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# REGULATION OF RETINOL-BINDING PROTEIN SECRETION AND EXPRESSION IN THE OVINE CONCEPTUS AND UTERUS BY RETINOIDS AND STEROIDS

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## DEDICATION

This thesis is lovingly dedicated to my parents, Warren and Pennie, and my sister, Wanda, who have given me their love and support throughout my life.

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# CHAPTER I

## LITERATURE REVIEW

In large domestic farm animals, quality of gametes, nutrition, environmental factors, or the failure to fertilize can all result in lower reproductive performance. Vitamin A and retinoid metabolites are known to be involved in reproductive processes (Takahashi et al., 1975; De Luca, 1991), but the mechanisms by which they function are not fully understood. Therefore, a greater understanding of the mechanisms that occur during the reproductive process is of value from an economic standpoint as well as contributing to our overall knowledge for a process that occurs in all animals.

### Vitamin A

The major dietary sources of vitamin A are carotenoids found in plants and retinyl esters found in animal tissues. Once ingested, vitamin A becomes emulsified with lipids in the stomach and travels to the intestine. In the intestine, retinyl esters are hydrolyzed to retinol and then are reesterified during absorption. Retinol and $\beta$-carotene are then solubilized in micelles in order to enter liver mucosal cells where $\beta$-carotene is converted to retinol in a reaction involving 15-15'-dioxygenase (Olson, 1989). Retinol is then

reesterified and stored in the lipid core of chylomicrons within the parenchymal cells of the liver and in stellate cells as retinyl esters. When vitamin A is needed, it is hydrolyzed to retinol and secreted from the liver bound to retinol-binding protein (RBP) in a 1:1 molar ratio (Kanai et al., 1968; Goss and McBurney, 1992; Olson, 1989).

The underlying action of retinoids is to influence cellular differentiation by inducing transcriptional activity of specific genes. Carcinoma stem cells (Breitman et al., 1980; Strickland and Mahdavi, 1978) and endothelial cells (Paige et al., 1991) have been known to differentiate when treated with retinoic acid. Further, the transcriptional activity of several genes, parathyroid hormone (Yasuda et al., 1989), transglutaminase (Chiocca et al., 1988; Nara et al., 1989), growth hormone receptor (Ohlsson et al., 1993), and cellular retinol-binding protein I, or CRBP I (Plateroti et al., 1993) have been shown to be affected by retinoic acid treatment resulting in either induction or inhibition.

The ability of retinoids to induce cellular proliferation has been less clear. Low doses of vitamin A in some studies induce proliferation, whereas in other studies, proliferation is suppressed. High doses of vitamin A were also found to inhibit cellular proliferation (Lotan, 1980). Daniel and coworkers (1991) found that $\beta$-carotene administered prior to calving enhanced lymphocyte

proliferation and suggested a role in protecting the mammary gland from infection.

Recently, retinoids (specifically synthetic retinoid analogs) have been used in cancer treatment (reviewed by Sporn et al., 1976) due to their effects on cellular proliferation and differentiation. They also have been recognized for their ability to act as free radical scavengers (Tesoriere et al., 1993; Davison et al., 1993; Olson, 1993; Bittrich et al., 1993).

## Vitamin A in reproduction

Vitamin A and retinoid binding proteins have been found in several extrahepatic sites. Vitamin A metabolites were found in bovine and porcine plasma, follicular fluid, and corpora lutea by Chew and coworkers (1984) indicating a role for vitamin A in reproduction. Several studies have confirmed that vitamin A-deficient animals or those with marginal vitamin A intakes had pregnancies that resulted in resorption, malformed fetuses, or decreased litter sizes (Takahashi et al., 1975; Mason, 1935; Wilson and Warkany, 1949; Wilson et al., 1953). Hypervitaminosis A may also result in fetal resorption and fetal abnormalities (Pillans et al., 1988; Kochhar, 1967; Lammer et al., 1985) due to the toxic effects of retinoids at high concentrations. Retinoic acid has been implicated as the most potent naturally occurring retinoid teratogen, but endogenous levels have

been detected in fetal tissues (Kraft and Juchau, 1993; Kraft et al., 1993). Neural crest cells have been suggested as a major target for retinoic acid during embryonic development (Dencker et al., 1990). The adverse effects of excess retinoic acid on embryonic development are varied and extensive (Yasuda et al., 1989; Steele et al., 1983; Thompson, 1969; Kraft and Juchau, 1993). At physiological concentrations, retinoic acid has been shown to induce polarizing activity in the chick limb bud (Noji et al., 1991), and to respecify pattern formation in the regenerating axolotl limb (Keeble and Maden, 1984).

## Retinol-binding protein

Serum RBP is initially synthesized in the liver as a preRBP (molecular weight=24 kD) and cotranslationally processed to a peptide with a molecular weight of approximately 21 kD (Kanai et al., 1968, Soprano et al., 1981). It functions to transport retinol in the blood (Kanai et al., 1968) and has been implicated as a buffer to protect cells against the toxic effects of high levels of retinol (Dingle et al., 1972). RBP accumulates in the liver during vitamin A deficiency. The factors which regulate its secretion are believed to be located in the endoplasmic reticulum. It has been shown that during retinol deficiency, RBP secretion is blocked in the endoplasmic reticulum before it can travel to the Golgi apparatus

(Soprano and Blaner, 1994; Smith and Goodman, 1979).

Plasma RBP has high amino acid sequence homology among several species with greater than 90% identity between human, bovine, and rabbit RBPs (Berni et al., 1990; Muto and Goodman, 1972). The major differences in the primary structure of RBP among different species are located on the surface of the molecule in the α-helix and the C-terminus. The high degree of conservation of the amino acid sequence may be due to the specific molecular interactions in which RBP participates (Berni et al., 1990).

RBP has binding affinity for most retinoids, but has highest affinity for retinol (Cogan et al., 1976). It has been suggested that an intact trimethylcyclohexenyl group found on most retinoid compounds is responsible for binding with RBP (Berni et al., 1993). It is believed that retinol must first bind RBP and this complex is then bound by transthyretin (TTR). In the reverse process in which retinol is released, retinol dissociates from RBP-TTR and then the RBP-TTR complex dissociates (Petersen, 1971; Noy et al., 1992).

Retinol-binding protein exists in a liganded form, holoRBP, or in an unliganded form, apoRBP. The crystal structure of bovine holoRBP has been determined and is structurally almost identical to human holoRBP (Zanotti et al., 1993a). The structure of RBP consists of a single domain which is composed of an N-terminal coil, eight

antiparallel β-strands, an α-helix and a C-terminal coil. Retinol binds at the β-barrel site causing a conformational change between the liganded and unliganded forms of RBP which results in the rotation of the side chain of Phenylalanine 36 toward the interior cavity of the β-barrel. This results in the displacement of the hydroxyl group of retinol in apoRBP (Zanotti et al., 1993b).

## RBP regulation in the liver

Most of our understanding of vitamin A and its metabolites have come from classical experiments performed in the rat. Within the liver and blood, RBP levels have been found to be controlled by retinoid status of the animal. Retinol must be present in order for RBP to be secreted by the liver. Vitamin A deficiency and depletion in rats caused a decrease in serum RBP levels, and concomitantly induced an increase in liver RBP levels as compared to normal rats (Muto and Goodman, 1972; Muto et al., 1972). Furthermore, if the vitamin A-depleted rats were supplemented with vitamin A, levels of RBP returned to normal. It has also been found that vitamin A-depleted rats have a lower rate of retinol utilization (Green et al., 1987). Vitamin A status does not however regulate the expression of RBP messenger ribonucleic acid (mRNA) within the liver (Soprano et al., 1986a, Soprano et al., 1982).

## Cellular retinoid-binding proteins

Within the cell, retinol is bound by cellular retinol-binding protein I or II (CRBP I or II), and retinoic acid is bound by cellular retinoic acid-binding protein I or II (CRABP I or II). The cellular retinoid-binding proteins belong to a family of low molecular weight carrier proteins which are found in most tissues (Wolf and Phil, 1991).

CRBP I (m.w.=15 kD) consists of 134 amino acids whose sequence is highly conserved among species. The tertiary structure of CRBP I and CRBP II are similar as determined by crystallographic studies (Ong et al., 1994). CRBP II has a molecular weight of approximately 16 kD and is composed of 134 amino acids (Ong, 1984; Ong et al., 1994). Both proteins bind retinol and retinal with high affinity, however CRBP I binds retinol with a higher affinity than retinal (Ong et al., 1994). In addition to regulating RBP secretion in the liver, it has been shown that secretion and expression of CRBP I mRNA are influenced by retinoid status (Plateroti et al., 1993; Kato et al., 1985; Rajan et al., 1990).

Several functional roles have been identified for these two proteins. Ottonello and coworkers (1993) found that retinol bound to CRBP I is used as a substrate for retinoic acid synthesis within the liver. In addition, CRBP I has been found to direct retinol to liver microsomes for esterification by an acyl-CoA independent enzyme reaction

(Yost et al., 1988). CRBP II is tissue-specific, found mostly within the intestinal mucosa (Ong, 1984). Free retinol undergoes esterification in the small intestine under *in vitro* conditions, and retinol bound to nonspecific serum proteins is esterified by an acyl-CoA:retinol acyltransferase activity. It is thought that in the *in vivo* system, esterification proceeds via lecithin-retinol acyltransferase when retinol is bound to CRBP II (Ong et al., 1987; Helgerud et al., 1983; MacDonald and Ong, 1988). It has been suggested that CRBP I and CRBP II play an important role in controlling the concentration and the type of retinoid found within a cell. CRBP I is mainly found in those tissues that require retinoic acid, while tissues primarily expressing CRBP II may have a lower tolerance for retinoic acid (Giguére, 1994). Expression of transcripts for CRBP and retinoic acid receptors (RARs) have also been detected in the uterine endometrium of pigs, indicating that CRBP may serve to facilitate the metabolism of retinol to retinoic acid for interaction with RARs within the nucleus (Harney et al., 1994a).

CRABP I and II differ in their N-terminal amino acid sequence and each have highly conserved amino acid sequences among different species (Ong et al., 1994). The levels of CRABP, in contrast to RBP and CRBP, have not been found to be controlled by retinoid status (Kato et al., 1985). The functional role of CRABP has been linked to embryogenesis.

It was suggested by Keeble and Maden (1984) that CRABP may be employed by retinoic acid to exert its action on pattern formation in the regenerating axolotl limb. Strong expression of CRABP was found in embryonic tissues that are colonized by neural crest cells, and Dencker and coworkers (1990) postulated that another role for CRABP may be to buffer the teratogenic effects caused by excess retinoic acid.

Gene knockout studies have shown interesting results for CRABP in mice. CRABP I null mutant mice appear normal and are indistinguishable from wild type mice (Gorry et al., 1994). Additionally, CRABP II mutant null mice also exhibit normal development, fertility, and life span, except for a limb malformation that is found to occur in approximately 50% of mutants. There seems to be no increase in expression of CRABP I or RAR transcripts in these CRABP II null mutant forms. Furthermore, CRABP I/CRABP II double null mutants appear to be normal, but display a limb malformation similar to the one mentioned above. Double mutant mice exhibit the same sensitivity to retinoic acid as wild type mice (Lampron et al., 1995). These studies demonstrated that CRABPs are not intrinsically involved in the retinoic acid signalling pathway.

## Retinoic acid receptors

Retinoic acid receptors (RARs) and retinoid X receptors (RXRs) are nuclear receptors that form homo- or hetero-dimers in order to bind to retinoic acid response elements (RAREs) on target genes. Many RAREs consist of a direct repeat of a core sequence AGGTCA spaced by 1 to 5-base pairs (Wan, 1993; Gudas, 1994). RARs can bind all-*trans* retinoic acid and 9-*cis* retinoic acid, whereas RXRs only bind 9-*cis* retinoic acid (Chambon, 1994). RAREs have been identified on the laminin B1 gene in mice, and transcription can be either induced or inhibited when receptor complexes bind to the RAREs (Vasios et al., 1989). MacDonald and coworkers (1994) found that parathyroid hormone expression is suppressed by retinoic acid which may occur via RARs or RXRs.

Currently, three RARs ($\alpha$, $\beta$, and $\gamma$) have been identified and multiple isoforms of each type exist (reviewed by Giguère, 1994). RARs consist of an N-terminus, a ligand-binding domain, a DNA-binding domain, a central region, and a C-terminus. The isoforms of each RAR differ in the N-terminal region (Chambon, 1994). Each of the RARs contains an RARE in their promoter region which indicates that RARs are retinoic acid responsive genes. The tissue distribution for the transcripts of each RAR varies; RAR$\alpha$ is found in most tissue types, RAR$\beta$ distribution is more restricted and found in abundance in the central nervous

system, and RARγ is mainly found in the skin and lungs (Giguère, 1994). In addition, RARs were localized in the uterus and ovary of several species (Zhuang et al., 1994).

During embryonic development and in adult forms, differential expression of the individual RARs exists suggesting that each RAR has a specific function (Yamagata et al., 1994; Giguère, 1994; Kato et al., 1992). The level of RARβ transcripts in rat tissues is regulated at the transcriptional level by retinoid status, whereas expression of RARα and γ mRNA does not seem to be as sensitive to retinoids (Kato et al., 1992; Harnish et al., 1992).

Several studies involving gene knockouts of the various RARs and their respective isoforms have been performed. RARβ null mutants appear and develop normally (Mendelsohn et al., 1994; Luo et al., 1995). In contrast, mutant mice lacking RARα or γ do not develop normally, and have reduced viability despite a normal appearance, suggesting that the retinoic acid signal is transduced through RARα and γ (Chambon, 1994; Lohnes et al., 1993). These studies have led several authors to suggest that a functional redundancy exists among RARs; if a particular RAR is lacking, another RAR can take over and perform its function (Chambon, 1994; Giguère, 1994; Luo et al., 1995)

To date, three RXRs (α, β, and γ) have been identified, but only RXRγ has been shown to have more than one isoform (Giguère, 1994). The RXRs and RARs vary quite considerably

in their amino acid sequences. The RXR proteins also consist of a DNA-binding domain and a ligand-binding domain and vary from each other in their N-terminus (Mangelsdorf et al., 1994). RXRs $\alpha$ and $\beta$ are ubiquitously expressed, while the distribution of RXR$\gamma$ is more restricted (Giguère, 1994). Gene knockouts for the RXRs have revealed that the RXR$\alpha$ null mutation is embryonic lethal, suggesting that RXR$\alpha$ is crucial during embryonic development (Kastner et al., 1994). RXRs have been shown to form either hetero- or homodimers with a number of nuclear receptors in order to mediate retinoic acid actions, indicating a role of the RXRs to create several pathways for retinoid signaling (Giguère, 1994).

## RBP membrane receptors

The existence of a membrane receptor for RBP for internalization of retinol into a cell has been somewhat controversial. While some investigators have found cells displaying cell-surface RBP binding activity, there has not been a thorough biochemical characterization of an RBP membrane receptor.

The most characterized membrane receptor has been found in the retinal pigment epithelium, which binds RBP in a 1:1 molar ratio. This protein has been described as having a molecular weight of approximately 63 kD (Båvik et al.,1992). Furthermore, the gene for this receptor appears to be highly

conserved among several species (Båvik et al., 1993). Additionally, high binding activity for RBP has been localized in the placenta, bone marrow, and kidneys, and lower binding activity exists in the small intestine, spleen, liver, lung, and testis (Smeland et al., 1995).

## Uterine RBP

The secretions of the uterus, or histotrophe, have a role in nutrition and in directing growth and development of the conceptus during pregnancy (Roberts and Bazer, 1988). These secretions are synthesized by the surface and glandular epithelium of the uterine endometrium (Roberts et al., 1993). In the uterus, RBP has been identified as a component of histotrophe (Roberts and Bazer, 1988).

The porcine uterine RBP has been found to be identical to serum RBP immunologically and by amino acid sequence analysis (Stallings-Mann et al., 1993). In cyclic pigs, expression of RBP mRNA was low or undetectable in the early to midluteal phase, increased during the late luteal phase, and decreased thereafter to the end of the estrous cycle. Pregnant gilts, on the other hand, maintain the increase in RBP mRNA expression, suggesting that the gene for RBP is regulated by progesterone (Harney et al., 1993). During pregnancy, RBP has been localized in the surface and glandular epithelium of the uterine endometrium (Harney et al., 1990). Ovariectomized gilts treated with progesterone

showed an increase in uterine secretion of RBP compared to those receiving corn oil vehicle alone (Adams et al., 1981). Furthermore, treatment of ovariectomized gilts with progesterone on days 3-16 and with estrogen on days 1-2 and 13-14 increased RBP expression, suggesting that estrogen secreted by elongating conceptuses may serve to increase uterine RBP expression as well as the signal for maternal recognition of pregnancy (Trout et al., 1992; Harney et al., 1994b).

Similar results have been obtained in the ovine and bovine species for the regulation of uterine expression of RBP. Bovine RBP has been characterized as a protein with a molecular weight of 21 kD consisting of several isoelectric variants (Liu and Godkin, 1992). The uterine species of RBP in the bovine shares complete sequence homology with placental and plasma RBP over the first 25 amino acids. Levels of RBP in uterine flushes of cows were elevated during midcycle, and barely detectable early and late in the estrous cycle, but remained elevated during early pregnancy (Liu and Godkin, 1992; Doré et al., 1995). Expression of RBP transcripts in cyclic cows declines early in the estrous cycle and reaches low levels on days 7-10, and then rises and peaks during proestrus (day 20). Furthermore, RBP mRNA expression is similar in pregnant and cyclic cows during diestrus, but levels of RBP mRNA in pregnant animals increase from day 17 to day 22 and remain elevated

thereafter through day 36 of pregnancy (MacKenzie et al., 1994). Studies involving primary luteal phase uterine epithelial cell cultures have shown that retinol and estrogen each cause an increase in RBP secretion, while retinoic acid and progesterone have no effect (Doré et al., 1995). Additionally, uterine concentrations of RBP are enhanced in ovariectomized cows by progesterone treatment (Thomas et al., 1992). Uterine RBP has been localized in the apical regions of surface epithelium and in the luminal regions of glandular epithelium suggesting that RBP may be secreted from the surface epithelium into the uterine lumen (Liu and Godkin, 1992).

Endometrial expression of RBP mRNA was elevated during midcycle in both cyclic and pregnant ewes and remained elevated in cyclic ewes, but decreased in pregnant ewes during the late luteal phase (days 14-16) (Harney et al., 1993). However, Doré and coworkers (1994) found RBP expression decreased from day 13 to day 16 in cyclic ewes, again suggesting control of RBP expression may be regulated by progesterone. Both authors suggested the decrease in RBP expression of day 16 pregnant ewes could be regulated by conceptus secretory products. After the decrease seen in RBP mRNA on day 16 in pregnant ewes, RBP transcript levels are stabilized through day 30 (Doré et al., 1994).

## Conceptus RBP

Another extrahepatic site of retinoid-binding proteins and of vitamin A that has recently been investigated is fetal tissues. Retinoic acid has been detected in second trimester fetal tissues of humans (Kraft et al., 1993), and seems to target neural crest cells and neural crest-derived tissues during fetal development in mice (Dencker et al., 1990).

In the rat, expression of RBP and TTR has been found in the fetal liver. In addition, the visceral yolk sac contained these transcripts. Explants of this tissue synthesized and secreted RBP and TTR (Soprano et al., 1986b). Expression of RBP increased from day 7 to 9 of development (Makover et al., 1989), while RBP protein concentrations remained low. A very high molar ratio of vitamin A:RBP was observed during this time (Takahashi et al., 1977). RBP increased from days 11-14 (Makover et al., 1989), and CRABP expression was maximal during this period (Dencker et al., 1990). The molar ratio of vitamin A:RBP decreased to approximately 1 between days 11 and 14 suggesting that vitamin A may have been transported to the conceptus independently of RBP earlier, but forms a complex with RBP on days 11-14 (Takahashi et al., 1977). Soprano and coworkers (1988) found that by day 14, the visceral yolk sac and fetal liver both synthesize and secrete RBP. Availability of vitamin A seems to play a role in the

regulation of conceptus RBP in the rat yolk sac similar to that seen in the liver. In a retinol-depleted state, the visceral yolk sac and fetal liver have greatly elevated levels of RBP, and repletion of retinol causes RBP levels to return to normal. In addition, RBP mRNA expression in the retinol-depleted tissues was not affected (Soprano et al., 1988). Takahashi and coworkers (1977) suggested that maternal retinol-RBP can cross the uterine-placental barrier from dam to fetus early in gestation.

Porcine conceptus RBP (pcRBP) has been found to have a molecular weight of 21 kD and to be identical to porcine serum RBP as determined by amino acid sequence analysis (Trout et al., 1991). Harney and coworkers (1994a) have found that pcRBP can bind retinol and retinoic acid *in vitro*, but has a higher affinity for retinol. In addition to RBP, porcine conceptuses express CRBP and RAR genes during the peri-implantation period of pregnancy. Secretion of RBP by conceptuses in culture increases between days 10 and 15 of pregnancy (Harney et al., 1990), and high levels of RBP mRNA have been detected in the day 15 conceptus (Harney et al., 1994b). Two other important events coincide with this time frame: the period of rapid elongation and maternal recognition of pregnancy which is signaled by secretion of estrogen by the conceptus (Roberts and Bazer, 1988). RBP mRNA was detected in the inner cell mass of day 11 spherical conceptuses (Trout et al., 1991). In addition,

RBP was detected in the trophectoderm and yolk sac of day 15 conceptuses and in allantoic fluid from day 30 of pregnancy (Harney et al., 1990).

Conceptus RBP in cows is also thought to be identical to plasma RBP as determined by amino acid sequence analysis and shares high sequence homology with pcRBP (Liu et al., 1990; Liu et al., 1993). Secretion of RBP by bovine embryos seems to be developmentally regulated in that RBP is detected in tissues and is synthesized and secreted by day 13 conceptuses that have elongated, but not by spherical day 13 conceptuses. During the period of elongation, RBP has been localized mainly within the trophectoderm (Liu et al., 1993). In situ hybridization studies have shown bovine placental RBP (bpRBP) to be a product of the chorion, allantois, and amnion. It has been located in the epithelial lining of these membranes, at days 45 and 135 of gestation (Gao et al., 1991; Liu et al., 1993). RBP has been immunolocalized in the basal and spinosum cell layers of day 160 amnion (Liu et al., 1994). Liu and coworkers (1990) found a low molar ratio of retinol:bpRBP, however it was not determined whether this occurred *in vivo* or was due to purification processes.

The protein profile of secreted proteins for ovine conceptuses closely resembles that of bovine conceptuses, however there are distinct temporal differences (Bartol et al., 1985; Godkin et al., 1988). For example, the

production of bTP-1 is twice as long in duration than oTP-1 production (Godkin et al., 1982). However it is reasonable to postulate that secretion of RBP by the ovine conceptus may be similar to that of the bovine conceptus. RBP has been detected at all time points examined during pregnancy and is therefore probably produced throughout pregnancy in both species (Liu et al., 1992; Liu et al., 1993). As in porcine and bovine conceptuses, secretion of ovine conceptus RBP (ocRBP) increased between day 13 and day 16 (Doré et al., 1994; Liu et al., 1992) which coincides with blastocyst elongation (Bindon, 1971) and maternal recognition of pregnancy (Godkin et al., 1982). At this time, ocRBP has been immunolocalized in the trophectoderm (Liu et al., 1992). Placental RBP has been detected in the chorion, allantois, amnion, and yolk sac during the first trimester of ovine (Liu et al., 1991; Liu et al., 1992) and caprine conceptuses (Liu et al., 1995). In the goat, RBP has been immunolocalized in the trophectoderm of the chorion, the endoderm of the allantois, and the ectoderm of the amnion (Liu et al., 1995). N-terminal sequence analysis of the first 34 residues indicates ovine placental RBP shares complete homology with bovine placental and plasma RBP and extensive homology with porcine conceptus and human plasma RBP (Liu et al., 1992). Similarly, caprine placental RBP shares complete amino acid sequence homology with bovine plasma RBP and extensive homology with caprine and bovine

placental RBP over the first 15 amino acids (Liu et al., 1995). At day 23 of gestation, ovine placental RBP is a major protein product and transcript (Doré et al., 1994; Liu et al., 1991). Donoghue and coworkers (1982) found that the carrier protein for the retinol-RBP complex in sheep was different in fetal circulation than in the adult; either TTR is modified or retinol-RBP forms a complex with another protein in the fetal circulation. As with bpRBP, a low molar ratio of retinol:opRBP was found suggesting RBP may be secreted in an apo- form (Liu et al., 1992).

## CHAPTER II

# REGULATION OF RETINOL-BINDING PROTEIN SECRETION BY RETINOIDS IN THE OVINE CONCEPTUS


### Abstract

During early pregnancy in sheep, implantation occurs relatively late and fetal-maternal interactions are critical to maintenance of pregnancy, embryonic survival and development. Reproductive processes are mediated in part by conceptus secretory proteins and uterine proteins. Vitamin A (retinol) plays a role in reproductive processes and must be present in precise amounts for normal embryonic development. Most retinol is stored in the liver and transported bound to retinol-binding protein (RBP). Within the liver, the levels of RBP are regulated by retinoid status. Previous studies have suggested that placental RBP may be secreted in an apo- form by the conceptus in order to buffer the teratogenic effects of retinoids. The purpose of this study was to determine if retinoids influence RBP secretion by the conceptus and to determine if conceptus RBP is secreted in an apo- or holo- form. Day 14 ovine conceptuses were obtained from uterine flushes and cultured in Dulbecco's minimal essential medium (DMEM) for 24 and 48 hours with addition of retinoids (10 $\mu$M all-trans retinol, 1 or 10 $\mu$M all-trans retinoic acid) in order to evaluate the

effects of retinoid treatment on secretion and synthesis of RBP by the conceptus. Concentration of RBP was determined by an enzyme linked immunosorbant assay (ELISA) in culture medium and solubilized conceptus tissues. Retinol concentration of control conceptus cultures (no addition of retinoids) was analysed by extracting culture medium and quantifying retinol-associated fluorescence of the extracts. There were no differences in RBP concentration due to treatments in either the culture medium or conceptus tissues. Retinol was undetectable in the medium of control conceptus cultures. The results of this study demonstrate regulation of RBP in the conceptus may be different than that of the liver since physiological doses of retinoids did not have an effect on RBP secretion or synthesis in the day 14 ovine conceptus. The failure to detect retinol in medium of control conceptus cultures indicates that either RBP is secreted in an apo- form or retinol concentrations were below the sensitivity of the assay.

## Introduction

In sheep, implantation is not fully completed until the fourth or fifth week of gestation, and definitive attachment of the trophoblast to maternal tissues is not intiated until about Day 15 (Boshier, 1969). At approximately day 13 of gestation, the conceptus undergoes a period of rapid elongation and changes in form from a spherical to

threadlike form (Bindon, 1971). During elongation, the conceptus initiates secretion of proteins in high concentrations (Godkin et al., 1982; Masters et al., 1982). The major secretory protein, interferon-$\tau$ (IFN-$\tau$), is now recognized as the conceptus antiluteolytic factor which is responsible for maintaining luteal function during early pregnancy (Godkin et al., 1982; Bazer et al., 1986). IFN-$\tau$ inhibits luteolysis by down-regulating estrogen and oxytocin receptors which cause the episodic secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), thereby extending progesterone secretion by the corpus luteum (Spencer et al., 1995b). This period of time is therefore a crucial stage in development for the conceptus.

RBP is first synthesized and secreted during the expansion period, but its regulation is not understood (Doré et al., 1994; Liu et al., 1992). RBP has been localized in the trophectoderm of preimplantaion stage ovine embryos, and it is secreted throughout much of pregnancy (Liu et al., 1992). Vitamin A is necessary for normal pregnancy and embryonic development (Takahashi et al., 1975; Mason, 1935; Wilson and Warkany, 1949; Wilson et al., 1953), however, excess vitamin A can lead to fetal malformations or resorption (Pillans et al., 1988; Kochhar et al., 1967; Lammer et al., 1985). Therefore RBP secretion by the conceptus may function to distribute an appropriate amount of retinol to conceptus tissues.

Retinol is stored mainly within the liver and secreted bound to RBP (Kanai et al., 1968). RBP secretion in the liver is controlled by retinoid status; retinol is necessary for RBP to be secreted. In a vitamin A-deficient state, RBP increases in the liver, while circulating levels in blood decrease. Upon retinol repletion, both liver and blood levels of RBP return to normal (Muto and Goodman, 1972). Hepatic expression of RBP messenger ribonucleic acid (mRNA) is not, however, regulated by dietary intake of vitamin A (Soporano et al., 1986a; Soprano et al., 1982).

Ovine placental RBP (opRBP) has a molecular weight of 23 kD and is synthesized by the trophectoderm during blastocyst elongation and well into pregnancy (Liu et al., 1991; Liu et al., 1992). Mechanisms which control RBP production by the conceptus are unknown. Secretion of RBP by the fetal yolk sac in rats is similarly controlled by retinoid status; secretion is inhibited in a vitamin A-deficient state and returns to normal upon retinol repletion (Soprano et al., 1988). The purpose of this study was to determine if RBP secretion by the day 14 conceptus is affected by retinoids and whether conceptus RBP is secreted in an apo- or holo- form. Previous studies in our laboratory have resulted in the discovery of a low molar ratio of retinol:RBP, but it was not determined whether RBP was secreted in an apo- form or if bound retinol had been lost during the purification process of RBP (Liu et al.,

1992).

## Materials and Methods

Culture medium supplies and buffers were purchased from GibcoBRL (New York). Culture dishes were obtained from Becton Dickinson (Lincoln Park, NJ). Microtiter plates (96 well) were obtained from Corning (Corning, NY). Conjugate was purchased from Bio-Rad (Hercules, CA). The p-nitrophenylphosphate (p-NPP) phosphatase substrate was obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Triton X-100 was scintillation grade and purchased from Amersham (Arlington Heights, IL). Protein assay reagents were obtained from Pierce (Rockford IL). The Superose-12 column was purchased from Pharmacia (Uppsala, Sweden). $^{3}$H-Retinol was obtained from NEN Research Products (Wilmington, DE). o-Xylene was purchased from Baxter. All other reagents were purchased from Sigma (St. Louis, MO).

*Collection of Embryos:* Sheep were bred at estrus (Day 0) with an intact ram. On day 14 of pregnancy, ewes were anesthetized, placed in a dorsal recombinant position and the uterus exteriorized through a midventral incision. The uterus was gently flushed by clamping the cervix with intestinal forceps. A cathetor was inserted in the contralateral uterine horn. Twenty ml Hank's buffered saline solution (HBSS) was injected into the uterine horn ipsilateral to the corpus luteum (CL), and the medium and

conceptus gently flushed around the uterine body and out through the catheter and collected in a small glass bottle. In most cases, a second 20 ml flush was performed to ensure the entire embryo had been collected. The contents of the bottle were transported to the laboratory within 15 minutes. Embryos were washed in HBSS and transferred to 35 X 10mm culture dishes containing 5 ml of a Dulbecco's modified minimum essential medium with treatments (Godkin et al., 1988) and cultured for 24-48 hours. Treatments were prepared in ethanol and consisted of all-trans retinol (10 $\mu$M), all-trans retinoic acid (1 or 10 $\mu$M), and controls (no addition of retinoids). Retinoid treatments were performed under subdued lighting due to the light-sensitivity of these compounds. Medium was removed after 24 hours and replaced with fresh culture medium and treatment, and the conceptus was cultured for an additional 24 hours. Uterine flushes and culture medium were centrifuged (2500 rpm) to remove cellular debris, the supernatant transferred to tubes wrapped in aluminum foil, and frozen at -80°C for analysis. Conceptus tissues were retained in the same manner.

*ELISA:* An ELISA was performed on the conceptus culture medium and the solubilized conceptus tissues to quantitate RBP. ELISA buffers are shown in Appendix 1. Standards were prepared from purified serum RBP (Liu et al., 1990) ranging from 0.1-10 ng/well and stored at -20°C. Microtiter plates were coated with RBP at a concentration of 20 ng/well and

allowed to incubate overnight in a humidified chamber at 4°C. The antigen-coated plates were washed with wash buffer using a Dynatech Ultrawash II Microplate Washer (Chantilly, VA), tapped dry, and stored in a sealed bag with dessicant at -20°C. Standards and diluted samples were mixed with a bovine placental RBP antibody (anti-bpRBP) (Liu et al., 1990) to a final dilution of 1:10,000 in 12 X 75mm borosilicate glass test tubes and allowed to incubate overnight at 4°C. The following morning, the sample-anti-bpRBP mixtures were transferred to the antigen-coated plate and incubated in a humidified chamber at 30°C for 2 hours. The plate was washed and tapped dry. Goat anti-rabbit IgG alkaline phosphatase conjugate was diluted 1:3,000 in room temperature diluent and transferred to the wells and incubated in a humidified chamber at 30°C for 2 hours. The wells were washed and tapped dry. p-NPP phosphatase substrate was prepared according to the manufacturer's directions (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Substrate was added to wells and incubated at 30°C in a humidified chamber until the 100% antibody wells (antigen-coated control wells with anti-bpRBP only) reached an optical density of approximately 0.8-0.9 when read at an absorbance of 405 nm. At that time, 5% $Na_2EDTA$ was added to the wells to stop the reaction and the plate was read at an absorbance of 405 nm on a BioTek Automated Microplate Reader (Winooski, VT) to calculate the results. The intra-assay

variation was <5% and the inter-assay variation was <10%.

*DNA Assay:* Cell lysis - Conceptus tissues were solubilized by adding 1 ml of 1% Triton. Cells were vortexed ½ hour, incubated at 40°C 1 hour, vortexed ½ hour, incubated at 40°C 1 hour, vortexed 2 hours, frozen overnight at -80°C, thawed the next day, and then centrifuged to pellet out cellular debris. The supernatant was drawn off for DNA analysis.

Procedure - Calf thymus DNA standards ranging from 0-1000 ng/ml and samples were prepared in buffer (150 mM NaCl, 5 mM HEPES, pH 7) and analyzed in triplicate in 1.5 ml eppendorf tubes. Six $\mu$l 4,6-Diamidino-2-phenylindole (DAPI) was added to the standards and samples (114 $\mu$l) at a final concentration of 200 ng/ml and these mixtures were transferred to a microtiter plate. All procedures involving DAPI were performed under subdued lighting because it is light-sensitive. After wells were loaded with the sample+ and standard+DAPI mixtures, fluorescence was determined using a Millipore Cytofluor 2350 (Bedford, MA) with excitation maximum set at 360 nm and emission maximum at 460 nm. The intra-assay variation was <5%.

*Protein Assay #1:* Conceptus culture medium - The Pierce Coomassie Protein Assay Reagent was used to determine total protein in conceptus culture medium. Directions for the Micro Assay Procedure using the Microtiter Plate Formula was followed according to the manufacturer's directions.

Briefly, bovine serum albumin (BSA) protein standards ranging from 0-25 μg/ml and samples were prepared in triplicate in 10 mM Tris buffer, pH 7.6, in 12 X 75mm borosilicate glass tubes. The Coomassie Protein Assay Reagent was mixed with standards or samples in a 1:1 ratio (volume:volume) to a final volume of 700 μl. Samples (200 μl/well) were transferred to a microtiter plate and absorbance was read on a platereader at 595 nm. The protein concentration of the samples was determined from the standard curve obtained by plotting absorbance vs. standard concentration. The intra-assay variation was <5%.

*Protein Assay #2:* Conceptus tissues - Because Triton interferes with the Coomassie Blue Dye assay described above, protein concentrations of the conceptus tissues were determined using the Pierce MicroBCA Protein Assay Reagent Kit following the Microtiter Plate Protocol. Briefly, BSA protein standards were prepared ranging from 0-50 μg/ml, and samples were diluted in HEPES buffer (150 mM NaCl, 5 mM HEPES, pH 7). Samples and standards were mixed in a 1:1 ratio (volume:volume) with the MicroBCA Protein Assay Reagent to a final volume of 700 μl in 12 X 75mm borosilicate glass tubes, and then transferred in triplicate to a microtiter plate (200 μl/well). The plate was covered and incubated at 60°C for 1 hour. The cover was removed, and the plate was read on a platereader at an absorbance of 570 nm. Sample protein concentrations were determined from

a standard curve constructed by plotting absorbance vs. protein standard concentrations. The intra-assay variation was <5%.

*HPLC:* Conceptus culture medium was chromatographed on a Superose-12 column utilizing an LKB HPLC unit (Bromma, Sweden) to determine the concentration of retinol-bound RBP by following the procedure of Trout and coworkers (1992). The elution buffer was 1.5 M NaCl, 0.02 M Tris-HCl, 0.02% azide, pH 8.2, and the flow rate was set at 0.5 ml/min for all chromatography. To determine the elution position of RBP, 1 ml of purified RBP was dried in a Savant Speed-Vac Concentrator (Farmingdale, NY) and resuspended in 300 $\mu$l of deionized water, 250 $\mu$l of which was submitted to column chromatography with protein being monitored at 280 nm. One ml of sample (conceptus culture media) was dried, prepared and chromatographed on the column as before. The two elution profiles were compared to find a common peak, however there were several peaks in common. The next step was to run several protein standards on the column to obtain a standard curve by plotting fraction # vs. log of the molecular weight of protein standards. The following were used as protein standards:

| | |
|---|---|
| $\gamma$-globulin, | mol. wt. 150,000Daltons |
| $\beta$-galactosidase, | mol. wt. 116,250 Daltons |
| pig albumin, | mol. wt. 67,000 Daltons |
| ovalbumin, | mol. wt. 43,000 Daltons |
| carbonic anhhydrase, | mol. wt. 31,000 Daltons |
| cytochrome C, | mol. wt. 29,000 Daltons |
| soybean trypsin inhibitor, | mol. wt. 20,000 Daltons |

| | |
|---|---|
| myoglobin, | mol. wt. 15,500 Daltons |
| lysozyme, | mol. wt. 14,400 Daltons |

The standard curve obtained was not very satisfactory, especially in the range of the lower standard molecular weights which was the area of interest. Therfore, a new elution buffer of lower salt concentration (0.15 M NaCl, 0.02 M Tris-HCl, 0.02% azide, pH 8.2) was prepared and used in the remainder of the experiment. The protein standards were rechromatographed, but the same standard curve was obtained. Another option was tried in which conceptus culture medium was dried down, prepared as before, and run on the column with the fractions being analyzed by ELISA (described earlier). RBP was not detected in any of the eluted fractions. Next, several fractions suspected to be associated with the RBP peak were run on a one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDS-PAGE, 12%) followed by Coomassie Brilliant Blue staining according to the Mini-Protean II Dual Slab Cell Instruction Manual (Richmond, CA). Again RBP protein bands were not detected in the lanes loaded with the sample fractions. Alternatively, purified RBP was incubated with retinol, dried, prepared as before, and chromatographed on the HPLC column. Fractions were collected in foil-wrapped tubes, and several fractions were read on a TKO 100 Dedicated Mini Fluorometer (Hoefer Scientific Instruments, San Francisco, CA) to determine if a peak of retinol

fluorescence coincided with a peak on the protein elution profile. There was no fluorescence peak associated with the fractions. A final attempt to determine the elution position of RBP was made by incubating purified RBP with $^{3}$H-retinol. $^{3}$H-Retinol and the RBP-$^{3}$H-retinol mixture were both chromatographed on the column and the protein absorbance peaks were compared with the radioactivity peaks and RBP was determined to elute at fractions 31-33. Several samples of each treatment (1 ml each) were dried, resuspended in 300 $\mu$l deionized water, and 200 $\mu$l was chromatographed with protein monitored at 280 nm. These runs were performed in subdued lighting and fractions 29-33 were collected in glass tubes and wrapped in aluminum foil. These fractions were subsequently analyzed by a Perkin Elmer Luminescence Spectrometer LS50B, with excitation wavelength set at 378 nm and emission wavelength set at 455 nm.

*Retinol Extraction and Quantitation:* Retinol concentrations in uterine flushes and control conceptus culture medium were to be determined by fluorescence analysis after xylene extraction using a procedure similar to that of Selvaraj and Susheela (1970). This entire procedure was performed in subdued lighting. Retinol standards were prepared in medium ranging from 0-1.0 $\mu$g/ml. Extraction of retinol from culture medium samples was performed by adding 1 ml of 95% ethanol to 1 ml standard or sample. After vortexing, tubes were placed in a 60°C heater

block for 20 minutes, and then allowed to cool. Xylene (1.8 ml) was added to the tubes and vortexed for 20 seconds. The tubes were centrifuged for approximately 2 minutes to separate the 2 layers and the upper xylene layer containing retinol was transferred into a separate glass tube. Fluorescence was determined by a Baxter Schimadzu Spectrofluorometer at an excitation wavelength of 325 nm and emission wavelength of 480 nm. A standard curve was prepared by plotting standard retinol concentrations vs. fluorescence.

For the uterine flushes, 5 ml of 95% ethanol were added to 5 ml standard or sample in a glass tube and vortexed. The tubes were incubated at 60°C for 20 minutes and allowed to cool. Six ml of xylene were added to the tubes and vortexed for 20 seconds. The layers were allowed to separate and the upper xylene layer was transferred to another glass tube. Fluorescence of standards and samples was determined the same as for the conceptus culture medium described above.

*Statistical Analysis:* Amount of conceptus RBP per amount of total conceptus cellular or total conceptus secreted protein was analyzed for effects of treatment, day, and treatment by day interactions. The experimental design was a completely randomized design and data were analyzed by the General Linear Models procedure of SAS (SAS, 1990). Means were separated by Fisher's least significant

difference test. Data was log transformed when necessary. Significance was considered at the 95% confidence level.

## Results

*RBP Secretion by Ovine Embryos:* The raw values for RBP in culture medium and in conceptus tissues are shown in Tables 1-1a, 1-1b, and 1-1c and Table 1-2, respectively. In order to determine the amount of RBP secreted per amount of conceptus tissue, RBP values were standardized with the total amount of conceptus protein or with the total amount of secreted conceptus protein. Initially, RBP values were to be standardized with the total DNA content of the conceptus, but Triton, in which conceptus tissues were solubilized, interferes with the DNA assay (data not shown). There were no differences due to treatments or treatment by day interaction in the amount of secreted RBP and the amount of RBP in conceptus tissues whether standardized by the total amount of secreted protein (TSP) (Table 1-3a, 1-3b, and 1-3c) or by the total amount of protein in conceptus tissues (TCP) (Table 1-4a, 1-4b, and 1-4c). There was no effect of day when RBP secretion was standardized with TCP (Table 1-4d), but there was a significant difference in the amount of RBP secreted by the conceptus from day 1 to day 2 when RBP values were standardized with TSP (Table 1-3d).

*RBP in Conceptus Tissues:* There were no differences in the amount of RBP within conceptus tissues when expressed as

a percentage of the total amount of protein in conceptus tissues (Table 1-5).

*Determination of apo- and holo-RBP:* HPLC fractions containing RBP were analyzed to determine if the fractions were associated with fluorescence characteristic of retinol. No fluorescence was detected in the fractions.

*Retinol extraction and quantitation:* No fluorescence was detected in the uterine flushes or control conceptus culture medium after being subjected to extraction.

**Table 1-1a.** Raw RBP Concentration Secreted by Day 14 Ovine Conceptus in Culture Medium on Day 1 of Culture.

| Treatment | N | RBP in culture medium (ng/ml) |
|---|---|---|
| Control | 16 | 65.888+/-6.146[a] |
| Retinol | 14 | 70.717+/-6.571[a] |
| 1μM RA | 12 | 65.793+/-7.097[a] |
| 10μM RA | 5 | 47.242+/-10.995[a] |
| [a]Mean+/-S.E.; no significant differences found P>0.05. | | |

**Table 1-1b.** Raw RBP Concentration Secreted by Day 14 Ovine Conceptus in Culture Medium on Day 2 of Culture.

| Treatment | N | RBP in culture medium (ng/ml) |
|---|---|---|
| Control | 16 | 68.445+/-6.146[a] |
| Retinol | 14 | 66.054+/-6.571[a] |
| 1μM RA | 12 | 62.788+/-7.097[a] |
| 10μM RA | 5 | 40.289+/-10.995[a] |
| [a]Mean+/-S.E.; no significant differences found P>0.05. | | |

**Table 1-2.** Raw RBP Concentration in Conceptus Tissues of Day 14 Ovine Conceptus at End of 48 Hour Culture Period.

| Treatment | N | RBP in conceptus tissues (ng/ml) |
|---|---|---|
| Control | 15 | 106.422+/-14.122[c] |
| Retinol | 13 | 163.750+/-15.169[a] |
| 1μM RA | 12 | 127.827+/-15.789[ab] |
| 10μM RA | 5 | 82.655+/-24.460[bc] |
| [a,b,c]Mean+/-S.E.;values with different superscripts are significantly different p<0.05. | | |

**Table 1-3a.** RBP Secretion by Day 14 Ovine Conceptus due to Treatment as % Total Protein (TSP) in Culture Medium on Day 1 of Culture.

| Treatment | N | RBP as % TSP in culture medium (ng RBP/ng TP X 100) |
|---|---|---|
| Control | 15 | 0.297+/-0.04[a] |
| Retinol | 12 | 0.229+/-0.05[a] |
| 1μM RA | 12 | 0.235+/-0.04[a] |
| 10μM RA | 5 | 0.172+/-0.07[a] |
| [a]Mean+/-S.E.; no significant differences found P>0.05. | | |

**Table 1-3b.** RBP Secretion by Day 14 Ovine Conceptus due to Treatment as % TSP in Culture Medium on Day 2 of Culture.

| Treatment | N | RBP as % TSP in culture medium (ng RBP/ng TP X 100) |
|---|---|---|
| Control | 12 | 0.680+/-0.05[a] |
| Retinol | 11 | 0.525+/-0.05[a] |
| 1μM RA | 9 | 0.578+/-0.06[a] |
| 10μM RA | 6 | 0.680+/-0.05[a] |
| [a]Mean+/-S.E.; no significant differences found P>0.05. | | |

**Table 1-3c.** RBP Secretion by Day 14 Ovine Conceptus as % TSP in Culture Medium on Day 1 and Day 2 of Culture.

| Day | N | RBP as % TSP in culture medium (ng RBP/ng TSP X 100) |
|---|---|---|
| 1 | 44 | 0.233+/-0.03[a] |
| 2 | 38 | 0.578+/-0.03[b] |
| [a,b]Mean+/-S.E.; values with different superscripts are significantly different p<0.001. | | |

**Table 1-4a.** RBP Secretion by Day 14 Ovine Conceptus due to Treatment as % Total Protein (TCP) in Conceptus Tissues on Day 1 of Culture.

| Treatment | N | RBP as % TCP in conceptus tissues (ng RBP/ng TCP X 100) |
|---|---|---|
| Control | 16 | 0.0228+/-0.003[a] |
| Retinol | 14 | 0.0201+/-0.003[a] |
| 1$\mu$M RA | 12 | 0.0259+/-0.003[a] |
| 10$\mu$M RA | 5 | 0.0293+/-0.005[a] |
| [a]Mean+/-S.E.; no significant differences found P>0.05. | | |

**Table 1-4b.** RBP Secretion by Day 14 Ovine Conceptus due to Treatment as % TCP in Conceptus Tissues on Day 2 of Culture.

| Treatment | N | RBP as % TCP in conceptus tissues (ng RBP/ng TCP X 100) |
|---|---|---|
| Control | 16 | 0.0286+/-0.003[a] |
| Retinol | 14 | 0.0175+/-0.003[a] |
| 1$\mu$M RA | 12 | 0.0249+/-0.003[a] |
| 10$\mu$M RA | 5 | 0.0266+/-0.005[a] |
| [a]Mean+/-S.E.; no significant differences found P>0.05. | | |

**Table 1-4c.** RBP Secretion by Day 14 Ovine Conceptus as % TCP in Conceptus Tissues on Day 1 and Day 2 of Culture.

| Day | N | RBP as % TCP in conceptus tissues (ng RBP/ng TCP X 100) |
|---|---|---|
| 1 | 47 | 0.0245+/-0.002[a] |
| 2 | 47 | 0.0244+/-0.002[a] |
| [a]Mean+/-S.E.; no significant differences found P>0.05. | | |

**Table 1-5.** RBP in Conceptus Tissues of Day 14 Ovine Conceptus due to Treatment as % TCP in Conceptus Tissues During 48 Hour Culture Period.

| Treatment | N | RBP as % TCP in conceptus tissues (ng RBP/ng TCP X 100) |
|---|---|---|
| Control | 15 | 0.0547+/-0.009[a] |
| Retinol | 13 | 0.0482+/-0.009[a] |
| 1$\mu$M RA | 12 | 0.0505+/-0.010[a] |
| 10$\mu$M RA | 5 | 0.0583+/-0.015[a] |
| [a]Mean+/-S.E.; no significant difference found P>0.05. | | |

## Discussion

This study examined the role of retinoids in RBP secretion in the Day 14 ovine embryo. The period between days 13 and 15 is crucial for the developing conceptus and many important events occur at this time. Embryo elongation is initiated between days 12 and 14 (Bindon, 1971) and proteins begin to be synthesized and secreted. Of major importance, ovine IFN-$\tau$ (oIFN-$\tau$), the protein responsible for maintenence of early pregnancy, is secreted at this time and RBP secretion also begins to occur during this period of

time. Because implantation is not completed until the fourth or fifth week of gestation (Boshier, 1969), there is no intimate contact between maternal and fetal tissues at day 14 of gestation.

Vitamin A, while necessary for normal development, can be embryotoxic at high concentrations so that the regulation of RBP is of major importance to the survival of the conceptus during early gestation. Liu and coworkers (1990) have previously determined that the molar ratio of retinol:RBP found in placental tissue cultures was low, indicating that RBP was secreted in the apo- form. However, it was not concluded whether this was a natural phenomena or an artifact that occurred during the purification of RBP. Initially, it was hypothesized that retinoids may cause RBP secretion to be elevated in order to buffer the teratogenic effects of these compounds. Clearly the results of this study have shown that retinoid treatment does not affect RBP secretion in the ovine conceptus at Day 14 of gestation.

It is well established that retinol is necessary for RBP secretion in the liver. The results of this study have shown that RBP secretion was not different in retinoid-treated embryos compared to controls leading to the conclusion that neither retinol nor retinoic acid affect RBP secretion by the conceptus as in the liver. There are several points to consider though before this conclusion can be made. First, the experimental design of this study was

not identical to those performed in the liver, in that the conceptus cultures were not vitamin A-deficient. Soprano and coworkers (1986a) fed rats a vitamin A-deficient diet for 8 weeks before rats were considered to be retinol-depleted as determined by serum retinol concentrations. Perhaps if the ewes had been fed a diet deficient in vitamin A, then the embryos could have been considered retinol-depleted and retinoid treatment may have effected different results in RBP secretion. However, as mentioned previously, animals maintained on a vitamin A-deficient diet are likely to have pregnancies that result in resorbed or malformed fetuses.

Second, it is possible that vitamin A may regulate RBP secretion in the conceptus and that retinol-depletion could be carried out to determine this possibility. Soprano and coworkers (1988) found that RBP secretion, as in the liver, was inhibited in a retinol-deficient state in the rat visceral yolk sac at Day 14 of gestation. Secretion of RBP returned to normal upon retinol-repletion. The dams in this study were maintained in a good state of health, and fetuses were considered normal since embryo resorption due to vitamin A-deficiency does not occur in rats until Day 16 of gestation.

In the liver, retinoids do not affect the level of expression of RBP mRNA. Several studies have found that excess retinol or retinoic acid elevates the level of

expression of retinoic acid receptor $\beta$ messenger ribonucleic acid (RAR$\beta$ mRNA), but physiological doses have no effect (Harnish et al., 1990; Harnish et al., 1992). The RAR$\beta$ gene has been found to have a retinoic acid response element (RARE) and therefore retinoic acid can induce expression of RAR$\beta$ mRNA, however this induction is cell specific and does not occur in all cell types when treated with retinoic acid (Davis and Lazar, 1993). No RARE has been found on the RBP gene. Retinoids were administered at physiological concentrations in this study. It may be interesting to see what effect teratogenic doses of retinoids have on RBP secretion in the ovine embryo. In conceptus cultures treated with 10$\mu$M RA, the raw RBP values were decreased, which may have been due to cell death since this concentration of retinoic acid may be somewhat toxic to the embryo.

There was no effect due to any of the variables on RBP values except for day when RBP secretion was expressed as a percentage of the total protein secreted by the conceptus. In this case, RBP was significantly greater on the second day of culture when compared to the initial day of culture. This occurred simply because the total amount of protein secreted by the conceptus decreased considerably on the second day of the experiment but RBP secretion remained the same.

In order to determine if RBP was secreted predominently

in an apo- or holo- form, retinol concentrations were to be determined to calculate the molar ratio of RBP:retinol. The retinol assay did not detect retinol in the conceptus cultures. This could be due to the fact that no retinol was present in the conceptus culture media, that the retinol in the culture media had been lost due to degradation, or that the level of retinol present in the media was below the sensitivity of the assay. The degradation of retinol while being stored is not provided as a possible explanation for the absence of retinol in the analyses since Comstock and coworkers (1993) found that retinol is stable for up to four years when stored at -70°C. Back calculation of retinol concentrations in control culture medium from corresponding RBP concentrations, assuming a 1:1 molar ratio of retinol to RBP, indicates that the levels of retinol could have been below the level of sensitivity of the assay used in this experiment. However, this also means it is possible that RBP could have been secreted in an apo- form.

In conclusion, RBP plays a crucial role in regulating the delivery of retinol to the developing conceptus, however the mechanism of control of RBP secretion in the conceptus is not understood. The present finding that RBP protein levels were not affected by physiological doses of retinoid treatments indicates that secretion of RBP by the ovine conceptus at Day 14 of gestation does not increase in order to protect itself from the potentially toxic effects of

vitamin A metabolites when added in physiological concentrations. However the *in vitro* studies employed here may not reflect the *in vivo* situation. Further studies to determine if teratogenic concentrations of retinoids have any effect on the secretion of RBP in the ovine conceptus would be of interest.

## CHAPTER III

# REGULATION OF RETINOL-BINDING PROTEIN MESSENGER RIBONUCLEIC ACID EXPRESSION BY THE SEX STEROIDS IN OVINE UTERINE ENDOMETRIUM


### Abstract

Vitamin A has been shown to play an integral role in reproductive processes, however it must be present within a defined range. Excess or deficiency in vitamin A may cause fetal malformations and resorption. In the liver, retinol is stored and secreted in a 1:1 molar ratio bound to retinol-binding protein (RBP). Secretion of RBP in the liver is regulated by retinoid status. Recently, several extrahepatic sites of RBP have been identified, including the uterus. RBP secretion by the uterine endometrium is thought to be regulated, in part, by steroid hormones. In the present study, ovariectomized ewes were treated with steroids in order to induce estrus (designated day 0) and then randomly assigned to the following treatments: progesterone (P), estrogen (E), or estrogen+progesterone (EP). Treatments were administered from day 1 to days 13-16. Blood samples were taken on days 3, 10, and the day of hysterectomy in order to determine circulating levels of the hormones. Hysterectomies were performed on the final day of treatment or on day 1 in the case of day 1 controls (D1).

Total uterine endometrial ribonucleic acid (RNA) was isolated and slot blot analysis was performed to quantify steady state RBP messenger RNA (mRNA) expression using an RBP cDNA probe. The results obtained from this study indicate that RBP mRNA expression in P-treated ewes is significantly higher than in C ewes ($P<0.05$), and E treatment tended ($P<0.16$) to be lower than C. Previous studies have found that uterine RBP mRNA expression in cyclic ewes decreases at the beginning of the luteal phase (day 5) when progesterone is secreted by the corpus luteum (CL). Expression of RBP increases to maximal level late in the luteal phase (day 13, 14) which coincides with progesterone receptor down-regulation. Expression either remains elevated, or begins to decrease during the follicular phase (days 15-16). These results support the theory that progesterone inhibits endometrial expression of RBP mRNA, and that long-term exposure to progesterone slightly stimulates RBP expression due to down-regulation of the progesterone receptor.

## Introduction

Uterine secretions prepare a suitable uterine environment in which to receive an embryo. Steroid hormones are believed to play a role in this preparation by regulating the secretions of the uterus, which are commonly known as histotrophe. The secretions of the uterus nourish

the developing conceptus prior to implantation.

Vitamin A is essential for reproductive processes. Vitamin A deficiency (Takahashi et al., 1975; Wilson et al., 1953) or excess (Pillans et al., 1988; Kochhar, 1967; Lammer et al., 1985) during pregnancy can result in fetal resorption or malformations. Therefore the amount of retinol that the conceptus is exposed to must be carefully regulated.

Most retinol is stored in the liver and is secreted in a 1:1 molar ratio bound to RBP (Kanai et al., 1968; Goss and McBurney, 1992; Olson, 1989). RBP secretion by the liver is dependent upon dietary vitamin A intake; retinol is necessary for RBP secretion. Hypovitaminosis A causes levels of RBP to be elevated within the liver and decreases circulating levels of RBP in the blood by inhibiting RBP secretion from the liver. Upon retinol repletion, hepatic RBP concentration decreases to normal levels, and plasma concentrations of RBP become elevated due to an increase in hepatic RBP secretion (Muto and Goodman, 1972).

Recently, several extrahepatic sites, including the conceptus and the uterus, have been found to synthesize and secrete RBP (Harney et al., 1993; Doré et al., 1994; Soprano et al., 1986b; Liu and Godkin, 1992; Liu et al., 1990; Liu et al., 1991). In the uterus, secretion of RBP is thought to be progesterone-induced. The concentration of RBP in uterine flushings of ovariectomized gilts and cows treated

with progesterone is elevated (Adams et al., 1981; Thomas et al., 1992). Endometrial expression of RBP mRNA is elevated during the mid and late luteal phases in cyclic ewes which coincides with maximal progesterone secretion (Harney et al., 1993; Doré et al., 1994). This experiment was designed to evaluate effects of ovarian steroid hormones on endometrial expression of RBP mRNA.

## Materials and Methods

Microtiter plates (96 well) were obtained from Corning (Corning, NY). Conjugate was purchased from Bio-Rad (Hercules, CA). The p-nitrophenylphosphate phosphatase (p-NPP) substrate was obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Silicone tubing was purchased from Baxter (McGaw Park, IL). Silicone sealant was purchased from Dow Corning (Midland, MI). Guanidine isothiocyanate (GTC), 4M, was prepared according to the methods of Sambrook and coworkers (1989), with Trizma Base, EDTA, and N-lauryl sarcosine all obtained from Sigma (St. Louis, MO). Cesium trifluoroacetate (CsTFA), was prepared following the manufacturer's directions to a have a specific gravity of 1.51 and obtained from Pharmacia (Uppsala, Sweden). S & S Nytran membrane and the Minifold II slot-blot system were purchased from Schleicher & Schuell (Keene, NH). $\alpha^{32}$P-dCTP and $\gamma^{32}$P-ATP were obtained from ICN (Costa Mesa, CA). All other reagents were purchased from Sigma

(St. Louis, MO).

*Treatment of animals:* Ewes were ovariectomized at least 6 weeks prior to the experiment to remove any effects of endogenous steroids. A steroid replacement regime developed by Homanics and Silvia (1988) was followed to induce estrus, except that progesterone was administered for 8 days instead of 4 as follows:

| Days before estrus | Time | Amount of hormone | |
|---|---|---|---|
| | | $P_4$ (mg)[a] | $E_2$ ($\mu$g)[b] |
| 10 | 8AM | 12 | - |
| 9 | 8AM | 12 | - |
| 8 | 8AM | 12 | - |
| 7 | 8AM | 12 | - |
| 6 | 8AM | 12 | - |
| 5 | 8AM | 12 | - |
| 4 | 8AM | 12 | - |
| 3 | 8AM | 12 | - |
| 2 | 4PM | - | 3.5 |
| | 12AM | - | 7.0 |
| 1 | 8AM | - | 14.0 |
| | 4PM | - | 7.0 |
| | 12AM | - | 3.5 |
| 0 | | | |

[a]Administered in corn oil vehicle (4.0 mg/ml).
[b]Administered in corn oil vehicle (7.0 $\mu$g/ml).

Ewes were checked for signs of behavioral estrus (designated day 0) on the day following steroid treatment. Ewes were randomly assigned to the following treatment groups: progesterone (P) (12 mg/day, i.m.), estrogen (E) administered via silastic implants (3 cm long, 3.35 mm inner diameter, 4.65 mm outer diameter) filled to capacity with estradiol-17$\beta$, estrogen and progesterone (EP) (12 mg/day $P_4$, i.m.+silastic implant containing estradiol-17$\beta$), day 1 controls (D1) (ewes that were hysterectomized on the first day (day 1) after behavioral estrus with no further steroid treatment), and controls (C) (ovariectomized ewes receiving no steroid treatment). Treatment was administered from day 1 to day 13-16 at which time the uterus was flushed with PBS and then surgically removed. Blood samples were taken on days 3, 10, and prior to hysterectomy to determine peripheral circulating concentrations of progesterone and estradiol-17$\beta$ by a validated RIA. The RIA for estradiol-17$\beta$ wa validated by Howard and coworkers (1990) and had 10% cross-reactivity with dehydroisoandrosterone and less than 1% cross-reactivity with all other steroids analyzed. The RIA for progesterone was validated by Jackson and coworkers (1989).

***RNA Isolation:*** Total RNA was isolated from the uterine endometrium in GTC according to Doré and coworkers (1994). Briefly, endometrium, containing both caruncular and intercaruncular tissue was isolated from the uterus by

dissection, homogenized in GTC buffer (4M) and then ultracentrifuged on a CsCl/TFA bed for 24 hours at 25,000rpm at 20°C to obtain a pellet of RNA. Ethanol (70%, RNAse-free) was added to wash the and then poured off. The RNA pellet was resuspended in 0.4% SDS (500 μl) and stored at -100°C.

*RNA Formaldehyde Gel:* To determine whether uterine RNA samples were intact, an RNA formaldehyde gel (1% agarose) was run. Absorbance of samples was read on a Beckman Spectrophotometer DU 640 to determine RNA concentrations. Each sample was prepared for electrophoresis as follows:

20 μg sample RNA
0.02 M 3-[N-morpholino]propanesulfonic acid (MOPS)
50% formamide
2.2 M formaldehyde

Prior to loading, samples were heated at 65°C for 10 minutes to denature RNA, then loaded and run on the gel. The gel was stained with 0.5 μg/ml ethidium bromide in 1X Tris borate, or TBE (0.09M Tris-borate, 0.002M EDTA, pH 8.3), and destained in 1X TBE.

*Slot Blot Analysis:* Steady state uterine RBP mRNA was quantified by slot blot analysis. The probe was a bovine conceptus RBP cDNA clone (Liu et al., 1993). Twenty μg of total RNA were applied to a nylon membrane with a Minifold slot blot apparatus following the manufacturer's instructions (Schleicher and Schuell). RNA was crosslinked to the membrane by UV irradiation (1.6 joules/$cm^2$). The

membrane was prehybridized (Appendix 2) in a sealed plastic bag at 42°C for 4 hours. The membrane was then hybridized (Appendix 2) in a sealed plastic bag with the RBP probe, prepared by random prime labelling with $\alpha^{32}$P-dCTP to a specific activity of 1.16 X $10^9$ cpm/$\mu$g DNA, at 42°C for 24 hours. The membrane was subjected to high-stringency washing (Appendix 3) and then exposed to Kodak X-Omat AR film for approximately 70 hours at -80°C. The membrane was stripped of the RBP probe by gently boiling in 0.1% SDS for 15 minutes and reprobed with a $\beta$-actin cDNA probe, prepared by random prime labelling to a specific activity of 1.38 X $10^9$ cpm/$\mu$g DNA as described previously for the RBP probe, to correct RBP values for loading error. Slot blot signal intensities were determined by computer assisted scanning laser densitometry using an LKB Ultrascan XL Enhanced Laser Densitometer (Bromma, Sweden). Slot blot sample signals were integrated and the area for each sample was calculated in arbitrary absorbance units (GSXL version 2 software, LKB). Sample areas were corrected for differences in total RNA loaded onto the membrane through use of the corresponding area from $\beta$-actin or oligo dT autoradiographs. Because temporal expression of $\beta$-actin seemed to change during the estrous cycle, the membrane was also reprobed with oligo dT to correct for loading error. The membrane was stripped of the $\beta$-actin probe. The oligo dT probe was prepared by random prime labelling with $\gamma^{32}$P-ATP to a

specific activity of 3.43 X $10^8$ cpm/$\mu$g DNA. The membrane was prehybridized (Appendix 4) for 2½ hours at 50°C in roller bottles. The membrane was then hybridized (Appendix 4) with the oligo dT probe. After high-stringency washing (Appendix 5), the membrane was exposed to film for approximately 2 hours at -80°C, and relative expression of RBP was determined as described above.

Steady state levels of uterine endometrial RBP mRNA were then determined by slot blot analysis using 10 $\mu$g total RNA instead of 20 $\mu$g. Twenty-five $\mu$g is the maximum amount of total RNA that can be applied to the nylon membrane used in this procedure. Therefore, since expression of RBP mRNA is fairly abundant in the uterus, 20 $\mu$g may overload the membrane and produce results outside the linear range of the membrane, therefore 10 $\mu$g of total RNA was used to obtain results that were well within the limits of this system. Ten $\mu$g total RNA from uterine endometrium was applied to a nylon membrane with a slot blot apparatus as described before. The RBP probe was prepared by random prime labelling with $\alpha^{32}$P-dCTP to a specific activity of 2.77 X $10^9$ cpm/$\mu$g DNA. The membrane was prehybridized (Appendix 6) for at least 4 hours at 65°C in roller bottles. The membrane was then hybridized (Appendix 6) with the RBP probe at 42°C for 18 hours in roller bottles. The membrane was subjected to high-stringency washes (Appendix 7) and then exposed to Kodak X-Omat AR film for approximately 40 hours at -80°C.

The membrane was stripped of the RBP probe and reprobed with an oligo dT probe prepared by random prime labelling to a specific activity of 1.67 X $10^8$ cpm/$\mu$g DNA as described previously to correct for loading error. The membrane was exposed to Kodak film as before and relative expression of RBP mRNA was determined as desribed above.

*Statistical Analysis:* Amounts of RBP in uterine flushes and relative quantities of RBP mRNA per equivalent amount of total RNA were analyzed for effect of treatment. A completely randomized design was the experimental design and data were analyzed by the General Linear Models procedure of SAS (SAS,1990). Means were separated by Fisher's least significant difference test. Data was log transformed when necessary. Significance was considered at the 95% confidence level unless otherwise stated.

## Results

*Steroid concentrations of blood samples:* $P_4$ and $E_2$ concentrations were measured in plasma of blood taken from the jugular vein during the treatment period. Table 2-1 shows $P_4$ concentrations due to treatment on the day of hysterectomy. $P_4$ concentrations were consistent with luteal $P_4$ concentrations (>1ng/ml) in the P and EP treatment groups and lowest ($P<0.05$) in the D1, E, and C treatment groups.

$E_2$ concentrations were as expected being highest ($P<0.001$) in the E and EP treatment groups and lowest in the

remaining treatment groups (Table 2-2). Estrogen and progesterone concentrations during the steroid treatment period are shown in Figures 2-1A and B, respectively.

*RBP Quantitation of Uterine Flushes:* The RBP content of uterine flushes collected at the time of hysterectomy is shown in Table 2-3. There were no differences in RBP values due to treatment. There was blood contamination in several of the uterine flushes (which were not used) and the degree of blood contamination in the flushes analyzed was not determined. There was also a large standard error associated with this data set.

*RBP mRNA expression in uterine endometrium:* Steady state expression of RBP mRNA was analyzed several ways. Uterine expression of RBP in the uterine endometrium was corrected for loading error with β-actin (Figure 2-2A) and oligo dT (Figures 2-2B and 2-3), and was analyzed using 10 (Figure 2-3) or 20 μg (Figures 2-2A and B) total RNA loaded onto nylon membranes. The values obtained by loading 10 μg of RNA onto a membrane and correcting loading error with oligo dT are those believed the most accurate, and were used. However all values are reported. RBP mRNA values corrected with β-actin and oligo dT using 20 μg total RNA are shown in Table 2-4 and Table 2-5, respectively. When RBP values were corrected with β-actin, the EP treatment group was significantly higher ($P<0.05$) than the C group. The P-treated group was greater ($P<0.05$) than the C group.

**Table 2-1.** $P_4$ Concentrations due to Treatment at Hysterectomy.

| Treatment | N | $P_4$ concentration (ng/ml) |
|---|---|---|
| C | 4 | 0.16+/-0.41[b] |
| P | 2 | 1.56+/-0.59[a] |
| E | 3 | 0.07+/-0.48[b] |
| EP | 3 | 2.32+/-0.48[a] |
| D1 | 2 | 0.83+/-0.59[a] |
| [a,b]Mean+/-S.E.; values with different superscripts are significantly different p<0.05. | | |

**Table 2-2.** $E_2$ Concentrations due to Treatment at Hysterectomy.

| Treatment | N | $E_2$ concentration (pg/ml) |
|---|---|---|
| C | 4 | 2.97+/-5.20[a] |
| P | 4 | 2.83+/-5.20[a] |
| E | 2 | 69.50+/-7.35[b] |
| EP | 2 | 65.73+/-7.35[b] |
| D1 | 2 | 4.19+/-7.35[a] |
| [a,b]Mean+/-S.E.; values with different superscripts are significantly different p<0.001. | | |

**Table 2-3.** Total RBP in Uterine Flushes due to Treatment.

| Treatment | N | RBP in uterine flush (ng) |
|---|---|---|
| C | 4 | 581.5+/-225.0[a] |
| P | 2 | 575.6+/-318.2[a] |
| E | 1 | 752.1+/-0[a] |
| EP | 2 | 1347.1+/-318.2[a] |
| D1 | 2 | 1238.3+/-318.2[a] |
| [a]Mean+/-S.E.; no significant differences found P>0.05. | | |

**Table 2-4.** Uterine endometrial expression of RBP mRNA standardized with $\beta$-actin. Values were obtained by loading 20 $\mu$g total RNA onto nylon membranes.

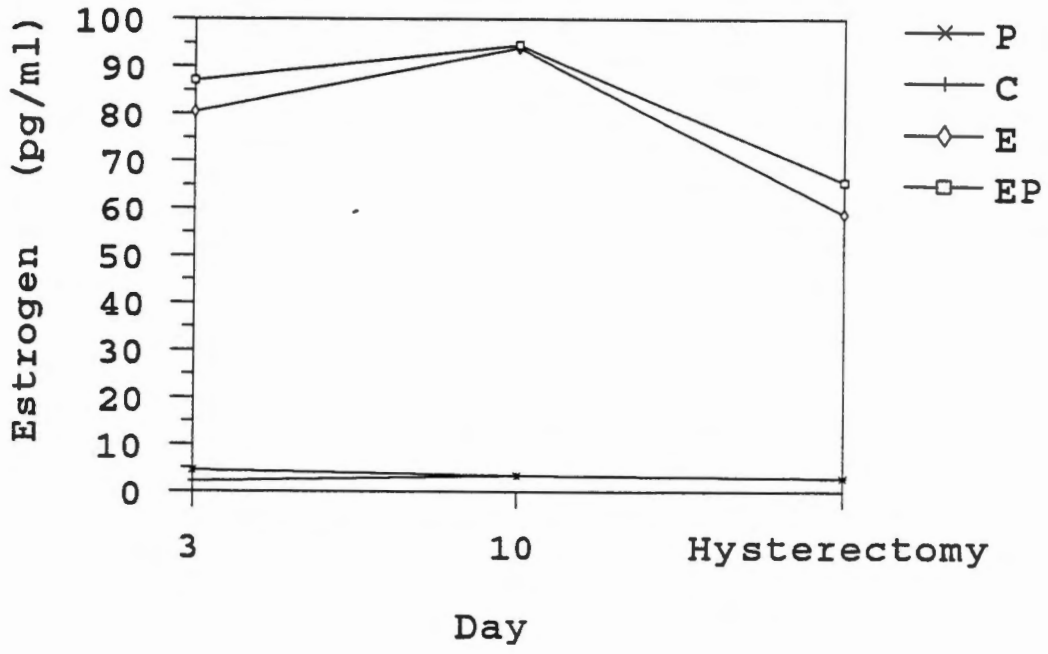
| Treatment | N | RBP mRNA (arbitrary units) |
|---|---|---|
| C | 4 | 0.230+/-0.056$^{c}$ |
| P | 3 | 0.417+/0.064$^{ab}$ |
| E | 3 | 0.199+/0.064$^{c}$ |
| EP | 3 | 0.480+/-0.064$^{a}$ |
| D1 | 2 | 0.247+/-0.079$^{bc}$ |
| $^{a,b,c}$Mean+/-S.E.; values with different superscripts are significantly different $p<0.05$. | | |

**Table 2-5.** Uterine endometrial expression of RBP mRNA standardized with oligo dT. Values were obtained by loading 20 $\mu$g total RNA onto nylon membranes.

| Treatment | N | RBP mRNA (arbitrary units) |
|---|---|---|
| C | 4 | 0.361+/-0.038$^{a}$ |
| P | 3 | 0.447+/-0.044$^{a}$ |
| E | 3 | 0.196+/-0.044$^{b}$ |
| EP | 3 | 0.397+/-0.044$^{a}$ |
| D1 | 2 | 0.420+/-0.053$^{a}$ |
| $^{a,b}$Mean+/-S.E.; values with different superscripts are significantly different $p<0.05$. | | |

**Figure 1.** Plasma $E_2$ **(A)** and $P_4$ **(B)** concentrations of ovariectomized ewes on days 3 and 10 of steroid treatment and on the day of hysterectomy.

A


Estrogen (pg/ml)
100
90
80
70
60
50
40
30
20
10
0
3
10
Hysterectomy
Day
P
C
E
EP

B

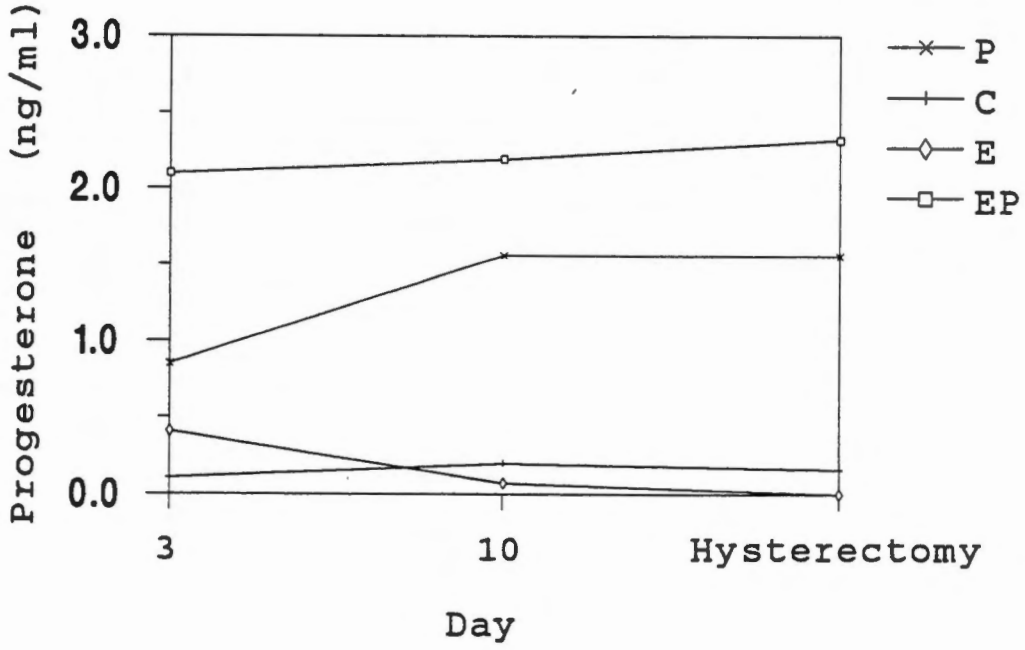
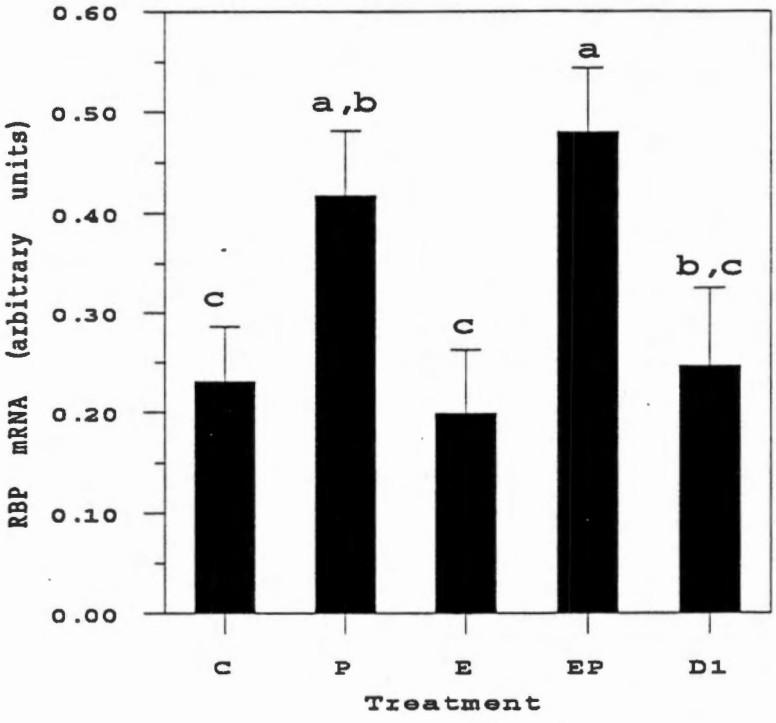

Progesterone (ng/ml)
3.0
2.0
1.0
0.0
3
10
Hysterectomy
Day
P
C
E
EP

**Figure 2.** Uterine endometrial expression of RBP mRNA standardized with $\beta$-actin **(A)** or oligo dT**(B)** (20 $\mu$g total RNA loaded onto membranes).

A


RBP mRNA (arbitrary units)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
c
a,b
c
a
b,c
C
P
E
EP
D1
Treatment

B

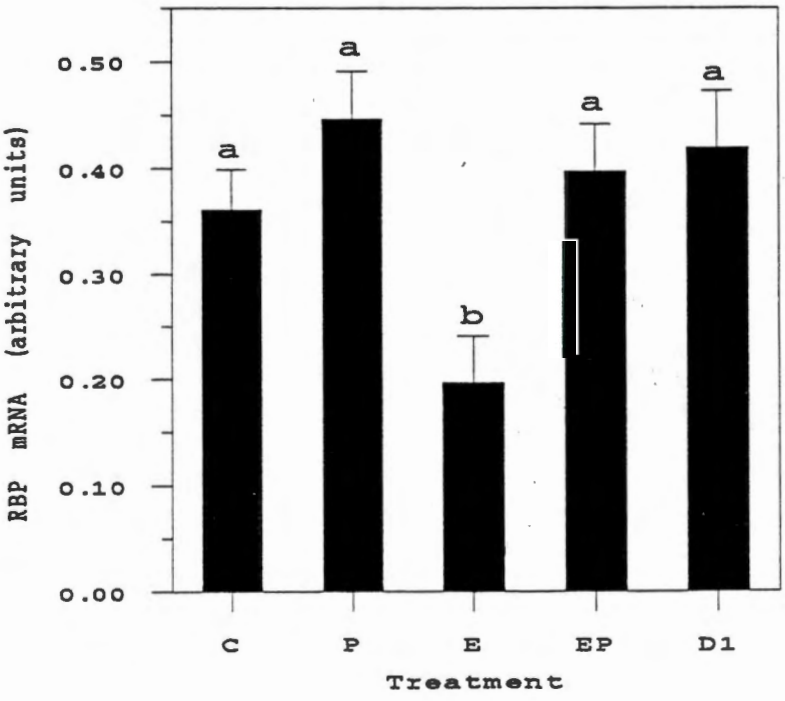

RBP mRNA (arbitrary units)
0.50
0.40
0.30
0.20
0.10
0.00
a
a
b
a
a
C
P
E
EP
D1
Treatment

**Table 2-6.** Uterine endometrial expression of RBP mRNA standardized with oligo dT. Values were obtained by loading 10 $\mu$g total RNA onto nylon membranes.

| Treatment | N | RBP mRNA (arbitrary units) |
|---|---|---|
| C | 4 | $0.439+/-0.073^{bc}$ |
| P | 2 | $0.879+/-0.104^{a}$ |
| E | 3 | $0.264+/-0.085^{bc}$ |
| EP | 2 | $0.541+/-0.104^{b}$ |
| D1 | 3 | $0.244+/-0.085^{c}$ |
| $^{a,b,c}$Mean +/-S.E.; values with different superscripts are significantly different $p<0.05$. | | |

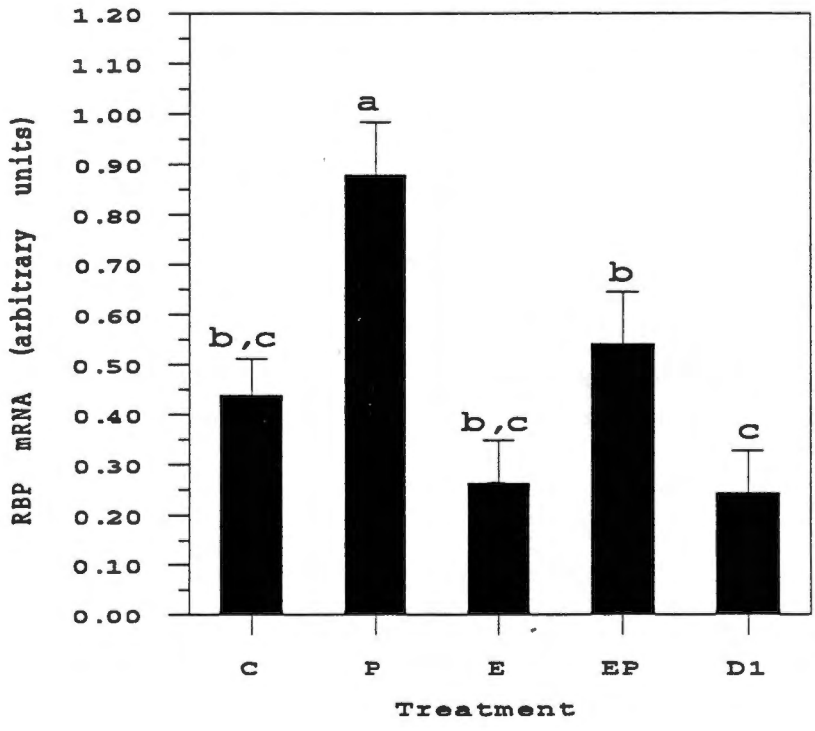



**Figure 3.** Uterine endometrial expression of RBP mRNA standardized with oligo dT (10 $\mu$g total RNA loaded onto the membrane).

Estrogen-treated animals were significantly lower ($P<0.05$) than all other treatment groups when RBP mRNA expression was standardized with oligo dT (Table 2-5). Steady state levels of RBP mRNA corrected with oligo dT using 10 $\mu$g total RNA are shown in Table 2-6 and Figure 3. The P treatment group was significantly higher ($P<0.05$) than the C group as can be seen in Figure 2-3. Ewes receiving EP treatment were slightly higher than C ewes, but this difference was not significant.

## Discussion

This study was undertaken in order to better understand the effects of individual steroids on uterine expression of RBP mRNA. The concentration of RBP secreted by the uterus was determined in uterine flushes. Due to high standard error, no differences due to treatment were found. Uterine flushes were obtained before hysterectomy, and therefore some degree of blood contamination was encountered. Because we could not be sure of how much blood was contained in each sample, the values presented in Table 2-3 cannot be trusted. Based on previous studies, ovariectomized gilts (Adams et al., 1981) and cows (Thomas et al., 1992) treated with progesterone had significantly higher levels of RBP in uterine flushes compared to control levels. Therefore, it is plausible to speculate that similar results might have been obtained in this experiment.

The serum levels of steroids were consistent with treatment in most cases. If the serum levels of the steroids did not correspond with its respective treatment, the sample was not included in the results. For progesterone, samples were excluded if the levels were below luteal concentrations (1.0 ng/ml). This occurred in 2 samples. Two samples from estrogen-ewes were excluded because the silastic implants were not recovered at the end of the experiment and the circulating levels of estradiol-17$\beta$ for these two samples were drastically lower than the remaining estrogen-treated ewes. Somewhat unexpectedly, estrogen concentrations were much higher in the ewes treated with estradiol-17$\beta$ than what was expected. The estrogen implants used in this experiment were found to release estrogen to maintain circulating concentrations at approximately 3 pg/ml by Karsch et al. (1980). Estrogen concentrations of the estrogen-treated ewes in this study had an average value of 65 pg/ml (Table 2). This may explain some of the results obtained for the levels of RBP mRNA which will be discussed later.

Steady state levels of RBP mRNA were determined several ways. Correcting RBP expression with $\beta$-actin did not result in accurate RBP values since $\beta$-actin expression changes throughout the estrous cycle. Alternatively, oligo dT was used to represent total RNA and correct for loading error. Initially, 20 $\mu$g total RNA was loaded onto membranes but

this amount of RNA is close to saturation of the membrane. Consequently, 10 μg total RNA was loaded onto the membrane. Therefore, the results obtained using oligo dT to correct for loading error and by applying 10 μg total RNA to nylon membranes (Table 2-6 and Figure 3) will be used for the purpose of discussion. The results of this experiment support the theory that progesterone inhibits endometrial expression of RBP mRNA, and that long-term progesterone treatment may have a negligible effect due to down-regulation of the progesterone receptor. According to this hypothesis, progesterone negatively regulates RBP expression early in the luteal phase (days 1-5). After approximately 8-10 days of progesterone exposure, progesterone has been shown to down-regulate the progesterone receptor (Spencer and Bazer, 1995; Spencer et al., 1995b). With this mechanism in place, progesterone no longer can bind to and form a complex with its receptor, and therefore cannot inhibit expression of RBP mRNA within the uterus.

Studies that characterized expression of RBP mRNA during the estrous cycle lend furhter support to this theory. During the estrous cycle in the ewe, levels of RBP mRNA decrease at the beginning of the luteal phase (day 5) when progesterone is being secreted by the corpus luteum, increase to maximal expression at the end of the luteal phase (days 13, 14), and are either maintained or slightly decrease during the follicular phase (days 15-16) when

estrogen levels are elevated (Harney et al., 1993; Doré et al., 1994). The increase in expression of RBP transcripts at the end of the luteal phase (days 13, 14) coincides with progesterone receptor down-regulation. The numbers of progesterone receptors increase during the follicular phase (days 15-16) (Ott et al., 1993), due to increasing levels of estrogen which up-regulate the progesterone receptor (Ing et al., 1996; Spencer et al., 1995a). Because progesterone levels are low to undetectable during the follicular phase due to luteolysis, progesterone is not available to bind to its receptor and affect RBP expression. As a result, the levels of endometrial RBP mRNA have been shown in previous studies to remain the same or slightly decrease compared to the levels obtained at the end of the luteal phase (Harney et al., 1993; Doré et al., 1994).

In this study, ovariectomized ewes were administered long-term treatment ( 13-16 days) of steroids. Expression of RBP mRNA was significantly higher in progesterone-treated ewes than any other treatment (Figure 3). Since ewes were treated with 13-16 days of progesterone, the progesterone receptor would have been down-regulated at this time, and progesterone therefore no longer would inhibit RBP expression. The fact that RBP mRNA levels of progesterone-treated ewes were actually greater than control levels was somewhat surprising. If long-term exposure to progesterone, through down-regulation of its receptor, does not affect

expression of RBP, we would expect no difference between progesterone treatment and controls, but long-term progesterone had a stimulatory effect on RBP mRNA. This suggests that regulation of RBP gene expression is complex and other factors besides the sex steroids may contribute to the regulatory mechanism of RBP mRNA expression.

In ewes treated with estrogen and progesterone, the levels of RBP mRNA were significantly lower than the levels in progesterone-treated ewes, but not significantly different than control levels. Estrogen, in contrast to progesterone, up-regulates expression of progesterone receptors. Therefore in ewes treated with progesterone and estrogen, we would not expect progesterone receptors to decrease as much as in progesterone-treated ewes allowing some degree of inhibition of RBP expression to occur, and hence the difference in expression of the two treatment groups.

The day 1 control group exhibited levels of RBP mRNA that were lower than controls, but this difference was not significant. In this group, the progesterone receptor would begin to be down-regulated from the initial progesterone treatment, and then steady state levels of RBP mRNA would increase after administration of estrogen in the absence of progesterone. As a result, expression of endometrial RBP transcripts would be somewhat inhibited. However, it should be pointed out that the results of this group are somewhat

confounded because the day 1 controls were treated with 6 or 8 days of progesterone which may have caused some variation within this group.

RBP mRNA expression in ewes treated with long-term estrogen was lower than control ewes, but this difference was not statistically significant. In ovariectomized rats, chronic exposure to estrogen actually decreased estrogen receptors (Manni et al., 1981), whereas at lower doses, estrogen increases estrogen receptor concentrations (Ing et al., 1996). In the present study, the plasma concentration of estrogen was high and might be considered to be chronic estrogen exposure which may have led to estrogen receptor down-regulation. Therefore, estrogen may not have exerted its full effect in the estrogen-treated group and RBP expression may have been significantly lower than controls if circulating levels of estrogen had not been so high.

Interestingly, estrogen receptors have been localized in the day 21 trophoblast (Wathes and Hamon, 1993). At the same time, levels of RBP mRNA peak during early pregnancy at day 23 of gestation (Doré et al., 1994). Low levels of estrogen are present during early pregnancy, suggesting that estrogen may up-regulate expression of RBP mRNA in the conceptus by binding to its receptor found in the day 21 conceptus. However it is not completely understood what the short-term effects of estrogen are on expression of RBP.

It is probable that there are many other factors

involved in the regulation of RBP mRNA besides the steroid hormones. Glover and coworkers (1976) have suggested that gonadal hormones are not involved at all, since RBP is found in ewes throughout the year and sheep exhibit an anestrous period in which the gonads are quiescent. In the present study, detectable levels of RBP mRNA were found in control ewes indicating that there must be other mechanisms of control besides steroids.

Ovine interferon-$\tau$ (oIFN-$\tau$) may play an indirect role in regulating RBP expression. oIFN-$\tau$ is believed to be the signal for maternal recognition of pregnancy and has been found to decrease estrogen and oxytocin receptors thereby inhibiting the luteolytic mechanism (Spencer et al., 1995a; Spencer et al., 1995b). This mechanism by which estrogen receptors are inhibited may also ensure down-regulation of the progesterone receptor enabling expression of endometrial RBP mRNA to remain high during early pregnancy.

In conclusion, this study lends support to the theory that long-term progesterone stimulates uterine expression of RBP mRNA by down-regulating its own receptor thereby negating inhibition of RBP mRNA expression caused by short-term progesterone exposure as seen on day 5 of the estrous cycle. The results also suggest that long-term estrogen treatment may inhibit RBP mRNA. Future studies involving short-term steroid treatment will be needed to prove this theory.

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**1. ELISA buffers.**

Diluent
100 ml 10% BSA
90 ml 10X PBS
5 ml 10% Tween 20
5 mg Bromphenol blue

Bring to 1 L with water.

10X TBS
350.64 g NaCl
96.88 g Trizma Base
8.0 g sodium azide

Adjust pH to 7.4. Bring to 4 L with water.

Wash buffer
400 ml 10X TBS
20 ml 10% Tween 20

Bring to 4 L with water.

**2. Prehybridization and hybridization buffers used with RBP probe and $\beta$-actin probe (20 $\mu$g total RNA loaded onto membrane).**

Prehybridization buffer
5X SSPE
2X Denhardt's
0.2% SDS
100 $\mu$g/ml yeast RNA

Hybridization buffer
5X SSPE
1X Denhardt's
0.1% SDS
100 $\mu$g/ml yeast RNA
50% formamide

**3. Wash procedure used with RBP probe and β-actin probe (20 μg total RNA loaded onto membranes).**

| Wash buffer | Time | Temperature |
|---|---|---|
| 2X SSPE/0.1% SDS | 5 minutes | room temperature |
| 2X SSPE/0.1% SDS | 5 minutes | room temperature |
| 1X SSPE/0.1% SDS | 5 minutes | room temperature |
| 1X SSPE/0.1% SDS | 15 minutes | 65°C |
| 0.1X SSPE/0.1% SDS | 20 minutes | 65°C |

**4. Prehybridization and hybridization buffer used with oligo dT probe (20 μg total RNA loaded onto membrane).**

Prehybridization and Hybridization buffer
5X SSPE
5% Dextran sulfate
5X Denhardt's
1 mM EDTA, pH 7.5
0.1 mg/ml tRNA
0.1% SDS

**5. Wash procedure used with oligo dT probe (20 μg total RNA loaded onto membrane).**

| Wash buffer | Time | Temperature |
|---|---|---|
| 5X SSPE/0.1% SDS | 15 minutes | 37°C in roller bottle |
| 5X SSPE/0.1% SDS | 15 minutes | 37°C in roller bottle |
| 5X SSPE/0.1% SDS | 15 minutes | 37°C in plastic container |
| 5X SSPE/0.1 % SDS | 15 minutes | 37°C in plastic container |

**6. Prehybridization and hybridization buffer used with oligo dT probe (10 μg total RNA loaded onto membrane).**

Prehybridization and Hybridization buffer
50% formamide
5X SSPE
5% Detran sulfate

pH mixture to 7-7.5

5X Denhardt's
0.1% SDS
0.1 mg/ml sheared salmon sperm ssDNA

**7. Wash procedure used with oligo dT probe (10 μg total RNA loaded onto membrane).**

| Wash buffer | Time | Temperature |
|---|---|---|
| 5X SSPE/0.1% SDS | 15 minutes | 42°C in roller bottle |
| 5X SSPE/0.1% SDS | 15 minutes | 42°C in roller bottle |
| 1X SSPE/0.1% SDS | 15 minutes | 42°C in plastic container |
| 0.1X SSPE/0.1% SDS | 15 minutes | 65°C in plastic container |
| 0.1X SSPE/0.1% SDS | 30 minutes | 65°C in plastic container |

# VITA

Wendy G. Jacobs was born in York, Pennsylvania on January 24, 1971. She attended schools in the Spring Grove Area School District and graduated from Spring Grove Area High School in May, 1989. She entered Elizabethtown College in the fall of 1989 where she received a partial four-year academic scholarship and graduated with a Bachelor of Science degree in Biology with a minor in Biochemistry in May of 1993. She entered the Master's program in Animal Science at The University of Tennessee, Knoxville in August, 1993 and officially received her Master's degree in December of 1996.

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