence (at bases 1 to 3 and 16 to 18 in Fig. 2-1), have purines at that position (G at base -3 and A at base 13). Both start sites would give rise to signal sequences that follow a general structure of basic N-terminal, hydrophobic central, and more polar C-terminal regions, that are of average signal sequence size (25 and 20 amino acids long), and that would be predicted to be cleaved at the same Cys$_{-1}$-Glu$_{1}$ site (von Heijne, 1986). The predicted peptide precursors of the UTMP from the nucleotide sequence were of $m = 49,222$ and 48,554 Da which would not be distinguishable by SDS-PAGE in the area of the product from in vitro translation. Therefore it is not possible to judge which of the two start sites is the one preferred for initiation of translation. Amino acid composition of the inferred sequence for the mature UTMP (Glu$_{1}$ to Glu$_{404}$ in Fig. 2-1) matches the experimental determinations (Table 1). Since the number of residues predicted from the latter were based on the assumption that the UTMP contained two Tyr residues and the inferred sequence has only one, slight disagreements in the comparison of amino acid analyses were expected.

Since no differences in peptide structure have been found that account for the two mature forms of the UTMP, the post-translational modification of the UTMP was studied to determine if differential processing of the precursor could be responsible for the size heterogeneity. The apparent $M_r$ of the mature UTMP were determined by SDS-PAGE (55,000 and 57,000) and equilibrium sedimentation analyses ($50,700 = \pm 4,800$; Moffatt et al., 1987a). We believe that three translated proteins, $M_r = 47,000$, 55,000 and 57,000, are related to the
UTMP. The $M_r = 47,000$ form, the major species translated by hybrid select translation and competed from immunoprecipitation by excess UTMP, may (1) contain the entire peptide precursor structure of the UTMP ($m = 49,222$ and $48,554$ Da depending upon the start site for translation), or (2) be the larger portion of the two forms created by a cleavage in the serpin loop structure ($m = 41,854$ Da, discussed later), or (3) be a product of premature termination of translation. The $M_r = 55,000$ translation product, present as a minor component in immunoprecipitations of products of total cellular RNA and hybrid select RNA translations, could be the form that carries the entire amino acid sequence of the UTMP precursor. With microsomes present in the assay, endometrial RNA translated considerably more of the product of $M_r = 57,000$ that was cotranslationally translocated to the interior of the microsomes and glycosylated. This protein band was of the same intensity as a band of $M_r = 55,000$ from translation without microsomes. It appeared, therefore, that the $M_r = 57,000$ protein may be a processed form of the $M_r = 55,000$ species. The $57,000$ form may differ from the $M_r = 55,000$ species by the addition of one carbohydrate chain (approximate $M_r$ change of 2000), or by the addition of two such chains and the loss of a signal sequence.

The results presented here are consistent with the presence of two possible glycosylation sites in the amino acid sequence inferred from the UTMP cDNAs and the analysis of Hansen et al. (1987a), which demonstrated the presence of one or two N-linked oligosaccharide chains of similar complexity on each secreted UTMP species. Uteroferrin of the pig is similar to the UTMP is many respects and is synthesized as a $M_r = 37,000$ form containing two carbohydrate chains. This newly
synthesized form is subsequently processed to a \( M_r = 35,000 \) form, by the removal of one carbohydrate chain, which is the form of uterofermin found in the uterus. The UTMP may be similarly processed, although less completely to yield the two forms found in utero. We conclude, as did Hansen et al. (1987a), that the secreted forms of the UTMP differ by possessing either one or two oligosaccharide chains.

In an additional post-translational processing event, mannose residues of the oligosaccharide chains of the UTMP become phosphorylated (Hansen et al., 1987a). Phosphorylation by the UDP-GlcNAc:glycoprotein N-acetylglucosamine 1-phosphotransferase reaction occurs in the Golgi and depends on structural determinants present on the polypeptide (Lang et al., 1984). The resultant mannose-6-phosphate residues are thought to be specific recognition signals for the mannose-6-phosphate receptor which is believed to direct hydrolases to lysosomes. However, molecules without known enzyme activities, such as thyroglobulin (Couso et al., 1986) and the UTMP also have been shown to carry them. Uteroferrin is similar to the UTMP in that it too is a glycoprotein secreted by the uterus in response to progesterone and possesses mannose-6-phosphate residues (Baumbach et al., 1984). In the case of uterofermin, it is known that the mannose-6-phosphate is masked by a covering GlcNAc residue. Whether or not the mannose-6-phosphate residues of the UTMP are masked has not been determined. It is interesting that the UTMP appear to be phosphorylated by UDP-GlcNAc:glycoprotein N-acetylglucosamine 1-phosphotransferase because (1) they are structurally related to ovalbumin, which is not a substrate for the enzyme (Lang et al., 1984), and (2) they have no identified activity as an acid hydrolase.
The primary sequence of the UTMP appears to place them in the serpin superfamily. Their primary sequence is most similar to sequences of baboon (and human) alpha-1 antitrypsin and human alpha-1 antichymotrypsin, with 31% and 27% positions with identical residues, respectively in the alignments as shown in Fig. 2-5. Among the regions of least similarity is the one that surrounding the residue Ala\textsubscript{365} which aligns with the reactive site of the serpins, characteristically one of the least conserved regions within the serpin superfamily (Hill et al., 1984). The predicted secondary structure predicted by the algorithm of Garnier (1978) for the UTMP sequence closely fits that determined by crystal structure studies of alpha-1 antitrypsin, even though the latter was performed on the serpin in its relaxed form after proteolysis at its reactive site (Loebermann et al., 1984). The potential site of UTMP glycosylation at Asn\textsubscript{243} (Fig. 2-5) aligns well with Asn\textsubscript{247} of human alpha-1 antitrypsin which carries one of the latter's three oligosaccharide chains (Kurachi et al., 1981).

It seems likely that the UTMP fold in the stressed conformation common to the serpins. Such structures carry an exposed loop of polypeptide that acts as a substrate cleavage site for the protease they inhibit (Carrell and Boswell, 1986). Upon cleavage of the reactive center on the loop, alpha-1 antitrypsin structure changes to a more stable, relaxed form with former neighboring residues, Met\textsubscript{358} and Ser\textsubscript{359}, now on opposite sides of the molecule. When this occurs, protease inhibitory activity is lost. Ovalbumin, a serpin produced by the reproductive tract of the chicken in response to estrogen, has no known protease inhibitory activity, but is cleaved by proteases at a site within the loop structure to generate a more stable derivative
called "plakalbumin" (Carrell, 1985). Angiotensinogen, another serpin that is not a known protease inhibitor, produces the vasoactive substances called angiotensins upon proteolytic cleavage of its reactive site. Cleavage of the UTMP at Ala\textsubscript{365}, a site that aligns with the reactive center of the other serpins, would produce peptides of molecular masses of 41,854 Da (365 amino acids) and 4,503 Da (39 amino acids) that would dissociate during SDS-PAGE. Although such a cleavage probably does not account for the two forms (M\textsubscript{r} = 55,000 and 57,000) of the UTMP, glycoproteins with apparent M\textsubscript{r} = 49,000 and 47,000 can be detected in varying amounts in most some uterine milk samples and increase in quantity with sample aging; these lower M\textsubscript{r} forms are thought to be the result of proteolytic action on the UTMP (Moffatt et al., 1987b).

In addition to structural similarities, the biosynthesis of the UTMP and the serpins may share common mechanisms of controlling their formation. The serpins alpha-1 antitrypsin, alpha-1 antichymotrypsin, and antithrombin III comprise 10\% (w/w) of total plasma protein and control cascades involved in blood coagulation, inflammation and immune reactions by their inhibition of proteolysis (Kidd and Woo, 1986). In the woman, alpha-1 antitrypsin production is also believed to increase with steroid hormone influence (Legge et al., 1984) and is a constituent of uterine fluid, although the uterus has not been determined to be its source (Fazleabas et al., 1987). The serpins alpha-1 antitrypsin and alpha-1 antichymotrypsin are said to be acute phase reactants, whose levels increase several fold in human serum during systemic stress states, such as inflammation and trauma (Heidtmann and Travis, 1986). During human pregnancy, alpha-1 antitrypsin serum levels rise, and have
been postulated to help control the invasiveness of trophoblast cells at aberrant sites, such as kidney and lung (Legge et al., 1984). The UTMP are the major protein components of the uterine fluid of a pregnant ewe, where they are present at concentrations greater than 10 mg/ml, and have been identified as minor components of maternal serum of the pregnant sheep (Newton et al., 1988). The UTMP may also be acute phase proteins because of the apparent induction of their synthesis by trauma to nonpregnant ovine endometrium prior to explant culture (Ing and Roberts, manuscript in preparation).

No specific function within the uterus during pregnancy has been determined for either the UTMP in the sheep or alpha-1 antitrypsin in the human. We have been unable to find a protease that was inhibited by the UTMP. This lack of activity may be the result of differences in single amino acids around the serpin reactive center or of the sequence duplication of 21 bases, resulting in an "extra" segment of seven amino acids, identical to a sequence only two residues away, that does not align with sequences of other serpins. Consequently, the UTMP may serve a passive role in reproduction such as that of a protein substrate for offspring nutrition. Ovalbumin, a serpin lacking activity as a protease inhibitor, appears to have such a function in the eggs of birds (Carrell and Boswell, 1986).

We conclude that the UTMP share many similar features in structure and control of biosynthesis with members of the serpin superfamily, while possessing several some unique characteristics of their own that may assist us in our understanding of the evolution of the UTMP and their role in reproduction of the sheep.
CHAPTER 3
PROGESTERONE INDUCTION OF THE UTPM

Introduction

Female sex steroids act on uterine endometrium to induce the secretion of proteins from the epithelium into the uterine lumen (for reviews see Beier, 1980; Roberts et al., 1987). Many functions have been ascribed to the secreted proteins, including those involving fetal nutrition, enzyme action and inhibition, and immunomodulation within the uterus (Roberts and Bazer, 1988). The secretions of the uterus, believed to be important in nurturing embryos, have been called "uterine milk" or "histiotroph" (Roberts and Bazer, 1980). Prior to implantation, uterine secretions provide the total environment bathing the embryo. The survival of the embryo depends upon its synchrony with the uterus as demonstrated by the poor success of pregnancy with transfer of asynchronous embryos (Surani and Fishel, 1980; Roberts et al., 1984b). Understanding the induction of secreted proteins of the uterus by steroid hormones may help determine the sequence of events in the uterus necessary to provide a successful pregnancy.

Few secreted uterine proteins have been investigated intensively. One exception is uteroferrin, the major progesterone-induced protein of porcine endometrium (Roberts and Bazer, 1980). Uteroferrin is a purple glycoprotein that contains iron and has acid phosphatase activity. Induction of uteroferrin is facilitated by the synergistic action of estrogen with progesterone in the ovariectomized gilt, and rates of
uteroferrin synthesis may exceed two grams per day at around midpregnancy when production is maximal (Roberts and Bazer, 1980). The amino acid sequence for uteroferrin has been determined by amino acid sequencing (Hunt et al., 1987) and sequencing of cDNA clones (Simmen et al., 1988a) and belongs to a large class of tartrate-resistant acid phosphatases (Ketcham et al., 1988) that are generally thought to reside in lysosomes. In addition, uteroferrin resembles most known lysosomal enzymes by carrying the mannose-6-phosphate marker residue when newly synthesized (Baumbach et al., 1984) which is believed to target acid hydrolases to the lysosome by binding to the so-called mannose-6-phosphate receptor. However, uteroferrin's mannose-6-phosphate is masked by a covering residue, which may account for its secretion from the uterus under the influence of progesterone.

The sheep uterus under progesterone influence secretes two major proteins called the UTMP (Moffatt et al., 1987a). The UTMP can be purified in quantity since amounts in excess of 15 g are present in the fluid of the uterine horn of unilaterally pregnant ewes at day 140 of gestation. In this unilaterally pregnant model, the fetus is surgically restricted to only one horn, allowing fluid to accumulate in the contralateral horn (Moffatt et al., 1987b). The production and secretion of uterine proteins in ovariectomized ewes is maximal when high levels of progesterone are given exogenously for long periods of time (Moffatt et al., 1987b). Previous studies have not identified the UTMP before 20 days of pregnancy or progesterone therapy nor have the proteins been found in any other species. The UTMP are a pair of basic glycoproteins that have been distinguished from each other only by using polyacrylamide gel electrophoresis in the presence of sodium dodecyl
sulfate (SDS-PAGE). They have closely similar $M_r$'s of 57,000 and 55,000 and seemingly identical pI's (Moffatt et al., 1987a). The UTMP have been shown to be closely related to each other in N-terminal primary sequence and peptide mapping, and in the size of their carbohydrate chains. They both carry the lysosomal enzyme marker, mannose-6-phosphate (Hansen et al., 1987a). Although the function of the UTMP remains to be elucidated, the large amounts produced following long term hormonal induction makes them useful models for study of steroid action.

The following study was carried out to describe the early progesterone induction of the UTMP in the uteri of ovariectomized sheep under steroid replacement therapy and also the time course in appearance of the proteins in of intact sheep during the estrous cycle and pregnancy. Additionally, tissues of the sheep at midpregnancy and uteri from the cow and gilt were analyzed for the presence of UTMP mRNA to determine the tissue specificity of UTMP production in response to progesterone.

**Experimental Procedures**

**Materials**

Standard laboratory chemicals, progesterone, amino acids, unstained molecular weight standards, and protein A-Sepharose were purchased from Sigma Chemicals (St. Louis, MO). Prestained $M_r$ standards were from Diversified Biotech (Newton Centre, MA). Medical grade Silastic 382 Elastomer was obtained from Dow Corning (Midland, MI). Polyuridine and cyanogen bromide-activated Sepharose were from Pharmacia (Piscataway, NJ). Antibiotic-antimycotic solution was purchased from Grand Island Biologicals, (Grand Island, NY) and globin mRNA was from Bethesda Research Laboratories (Gaithersburg, MD). Kits were employed for
immunostaining tissue (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) and blots (Protoblot kit, Promega, Madison, WI), and for labelling DNA by nick translation (Amersham, Arlington Heights, IL). Herring sperm DNA and goat-antirabbit antibody linked to alkaline phosphatase were purchased from Boehringer Mannheim (Indianapolis, IN). XAR and XRP films were purchased from Eastman Kodak (Rochester, NY). Radiochemicals were obtained from New England Nuclear (Boston, MA) except L-[2,3,4,5-3H(N)]-leucine (110 Ci/mmol) was from ICN (Irvine, CA) which was also the source of BIOTRANS nylon membranes. The actin cDNA clone was the generous gift of Dr. L. Kedes.

Animals and Sample Collection

Eighteen crossbred ewes were ovariectomized at least four months prior to the start of progesterone treatment (day 0). Ten ewes were given 5 μg of E2 in sesame oil in a subcutaneous injection on day -4. On day 0, silastic implants containing a total of 1.6 g progesterone were placed subcutaneously in all but four ewes (two E2-primed and two untreated animals). These four ewes underwent blood sampling from the jugular vein, prior to induction of general anesthesia and midventral laparotomy. After flushing their uteri (Moffatt et al., 1987a) with 30 ml of Earle's minimal essential medium, the four animals were hysterectomized. Segments of uterine horns were fixed in Bouin's fixative for immunocytochemistry. Endometrium was harvested for explant cultures and RNA isolation (Basha et al., 1980b). Groups of four ovariectomized ewes given P4 implants (two E2-primed and two unprimed) were subjected to identical sampling on days 6, 14 and 30 of
progesterone therapy. A group of two $E_2$-primed ewes was sampled on day 2 of the experiment. Progesterone implants were removed on the day of surgery.

Intact crossbred ewes were checked daily for estrus with vasectomized rams. Cyclic ewes underwent surgical uterine flushing and hysterectomy with collection of endometrium on day 12, 14 or 16 of the estrous cycle (one ewe per day). Similar samples were obtained on days 12, 14, 16, 18, 20 or 22 of pregnancy from single ewes that had been bred by fertile rams, with presence of embryos in the uterine flush as the criterion for pregnancy. Three ewes were unilaterally ovariectomized and surgically ligated across their ipsilateral uterine horn (Bazer et al., 1979) were bred by fertile rams. Samples were collected separately for each uterine horn from one unilaterally pregnant ewe at day 14, 16 or 18 of gestation.

A ewe at day 100 of gestation was hysterectomized and a liver biopsy was taken. The endometrium, separated into caruncular and intercaruncular tissues, and liver tissue were used for RNA isolation. A bovine uterus was collected at slaughter at day 18 of gestation and another at day 45. Porcine endometrium was harvested at surgery from a gilt at day 47 post-estrus that had been rendered pseudopregnant by estradiol administration at days 11-15 of her cycle (Basha et al., 1980a). All animal work was done with strict adherence to NIH guidelines for animal care.

Antiserum Preparation

A New Zealand White rabbit was immunized with 100 ug purified UTMP in complete Freund's adjuvant. Blood was collected from a marginal ear vein about six days after each monthly boost of 100 ug of UTMP in
saline. After clotting, blood was centrifuged (2000 X g, 10 minutes). Serum was harvested and stored in aliquots at -20° C until use.

**Progesterone RIA**

Progesterone concentration was determined in plasma from jugular vein blood samples in a validated radioimmunoassay (Cantley et al., 1975) with an $^3$H-progesterone standard.

**Immunocytochemistry**

Segments of uterus fixed in Bouin's solution were embedded in paraffin, sectioned and mounted on poly L-lysine coated slides. Tissue sections were stained by using the avidin-biotin-immunoperoxidase complex method (Hsu et al., 1981). Adjacent sections were developed with 1:400 (v/v) dilution of immune rabbit serum, with similarly dilute normal rabbit serum, or with dilute antiserum cleared of UTMP-specific antibodies by two passages through an 8 ml column of UTMP-Sepharose (made from cyanogen bromide-activated Sepharose and UTMP following Pharmacia's protocol). Biotinylated goat anti-rabbit immunoglobulin G (ABC Vectastain kit) was used as the second antibody. The peroxidase was developed by using the chromogen 3,3'-diaminobenzidine tetra-chloride. Control sections were stained with hematoxylin and eosin to assess maintenance of tissue morphology after fixation. Positive immunostaining, noted as brown color, was graded on a 0 to ++++ scale, with 0 denoting background intensity of reaction and ++++ denoting the heaviest immunoreactivity and involvement of glandular epithelium. Representative sections from day 6 and day 30 endometrial samples were photographed.
Explant Cultures and SDS-PAGE

Pieces of endometrium (8 mm³) with a total weight of 0.5 g were cultured in 10 ml of Earle's modified minimal essential medium containing 4 mg/ml glucose, 2 U/ml insulin, essential and nonessential amino acids except leucine and antibiotic-antimycotic, and 100 uCi [³H]-leucine under an atmosphere of 50% N₂, 45% O₂ and 5% CO₂ on a rocking platform (24 hours, 37° C). Cultures were centrifuged at 2,000 X g (10 minutes, 4° C) to separate tissue and debris from the medium. Tissue (0.1 g) was homogenized in 1 ml of immunoprecipitation buffer (0.3 M NaCl, 50 mM Tris acetate, pH 7.5, 0.5% (v/v) Nonidet P-40, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.02% (w/v) sodium azide) with four 15 second bursts at 40% full power from a microprobe of a sonicator. Medium samples were dialyzed against water. To identify proteins synthesized in culture, samples of tissue homogenate and dialyzed media (75 ul) were subjected to 7.5% (w/v) SDS-PAGE (Roberts et al., 1984a). Gels were stained with Coomassie Blue and then successively soaked 30 minutes in water and 30 minutes in Enhance (New England Nuclear) and dried. Gels were subjected to fluorography (4 days, -70° C) in the presence of enhancing screens and XAR film.

Immunoprecipitation was performed on dialyzed samples of media and solubilized tissue samples to identify the UTMP. Samples (500 ul) were combined with 5 ul antiserum, nonimmune serum, or antiserum plus 5 ul of 10 mg/ml UTMP and incubated at 4° C overnight. After the addition of 100 ul of 10% (v/v) protein A-Sepharose, samples were incubated at room temperature for 4 hours. Sepharose was pelleted by a brief spin in a microcetrifuge and washed 3 times with 1 ml volumes of wash buffer (0.3 M NaCl, 50 mM Tris/acetate, pH 7.5, 0.5% (v/v) Nonidet P-40,
0.1% (w/v) SDS and 0.02% (w/v) sodium azide. Immunoprecipitated proteins were eluted from the gel beads by boiling in 30 ul of electrophoresis buffer and then subjected to SDS-PAGE and fluorography (Roberts et al., 1984).

**Western Blots**

Uterine flushes were centrifuged at 2,000 X g (10 minutes, 4° C) to remove cellular debris. Uterine flush and medium samples were dialyzed against water. To allow analyzed samples to be prepared with equivalent protein content, the total amounts of protein in uterine flush, and homogenized tissue and medium from explant cultures were analyzed by the method of Lowry (1951). A selected volume was lyophilized and resuspended in water. Protein samples (100 ug) were separated by SDS-PAGE and electroblotted onto nitrocellulose filters. Western blots were developed by using rabbit antiserum to the UTMP, diluted 1:1000 (v/v). Bound antibodies were detected with a goat anti-rabbit antibody linked to alkaline phosphatase, diluted 1:3000 (v/v), and the ProtoBlot kit. Prestained standards and samples of purified UTMP (200 ng; Moffatt et al., 1987a) were included on each gel to determine the success of SDS-PAGE and electroblotting and to identify the extent of UTMP migration and immunostaining. Blots were scored 0 to ++++ for the presence and intensity of bands in the UTMP region, with 0 denoting absence of immunostaining and ++++ indicating that UTMP reaction was maximal. The lowest level of UTMP detection, tested on nitrocellulose dot blots of purified UTMP developed with the immunostaining system, was 1 ng in 1 ul samples.
RNA Isolation

RNA was isolated from endometrial tissue of the ewes, cow, and pig, and from ovine liver according to the method of Chirgwin et al. (1979). After ethanol precipitation, RNA was dissolved in water and its concentration was determined by measuring the absorbance of the solution at 260 nm. RNA was reprecipitated with ethanol and dissolved in water so that samples were of equal concentration.

RNA Dot and Northern Blots

A dot blot apparatus was used with low vacuum to place 4 ul volumes of solution containing 2 ug of RNA onto 0.2 um BIOTRANS nylon membranes. Positive control RNA (from day 140 pregnant ewe endometrium) was spotted similarly in 1, 0.1 and 0.01 ug amounts. Negative control RNA (globin mRNA) was spotted in 200, 20 and 2 ng amounts. After drying (1 hour), blots were baked (2 hours, 80° C). Blots were prehybridized in a buffer containing 50% (v/v) formamide, 5X SSC (1 X SSC = 150 mM NaCl, 15 mM Na-citrate, pH 7.0), 5X Denhardt's solution (Maniatis et al., 1982), 10 mM sodium phosphate, pH 6.5, 0.05% (w/v) SDS, 400 ug/ml heat-denatured herring sperm DNA and 250 ug/ml polyuridine (2 hours, 42° C). Blots were then hybridized in buffer in the presence of a 32P-labeled cDNA probe for the UTMP, "pUTMP", (N. Ing and R. M. Roberts, manuscript in preparation). This cDNA had been radiolabeled by nick translation with [alpha-32P]-deoxycytidine triphosphate (800 Ci/mmol) and the Amersham kit. Blots were washed four times in hybridization buffer lacking DNA and RNA (10 minutes, 42° C) and exposed to XRP film (4 days, -70° C). The pUTMP probe was removed from the blots according to ICN's protocol, and was confirmed by film exposed to the blots. After prehybridization, blots were rehybridized to a 32P-labeled cDNA
probe encoding almost the full length of human gamma-actin (Gunning et al., 1983) and autoradiographs were developed.

Northern blot analysis was performed on RNA samples (4.5 to 12 ug) run on 1.5% (w/v) agarose gels containing 1.1 M formaldehyde and 5 ug/ml ethidium bromide in 20 mM 3-(4-morpholino) propane sulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0. DNA size standards (lambda DNA cleaved with EcoRI and Hind III enzymes, and radiolabeled with [alpha-32P]-deoxyadenosine triphosphate and Klenow enzyme) were run concurrently. After gels were soaked in 20X SSC (30 minutes), RNA was transferred to nitrocellulose membranes by capillary blotting in 10X SSC overnight. Blots were photographed with a UV transilluminator system to assess the success of transfer and to ensure that roughly equivalent amounts of RNA were present in the lanes. They were then dried, baked and hybridized as described for the RNA dot blots. Autoradiograph development times varied from 1 to 11 days.

Results

Effectiveness of Steroid Therapy Measured by Progesterone Levels in the Plasma of Ovariectomized Ewes

Plasma progesterone levels rose from 0.49 ± 0.08 (S.E.) ng/ml prior to placing the implants to 1.24 ± 0.11 ng/ml in ewes sampled at day 2. Similar levels to those at day 2 were found in ewes sampled on days 6 (1.27 ± 0.22 ng/ml) and 14 (1.87 ± 0.97 ng/ml). The ewes sampled at day 30 had lower plasma progesterone levels of 0.73 ± 0.06 ng/ml. Since estrogen priming appeared to have no significant effect on circulating progesterone levels, the amount of UTMP produced or on the onset of UTMP production, subsequent results from E2-primed and nonprimed animals were pooled.
UTMP Detection in Uterine Flush and Explant Culture Samples From Ovariectomized Ewes

Samples containing equal volumes of dialyzed uterine flush, and medium and homogenized tissue from 24 hour explant cultures run on SDS-PAGE all showed many bands after Coomassie staining (results not shown). Fluorographs of the gels with explant culture samples showed many radiolabeled protein bands signifying production of many proteins in vitro. Fig. 3-1 shows lanes from fluorographs of homogenized tissue (T) and medium (M) samples from an ovariectomized ewe without progesterone (day 0) and one after 30 days of progesterone influence (day 30) that demonstrate the patterns of proteins produced before and after long term progesterone induction. The fluorograph of the medium sample (M) from the latter ewe shows a large band of radiolabeled protein that comigrates with the UTMP standard indicating that the UTMP are the major products synthesized and released during the culture of the endometrium. Radiolabeled UTMP were detected by immunoprecipitation of culture medium of samples from two out of four day 14 ewes and three out of four day 30 ewes, but not in controls samples tested with with nonimmune serum or excess UTMP (results not shown).

Since results from other procedures (immunostaining and RNA dot blots) of samples from the same animals indicated that the UTMP were produced after 6 days of progesterone treatment, it was clear that the sensitivity of UTMP detection by immunoprecipitation was inadequate. For greater sensitivity of detection, equal amounts of tissue, medium, and flush proteins were analyzed by immunostaining on Western blots. Bands comigrating with UTMP were scored for their intensity (Table 2). All tissue and medium samples from 24 hour explant culture demonstrated the presence of the UTMP. The amounts detected in explant tissue were
Fig. 3-1. Progesterone induction of the UTMP in endometrial explant cultures and uterine flushes.

An ovariectomized ewe was treated with progesterone for 30 days. Samples were collected by uterine flush (f) and endometrial tissue (T,t) explant culture in medium (M,m) for 24 hours in the presence of $^3$H-leucine. Proteins from these samples and ones from an untreated ovariectomized ewe were separated by SDS-PAGE. Gels with tissue (T) and medium (M) samples were dried and fluorographs were developed. Gels with all three sample types were electroblotted and immunostained (t,m,f). Extents of migration of molecular weight standards are indicated at left. An immunostained UTMP standard is shown in the first lane.
Table 2. Immunodetection of UTMP in samples from endometrial explant cultures, uterine flushes, and uteri of ovariectomized ewes given progesterone for the indicated number of days.

<table>
<thead>
<tr>
<th>Day</th>
<th>Tissue</th>
<th>Western Blots</th>
<th>Cytochemistry</th>
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<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>Flush</td>
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<tr>
<td>0</td>
<td>+</td>
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<td>6</td>
<td>+½</td>
<td>+</td>
<td>0</td>
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<tr>
<td>14</td>
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Intensities of immune reactions were graded on a 0 to ++++ scale for samples from each ewe on Western blots and with cytochemistry. Reported scores are averages to the nearest half of a + for samples from four animals in each treatment group but day 2, which had two animals.
very low at days 0 and 2, slightly higher at days 6 and 14, and reached moderate levels at day 30. Medium samples followed a similar trend in UTMP prevalence except that the two day 2 samples demonstrated moderately high levels of the UTMP.

Uterine flush samples were variable in UTMP detection on Western blots in day 0, 2, and 6 samples, with either the detection of very low levels or none at all. Uterine flush samples taken from ewes after 14 and 30 days of progesterone influence showed high levels of UTMP, reflecting the presence of large amounts of the UTMP in utero. The Western blot analyses consistently showed that low levels of UTMP were present in endometrial explant culture samples from ewes that were free of exogenous progesterone influence. The uterine flush samples, however, detected only low levels of the UTMP in 6 out of 10 ewes before 14 days of progesterone influence. Therefore the veracity of the explant culture results in relating to the UTMP production status of the ewe was questioned, and the possibility exists that UTMP production is an artifact of in vitro culture.

The change in amount of UTMP detected on Western blots of explant culture and uterine flush samples from ewes with and without long term progesterone influence is demonstrated in Fig. 3-1. Only very faint bands are detected with immunostaining of explant culture samples from day 0 ewes. In contrast, explant culture and uterine flush samples from a ewe after 30 days of progesterone treatment all show the presence of high levels of the UTMP. The samples of the day 30 ewe demonstrated the predominance of the UTMP at this stage compared to the minimal amount of the UTMP in the three day 0 samples when equal amounts of protein were
compared (Fig. 3-1). Immunoreactive products of lower M_r than the UTMP are noted in the analysis of medium and flush proteins of the day 30 ewe.

UTMP mRNA Detection on Dot and Northern Blots of Endometrial RNA from Ovariectomized Ewes

UTMP mRNA was detected in only one RNA sample from a ewe with less than 6 days of progesterone therapy (Fig. 3-2). Samples taken at day 6 showed low levels of hybridization to the ^32P-labeled pUTMP probe, while some day 14 samples showed moderate hybridization. Samples taken at day 30 all demonstrated hybridization signals, but of variable intensities. Positive control RNA from late pregnant ovine endometrium showed strong hybridization signals with 1.0 and 0.1 ug amounts and a weak signal with 0.01 ug. To confirm that samples contained intact RNA in similar amounts, the blot was rehybridized with an actin probe. With actin probe, all sample dots had moderate hybridization signals, as did the 1.0 ug positive control RNA sample, except for two of the day 0 samples; one day 0 sample lacked a signal, while another showed an unusually strong hybridization with the actin probe. The latter had shown moderate hybridization signal previously with the pUTMP probe, and probably bound both probes more extensively than other samples because of an increased amount of RNA or a contaminant. Globin mRNA, chosen as a negative control to ensure that the pUTMP probe did not hybridize nonspecifically to other mRNAs, showed no hybridization to either pUTMP or actin probes in samples with 200, 20 and 2 ng amounts. These amounts of mRNA relate to amounts of 4000, 400, and 40 ng of total cellular RNA.
Fig. 3-2. Autoradiographs from dot blots of endometrial RNA from ovariectomized ewes.

Samples of endometrial RNA (2 ug) from ovariectomized ewes, untreated (day 0) or treated with progesterone for a number of days (day 2, 6, 14 or 30) were spotted onto a membrane, bound irreversibly, and hybridized to the radiolabeled pUTMP probe (u). Samples from ewes that received a priming dose of estrogen appear in the upper two positions (EP) while those receiving no estrogen are below them (P). After probe removal, blots were rehybridized with an actin probe (a). Controls were RNA from endometrium of a ewe at day 100 of gestation (+) and globin mRNA (-). Spots on autoradiographs developed from different probes on the same RNA dot appear side by side.
if mRNA comprises 5% of the RNA of a cell (range 1 to 5%, Maniatis et al., 1982). Therefore, the control and sample mRNA amounts were similar.

Northern blot analysis of endometrial RNA from ovariectomized ewes of the day 0, 2, and 6 groups failed to detect UTMP mRNA in 6 ug samples of total cellular RNA. The pUTMP probe detected UTMP mRNA on a Northern blot in two of four day 14 samples and all four day 30 samples, with hybridization signals varying considerably in the latter group. Results from RNA analysis of endometrium from ovariectomized ewes at day 14 and day 30 are shown in Fig. 3-3. The single UTMP mRNA band was approximately 1600 bases in length when compared to double-stranded DNA standards. The RNA in all samples was judged to be intact and in equivalent amounts by visualization of the ethidium bromide-stained RNA on the blot after transfer (as in Fig. 3-3, lane 8) except for two of four day 0 and day 6 samples.

**Immunocytochemical Detection of UTMP in Endometrium of Ovariectomized Ewes**

The UTMP were detected in endometrial samples from all ovariectomized ewes after 6 days of progesterone therapy (Table 2). The day 6 samples showed light supranuclear staining in cells in the deep and middle glandular epithelium, but not in the luminal epithelium (Fig. 3-4, panel A). Staining varied in extent from gland to gland and from cell to cell within the gland (Fig. 3-4, panel B). One animal receiving no progesterone also showed very light immunostaining for UTMP in a limited number of glands (not shown).
Fig. 3-3. Autoradiographs from Northern blot analysis of various RNA samples with the pUTMP probe. RNA samples were separated by gel electrophoresis, and blotted and bound to nitrocellulose. Blots were hybridized with the pUTMP probe and autoradiographs were developed. RNA samples were from endometrium of ovariectomized ewes after 14 days (1) and 30 days (2) of progesterone treatment, caruncular (3) and intercaruncular (4) endometrium and liver (5) of a ewe at day 100 of gestation, endometrium of a cow at day 45 of gestation (6), and endometrium of an unbred ewe at day 16 of the estrous cycle (7). Lane 8 shows the RNA of lane 7, visible with ethidium bromide staining of the blot. Lanes shows radiolabeled DNA standards with their sizes indicated at left in base pairs (bp). Sample size and blot development time varied (4.5 ug and 1 day for lanes 1 and 2, 6 ug and 4 days for lanes 3 through 6, and 12 ug and 11 days for lane 7).
Fig. 3-4. Immunolocalization of the UTMP in endometrium from ovariectomized ewes treated with progesterone for 6 days.

Cross sections of uterus, taken in close proximity to each other, from ovariectomized ewes treated with progesterone for 6 days (A-D). Antiserum to the UTMP was used to stain tissues in A and B. B is a higher magnification of the slide from which A was photographed. A negative controls was done with nonimmune serum (C). A section was stained with hematoxylin and eosin (D) to demonstrate maintenance of tissue morphology during the fixation process. Magnification was 68X for all except B and F, for which it is 276X. Bars denote 100 um.
Fig. 3-5. Immunolocalization of the UTMP in endometrium from ovariectomized ewes treated with progesterone for 30 days.

Techniques were similar to those in Fig. 3-4. Panels E and F show uterine cross-sections stained with immune serum low and high power, respectively and H is stained with hematoxylin and eosin. Panel G shows a negative control stained with immune serum from which UTMP antibodies were removed by adsorption to UTMP-Sepharose. Bars indicate 100 um.
At day 14, immunostaining was much more intense than at day 6. However some glands and some glandular epithelial cells within a region of gland that showed positive staining in some cells still failed to stain at all. Immunostaining of day 30 samples of endometrium was heavy in the epithelial cells of deep and middle gland area. Most epithelial cells were involved, with intense immunostaining seen throughout the apical cytoplasm and within the lumen of the glands (Fig. 3-5, panel E). Epithelial cells along the uterine lumen did not stain except for an area along the brush border which might indicate trapping of the UTMP produced initially in the glands. No staining was noted in the myometrium, in uterine structures other than those noted above, or in negative controls treated with equivalently diluted normal rabbit serum (Fig. 3-4, panel C) or with immunoadsorbed antiserum (Fig. 3-5, panel C). Tissue sections stained with hematoxylin and eosin demonstrate normal uterine morphology and adequate tissue fixation (Fig. 3-4 and 3-5, panels D and H).

UTMP Detection in the Intact Ewe, Cow and Gilt

Uterine flush samples taken as early as 16 days post-breeding from intact ewes during early pregnancy showed immunostaining for UTMP on Western blots (results not shown). Western blot analysis failed to detect the UTMP in uterine flush samples taken at day 12 or 14 of pregnancy, or in samples from cyclic ewes at days 12, 14 and 16 of their 17-day long estrous cycles. The UTMP were present in low levels in medium from 24 hour culture of endometrial explants taken at day 14, 16 and 18 of pregnancy. Unilaterally pregnant animals showed no
significant difference in UTMP production between gravid and nongravid horns by Western analysis of uterine flush and explant culture medium samples.

Northern blot analyses showed that low levels of UTMP mRNA were present in the endometrium taken from ewes as early as day 14 of pregnancy and in all samples from day 16 to 22. In a single ewe tested at day 12 of pregnancy, UTMP mRNA was not detected. Messenger RNA for the UTMP was detected at day 16 of the estrous cycle (Fig. 3-3, lane 7), but neither Northern or dot RNA analyses demonstrated UTMP mRNA at days 12 or 14 of the cycle. Caruncular and intercaruncular endometrium and liver RNA samples from a ewe at day 100 of gestation showed moderate, strong, and no hybridization signals, respectively (Fig. 3-3, lanes 3-5). These results demonstrate that UTMP mRNA levels are higher in intercaruncular endometrium, where the majority of uterine glands exist, than in caruncles, and is not prevalent in the liver of the ewe at midpregnancy.

The UTMP were identified in a uterine flush sample taken from a cow at day 18 of pregnancy as a single band of immunostaining material that comigrated with an ovine UTMP standard on a Western blot was detected (results not shown). Northern blot analysis of RNA from day 45 pregnant cow endometrium showed weak signal to a single band of identical size to ovine UTMP mRNA (Fig. 3-3, lane 6). RNA from endometrium of a day 47 pseudopregnant pig showed no hybridization signal with similar Northern analysis (not shown).

Discussion

Progesterone administration by the implants was successful in maintaining physiological levels of hormone in the ovariectomized ewes
until the end of the 30 day experiment. Similar plasma levels are found in ewes in the luteal phase of the estrous cycle (1.5 to 2.5 ng/ml; Thorburn et al., 1969) or during early pregnancy (2 to 3 ng/ml; Bassett et al., 1969). The lower progesterone levels in the day 30 animals may be explained by decreasing potency of the implants or by the loss of one of four implants by three animals in the last week of treatment. Interestingly, the animal in the day 30 group with the lowest progesterone in its serum and had lost one implant but still produced more uterine milk and UTMP than any other animal in the study. No effect of estrogen priming was noted in sample analysis, possibly due to large variation in UTMP production between animals and the small numbers of animals in the treatment groups. Also, the estrogen priming dose (5 ug) was slightly smaller than that used by others (25 ug, Moore et al., 1983).

Production of the UTMP was detected in ewes that had been ovariectomized for over four months and had received no exogenous progesterone. From the four ewes tested, uterine flush and explant culture samples consistently demonstrated the presence of low levels of the UTMP. Immunocytochemical analysis of the uteri of the four animals revealed that the UTMP were present in sections prepared from one sample. It was located in the supranuclear region of epithelial cells in the middle and deep segments of a few uterine glands. However, analyses of RNA from the endometrium of the three animals failed to detect UTMP mRNA. A fourth ewe provided dot blots that gave a detectable signal with the pUTMP probe and an unusually strong signal with the actin probe. However, this was not the ewe that gave the positive immunocytochemical response and it is possible that the dot
blots were artifacts. It is also possible that there are basal levels of UTMP production in the absence of ovarian or exogenous progesterone. Alternatively, low levels of UTMP production in ovariectomized ewes may be the result of endogenous progesterone from their adrenal glands.

Samples taken from ovariectomized ewes after two days of progesterone therapy did not consistently indicate the production of the UTMP, except for those used for 24 hour explant culture. Explant culture medium samples from both day 2 ewes contained moderate levels of the UTMP. It is possible that the UTMP production detected in explant cultures but not by other techniques employed on tissue from the same animals is an artifact arising from the procedure of explant culture. A similar occurrence was found for avidin, a protein secreted by the chick oviduct in response to progesterone. When tissue trauma occurred, such as mincing or when the oviduct was damaged by burning, avidin production was induced, although the mechanism of induction was believed to be different than that for its progesterone induction (Elo, 1979). Such induction of the UTMP is consistent with their relationship to the serpin family of protease inhibitors (N. H. Ing and R. M. Roberts, manuscript in preparation), which are acute phase proteins that, in response to trauma, increase several-fold in their plasma levels. Conceivably UTMP production may be induced by the trauma of mincing the endometrium during explant preparation or by other types of tissue damage. Thus, presence of the UTMP in endometrial explant cultures from the early stage treatments (days 0 and 2) may be an artifact unrelated to the effects of progesterone.
In ovariectomized ewes after six days of progesterone therapy, immunocytochemistry consistently demonstrated low levels of UTMP production in the epithelial cells of middle and deep uterine glands. The faint staining in the supranuclear region of the cells is consistent with the region occupied by the Golgi apparati. Endometrial RNA samples at day 6 also bound the UTMP probe on dot blots. However, western blot analysis of uterine flush samples from ewes after six days of progesterone therapy failed to detect the UTMP. These results suggest that UTMP synthesis, but not secretion, occurs in the endometrium of ovariectomized ewes after 6 days of progesterone therapy. Alternatively secretion may occur, but the proteins are rapidly turned over.

Strong evidence for the secretion of the UTMP in utero was seen in ovariectomized ewes after 14 days of progesterone treatment. Uterine flush samples demonstrated the presence of high levels of the UTMP on Western blots. Immunocytochemistry of the uteri of all four day 14 ewes showed epithelial cells of the endometrium that stained heavily throughout their apical cytoplasm. The cells containing the UTMP were interspersed with unstained epithelial cells, and regions of the middle and deep uterine glands varied greatly in the number of stained cells. Moderate levels of UTMP mRNA were detected in endometrial RNA samples of the ovariectomized ewes treated for 1 days. However, the results showed that the amount of UTMP mRNA had increased markedly since day 6.

After 30 days of progesterone therapy, ovariectomized ewes were clearly producing and secreting large amounts of the UTMP. High levels of UTMP mRNA were detected in the endometrium. The UTMP were also present in appreciable quantities in uterine flushes and appeared to be the major protein products detected in explant culture medium by
fluorography. The majority of epithelial cells of middle and deep uterine glands were stained heavily for the UTMP by immunocytochemistry, suggesting that the cells were dedicated to production of large amount of UTMP. The UTMP were also detected as amorphous material in the lumina of the uterus and uterine glands, and along the brush borders of their epithelia.

The pattern of immunostaining contrasts with that of another protein, the so-called 14K protein ($M_r = 14,000$), which is also believed to be produced by ovine endometrium in response to progesterone (M. Kazemi and R. M. Roberts, manuscript in preparation). Antiserum to the 14K protein only stained the intercaruncular surface epithelium and to some extent the glandular epithelium of the necks of the glands. A similar contrast between the immunolocalization of progesterone-induced proteins within the endometrium has been reported for secreted proteins of the pig. There, uteroferrin appears to be a product of the glands whereas a progesterone-induced plasmin/trypsin inhibitor is produced primarily by the surface epithelium (Fazleabas et al., 1985). The results in both species indicate that distinct epithelial cell populations exist in the uterine glands and lumen, and that these respond very differently to the same steroid hormone in their production of protein.

The increase in the production of the UTMP with increasing duration of progesterone therapy was fairly consistent between the various methods employed. All procedures showed that the UTMP were being synthesized and secreted after 14 days of progesterone therapy. The immunocytochemical studies suggested that active synthesis was also underway as early as day 6. Since the plasma progesterone levels of the
ovariectomized ewes given progesterone were similar to peak progesterone levels in the cyclic ewe and levels in the early pregnant ewe, the results presented are believed to be relevant to early UTMP production in intact animals. The earliest production of the UTMP in intact ewes was detected at day 16 of the estrous cycle and at day 14 of pregnancy (both by Northern blot analysis). These results are consistent with the time frame of progesterone induction of the UTMP detected in the ovariectomized ewe study. The presence of a conceptus did not appear to affect the production of the UTMP by endometrium of the gravid horns of unilaterally pregnant ewes when compared to that of the nongravid horns. This result also suggests that UTMP production studies in nonpregnant animals are relevant to the production of the UTMP in pregnant animals.

Intercaruncular endometrium of a ewe at day 100 of gestation showed a greater level of UTMP mRNA than caruncular endometrium, a result which is consistent with the immunocytochemical evidence for UTMP production in the uterine glands of ovariectomized ewes given progesterone presented here and that by Moffatt et al. (1987a). Recently, the UTMP have been tentatively identified in the serum of pregnant ewes (Newton et al., 1988). Liver of a day 100 pregnant ewe was analyzed for UTMP mRNA as a potential source of the UTMP but none was detected, indicating that there is probably not a hepatic source for the UTMP in the sheep. Therefore, UTMP detected in the serum of pregnant ewes (Newton et al., 1988) may be from the uterus.

The bovine uterus was an apparent source of the UTMP during pregnancy. Both protein and UTMP mRNA were detected. However, at day 45 of pregnancy the UTMP mRNA levels in the bovine endometrium were
much lower than those in the endometrium of ovariectomized ewes given progesterone for 30 days. Lack of detection of UTMP mRNA in the pig could have been due to very low levels of production or divergence of the mRNA sequence (the pig is evolutionarily more distantly related to the sheep than is the cow), as well as lack of production. The hybridization conditions used in Northern blot analysis were of low stringency, so similar mRNAs would most likely have been detected. Detection of UTMP production in cow endometrium proved that the UTMP were not unique to the sheep, but lack of detection in the pig suggested that the UTMP are not produced by all ungulates. Production of large amounts of the UTMP by the endometrium in response to progesterone does appear to occur only in the sheep.

Normal physiology of the endometrium appears to depend on long-term cycles of steroid influence and not merely on the immediate levels of circulating hormones. Evidence for this concept is demonstrated by embryo transfer experiments using ovariectomized ewes as recipients (Moore et al., 1983). In such experiments, greatest success is observed if the steroid regimen, designed to mimic the normal steroid influences of the estrous cycle, is given for a full cycle before the cycle in which transfer is performed. The results of the present study describe the induction of the UTMP by progesterone in the ovariectomized ewe and are believed to relate to the normal ewe during the estrous cycle and early pregnancy. UTMP production appears to require weeks of progesterone therapy to be maximal. The events that transpire during long steroid influence that induce the secretion of uterine proteins and make the uterus suitable for nurturing a conceptus are not understood. The secretion of uterine proteins may be essential for successful
pregnancy. The UTMP may be a good marker protein for the elucidation of progesterone's action in the slow process of inducing a tissue to produce a secreted protein.
CHAPTER 4
DISCUSSION

Background

The UTMP are the major progesterone-induced secreted proteins of the sheep uterus. They are basic glycoproteins of $M_r = 57,000$ and 55,000 as determined by SDS-PAGE. Their purification and preliminary characterization were performed in previous studies (Moffatt et al., 1987a; Hansen et al., 1987a). Conclusions of the early studies were:

1. the UTMP are similar in peptide structure,
2. both forms of the UTMP are glycosylated,
3. the extent of glycosylation may account for the two $M_r$ forms of the UTMP; one oligosaccharide chain on the $M_r = 55,000$ and two on the $M_r = 57,000$, and
4. the UTMP carry mannose-6-phosphate residues on their oligosaccharide chains.

Structure

In the present study, the description of the UTMP was extended by molecular cloning. Endometrial mRNA from ewes in late pregnancy was used to synthesize cDNA, which was cloned and sequenced. The 5' region of the mRNA was sequenced directly. The entire UTMP mRNA sequence was determined and used to deduce the amino acid sequence of the UTMP. Also, the two $M_r$ forms of the UTMP were individually analysed for N-terminal amino acid sequence. The sequence was identical for the two forms and agreed well with the inferred amino acid sequence.

The UTMP mRNA sequence demonstrates several interesting features. Two start codons of probable function are present. This is a rare
characteristic of eukaryotic mRNAs. However, Ketcham et al. (1988) similarly found a pair of start codons in the mRNA of an acid phosphatase from human placenta that is closely related to uteroferrin of the pig uterus. The biological significance of the two start codons in close proximity is unknown. It would be interesting but technically difficult to determine the actual sites of translation initiation for the UTMP.

Another interesting feature of the UTMP mRNA is the repeat region of 21 bases within the open reading frame. The repeat region encodes two repeats of seven amino acids that are only two residues apart. One repeat does not align with sequences of the other serpins in a comparison of UTMP and serpin amino acid sequences. Ovalbumin, a serpin secreted by the chicken oviduct in response to E<sub>2</sub>, contains several "extra" segments that do not align with sequences of the serpins. Since no protease inhibitory activity has been demonstrated for either ovalbumin or the UTMP, it is possible that the non-aligning segments are responsible. The extra segments may disrupt protein structure so that activity as protase inhibitors is lost. A deletion mutant of the UTMP that lacks the repeat could be made with cloning techniques. It would be very exciting if such a mutant gained function as an inhibitor of a protease. Thus, the duplication of a short segment of a gene, a single event in molecular evolution, might be shown to account for loss of a molecular function.

The most remarkable discovery involving the description of the UTMP mRNA and amino acid sequences is that they show strong homology with several members of the serpin superfamily of plasma serine protease inhibitors. The amino acid sequences of the UTMP and baboon alpha-1
antitrypsin are identical at 31% of the positions in an alignment of the UTMP sequence with those of five serpins. In addition to this similarity in primary amino acid structure, features of secondary amino acid structure appear to be comparable between those predicted for the UTMP by an algorithm and those identified by x-ray diffraction of crystallized human alpha-l antitrypsin (cleaved at its reactive site). The glycosylation of the UTMP is probably similar to that of human alpha-l antitrypsin. The UTMP sequence contains two possible glycosylation sites, one of which (Asn\textsubscript{243}) aligns with known glycosylation site of human alpha-l antitrypsin (Asn\textsubscript{247}). The UTMP may be synthesized in a tertiary conformation similar to other serpins as discussed in the next section.

**Biosynthesis**

No evidence for the two \(M_r\) forms of the UTMP (57,000 and 55,000) arising from multiple genes has been found. By peptide mapping and N-terminal amino acid sequencing the two forms appear identical. By mRNA sequencing and Northern blot analysis the UTMP mRNA appears to be homogeneous. To determine if the UTMP are composed of products from more than one gene, genomic DNA of the sheep could be digested by restriction enzymes and blotted to nitrocellulose by the method of Southern (Maniatis et al., 1982). The pUTMP and U-5 cDNAs could be radiolabeled and used as probes to identify fragments of DNA containing the UTMP gene(s).

The UTMP products of in vitro translation of endometrial RNA were examined in the present study. The results identified two possible UTMP peptide precursors (\(M_r = 55,000\) and 47,000) from translation reactions. The \(M_r = 55,000\) form is believed to be the UTMP precursor.
The $M_r = 47,000$ form probably arises from limited proteolysis of an exposed loop of polypeptide common to serpin structures that is contained on the $M_r = 55,000$ precursor. To confirm that the $M_r = 47,000$ product is a cleaved form of the UTMP, the translation assay could be done on a preparative scale. Products of translation could be separated by SDS-PAGE or by high pressure liquid chromatography and $M_r = 55,000$, 47,000 and 5,000 products isolated. These could be subjected to N-terminal amino acid sequence analysis to determine if they originate from the UTMP and to identify the site of cleavage. It would be interesting to see if serpins translated in rabbit reticulocyte assays undergo cleavage at their reactive sites.

The addition of canine pancreatic microsomes in the translation reaction resulted in the production of a glycosylated and translocated form of the UTMP. This $M_r = 57,000$ form is believed to arise from the $M_r = 55,000$ form by addition of one oligosaccharide chain or by the addition of two chains and removal of the signal peptide. Evidence presented in this thesis is consistent with earlier evidence which concluded that the two secreted forms of the UTMP differed in the number of carbohydrate chains they carry ($M_r = 55,000$, one chain; $M_r = 57,000$, two chains). Limited proteolysis of the serpin UTMP at its "reactive site" could explain the presence and accumulation of $M_r = 49,000$ and 47,000 forms identified in UTM samples. An easy experiment to definitively determine if the $M_r = 57,000$ and 55,000 forms of the UTMP differ by carrying two and one oligosaccharide chains, respectively, would be to separate the two $M_r$ forms by preparative SDS-PAGE and analyze them for carbohydrate content relative to peptide content.
The resemblance of the post-translational processing of the UTMP and uteroferin encourages speculation. Newly synthesized uteroferin molecules carry two oligosaccharide chains. The sheep endometrial RNA in translation with microsomes produces a UTMP of \( M_r = 57,000 \) which may also carry two chains. Both uteroferin and the UTMP contain two sites of possible glycosylation on their amino acid sequences. Once secreted into the uterus, most of the uteroferin molecules either lose one of their oligosaccharide chains or one chain becomes severely truncated. The UTMP may be similarly but less completely processed to form a population of molecules carrying either one and two oligosaccharide chains.

In explant culture, uteroferin and the UTMP incorporate \(^{32}\)P from inorganic phosphate. The radiolabel is present on their oligosaccharide chains in a mannose-6-phosphate linkage. The UDP-GlcNAc:glycoprotein N-acetylglucosamine 1-phosphotransferase responsible for transferring phosphate groups to terminal mannosyl residues selects its substrates by determinants on their protein portions. The primary structures of the UTMP and uteroferin are dissimilar as are the families of proteins to which they belong. However, the phosphotransferase of the Golgi system acts upon both of their protein structural determinants. Therefore, the UTMP and uteroferin structures may contain subtle similarities that the phosphotransferase recognizes. Phosphorylation may be involved in the control mechanisms of the biosynthesis of both the UTMP and uteroferin. Both the UTMP and uteroferin are phosphorylated and are the major progesterone-induced secretory proteins from the uteri of the sheep and the pig, respectively. Although the UTMP and ovalbumin are very similar in structure, site of synthesis and inducing agents, the
UDP-GlcNAc:glycoprotein N-acetylglucosamine 1-phosphotransferase phosphorylates the oligosaccharide chains on the UTMP but not on ovalbumin. By comparing the structures of the UTMP, uteroferrin, and ovalbumin, the specific protein determinants that the phosphotransferase recognizes might be discerned.

**Progesterone-induction**

The P₄-induction of the UTMP occurred in ovariectomized ewes given exogenous P₄ by an implant system that was believed to release a constant physiological level of hormone. Estrogen-priming did not significantly affect UTMP production, probably due to large variations in UTMP production between animals and small numbers of animals tested. UTMP production was first detected by the presence of its mRNA and intracellular localization after six days of progesterone therapy. Secretion of the UTMP was detected after 14 days of P₄ treatment and was enhanced after 30 days of hormone therapy.

The ovariectomized ewes given P₄ resembled cyclic ewes in the luteal phase (day 16) and early pregnant ewes (day 14) in plasma P₄ levels and the time of first UTMP secretion. Because of this and because the presence of conceptuses in unilaterally pregnant ewes did not affect UTMP production by the local endometrium, the ovariectomized ewe model for P₄ induction was proposed to be relevant to the study in sheep during normal reproductive physiology.

A multitude of questions remain to be answered concerning UTMP production in the ewe. A few are mentioned here. What are the precise levels of UTMP production and UTMP mRNA transcription in the ewe during the estrous cycle, pregnancy and anestrous periods? Is UTMP production under transcriptional or translational control? Antibody could be used
to quantitate UTMP in samples such as those obtained in the ovariectomized ewe study. In situ hybridization could be used to measure levels of UTMP mRNA and to localize the mRNA to specific cells. Does the chorioallantois affect UTMP production? What are the levels of UTMP mRNA and UTMP production in endometrium of gravid and nongravid horns of unilaterally pregnant animals? What is the sequence of the gene of the UTMP? Especially interesting are the flanking sequences involved in the control of transcription of the UTMP. Are progesterone response elements (Compton et al., 1983) present? Are sequences identified as responding to other messengers present so that the lag in P4 induction of UTMP synthesis may be explained by delay in production of a second messenger? The major P4-induced protein of the rabbit uterus, uteroglobin, shows an increase in its gene transcription after two days of P4-treatment in ovariectomized animals although total transcription increases within one day (Shen et al., 1983). The lag in uteroglobin mRNA production is reduced in intact animals given P4. The molecular events responsible for the lags in P4-induced uteroglobin and UTMP production are unknown. With the characterization of the UTMP and the available antibody and nucleic acid probes, the UTMP provide the best model system for the study of P4-induction of secreted proteins in a large mammal.

The synthesis of the UTMP may be induced by tissue trauma. Endometrial explants of the two ovariectomized ewes in the day 2 P4-treatment group showed high levels of UTMP secretion that were not reflected by any other methods of detection. Avidin, a P4-induced protein of the chick oviduct, in induced by tissue trauma of mincing and
burns (Elo, 1979). It is believed that the mechanism of avidin induction by $P_4$ differs from that by trauma. It would be informative to see if this were also true for the UTMP.

The apparent induction of the UTMP by trauma may relate to their homology with the serpin superfamily. In human serum, the levels of alpha-1 antitrypsin rise in response to trauma or inflammation. The UTMP may be acute phase proteins, as is alpha-1 antitrypsin. Ovariectomized ewes could be subjected to temperature stress and UTMP production could be quantitated in the uterus by Western blotting or immunocytochemistry. The sequence of the UTMP gene(s) could be searched for regulatory sequences common to acute phase proteins or inflammatory mediators (Caput et al., 1986).

The first detection of the UTMP and UTMP mRNA in a species other than the sheep is presented in this thesis. Both were identified in the pregnant cow. However, at early pregnancy the level of detection of UTMP mRNA in bovine endometrium was much less than that in ovine endometrium at a similar stage. Under a similar duration of $P_4$ influence, no UTMP mRNA was detected in pig endometrium. In ungulate ancestry, the porcine family emerged about 60 million years ago and the bovine and ovine families about 30 million years ago (Romer, 1959). The rate of divergence of nucleotide sequences coding for proteins has been estimated as 0.5% per million years (Jukes, 1980). However, the conservation of sequences does depend upon the protein and selection pressure on the species. The divergence of porcine and bovine "UTMP" mRNA sequences from that of the sheep would be predicted to be 3% and 1.5% respectively. Northern blot analysis was performed in hybridization conditions of low stringency in order to detect
heterologous mRNA. The detection may have failed because of a large amount of sequence divergence between species. Alternatively, the mRNA level may have been too low. However, the production of large amounts of the UTMP in response to P_4 appears to be specific to the sheep.

**Function**

Previous investigations were unsuccessful in finding a specific function for the UTMP. Although the UTMP are homologous to the serpin superfamily, no inhibition of proteolysis was apparent in the tests presented here. The UTMP should be retested against thrombin and plasmin because of the low UTMP ('inhibitor'):protease molar ratio used. The UTMP may be inhibitory to a protease with which they have not been tested. Sensitive substrates specific for proteases are available.

The P_4-induced proteins synthesized by porcine and bovine endometrium have been characterized and they are not a pair of molecules that resemble the UTMP (Basha et al., 1980b; Bartol et al., 1985). The production of UTMP by the cow may signify its importance in reproduction. The UTMP may be active in the apparently low levels found in the cow uterine secretion as well as the low levels identified in the uteri of cyclic and early pregnant ewes.

It is remarkable that in many species examined, long term progesterone influence causes the endometrium to produce a spectrum of secreted proteins. However, the pattern of proteins induced appear to be unique to the species. One might conclude that this failure to produce an identical set of proteins may be a reproductive barrier that separates species. However, several other reproductive barriers inhibit interbreeding of different species prior to the induction of uterine
protein secretion by long term progesterone during pregnancy. An alternative explanation of the different protein patterns produced by the uteri of species is that the response is a random one with respect to the choice of proteins produced. As long as the recruited proteins are relatively harmless to tissues in high concentration, they may be able to confer reproductive advantages of control of the osmotic character and pH of the uterine fluid, transport of limiting nutrients, or nutrition of the embryo.

Serpins are candidates for secretory proteins in reproductive tracts. Most serpins are active as inhibitors of proteases and can be inactivated by proteases for which they are not inhibitory or by overwhelming amounts of proteases. They are harmless to tissues as demonstrated by their presence in blood serum in high concentrations. The pig uterus secretes a uteroferrin-associated protein that is highly homologous to human protein C inhibitor of serum (P. V. Malathy and R. M. Roberts, unpublished results). The homology is so complete that the inhibitor may represent the porcine complement of the inhibitor. This is yet another example of a serpin being secreted into the uterus in response to P₄.

Two species have been shown to produce serpins as major secretory proteins of their reproductive tracts. The chicken oviduct produces ovalbumin in response to E₂ and the sheep uterus produces the UTMP in response to P₄. In this thesis I have shown that, in addition to their similarity in the source of their biosynthesis, the UTMP are structurally similar to ovalbumin. Although specific functions remain to be elucidated for these homologous proteins, their role in reproduction is undisputable because of their prevalence. Ovalbumin
serves a role in nutrition of the avian embryo. The UTMP are not found in large quantities in the uterus of the normally pregnant sheep. The UTMP are known to be taken up by the fetus and are found in allantoic and amniotic fluids. The fetus probably makes use of this protein source. Thus, the UTMP may be appropriately named for their nutritive role.

I believe that the UTMP and ovalbumin perform vital roles in the uterus and in the egg, respectively, that are similar to the roles of albumin in the blood serum. By their presence in high concentration, the proteins must affect the osmotic character of the fluid of the reproductive tract. Also because of their prevalence, they are likely to function in transport of vital nutrients from the mother to the conceptus. Such functions remain to be identified for the UTMP and provide intriguing areas for future study of the reproduction of the ewe.
REFERENCES


I, Nancy Hughes Ing, was born on December 17, 1957 in Cleveland, Ohio. My family included my father (Edwin Bruce Ing) and my mother (Mary Hughes Ing) and my sister (Katherine Ann Ing). My father was an officer in the U. S. Coast Guard and we moved to different parts of the United States every three years until his retirement in 1967. We then moved to Stuart, Florida, where I went to school from grades 5 to 12.

When I was in my early teens, I wanted to be a veterinarian. In 1975, between my junior and senior years in high school, I participated in the Summer Science Research Program of the Florida Foundation for Future Scientists at the University of Florida. With exposure to scientific research, I saw the value of having a strong background in medicine and being able to apply it to research. Thus, I became interested in veterinary research.

I chose to go to college at the University of Florida because of my previous introduction to it and the presence of the state veterinary college. I completed a B.S. degree in zoology in 1979 but failed to be accepted by the College of Veterinary Medicine at that time. I did post-baccalaureate studies for a year, in which I took the core biochemistry graduate courses, applied to the Department of Biochemistry and Molecular Biology as a graduate student, and reapplied for the veterinary class that would graduate in 1984.
In 1980, I accepted positions both as a biochemistry graduate student and a veterinary medicine student. Minimal cooperation of the veterinary program was possible due to its rigid class schedule. However, I was exempt from the veterinary school biochemistry course, during which time I did biochemistry research, and I participated in the biochemistry graduate student seminars. I spent summers and some holidays working as a biochemistry graduate student until I graduated from veterinary school in 1984.

After I earned my D.V.M. degree, I became a full time graduate student and worked in the laboratory of Dr. R. Michael Roberts, under whose supervision my thesis was done. At the end of 1985, I followed Dr. Roberts and his laboratory to Columbia, Missouri, to complete my research at the University of Missouri. Although the move was costly in time, I am very grateful for the move to Missouri because I learned how to set up laboratories and met William Harold Cox, whom I married on October 2, 1987.

Currently, I am involved in post-doctoral training in the laboratory of Dr. Bert W. O'Malley in the Department of Cell Biology in Houston, Texas. Although my project is not yet defined, it will involve the transcriptional control of gene regulation. My career goal is to obtain a faculty position, possibly at a college of veterinary medicine, where I can teach students and direct my own research projects.
I certify that I have read this study and that in my opinion it
conforms to acceptable standards of scholarly presentation and is fully
adequate, in scope and quality, as a dissertation for the degree of
Doctor of Philosophy.

R. Michael Roberts, Chair
Professor of Biochemistry
and Molecular Biology

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conforms to acceptable standards of scholarly presentation and is fully
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Fuller W. Bazer
Professor of Animal Science

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1988

Dean, Graduate School

[Signature]

Dean, College of Medicine

[Signature]