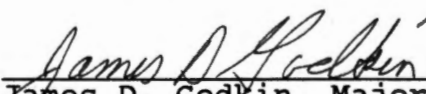
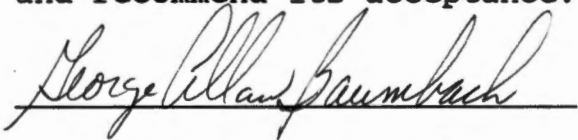


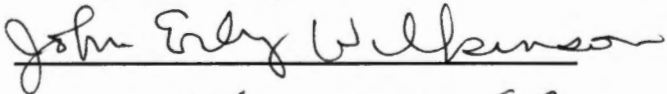
To the Graduate Council:

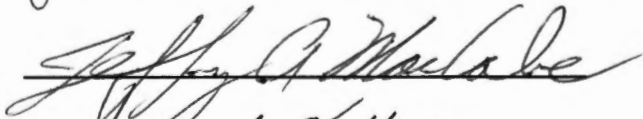
I am submitting herewith a dissertation written by Kaung Huei Liu entitled "Purification, Characterization and Immunolocalization of bovine and ovine placental retinol-binding protein." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

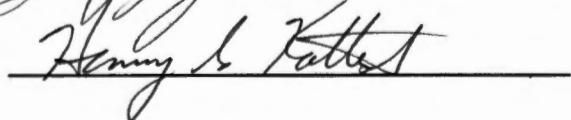

James D. Godkin, Major Professor

We have read this dissertation
and recommend its acceptance:


George Allan Baumbach


John Erby Wilkinson


Jeffery A. Moulton


Henry L. Kottel

Accepted for the Council:


Associate Vice Chancellor
and Dean of The Graduate
School

**PURIFICATION, CHARACTERIZATION AND IMMUNOLocalIZATION
OF BOVINE AND OVINE PLACENTAL
RETINOL-BINDING PROTEIN**

A DISSERTATION

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Kaung Huei Liu

August 1991

AD-VET-MED

THESIS

91b

.4583

ACKNOWLEDGEMENTS

I would like to express sincere gratitude to my major professor, Dr. James Godkin for his encouragement, friendship and guidance throughout my doctoral program. Special thanks also go to Dr. George Baumbach for the guidance and assistance in all phases of this project. I would also like to thank Dr. Henry Kattesh, Dr. Jeffrey MacCabe and Dr. John Wilkinson, for their constructive suggestions, assistance and support as members of my graduate committee.

I am also very grateful to the following people:

Dr. Randy Brewton, Ben Lifsey, Linda Smith and Barbara Gillespie for their assistance in the laboratory work and for their friendship.

Dr. Kuixiong Gao for his assistance in the immunohistological study of the project.

My fellow graduate students, Roger Johnson, Scott Smith and Jules Dore, Jr., who in one way or the other helped in data collection.

Teresa Herring and Sandra Dailey for their secretarial help through out my educational career at Tennessee.

My parents, Mr. and Mrs. Chen Liu for allowing me the opportunity to study in the United States.

My wife Shirley for her support and understanding during the course of the research.

ABSTRACT

A retinol-binding protein (RBP) synthesized and secreted by bovine and ovine allantois in vitro was purified from culture medium. The protein consisted of three isoelectric variants (pI 5.3-6.1) of identical molecular weight of about 23,000 as determined by two-dimensional polyacrylamide gel electrophoresis. Thirty-one of the first 34 N-terminal amino acids of the purified protein from both sources were sequenced and shown to have complete homology with bovine plasma RBP. The UV absorption spectrum and fluorescence excitation and emission spectra of the purified bovine and ovine placental RBP indicated the presence of bound retinol. Rabbit antiserum was raised against purified bovine placental RBP. Metabolic labelling, immunoprecipitation studies demonstrated that the protein was synthesized by bovine and ovine extraembryonic membranes (allantois, chorion and amnion). The protein was also detected in allantoic and amniotic fluids by immunoblotting. With immunocytochemical procedures, the placental RBP was immunolocalized in trophoctodermal cells of the chorion, endodermal cells lining the allantois and ectodermal cells lining the amnion. Results from this study suggest that bovine and ovine placental membrane epithelia synthesize and secrete RBP. Transport, storage, and metabolism of retinol mediated by placental RBP may be essential for normal embryonic development during pregnancy.

TABLE OF CONTENTS

CHAPTER	PAGE
PART 1: INTRODUCTION AND LITERATURE REVIEW	
1. INTRODUCTION.....	1
2. LITERATURE REVIEW.....	3
LIST OF REFERENCES.....	10
PART 2: PURIFICATION AND CHARACTERIZATION OF BOVINE PLACENTAL RETINOL-BINDING PROTEIN	
1. ABSTRACT.....	21
2. INTRODUCTION.....	22
3. MATERIALS AND METHODS.....	25
4. RESULTS.....	31
5. DISCUSSION.....	55
LIST OF REFERENCES.....	61
PART 3: IMMUNOHISTOCHEMICAL LOCALIZATION OF BOVINE PLACENTAL RETINOL-BINDING PROTEIN	
1. ABSTRACT.....	71
2. INTRODUCTION.....	72
3. MATERIALS AND METHODS.....	74
4. RESULTS.....	78
5. DISCUSSION.....	83
LIST OF REFERENCES.....	87

PART 4: CHARACTERIZATION OF PROTEIN PRODUCTION BY OVINE**PLACENTAL MEMBRANES: IDENTIFICATION OF A****PLACENTAL RETINOL-BINDING PROTEIN**

1. ABSTRACT.....	95
2. INTRODUCTION.....	97
3. MATERIALS AND METHODS.....	100
4. RESULTS.....	103
5. DISCUSSION.....	115
LIST OF REFERENCES.....	123

PART 5: PURIFICATION AND IMMUNOLocalIZATION OF OVINE**PLACENTAL RETINOL-BINDING PROTEIN**

1. ABSTRACT.....	134
2. INTRODUCTION.....	135
3. MATERIALS AND METHODS.....	137
4. RESULTS.....	141
5. DISCUSSION.....	155
LIST OF REFERENCES.....	160

PART 6: GENERAL DISCUSSION

1. DISCUSSION OF PARTS 2 - 5.....	171
LIST OF REFERENCES.....	177

PART 7: GENERAL CONCLUSIONS

1. SUMMARY OF PARTS 2 - 5.....	184
VITA.....	185

LIST OF FIGURES

FIGURE

PAGE

PART 2: PURIFICATION AND CHARACTERIZATION OF BOVINE

PLACENTAL RETINOL-BINDING PROTEIN

1. Fluorograms of two-dimensional gels for analysis of radioisotopically labeled proteins synthesized and released by allantois (Panel A) and chorion (Panel B) from Day 53 of pregnancy.....32
2. Stages in the purification of 3B₃ from bovine allantois culture medium.....34
3. Q-Sepharose ion exchange chromatography of dialyzed medium following a 24-hour incubation of Day 29-90 bovine allantois in L-[³H]Leucine.....36
4. Sephadex G-75 gel chromatography of peak B from Figure 3.....39
5. DEAE-HPLC ion exchange chromatography of Sephadex G-75 gel chromatography fraction containing 3B₃.....41
6. Biogel P-30 gel chromatography of the DEAE-HPLC-enriched 3B₃.....43
7. Analysis of immunoprecipitates from allantois and chorion secretory proteins of cow by one-dimensional polyacrylamide gel electrophoresis and fluorography.....46
8. Coomassie Brilliant Blue-stained two-dimensional gel containing total Day 53 bovine allantoic culture medium proteins (Panel A) and Day 60 allantoic fluid proteins (Panel B).....48
9. Fluorescence excitation and emission spectra of purified 3B₃ from allantoic fluid in Tris-HCl buffer, pH 7.6.....53

PART 3: IMMUNOHISTOCHEMICAL LOCALIZATION OF BOVINE**PLACENTAL RETINOL-BINDING PROTEIN**

1. Bovine chorionic membrane stained with hematoxylin and eosin (H & E).....79
2. Keratin immunostain in bovine chorionic membrane.....79
3. Localization of bpRBP in bovine allantois.....79
4. The same area as in Figure 3 showing overall nonspecific fluorescence in tissue with an FITC Filter.....79
5. Chorionic membrane immunostained with pre-immune rabbit serum (1:100) followed by IgG-Au (1:30), IGSS, H & E (as a negative control for Figure 6).....79
6. Localization of bpRBP in bovine chorionic membrane...80
7. Distribution of bpRBP antigenic sites in apical region of chorionic epithelial cells.....80
8. Same area as shown in Figure 6.....80
9. Immunostain of bpRBP antigenic sites in bovine amniotic membrane.....80
10. Negative control for Figure 3.....80

PART 4: CHARACTERIZATION OF PROTEIN PRODUCTION BY OVINE**PLACENTAL MEMBRANES: IDENTIFICATION OF A****PLACENTAL RETINOL-BINDING PROTEIN**

1. Fluorogram of two-dimensional gels for analysis of ³⁵S-methionine-labeled secretory proteins from Day 35 chorion (A), allantois (B) and amnion (C).....104
2. Fluorogram of two-dimensional gel for analysis of ³⁵S-methionine-labeled proteins synthesized and released by yolk sac isolated from a Day 23 conceptus.....106

3. Ovine allantoic fluid proteins (60 ug) analyzed by two-dimensional polyacrylamide gel electrophoresis (A) and Western blot of panel A using 1:1000 dilution of anti-bovine placental retinol-binding protein antiserum.....113

PART 5: PURIFICATION AND IMMUNOLocalIZATION OF OVINE

PLACENTAL RETINOL-BINDING PROTEIN

1. Two-dimensional polyacrylamide gel electrophoresis analyses of medium proteins from Day 45 allantois cultures (left) and purified placental RBP.....142
2. Q-Sepharose ion exchange chromatography of proteins released into culture medium by ovine allantois at 35-45 days of pregnancy.....144
3. Sephadex G-75 gel chromatography of retinol-containing fractions from Figure 2.....146
4. Purification of protein D₄ by immunoaffinity chromatography.....148
5. Photomicrographs of ovine conceptus tissues immunostained for identification of RBP.....153

PART 1: INTRODUCTION AND LITERATURE REVIEW

CHAPTER 1

INTRODUCTION

Early embryonic mortality is a major cause of reproductive failure and estimated to occur in approximately 30% of all fertile matings in domestic farm animals (Januideen and Hafez, 1980). Over the past two decades, numerous studies have been performed with the objective of elucidating the mechanisms contributing to embryonic mortality. It is generally accepted that chemical interactions that occur between the conceptus and maternal tissues are essential to the establishment and maintenance of pregnancy. Proteins are believed to be important mediators of fetal-maternal interactions, however, few placental secretory proteins have been isolated and their function identified. Chemical interactions mediated by proteins occur between the maternal and fetal units which are essential to the establishment and maintenance of pregnancy and the development of the embryo. However, little is known of the role of placental secretory proteins during early pregnancy.

The overall theme of this project was to study a bovine and ovine placental protein, designated 3B₃ in the cow (Godkin et al., 1988a; 1988b). The synthesis of 3B₃ was observed to initiate at a time coincident with the outgrowth

of allantois, its apposition to the chorion, and the progressive attachment of the allanto-chorion to the endometrium. Protein 3B₃ synthesis continue through late gestation even after placentome formation (Godkin et al., 1988a; 1988b). The appearance of 3B₃ during critical periods of early pregnancy suggested its importance, however, the function of 3B₃ was unknown.

The objectives of this study were to:

1. purify protein 3B₃ to homogeneity,
2. characterize 3B₃ biochemically,
3. determine its N-terminal amino acid sequence,
4. identify 3B₃ by comparison of its sequence with known proteins,
5. generate specific antiserum against 3B₃, and
6. immunolocalize 3B₃ in placental tissues.

CHAPTER 2

LITERATURE REVIEW

Early Embryonic Development

In bovine and ovine, after onset of estrus (day 0), fertilization occurs on day 1 in the oviduct, at the ampullary-isthmic junction (Hunter, 1985; Hunter and Wilmut, 1984). The fertilized egg is surrounded initially by a transparent cell coat, the zona pellucida. By day 4-5, the embryo has developed to the 8-16 cell morula stage and passed from the oviduct into the uterus (Bazer and First, 1983). The compact morula develops into the fluid-filled blastocyst on day 6-8 (Lindner and Wright, 1983). Hatching from the zona pellucida occurs on day 9-10 (Flechon and Renard, 1978). By day 11, the spherical blastocyst consists of inner cell mass (ICM), the surrounding trophectoderm and a fluid-filled cavity, the blastocoele (Renard et al., 1978). At day 12 of gestation, the hatched blastocyst begins to elongate. Over the next 10 days the blastocyst undergoes a tremendous growth resulting in a filamentous structure. By day 17 in sheep and day 24 in cattle, the conceptus fills the gravid horn and extends well into the contralateral horn (Bazer and First, 1983).

Endoderm cells spread out from beneath the inner cell mass at about day 8 and completely line trophectodermal

cells by Day 10 (Greenstein and Foley, 1958; Linares and Ploen, 1981; Massip et al., 1981). Mesodermal cells begin to migrate out from the ICM between the trophoctoderm and endoderm at about Day 14-16. The outer layer of mesoderm lines the trophoctoderm to form the chorion while the inner layer covers the endoderm to constitute the wall of the yolk sac (Greenstein and Foley, 1958). Yolk sac reaches peak development around Day 20 and then undergoes rapid regression (Greenstein et al., 1958). Amnion forms by cavitation from ICM, as the trophoderm folds around the embryo. On day 13-16, the amnion contains fluids which suspend the embryo, protecting it and permitting its free growth (Greenstein et al., 1958). Allantois is first detectable at about day 17 in sheep and day 20 in cattle. Allantois is composed of an inner layer of endoderm and an outer layer of vascular mesoderm which migrates into the space formed between the two mesoderm layers (Flechon, 1978). By day 22-27, the allantois undergoes a dramatic expansion and fuses with chorion until the chorioallantois is completely formed around the amnion by days 31-33 (Greenstein et al., 1958).

The attachment of the fetal membranes is a gradual process that begins with initial loose attachment by interdigitating microvilli of the trophoblast and endometrial epithelia at about Day 20 and progresses to an

attachment of the chorioallantois to the endometrial caruncles at day 30-36 of pregnancy (Atkinson et al., 1984; King et al., 1979; 1980; 1981). Over the next 30 to 40 days the chorion and endometrium form an interdigitating complex mature interplacentome placenta (King et al., 1979).

Secretory Proteins of Preimplantation Conceptus

In large domestic animals, communication between the developing conceptus and maternal tissues is essential for the establishment and maintenance of pregnancy. The major classes of compounds that may serve as signals for fetomaternal interactions include steroids, prostanoid, and proteins (Bazer and First, 1983; Bazer et al., 1986; Thatcher et al., 1986). The presence of conceptuses must be signaled before implantation begins to prevent the regression of the corpora lutea. The phenomenon, known as maternal recognition of pregnancy (Short, 1969), occurs between days 12 and 13 in ewes (Moor and Rowson, 1966a, 1966b), days 16 and 17 in cows (Betteridge et al., 1980; Northey and French, 1980) and days 15 and 17 in goats (Gnatek et al., 1989). Ovine, bovine and caprine maternal recognition of pregnancy is thought to be initiated by conceptus secretory proteins, ovine throphoblast protein-1 (oTP-1), bovine TP-1 (bTP-1) and caprine TP-1 (cTP-1), respectively. These proteins are major secretory products of conceptuses between days 13-21 in sheep (Godkin et al.,

1982), days 15-24 in cattle (Bartol et al., 1985), and days 16-21 in goats (Gnatek et al., 1989).

Bovine TP-1 and cTP-1 have physical and immunological characteristics similar to oTP-1 (Gnatek et al., 1989, Bartol et al., 1985; Godkin et al., 1988a; 1988b; Helmer et al., 1987). Ovine TP-1 appears to act locally as a paracrine hormone at the level of the uterine endometrium (Godkin et al., 1984a). Ovine TP-1 triggers a series of maternal responses to the presence of the conceptus including prevention of corpus luteum (CL) regression (Godkin et al., 1984b), changes in endometrial protein synthesis (Godkin et al., 1984a) and prostaglandin metabolism (Salamonsen et al., 1988; Vallet et al., 1987). As with oTP-1 in the ewe, bTP-1 can influence uterine prostaglandin metabolism (Knickerbocker et al., 1986), possibly by inducing an inhibitor of $\text{PGF}_{2\alpha}$ synthesis (Gross et al., 1988) and prolong the lifespan of the CL (Thatcher et al., 1989).

Recently, oTP-1 and bTP-1 have been identified as a member of the interferon- α (IFN) family by N-terminal amino acid analysis and cDNA sequencing (Imakawa et al., 1989; Stewart et al., 1987). Infusion of bovine recombinant IFN- α into the uteri of nonpregnant ewes (Stewart et al., 1989) or cows (Plante et al., 1988) can extend luteal lifespan. Furthermore, INF- α can compete with oTP-1 for binding to

endometrial receptors (Stewart et al., 1987; Hansen et al., 1989), and mimic the effects of oTP-1 on endometrial protein and prostaglandin biosynthesis (Thatcher et al., 1989; Salamonsen et al., 1988; Silcox et al., 1988). In addition, oTP-1 appears to possess antiviral (Roberts et al., 1989; Pontzer et al., 1988) and antiproliferative activities (Roberts et al., 1989) characteristic of INFs.

In addition to the trophoblastic proteins, preimplantation cow and ewe conceptuses also secrete a high molecular weight glycoprotein with a carbohydrate structure common to lactosaminoglycans (Fukuda et al., 1979; ^{human} Jarnefelt et al., 1978; Krusius et al., 1978; Masters et al., 1982; Newton and Hansen, 1988; Newton et al., 1988). The protein was demonstrated to have immunosuppressive activity (Murray et al., 1987) and thus it may form a glycocalyx layer at the maternal-conceptus interface to function in immune protection of the fetal allograft. It has also been speculated that the protein may be involved in trophoblast cell movement or attachment to the endometrium (Masters et al., 1982).

As gestation advances, patterns of protein production by the conceptus become more complex. Dramatical changes in the patterns of conceptus protein synthesis occur at about day 24-29 in cow (Godkin et al., 1988a; 1988b), day 23 in sheep (Godkin et al., 1982) and in goats (Gnatek et al.,

1989). These changes are coincident with the outgrowth of the allantois, its subsequent fusion with chorion and the progressive attachment of the chorion to the uterine endometrium (Greenstein et al., 1958). Production of oTP-1, bTP-1 and cTP-1 diminishes over this time period and synthesis of another discrete low molecular weight acidic protein (LMWAP), designated 3B₃ in bovine (Godkin et al., 1988a; 1988b), becomes prominent. The physical characteristics of the LMWAP are very similar among these three species, however, it remains to be determined if these LMWAPs are identical. The protein 3B₃ is also a major LMWAP produced by bovine placental membranes from at least Day 22 through Day 40 of pregnancy and a major component of allantoic fluid (Godkin et al., 1988a).

Secretory Proteins of Placenta

Placenta has been considered an endocrine organ because of the unique quantity and variety of hormones it secretes or metabolizes. In addition to its central role in steroidogenesis, it is also the source of several polypeptide hormones. Proteinaceous products from extraembryonic membranes have been reported to have thyrotropic (Avivi et al., 1982), luteotropic (Alilenberg and Shemesh, 1983), antiprostanoic (Shemesh et al., 1984; Knickerbocker et al., 1986), lactogenic (Beckers et al., 1980; Bolander and Fellows, 1976; Eakle et al., 1982; Murthy

et al., 1982), and immunosuppressive (Murray et al., 1987) activities. Clearly, proteins produced by placental membranes are essential in the establishment and maintenance of pregnancy and development of the conceptus.

Protein production by bovine allantois and chorion during early pregnancy has been characterized by Godkin et al. (1988a). In general, chorion was the source of a number of basic-to-neutral proteins, allantois and amnion were the sources of more acidic proteins. Both bovine chorion and allantois are active in protein synthesis. Major qualitative alterations in proteins synthesized occurred between Days 24-29, coincident with the development of the allantois and its subsequent fusion with chorion. Production of bTP-1 diminished over this time period and synthesis of the LMWAP, designated 3B₃ (Godkin et al., 1988a; 1988b) became prominent. In addition, Godkin et al. (1982) reported that Day 23 ovine conceptuses also produce a protein with electrophoretic properties identical to 3B₃. The goal of this study was to define the physiological function of this protein during early pregnancy in sheep and cattle.

LIST OF REFERENCES

LIST OF REFERENCES

- Ailenberg M. and M. Shemesh. 1983. Partial purification of a chorionic gonadotropin-like protein from bovine cotyledons. *Biol. Reprod.* 28:517.
- Atkinson B. A., C. J. King, and E. C. Amoroso. 1984. Development of the caruncular and intercaruncular regions in the bovine endometrium. *Biol Reprod.* 30: 763.
- Avivi A., A. B. Schreiber, and M. Shemesh. 1982. Isolation and characterization of a 94,000-dalton protein with thyrotropic activity from early bovine placenta. *J. Biol. Chem.* 257:11384-11389.
- Bartol F. F., R. M. Roberts, F. W. Bazer, G. S. Lewis, J. D. Godkin, and W. W. Thatcher. 1985. Characterization of proteins produced in vitro by periattachment bovine conceptuses. *Biol. Reprod.* 32:681-693.
- Betteridge K. J., M. D. Eaglesome, G. C. B. Randal, and D. Mitchell. 1980. Collection, description and transfer of embryos from cattle 10-16 days after oestrus. *J. Reprod. Fert.* 59:205-216.
- Bazer F. W., N. L. First. 1983. Pregnancy and parturition. *J. Anim. Sci.* 57:425-460.

- Bazer F. W., J. L. Vallet, R. M. Roberts, D. C. Sharp, and W. W. Thatcher. 1986. Role of conceptus secretory products in establishment of pregnancy. *J. Reprod. Fert.* 76:841-850.
- Beckers J. F., C. Fromont-Lienard, P. van der Zwalnen, P. Wouters-Ballman, and F. Ectors. 1980. *Ann. Med. Vet.* 124:585-592.
- Bolander F. F., and R. E. Fellows. 1976. Purification and characterization of bovine placental lactogen. *J. Biol. Chem.* 251:2703-2707.
- Eakle K. A., Y. Arima, P. Swanson, H. Grimek, and R. D. Bremel. 1982. A 32000 molecular weight protein from bovine placenta with placental lactogen-like activity in radioreceptor assays. *Endocrinology* 110:1758-1765.
- Flechon, J. E. 1978. Morphological aspects of embryonic disc at the time of its appearance in the blastocyst of farm mammals. *Scanning Electron Microscopy*. vol 2, 54:548.
- Flechon J. E. and J. P. Renard. 1978. A scanning electron microscope study of the hatching of bovine blastocyst in vitro. *J. Reprod. Fert.* 53:9-12.
- Fukuda M., M. N. Fukuda, S. Hakomori. 1979. Developmental changes and genetic defect in the carbohydrate structure of band 3 glycoprotein of human erythrocyte membrane. *J. Biol. Chem.* 254:3700-3703.

- Gnatek G. G., L. D. Smith, R. T. Duby, and J. D. Godkin.
1989. Maternal recognition of pregnancy in the goat:
effects of conceptus removal on interestrus intervals
and characterization of conceptus protein production
during early pregnancy. Biol. Reprod. 42:655-663.
- Godkin J. D., F. W. Bazer, J. Moffatt, F. Sessions, and R.
M. Roberts. 1982. Purification and properties of a major
low molecular weight protein released by the
trophoblast of sheep blastocysts at Day 13-20. J.
Reprod. Fert. 65:141-150.
- Godkin J. D., F. W. Bazer, and R. M. Roberts. 1984a. Ovine
trophoblast protein-1, an early secreted blastocyst
protein, binds specifically to uterine endometrium and
affects protein synthesis. Endocrinology 114:120-130.
- Godkin J. D., F. W. Bazer, W. W. Thatcher, and R. M.
Roberts. 1984b. Proteins released by Day 15-16
conceptuses prolong luteal maintenance when introduced
into the uterine lumen of cyclic ewes. J. Reprod.
Fertil. 71:57-64.
- Godkin J. D., B. J. Lifsey, and B. E. Gillespie. 1988a.
Characterization of bovine conceptus proteins produced
during the peri- and postattachment periods of early
pregnancy. Biol. Reprod. 38:703-711.

- Godkin J. D., B. J. Lifsey, and G. A. Baumbach. 1988b. Characterization of protein production by bovine chorionic and allantoic membranes. *Biol. Reprod.* 39:195-204.
- Greenstein J. S., and R. C. Foley. 1958. Early embryology of the cow. I. Gastrula and primitive streak stages. *J. Dairy Sci.* 41:409-421.
- Greenstein J. S., R. W. Murray, and R. C. Foley. 1958. Observations on the morphogenesis and histochemistry of the bovine preattachment placenta between 16 and 33 days of gestation. *Anat. Rec.* 132:321-341.
- Hansen T. R., M. Kazemi, D. H. Keisler, P. V. Malathy, K. Imakawa, and R. M. Roberts. 1989. Complex binding of the embryonic interferon, ovine trophoblast protein-1, to endometrial receptors. *J. Interferon Res.* 9:211-221.
- Helmer S. D., P. J. Hansen, R. V. Anthony, W. W. Thatcher, F. W. Bazer, and R. M. Roberts. 1987 Identification of bovine trophoblast protein-1, a secretory protein immunologically related to ovine trophoblast protein-1. *J. Reprod. Fertil.* 79:83-91.
- Hunter R. H. F., and I. Wilmut. 1984. Sperm transport in the cow: periovulatory redistribution of viable cells within the oviduct. *Reprod. Nutr. Develop.* 24:597-608.
- Hunter R. H. F. 1985. Experimental studies of sperm transport in sheep, cows and pigs. *Vet. Rec.* 116:188.

- Imakawa K., T. R. Hansen, P. V. Malathy, R. V. Anthony, H. G. Polites, K. R. Marotti, and R. M. Roberts. 1989
Molecular cloning and characterization of cDNAs
corresponding to bovine trophoblast protein-1. A
comparison with ovine trophoblast protein-1 and bovine
interferon- α_{II} . Mol. Endocrinol. 3:127-139.
- Januideen M. R., and E. S. E. Hafez. 1980. Reproductive
failure in females. In: Reproduction in Farm Animals
(4th ed), E. S. E. Hafez, (eds.) pp 449-470, Lea and
Febiger, Philadelphia, PA.
- Jarnefelt J., J. Rush, Y-T Li, R. A. Laine. 1978.
Erythroglycan, a high molecular weight glycopeptide
with the repeating structure [galactosyl-(1-4)-2-deoxy-
2-acetamido-glucosyl (1-3)] comprising more than one-
third of the protein-bound carbohydrate of the human
erythrocyte stroma. J. Biol. Chem. 253:8006-8009.
- King C. J., B. A. Atkinson, and H. A. Robertson. 1980.
Development of the bovine placentome from Days 20 to 29
of gestation. J. Reprod. Fert. 59:95-100.
- King C. J., B. A. Atkinson and H. A. Robertson. ~~1982~~ 1970
Development of the bovine placentome during the second
month of gestation. J. Reprod. Fert. 55:173-180.

King C. J., B. A. Atkinson, and H. A. Robertson. 1981.

Development of the intercaruncular areas during early gestation and establishment of the bovine placenta. J. Reprod. Fert. 61:469-474.

Knickerbocker J. J., W. W. Thatcher, F. W. Bazer, D. H.

Barron, and R. M. Roberts. 1986a. Inhibition of uterine prostaglandin $F_{2\alpha}$ production by bovine conceptus secretory proteins. Prostaglandins 31:777-793.

Knickerbocker J. J., W. W. Thatcher, F. W. Bazer, M. Drost,

D. H. Barron, K. B. Fincher, and R. M. Roberts. 1986b. Proteins secreted by Day-16 to 18 bovine conceptuses extend corpus luteum function in cow. J. Reprod. Fert. 77:381-391.

Krusius T., J. Finne, and H. Rauvala. 1978. The poly

(glycosyl) chains of glycoproteins. Eur. J. Biochem. 92:289-300.

Linares T., and L. Ploen. 1981. On the ultrastructure of seven day old normal (blastocysts) and abnormal bovine embryos. Zbl. Vet. Med., C. Anat. Histol. Embryol. 10:212-226.

Lindner G. M., and R. W. Wright (Jr.) 1983. Bovine embryo morphology and evaluation. Theriogenology 20:407-416.

Massip A., J. Mulnard, R. Huygens, C. Hanzen, P. Van der Zwalm, and F. Ectors. 1981. Ultrastructure of the cow blastocyst. J. Submicrosc. Cytol. 13:31-40.

Masters R. A., R. M. Roberts, G. S. Lewis GS, W. W.

Thatcher, F. W. Bazer, and J. D. Godkin. 1983. High molecular weight glycoproteins released by expanding preattachment sheep, pig, and cow blastocysts in culture. *J. Reprod. Fertil.* 66:571-583.

Moor, R. M. and L. E. A. Rowson. 1966a. The corpus luteum of the sheep: effect of the removal of embryos on luteal function. *J. Endocr.* 34:487-502.

Moor, R. M. and L. E. A. Rowson. 1966b. The corpus luteum of the sheep: functional relationship between the embryo and the corpus luteum. *J. Endocr.* 34:233-239.

Murray M. K., E. C. Segerson, P. J. Hansen, F. W. Bazer, and R. M. Roberts. 1987. Suppression of lymphocyte activation by a high molecular weight glycoprotein released from preimplantation ovine and porcine conceptuses. *Am. J. Reprod. Immunol. Microbiol.* 14:38-44.

Murthy G. S., C. Schellenberg, and H. G. Friesen. 1982. Purification and characterization of placental lactogen. *Endocrinology* 111:2117-2228.

Newton G. R., P. J. Hansen, and B. G. Low. 1988. Characterization of a high molecular weight glycoprotein secreted by the peri-implantation bovine conceptus. *Biol. Reprod.* 39:553-560.

- Newton G. R., and P. J. Hansen. 1988. Secretion of a lactosaminoglycan- containing glycoprotein by peri-implantation sheep conceptuses. *Biochem. Biophys. Acta.* 996:56-64.
- Northey D. L., and L. R. French. 1980. Effect of embryo removal and intrauterine infusion of embryonic homogenates on the lifespan of the bovine corpus luteum. *J. Anim. Sci.* 50:298-302.
- Plante C., P. J. Hansen, and W. W. Thatcher. 1988. Prolongation of luteal lifespan in cows by intrauterine infusion of recombinant bovine alpha-interferon. *J. Endocrinol.* 122:2342-2344.
- Pontzer C. H., B. A. Torres, J. L. Vallet, F. W. Bazer, H. M. Johnson. 1988. Antiviral activity of the pregnancy recognition hormone ovine trophoblast protein-1. *Biochem. Biophys. Res. Commun.* 152:801-807.
- Renard J. P., Y. Menezo, F. Saumande and Y. Heyman. 1978. Attempts to predict the viability of cattle embryos produced by superovulation. In *Control of Reproduction in the Cow*, Ed. J. M. Sreenan, Martinus Nijhoff, The Hague, pp. 398-417.
- Roberts R. M., K. Imakawa, Y. Niwano, M. Kazemi, P. V. Malathy, T. R. Hansen, A. A. Glass, and L. H. Kronenberg. 1989. Interferon production by the preimplantation sheep embryo. *J. Interf. Res.* 9:171-83.

- Salamonsen L. A., S. J. Stuchbery, C. M. O'Grady, J. D. Godkin and J. K. Findlay. 1988. Interferon-alpha mimics effects of ovine trophoblast protein-1 on prostaglandin and protein secretion by ovine endometrial cells in vitro. *J. Endocr.* 117:R1-4.
- Short R. V, 1969. Implantation and the maternal recognition of pregnancy. In Wolstenholme G. E. W. and M. O'Connor (eds.) p 2. *Foetal Autonomy*. J and A Churchill Ltd.
- Silcox R. W., H. Francis, and R. M. Roberts. 1988. The effects of ovine trophoblast protein-1 (oTp-1) on ovine endometrial protein synthesis are mimicked by human alpha-1 interferon. *J. Anim. Sci.* 66(Suppl. 1):153-154.
- Shemesh M., W. Hansel, and J. F. Strauss. 1984. Modulation of bovine placental prostaglandin synthesis by an endogenous inhibitor. *Endocrinology* 115:1401-1410.
- Stewart H. J., A. P. F. Flint, G. E. Lamming, S. H. E. McCann, and T. J. Parkinson. 1989. Antiluteolytic effects of blastocyst-secreted interferon investigated in vitro and in vivo in the sheep. *J. Reprod. Fertil.* Suppl. 37:127-138.
- Stewart H. J., S. H. E. McCann, P. J. Barker, K. E. Lee, G. E. Lamming, and A. P. F. Flint. 1987. Interferon sequence homology and receptor binding activity of ovine trophoblast antiluteolytic protein. *J. Endocrinol.* 115:R13-15.

- Thatcher W. W., P. J. Hansen, T. S. Gross, S. D. Helmer, C. Plante, and F. W. Bazer. 1989. Antiluteolytic effects of bovine trophoblast protein-1. J. Reprod. Fert. Suppl. 37:91-99.
- Thatcher W. W., F. W. Bazer, D. C. Sharp, and R. M. Roberts. 1986. Inter-relationships between uterus and conceptus to maintain corpus luteum function in early pregnancy: sheep, cattle, pigs and horses. J. Anim. Sci. 62:25-46.
- Vallet J. L., F. W. Bazer and R. M. Roberts. 1987. The effect of ovine trophoblast protein-1 on endometrial protein secretion and cyclic nucleotides. Biol. Reprod. 37:1307-1315.

**PART 2: PURIFICATION AND CHARACTERIZATION OF BOVINE
PLACENTAL RETINOL-BINDING PROTEIN**

CHAPTER 1

ABSTRACT

A major low molecular weight acidic protein, 3B₃, produced from cultures of Days 29-90 bovine allantoic membranes (in the presence of [³H]-leucine or [³⁵S]-methionine) and from Days 29-60 allantoic fluid, has been purified. The protein consisted of three isoelectric variants (pI 5.3 - 6.1) of identical molecular weight (23,200 ± 900) when analyzed by 2D-SDS-PAGE. Amino-terminal sequence analysis of 3B₃ isolated from allantoic fluid on the first 43 amino acids showed that 3B₃ had 93% and 91% homology with rabbit and human plasma retinol-binding protein (RBP), respectively. The ultraviolet absorption spectrum and the fluorescence excitation and emission spectra of purified 3B₃ from both sources indicated the presence of bound retinol. Rabbit antiserum was raised against placental RBP (3B₃) isolated from allantoic membrane culture medium. Placental RBP was immunoprecipitated from radiolabeled allantois and chorion culture medium and was detected in allantoic membrane culture medium and allantoic fluid by Western blotting. These results suggested that bovine placental membranes secrete RBP into allantoic fluid and that placental RBP may play important roles in vitamin A metabolism in the developing embryo.

CHAPTER 2

INTRODUCTION

Proteins produced by placental membranes play essential functions in the establishment and maintenance of pregnancy and development of the conceptus. Synthesis of proteins in vitro by bovine conceptuses and isolated extraembryonic membranes has been characterized (Bartol et al., 1985; Godkin et al., 1988a; 1988b). During the pre- and peri-attachment periods, bovine conceptuses produce a complex of low molecular weight, acidic polypeptides named bovine trophoblast protein-1 (bTP-1), that has been identified as a member of the interferon- α family (Imakawa et al., 1989). Bovine TP-1 (Helmer et al., 1987) along with similar conceptus products of sheep (oTP-1) (Godkin et al., 1984a; 1984b) has been implicated as an embryonic signal in the maintenance of corpora lutea function during early pregnancy (Reviewed by Roberts, 1989). Similar interferon-like proteins have been detected as secretory products of goat (Gnatek et al., 1989) and pig (Cross and Roberts, 1989) conceptuses. Production of a very high molecular weight glycoprotein by preimplantation cow (Bartol et al., 1985) conceptuses has been reported. A similar protein (Masters et al., 1983) from sheep and pig conceptuses was demonstrated to have immunosuppressive activity in vitro

(Murray et al., 1987) and a carbohydrate structure common to lactosaminoglycans (Newton et al., 1988).

Several laboratories have reported the presence of pregnancy associated proteins in uterine flushings from pregnant cows (Roberts and Parker, 1974) or extracts of extraembryonic membranes (Avivi et al., 1982; Laster, 1977) but these have not been well characterized. A radioimmunoassay for a protein designated pregnancy specific protein B has been reported to be an effective assay for pregnancy diagnosis in cattle (Sasser et al., 1986). To date, bTP-1 (Lifsey et al., 1989) and bovine placental lactogen (Murthy et al., 1982) are the only well characterized, purified bovine placental proteins demonstrated to have specific target organs and functions.

Proteins produced during in vitro culture by bovine conceptuses obtained during the peri-and early postattachment periods have been characterized by 2D-SDS-PAGE and fluorography (Bartol et al., 1985; Godkin et al., 1988a; 1988b). Major qualitative alterations in proteins synthesized occurred between Days 24-29, coincident with the development of the allantois and its subsequent fusion with chorion. Production of bTP-1 diminished over this time period and synthesis of another low molecular weight acidic protein, designated 3B₁ (Godkin et al., 1988a; 1988b) became prominent. This protein was observed to be a

major low molecular weight secretory polypeptide produced by placental membranes from about Day 22 (Godkin et al., 1988a) through at least Day 90 of pregnancy (Liu and Godkin unpublished observation) and a major component of allantoic fluid protein (Godkin et al., 1988b). Here, we report the purification and characterization of 3B₃ synthesized by allantoic membrane cultures and from allantoic fluid. In addition, evidence is presented to demonstrate that 3B₃ is a placental RBP.

CHAPTER 3

MATERIALS AND METHODS

Materials

Supplies for tissue culture and electrophoresis were as described previously (Godkin et al., 1988a; 1988b). Biogel P-30, Immunoblot Assay Kit and Silver Stain Kit were from BioRad Laboratories, Richmond, CA; Sephadex G-75 was from Pharmacia LKB Biotechnology, Inc., Piscataway, NJ. All other supplies for gel and high performance liquid chromatography, protein blotting and immune complex precipitation, antibody production, ultrafiltration and dialysis were as described by Lifsey et al. (1989). L-[2, 4, 5-³H]-leucine (53 Ci/mmol) and L-[³⁵S]-methionine (1134 Ci/mmol) were purchased from New England Nuclear.

Animals

Estrous cycle synchronization and breeding of crossbred beef cows (n=9) by artificial insemination was as described by Godkin et al. (1988a). Reproductive tracts were obtained at slaughter from a local packing plant and transported to the laboratory on ice within 40 min after death. Additional reproductive tracts (n=8) were obtained at slaughter from dairy and beef cows with unknown breeding dates. Ages of embryos from these animals were estimated by crown-rump measurements (Bongso and Basrur et al., 1976). Ages of all embryos were determined or estimated to be

between 29 and 90 days.

In Vitro Culture of Allantoic Membranes

Allantoic and chorionic tissues were carefully dissected apart under sterile conditions with the aid of a stereo zoom microscope in a laminar flow tissue culture hood as described by Godkin et al. (1988b). Tissues were placed in 100 x 20 cm sterile Petri dishes containing MEM and cut into 2-3 mm pieces. Approximately 200 mg of tissue were transferred to 60 x 15 mm tissue culture dishes containing 6 ml MEM and 5 uCi/ml [³⁵S]-methionine. Two grams of tissue were transferred to 150 x 25 mm tissue culture dishes containing 60 ml MEM and 3 uCi/ml [³H]-leucine. Culture conditions were as described by Godkin et al. (1988b). Proteins labeled with [³H]-leucine were used in protein purification while those labeled with [³⁵S]-methionine were used in the 2D-SDS-PAGE and immunoprecipitation studies (see below).

Isolation of 3B₃

All steps were performed at 4 C unless noted otherwise. Protein was monitored by absorbance at 280 nm and/or liquid scintillation spectrometry. The presence of 3B₃ in column fractions was determined by 1D-SDS-PAGE (see below). Protein concentrations were determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard.

Medium from Day 29 to 90 bovine allantoic membrane cultures was pooled (approximately 500 ml) and concentrated 5-fold by positive pressure ultrafiltration utilizing a Spectra/Por stirred cell apparatus with a membrane having a nominal molecular weight cutoff of 10,000. Removal of salts and phenol red was achieved by gel filtration on a column (5 x 30 cm) of Spectra/Gel AcA 202 equilibrated in 10 mM Tris-HCl, pH 7.6, 0.02% (w/v) NaN₃ at a flow rate of 0.8 ml/min. Protein eluting in the excluded volume was directly adsorbed onto a column (2 x 7 cm) of Q-Sepharose anion-exchange medium equilibrated with the same buffer. Protein was eluted with a linear gradient of NaCl from 70 ml of starting buffer to 70 ml of the same buffer containing 600 mM NaCl. Fractions (2.5 ml) were collected at a flow rate 1.0 ml/min. The fractions containing 3B₃ were pooled, dialyzed against 1 mM Tris-HCl buffer and lyophilized. The sample was redissolved in 2 ml Tris-HCl buffer and applied to a Sephadex G-75 column (1.7 x 100 cm) equilibrated in 10 mM Tris-HCl, pH 7.6, and 0.02% (w/v) NaN₃. Fractions (2 ml) containing the low molecular weight protein peak (M_r = 13,000 - 30,000) were pooled and dialyzed (M_r cutoff 12,000 - 14,000) against 10 mM Tris-HCl, pH 7.6 (4 changes at 12-h intervals). Proteins in the low molecular weight peak were fractionated on a TSK DEAE column (7.5 x 75 mm) equilibrated in 10 mM Tris-HCl, pH 7.6, at 23 C. Flow rate was

maintained at 0.8 ml/min. Bound proteins were eluted with a linear gradient of NaCl (0 - 0.35 M). Fractions (0.4 ml) containing 3B₃ were pooled and lyophilized. Protein was then redissolved in 1 ml Tris-HCl buffer and applied to Biogel P-30 column (1.4 x 120 cm) which had been equilibrated with the same buffer as G-75 column and collected in 1 ml fractions. The final solution containing 3B₃ was dialyzed against 1 mM Tris-HCl, pH 7.6 (4 changes at 8-h intervals), concentrated and stored at -20 C for further analysis.

Protein Electrophoresis

One-dimensional SDS-PAGE was performed according to the method of Laemmli (22) in 12.5% (w/v) polyacrylamide gels and 5% of stacking gel. Fixation and silver-staining of proteins was performed according to the manufacturer's instructions. The method of Roberts et al. (23) was used for 2D-SDS-PAGE. After electrophoresis, Coomassie Brilliant Blue R-250-stained gels were impregnated with 1 M sodium salicylate for fluorography and dried (24). Radiolabeled proteins were detected using Kodak XAR film.

Production of Antiserum

Purified 3B₃ protein (150 ug) was emulsified in Complete Freund's Adjuvant, and injected s. c. into the shoulders and back of a New Zealand White rabbit. Serum was first obtained 5 weeks after immunization. The rabbit was given a second immunization of 50 ug 3B₃ in Incomplete

Freund's Adjuvant and five subsequent bleedings were performed at 2-week intervals. Prior to immunization, serum was collected from the rabbit (preimmune serum) to detect nonspecific binding in Western blotting, and immunoprecipitation procedures.

Immunoprecipitation of 3B₃

One milliliter aliquots of ³⁵S-labeled, Day 29 cow allantoic and chorionic membrane culture medium were incubated with anti-3B₃ serum or preimmune rabbit serum. Immune complexes were absorbed onto Protein A-Sepharose as described by Lifsey et al. (1989) and analyzed by 1D-SDS-PAGE.

Immunoblot Analysis

Aliquots of unfractionated protein (60 ug) from Day 53 bovine allantoic membrane culture medium and Day 60 allantoic fluid were separated by 2D-SDS-PAGE and stained with Coomassie Brilliant Blue. Proteins were electrophoretically transferred to nitrocellulose membranes (Roberts et al., 1984). Bovine serum albumin (3% w/v) was used to block nitrocellulose. Membranes were incubated for 15 h at 4 C (with rocking) in anti-3B₃ serum or nonimmune rabbit serum at a dilution of 1:1000. Bound antibody was visualized using a 1:1000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase according to the Immunoblot Assay Kit instructions.

Amino-terminal Protein Microsequencing

Sixty micrograms of purified 3B₃ from allantoic fluid in 0.1 ml of 0.1% trifluoroacetic acid, 50% acetonitrile were spotted onto Immobilon P membranes and subjected to amino-terminal amino acid microsequencing by gas-phase Edman degradation (Applied Biosystems 470A). Cysteines were not reduced and alkylated and thus could not be identified. Amino acid sequences obtained for 3B₃ were analyzed on the National Biomedical Research Foundation-Protein Identification Resource data base for comparison with documented protein sequences.

Retinol Determination

Fluorescence spectra were obtained on a Perkin-Elmer fluorescence spectrophotometer to determine the excitation and emission maxima of 3B₃.

CHAPTER 4

RESULTS

Two-Dimensional PAGE of Synthesized Protein

Figure 1 illustrates the electrophoretic patterns of ^{35}S -labeled protein synthesized and released by allantois and chorion at Day 53 of pregnancy. The pattern of proteins synthesized at Day 53 was identical to that observed at Day 40 (Godkin et al., 1988b). Cultures that contained [^3H]-leucine produced identical fluorograms as those containing [^{35}S]-methionine. Protein 3B₃ appeared to consist of three isoelectric variants (pI 5.3 - 6.1), with the slightly more basic isotypes being more abundant.

Purification of 3B₃

Allantoic membrane culture medium contained a less complex array of radiolabeled (Fig. 1) or coomassie-stainable (not shown) polypeptides than chorionic membrane culture medium and was therefore utilized as the starting material for isolation of protein 3B₃. Figure 2a shows a typical electrophoretic pattern of radiolabeled protein in the starting material. Approximately 500 ml of allantoic membrane culture medium (^3H -labeled, Days 29-90) were concentrated, isolated and applied to Q-Sepharose. Bound proteins were eluted as three radiolabeled peaks designated "A", "B", and "C" by employing a salt gradient (Fig. 3). As

Figure 1. Fluorograms of two-dimensional gels for analysis of radioisotopically labeled proteins synthesized and released by allantois (Panel A) and chorion (Panel B) from Day 53 of pregnancy. Both membranes had been cultured in the presence of L-[³⁵S]-methionine for 24 h. 150,000 cpm were loaded onto each gel and fluorographs were exposed to dried gels for 7 days. A group of low molecular weight proteins, named 3B₃, are indicated by brackets. Rabbit muscle phosphorylase b, bovine serum albumin, hen egg white albumin, bovine carbonic anhydrase, soybean trypsin inhibitor, and hen egg white lysozyme were used as protein molecular weight standards. Horizontal scale represents pH values.

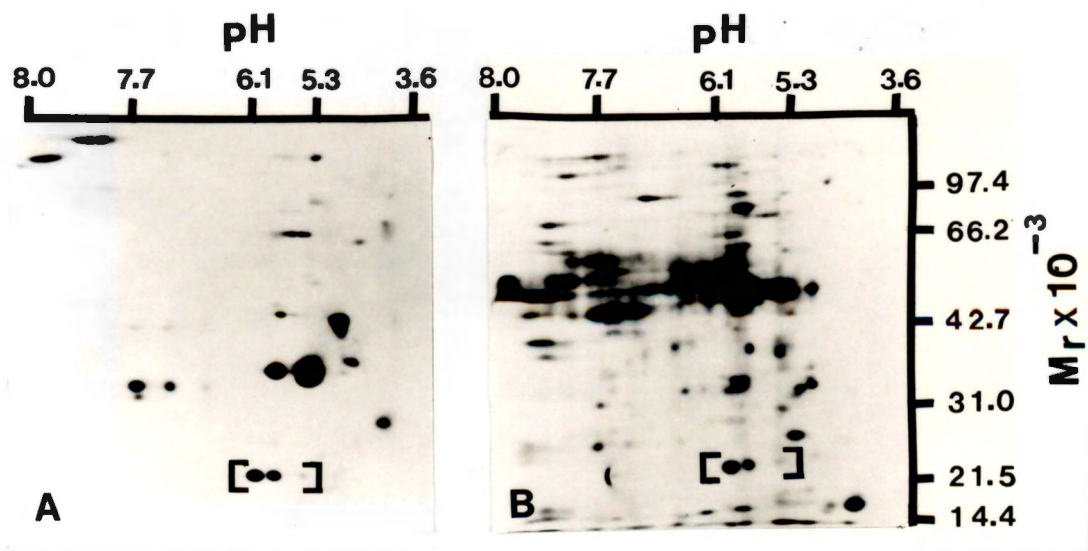


Figure 2. Stages in the purification of 3B₃ from bovine allantois culture medium. Samples of 3B₃ containing fractions were analyzed by 1D-SDS-PAGE using 12.5% (w/v) polyacrylamide gels in the presence (Lane a-d) or absence (Lane e) of 2-mercaptoethanol. Radiolabeled proteins of Day 36 total bovine allantois culture medium in [³H]-leucine (50,000 cpm, Lane a) and Q-Sepharose fraction (20,000 cpm, Lane b) were detected by fluorography. DEAE-HPLC-enriched 3B₃ fractions (8 ug, Lane c) and Sephadex P-30-purified 3B₃ fractions (6 ug, Lane d; e) were silver-stained.

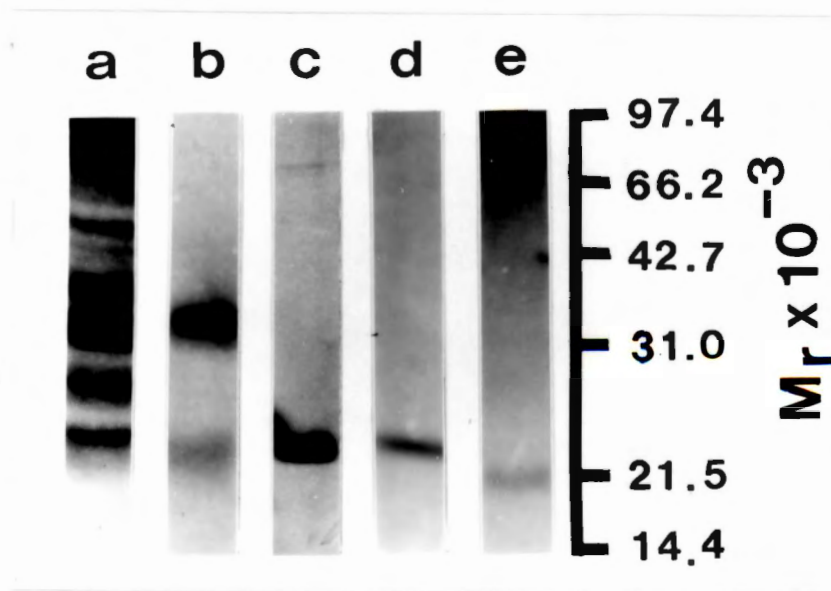
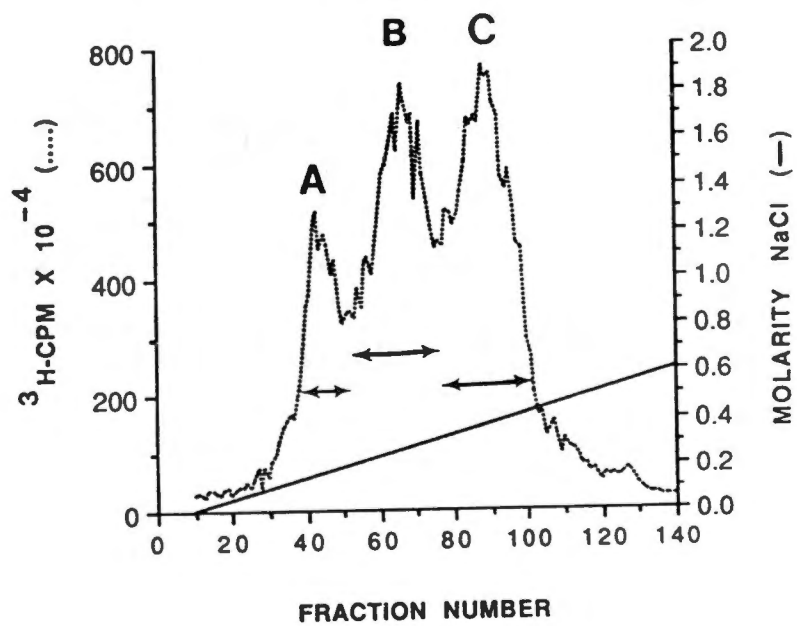


Figure 3. Q-Sepharose ion exchange chromatography of dialyzed medium following a 24-hour incubation of Day 29-90 bovine allantois in L- $[^3\text{H}]$ -leucine. Bound proteins were eluted with a 0.0 to 0.6 M gradient of NaCl. Samples (30 μl) were removed from each fraction (2.5 ml) and analysed for radioactivity. Fractions containing peak radioactivity were combined into three pools designated "A", "B", and "C" as indicated by double-headed arrows. As shown in Figure 1b, the radioactive peak B contained 3B_3 .



shown in Figure 2b, peak B contained two major radiolabeled polypeptides with $M_r = 23,000$ and $33,000$. The two were separated by Sephadex G-75 chromatography (Fig. 4). Fractions eluting in the molecular weight range of $17,000$ to $30,000$ were pooled, dialyzed and chromatographed by DEAE-HPLC (Fig. 5). Bound protein was eluted with a linear gradient of NaCl. The major radiolabeled peak was eluted at 0.12 - 0.14 M NaCl coincident with a major protein absorbance (280 nm) peak. Analysis of this pooled peak by 1D-SDS-PAGE and silver-staining demonstrated the presence of a major polypeptide with a molecular weight of $22,000 - 24,000$ ($3B_3$) and a small amount of a protein with a molecular weight of about $70,000$ (Fig. 2c). Final purification was achieved by gel filtration on Biogel P-30 column. A single symmetrical peak was eluted in the molecular weight range of $22,000$ - $24,000$ (Fig. 6). The material in the eluted peak migrated as a single band after 1D-SDS-PAGE and silver-staining (Fig. 2d). Yield of the protein $3B_3$ varied between preparations. Typically 80 - 100 ug of $3B_3$ were isolated from 500 ml of culture medium.

Molecular Weight Estimation of $3B_3$

The molecular weight of purified $3B_3$ was estimated to be $23,200 \pm 900$ (mean $\pm$ SD; $n = 7$ separations) by 1D-SDS-PAGE (reducing conditions) (Fig. 2c; 2d). When 2-mercaptoethanol was omitted during sample preparation (nonreducing

Figure 4. Sephadex G-75 gel chromatography of peak B from Figure 3. Pooled fractions of peak B from Q-Sepharose were dialyzed, concentrated and applied to a column (1,7 x 100 cm) of Sephadex G-75. The column was eluted with 10 mM Tris-HCl (pH 7.6). Arrows indicate the elution positions of the molecular weight markers: (1) Transferrin (78,000), (2) ovalbumin (42,699), (3) myoglobin (16,900), and (4) cytochrome C (12,900). The indicated fractions containing 3B₃ were pooled and dialyzed in preparation for further fractionation by DEAE-HPLC.

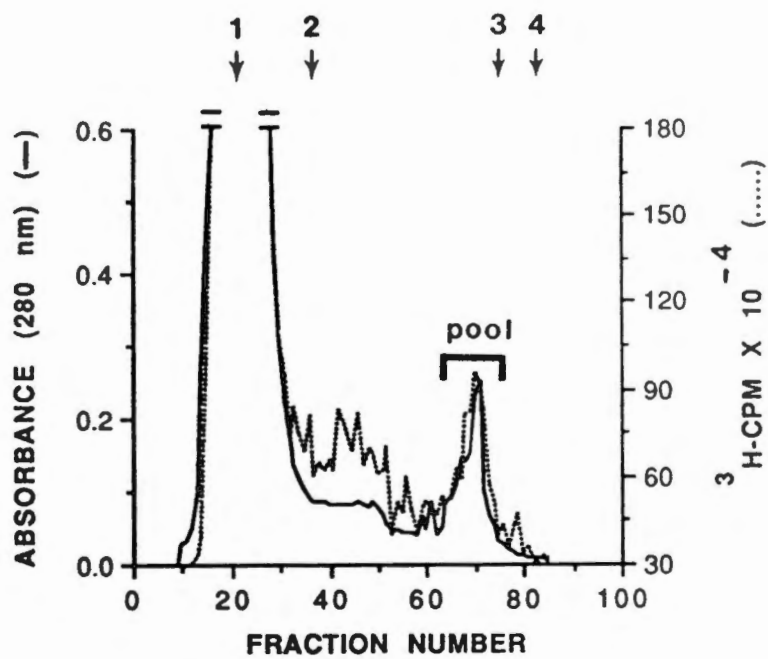


Figure 5. DEAE-HPLC ion exchange chromatography of Sephadex G-75 gel chromatography fraction containing $3B_3$. Dialyzed samples were loaded and the column washed with approximately 8 ml of 10 mM Tris-HCl, pH 7.6, prior to elution with a gradient of 0-0.34 M NaCl (----). Ten μ l samples of each fraction (0.4 ml) of the eluate were analyzed for radioactivity. $3B_3$ enriched fractions were eluted with approximate 0.12-0.14 M NaCl. The indicated fractions containing $3B_3$ were pooled.

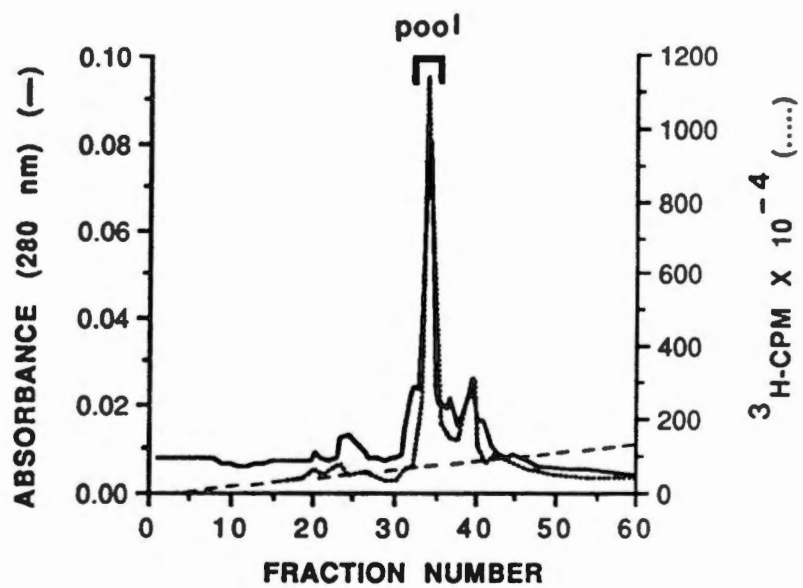
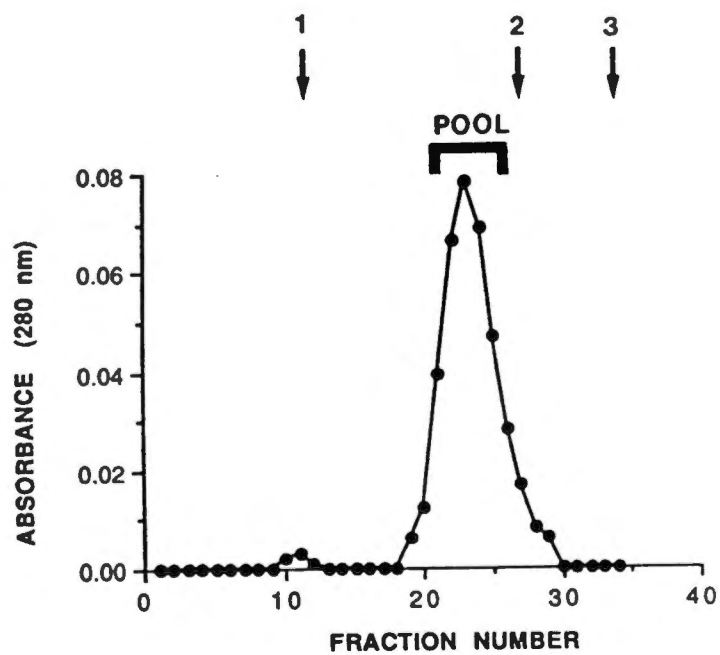


Figure 6. Biogel P-30 gel chromatography of the DEAE-HPLC-enriched 3B₃. Pooled 3B₃ enriched fractions from DEAE-HPLC run were concentrated and applied onto a Biogel P-30 columnn (1.4 x 120 cm) eluted with 10 mM Tris-Hcl (pH 7.6). Arrows indicate the elution positions of the molcular weight markers: (1) Transferrin (78,000), (2) myoglobin (16,900), and (3) cytochrome C (12,900). The indicated fractions containing 3B₃ were pooled and dialyzed for the further analysis.



conditions) the apparent molecular weight was $21,200 \pm 400$ ($n = 5$) (Fig. 2e).

Immunoprecipitation and Immunoblotting of 3B₃

3B₃ was immunoprecipitated with anti-3B₃ serum from ³⁵S-labeled proteins of Day 29 cow allantois and chorion culture medium. A single radiolabeled band ($M_r = 23,200$) was detected by 1D-SDS-PAGE and fluorography (Fig 7. Lane 1 and 3). Preimmune rabbit serum did not react with radiolabeled cow allantoic and chorionic proteins (Lane 2 and 4).

Sixty micrograms of bovine allantoic culture medium proteins (Day 53) and allantoic fluid proteins (Day 60) were analyzed on Western blot of 2D gel for antigens (Fig. 8, Panel A and B) that were immunoreactive with the anti-3B₃ serum. Anti-3B₃ serum reacted with an identical group of proteins from both culture medium and fluid composed of three polypeptides with different pI s (5.3 - 6.1) but with the same molecular weight ($23,200 \pm 900$) (Fig. 8, Panel C and D). No reaction was observed when preimmune rabbit serum was used (data not shown). These results suggested that the antiserum was specific for 3B₃ which appeared to consist of three electrophoretic isoforms and was present in allantoic fluid.

Since the electrophoretic mobilities of 3B₃ and bTP-1 were very similar, we performed analyses to demonstrate that

Figure 7. Analysis of immunoprecipitates from allantois and chorion secretory proteins of cow by one-dimensional polyacrylamide gel electrophoresis and fluorography. Lane 1 and 3 contain ^{35}S -labeled 3B₃ immunoprecipitated from Day 29 cow allantois and chorion culture medium with anti-3B₃ serum, respectively. Lane 2 and 4 are the preimmune serum control for lane 1 and 3, respectively.

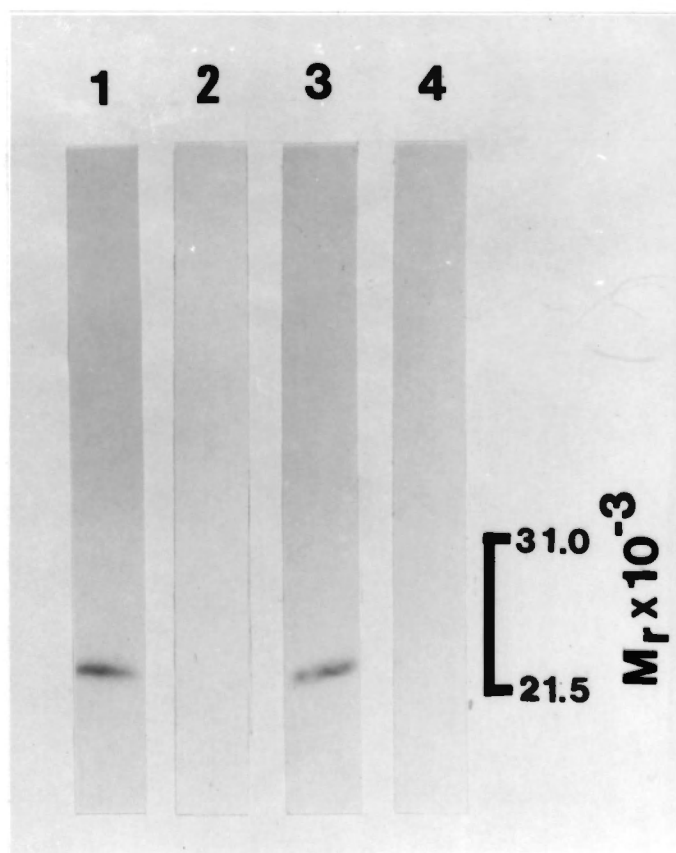
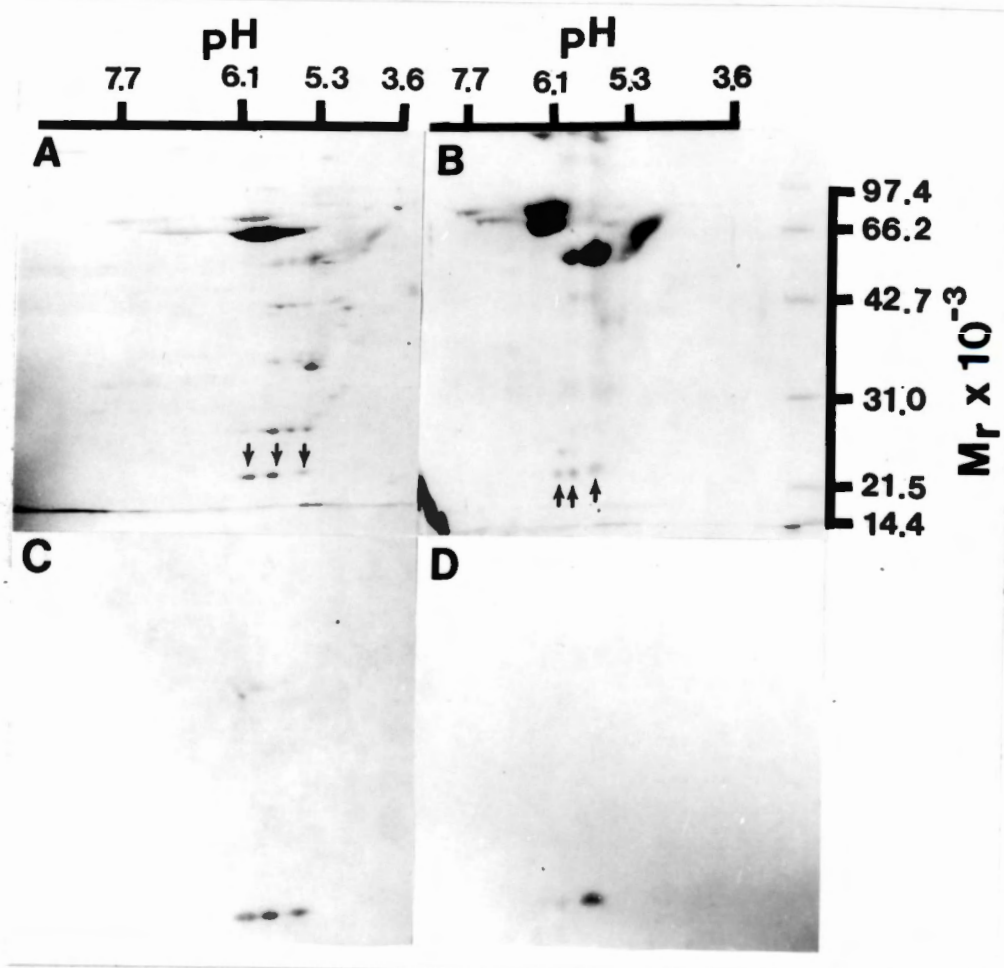


Figure 8. Coomassie Brilliant Blue-stained two-dimensional gel containing total Day 53 bovine allantoic culture medium proteins (Panel A) and Day 60 allantoic fluid proteins (Panel B). The position of 3B₃ isoelectric variants are indicated by arrows. The stained polypeptides were Western blotted (Panel C and Panel D, respectively) using 1:1000 dilution of anti-3B₃ serum. Three isoelectric variants of the protein were detected (lower portion of Panels C and D). Preimmune serum failed to react with both the bovine allantois culture medium proteins and allantoic fluid proteins (not shown).



the proteins were distinct. Antiserum raised against purified 3B₃ failed to cross-react with purified bTP-1 by Western blotting; likewise anti-bTP-1 serum failed to cross-react with purified 3B₃ (results not shown). These results demonstrated that 3B₃ and bTP-1 were distinct proteins.

Purification of 3B₃ from Allantoic Fluid

Allantoic fluid from cows in early pregnancy (Day 29-60) was a readily available source for isolation of 3B₃. When the same procedure was used to purify 3B₃ from allantoic fluid, a 23,000 protein was isolated. Generally, 150-200 ug of purified 3B₃ were obtained from 500 ml of allantoic fluid.

Amino-terminal Sequence of Purified 3B₃

The amino-terminal sequence of the first 43 amino acids of purified 3B₃ from allantoic fluid is presented in Table 1. Three cycles (4, 8 and 30) did not contain an identifiable amino acid residue. Since cysteines were not reduced and alkylated prior to sequencing, the fourth amino acid was most likely cysteine. RBP from rabbit (Sundelin et al., 1985) and human (Rask et al., 1979) plasma and pig conceptus (Harney et al., 1990) all contained cysteine at position 4 (Table 1). Assuming that unidentified amino acids represented mismatches, 3B₃ had 93% and 91% homology with rabbit and human plasma RBP, respectively. Significant sequence homology (81%) also existed between 3B₃

Table 1. The amino-terminal amino acid sequence (one letter abbreviation) of purified 3B₃ from bovine allantoic fluid, pig conceptus retinol-binding protein (pcRBP) and rabbit and human plasma retinol-binding proteins (rpRBP and hpRBP, respectively).

Protein	Amino acid position									
	1	5	10	15	20	25	30	35	40	43
3B ₃	ERD-R	VS-FR	VKENF	DKARF	AGTWY	AMAK-	DPEGL	FLQDN	IVA	
pcRBP	ERDCR	VSSFR	VKENF	DKARF	SGTWY	AM-KK	-PE			
rpRBP	ERDCR	VSSFR	VKENF	DKARF	AGTWY	AMAKK	DPEGL	FLQDN	IVA	
hpRBP	ERDCR	VSSFR	VKENF	DKARF	SGTWY	AMAKK	DPEGL	FLQDN	IVA	

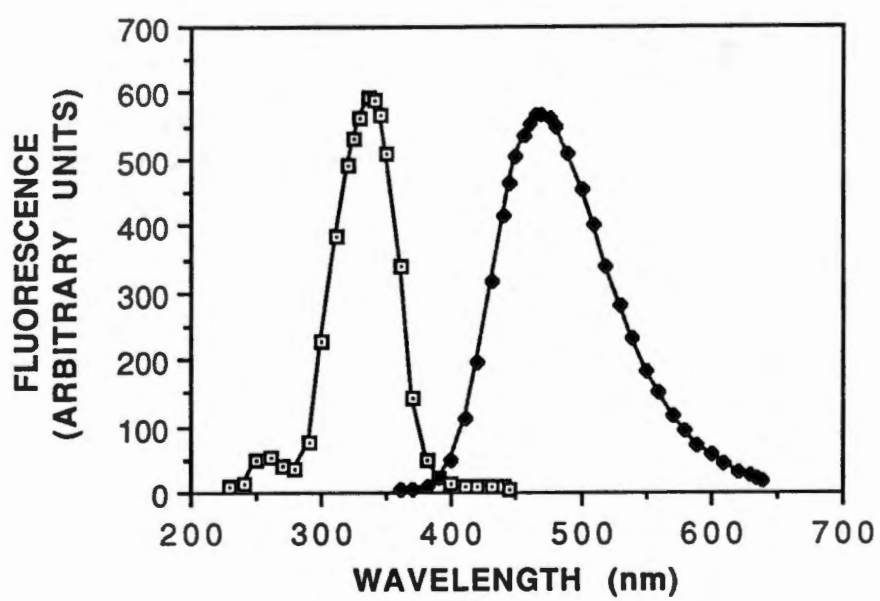
The dashes (-) indicate unidentified amino acid residues.

and putative pig conceptus RBP (Harney et al., 1990). The two proteins contained identical amino acid residues in 27 of the first 33 positions except for 5 unknown residues (3 for 3B₃; 2 for pig conceptus RBP) and an alanine/serine interchange at position 21.

Presence of Retinol in Purified 3B₃

The ultraviolet absorption spectrum of the purified 3B₃ from both allantois culture medium and allantoic fluid exhibited two peaks, with maxima at 280 nm (protein) and 330 nm (retinol). The A₃₃₀/A₂₈₀ ratio was 0.26 for the 3B₃ purified from allantoic membrane cultures and 0.28 for the 3B₃ purified from allantoic fluid. Purified 3B₃ from both sources displayed fluorescence spectra with an excitation maximum at 335 nm and emission maximum at 470 nm (Fig. 9). These spectral properties are unique for retinol (McGuire and Chytil, 1980; Muto and Goodman, 1972; Peterson and Berggard, 1971). These results, taken together, indicated the presence of a chromophore, retinol, bound to the 3B₃.

Figure 9. Fluorescence excitation and emission spectra of purified 3B₃ from allantoic fluid in Tris-HCl buffer, pH 7.6. The excitation spectrum (open boxes) was measured with emission set at 470 nm and the emission spectrum (closed diamonds) was measured with excitation set at 330 nm.



CHAPTER 5

DISCUSSION

In early studies, we identified a protein, designated 3B₃, as a major low molecular weight acidic protein present in allantoic fluid and synthesized in vitro by bovine conceptuses and isolated allantoic and chorionic membranes during early pregnancy (Godkin et al., 1988a; 1988b). In the present study, protein 3B₃ produced from the culture of bovine allantoic membrane and from allantoic fluid has been purified to homogeneity. The degree of purity obtained for 3B₃ permitted the determination of the amino-terminal sequence up to 43 amino acids which revealed that 3B₃ had significant homology with human and rabbit plasma RBP and porcine conceptus RBP. In addition, the absorbance ratio (330 nm/280 nm) and fluorescence excitation and emission spectra of purified 3B₃ indicated that the isolated protein, 3B₃, was capable of combining with retinol (vitamin A alcohol) in vitro (allantoic membrane culture medium) and in vivo (allantoic fluid). These data suggest that the purified protein, 3B₃, is a bovine placental RBP.

Bovine placental RBP (3B₃) had molecular weight similar to RBP from human plasma ($M_r = 21,000$) (Peterson and Berggard, 1971). The molecular weight of the placental RBP was approximately 23,200 and 21,200 by 1D-SDS-PAGE under

reducing and nonreducing conditions, respectively. A similar observation for human plasma RBP was made and it was suggested that the lower mobility of the reduced RBP compared to nonreduced RBP was due to complete unfolding of the protein molecule after reduction of intrachain disulfide bond(s) (Peterson and Berggard, 1971). Thus bovine placental RBP may contain one or more intrachain disulfides.

A monospecific antiserum was generated in rabbit against placental RBP isolated from allantoic membrane culture medium. Monospecificity of the antiserum was demonstrated by selective immune precipitation of the protein from Day 29 bovine allantois and chorion culture medium and Western blot analysis of Day 53 allantois culture medium proteins and Day 60 allantoic fluid proteins. Results from these studies demonstrated bovine placental RBP consisted of three isoelectric variants ($pI = 5.3 - 6.1$) of identical molecular weight ($23,200 \pm 900$). Molecular microheterogeneity on certain polyacrylamide gel systems is a general characteristic of plasma RBP (Peterson et al., 1973). Peterson and Berggard (Peterson and Berggard, 1971) reported that human RBP did not contain neutral sugars or hexosamine. This finding is consistent with the observation reported by Godkin et al. (1988b) that addition to the culture medium of tunicamycin, an inhibitor of N-linked glycosylation, had no effect on 3B₃ mass or production by

allantoic or chorionic membranes. A tentative explanation of the microheterogeneity of RBP could be due to different amounts of retinol bound to the protein as suggested by Muto et al. (1973).

The major site of plasma RBP synthesis is the liver (Smith and Goodman, 1971), although several other extrahepatic tissue sites of RBP mRNA have been identified in the adult rat (Soprano et al., 1986). Recently, Soprano et al. (1987) and Makover et al. (1989) demonstrated the presence of RBP mRNA in rat visceral yolk sac. Harney et al. (1990) presented evidence indicating production of RBP by pig conceptuses at Days 10-15 of pregnancy. In addition, human RBP antiserum was used to immunolocalize the protein in pig trophectoderm and yolk sac. The present study demonstrated by radiolabeled amino acid incorporation into protein, immunoprecipitation and Western blot analysis that bovine placental RBP is a product of the chorion and allantois from at least Days 29 through 53 of pregnancy. Polypeptides with electrophoretic mobilities identical to RBP were first detectable on fluorograms of 2D gels around Day 22 and increased in relative amount up to Day 33 (Godkin et al., 1988a; 1988b), a time period coincident with allantois outgrowth and yolk sac regression. We have not analyzed bovine yolk sac for RBP production and can only point out that the synthesis of the protein appears maximal

during and after atrophy of this membrane. A preliminary study demonstrated immunocytochemical localization of placental RBP in the ectodermal cell lining of the amnion, endodermal cell lining of the allantois and outer ectoderm of the chorion at Day 90 of pregnancy (Liu and Godkin, unpublished data). These three extraembryonic membranes were also shown to synthesize protein with physical characteristics (Mr, pI) identical to placental RBP. In addition, RBP was shown to be present in amniotic (Liu and Godkin, unpublished data) and allantoic fluids at Day 60 by Western blot analysis. Taken together, these results suggest that placental RBP is indeed a product of extraembryonic membranes that is probably released into the allantoic and amniotic fluids. The synthesis of placental RBP appears not to be terminated later in gestation after placentome formation.

Although the binding affinities of bovine placental RBP for the various retinoids have not been established, the spectral data shown in this study indicate that placental RBP has affinity for all-trans-retinol in vitro and in vivo. This finding gains some support from the reports of Heller and Horwitz (1973) that all-trans-retinol is the only physiological ligand of plasma RBP. The interaction of retinol with plasma RBP serves to solubilize the water-insoluble retinol molecule, to stabilize the retinol

against chemical degradation and to protect tissues from the toxic action of free retinol (Kanai et al., 1968). The molar ratio of RBP:retinol in human serum is 1:1 (Heller and Horwitz, 1973) and spectra analysis (A330/A280) is generally in the range of 0.8-1.05 (Raz et al., 1970; Heller et al., 1975). In present study, the low value of bound retinol to the purified placental RBP (A330/A280 = 0.26 - 0.28) is probably the result of the purification procedures which exposed the protein to low ionic strength which leads to a rapid release of retinol from RBP (Perterson, 1971).

Vitamin A is essential for normal fetal growth and development (Moore, 1957). Sporn et al. (1981) suggested that effects of retinoids on cellular differentiation, and proliferation of both epithelial and mesenchymal cells may be closely related with peptide growth factors. The expression of transforming growth factor- α (fetal analog of epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) in mouse blastocysts suggests an essential role for these factors in mammalian embryonic development (Rappolee et al., 1988). Retinoids have been shown to have specific effects on regulation of cellular receptors for EGF (Rees et al., 1979; Jetten, 1981), PDGF and production of PDGF (Rizzino and Bowen-Pone, 1985) in certain embryonic cells. In addition, retinoids are important in embryonic morphogenesis. A number of recent reports indicate that

retinoids influence the gene expression that regulates many proteins associated with formation of cytoskeleton and extracellular matrix (Brinckerhoff et al., 1981; Fuchs and Green, 1981; Strickland et al., 1980; Yuspa et al., 1982). Many processes occur during the time of placental RBP secretion including: outgrowth of chorion, allantois and amnion; placentation; growth and differentiation of embryo as well as growth of the uterus and placentome formation. Therefore, retinoids may play an important function in controlling these processes to establish and maintain a successful pregnancy.

Among the domestic farm animals, production of a placental RBP is not unique to the cow. We have recently identified the presence of newly synthesized (radiolabeled) RBP in medium from cultures of ovine and caprine extra embryonic placental membranes (chorion, allantois, amnion) by immunoprecipitation using antiserum generated against bovine placental RBP (Liu and Godkin, unpublished data). Harney et al. (1990) identified RBP synthesized and released into the medium of Days 10-15 porcine conceptus cultures. These results may indicate a common role for placental RBP in conceptus development in large domestic farm animals and species with epitheliochorial placentation.

LIST OF REFERENCES

LIST OF REFERENCES

- Avivi A., A. B. Schreiber, and M. Shemesh. 1982. Isolation and characterization of a 94,000-dalton protein with thyrotropic activity from early bovine placenta. *J. Biol. Chem.* 257:11384-11389.
- Bartol F. F., R. M. Roberts, F. W. Bazer, G. S. Lewis, J. D. Godkin, and W. W. Thatcher. 1985. Characterization of proteins produced in vitro by periattachment bovine conceptuses. *Biol. Reprod.* 32:681-693.
- Bongso T. A., and P. K. Basrur. 1976. Foetal fluids in cattle. *Can. Vet. J.* 17:38-41.
- Brinckerhoff C. E., and E. D. Harris. 1981. Modulation by retinoic acid and corticosteroids of collagenase production by rabbit synovial fibroblasts treated with phorbol myristate acetate or poly(ethylene glycol). *Biochim. Biophys. Acta.* 677:424-432.
- Chamberlain J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water soluble fluor, sodium salicylate. *Anal. Biochem.* 98:132-135.
- Cross J. C., and R. M. Roberts. 1989. Porcine conceptuses secrete an interferon during the pre-attachment period of early pregnancy. *Biol. Reprod.* 40:1109-1118.

- Fuchs E., and H. Green. 1981. Regulation of terminal differentiation of cultured human keratinocytes by vitamin A. *Cell* 25:617-625.
- Gnatek G. G., L. D. Smith, R. T. Duby, and J. D. Godkin. 1989. Maternal recognition of pregnancy in the goat: effects of conceptus removal on interestrus intervals and characterization of conceptus protein production during early pregnancy. *Biol. Reprod.* 42:655-663.
- Godkin J. D., F. W. Bazer, and R. M. Roberts. 1984. Ovine trophoblast protein-1, an early secreted blastocyst protein, binds specifically to uterine endometrium and affects protein synthesis. *Endocrinology* 114:120-130.
- Godkin J. D., F. W. Bazer, W. W. Thatcher, and R. M. Roberts. 1984. Proteins released by Day 15-16 conceptuses prolong luteal maintenance when introduced into the uterine lumen of cyclic ewes. *J. Reprod. Fertil.* 71:57-64.
- Godkin J. D., B. J. Lifsey, and B. E. Gillespie. 1988. Characterization of bovine conceptus proteins produced during the peri- and postattachment periods of early pregnancy. *Biol. Reprod.* 38:703-711.
- Godkin J. D., B. J. Lifsey, and G. A. Baumbach. 1988. Characterization of protein production by bovine chorionic and allantoic membranes. *Biol. Reprod.* 39:195-204.

- Harney J. P., M. A. Mirando, L. C. Smith, and F. W. Bazer. 1990. Retinol-binding protein: a major secretory product of the pig conceptus. *Biol. Reprod.* 42:523-532.
- Heller J. 1975. Characterization of bovine plasma retinol-binding protein and evidence for lack of binding between it and other bovine plasma proteins. *J. Biol. Chem.* 250:6549-6554.
- Heller J., and J. Horwitz. 1973. Conformational changes following interaction between retinol isomers and human retinol-binding protein and between the retinol-binding protein and prealbumin. *J. Biol. Chem.* 248:6308-6316.
- Helmer S. D., P. J. Hansen, R. V. Anthony, W. W. Thatcher, F. W. Bazer, and R. M. Roberts. 1987. Identification of bovine trophoblast protein-1, a secretory protein immunologically related to ovine trophoblast protein-1. *J. Reprod. Fertil.* 79:83-91.
- Imakawa K., T. R. Hansen, P. V. Malathy, R. V. Anthony, H. G. Polites, K. R. Marotti, and R. M. Roberts. 1989. Molecular cloning and characterization of cDNAs corresponding to bovine trophoblast protein-1. A comparison with ovine trophoblast protein-1 and bovine interferon- α _{II}. *Mol. Endocrinol.* 3:127-139.
- Jetten A. M. 1981. Action of retinoids and phorbol esters on cell growth and the binding of epidermal growth factor. *Ann. N. Y. Acad. Sci.* 359:200-216.

Kanai M. A., A. Raz, and D. S. Goodman. 1968.

Retinol-binding protein: the transport protein for vitamin A in human plasma. J. Clin. Invest. 47:2025-2044.

Laemmli U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227:680-685.

Laster D. B. 1977. A pregnancy specific protein in the bovine uterus. Biol. Reprod. 16:682-690.

Lifsey B. J., G. A. Baumbach, and J. D. Godkin. 1989. Isolation, characterization and immunocytochemical localization of bovine trophoblast protein-1. Biol. Reprod. 40:343-352.

Lowry O. H., N. C. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Makover A., D. R. Soprano, M. L. Wyatt, and D. S. Goodman. 1989. An in situ-hybridization study of the localization of retinol-binding protein and transthyretin messenger RNAs during fetal development in the rat. Differentiation 40:17-25.

- Masters R. A., R. M. Roberts, G. S. Lewis GS, W. W. Thatcher, F. W. Bazer, and J. D. Godkin. 1983. High molecular weight glycoproteins released by expanding preattachment sheep, pig, and cow blastocysts in culture. *J. Reprod. Fertil.* 66:571-583.
- McGuire B. W., and F. Chytil. 1980. Three-step purification of retinol-binding protein from rat serum. *Biochim. Biophys. Acta.* 621:324-331.
- Moore T. 1957. Vitamin A, pp. 237-262, Elsevier Publishing Co., New York.
- Murray M. K., E. C. Segerson, P. J. Hansen, F. W. Bazer, R. M. Roberts. 1987. Suppression of lymphocyte activation by a high molecular weight glycoprotein released from preimplantation ovine and porcine conceptuses. *Am. J. Reprod. Immunol. Microbiol.* 14:38-44.
- Murthy G. S., C. Schellenberg, and H. G. Friesen. 1982. Purification and characterization of placental lactogen. *Endocrinology* 111:2117-2228.
- Muto Y, and D. S. Goodman. 1972. Vitamin A transport in rat plasma. Isolation and characterization of retinol-binding protein. *J. Biol. Chem.* 247-2533-2541
- Muto Y., F. R. Smith, and D. S. Goodman. 1973. Comparative studies of retinol transport in plasma. *J. Lipid. Res.* 14:525-532.

Newton G. R., and P, J. Hansen. 1988. Secretion of a lactosaminoglycan- containing glycoprotein by peri-implantation sheep conceptuses. Biochem. Biophys. Acta. 996:56-64.

Peterson P. A. 1971. Studies on the interaction between prealbumin, retinol-binding protein and vitamin A. J. Biol. Chem. 246:44-49.

Peterson P. A., and I. Berggard. 1971. Isolation and properties of a human retinol-transporting protein. J. Biol. Chem. 246:25-3331.

Peterson P. A., L. Rask, L. Ostberg, L. Anderson, F. Kamwendo, and H. Pertoft. 1973. Studies on the transport and cellular distribution of vitamin A in normal and vitamin-A-dificient rats with special reference to the vitamin A binding plasma protein. J. Biol. Chem. 248:4009-4022.

Rappolee D. A., C. A. Brenner, R. Schultz, D. Mark D, and Z. Werb. 1988. Developmental expression of PDGF, TGF-a, and TGF-b genes in preimplantation mouse embryos. Science 241:1823-1825.

Rask L., H. Anundi, P. A. Peterson. 1979. The primary structure of the human retinol-binding protein. FEBS Lett. 104:55-58.

- Raz A., T. Shiratori, and D. S. Goodman. 1970. Studies on the protein- protein and protein-ligand interactions involved in retinol transport in plasma. J. Biol. Chem. 245:1903-1912.
- Rees A. R., E. D. Adamson, and C. F. Graham. 1979. Epidermal growth factor receptors increase during the differentiation of embryonal carcinoma cells. Nature 281:309-311.
- Rizzino A., and D. F. Bowen-Pone. 1985. Production of PDGF-like factors by embryonal carcinoma cells and response to PDGF by endoderm like cells. Dev. Biol. 110:15-22.
- Roberts R. M. 1989. Conceptus interferons and maternal recognition of pregnancy. Biol. Reprod. 40:449-452.
- Roberts R. M., G. A. Baumbach, W. C. Buhi, F. B. Denny, L. A. Fitzgerald, S. F. Babelyn, and M. N. Horst. 1984. Analysis of membrane polypeptides by two-dimensional polyacrylamide gel electrophoresis. In: Ventor JC, Harrison LC (eds.), Molecular and Chemical Characterization of Membrane Receptors. pp 61-113. Alan R. Liss, Inc. New York.
- Roberts G. P., and J. M. Parker. 1974. Macromolecular components of the luminal fluid from the bovine uterus. J. Reprod. Fertil. 40:241-303.

- Sasser R. G., C. A. Ruder CA, K. A. Ivani KA, J. E. Butler, and W. C. Hamilton. 1986. Detection of pregnancy by radioimmunoassay of a novel pregnancy specific protein in serum of cows and a profile of serum concentrations during gestation. Biol. Reprod. 35:936-942.
- Smith F. R., and D. S. Goodman. 1971. The effects of diseases of the liver, thyroid and kidneys on the transport of vitamin A in human plasma. J. Clin. Invest. 50:2426-2436.
- Soprano D. R., M. L. Wyatt, J. L. Dixon, K. L. Soprano, and D. S. Goodman. 1987. Retinol-binding protein synthesis and secretion by the rat visceral yolk sac. J. Biol. Chem. 263:2934-2938.
- Sporn M. B., D. L. Newton, A. B. Roberts, J. E. De Larco, and G. J. Todaro. 1981. Retinoids and suppression of the effects of polypeptide transforming factors-a new molecular approach to chemoprevention of cancer. In: A. C. Sartorelli, J.S. Lazo, and J.R. Bertino (ed.), Molecular Actions and Targets for Cancer Chemotherapeutic Agents, pp. 541-554. Academic Press, Inc. New York and London.
- Soprano D. S., K. J. Soprano, and D. S. Goodman. 1986. Retinol-binding protein messenger RNA levels in the liver and in extrahepatic tissues of the rat. J. Lipid. Res. 27:166-171.

Strickland S., K. K. Smith, and K. R. Marotti. 1980.

Hormonal induction of differentiation in
teratocarcinoma stem cells: generation of parietal
endoderm by retinoic acid and dibutyryl cAMP. Cell
21:347-355.

Sundelin J., B. C. Laurent, H. Anundi, L. Tragardh, D.

Larhammar, L. Bjorck, U. Eriksson, B. Akerstrom, A.
Jones, M. Newcomer. 1985. Amino acid sequence homologies
between rabbit, rat, and human serum retinol-binding
proteins. J. Biol. Chem. 260:6472-6480.

Yuspa S. H., T. Ben, and P. Steinert. 1982. Retinoic acid
induces transglutaminase activity but inhibits
cornification of cultured epidermal cells. J. Biol.
Chem. 257:9906-9908.

**PART 3: IMMUNOHISTOCHEMICAL LOCALIZATION OF BOVINE
PLACENTAL RETINOL-BINDING PROTEIN**

CHAPTER 1

ABSTRACT

An immunogold silver enhanced staining method was used in combination with epipolarization microscopy to demonstrate the localization of bovine placental retinol-binding protein (RBP) in bovine extraembryonic membranes. Amnion, chorion and allantois from a 35 cm fetus were fixed in Bouins fixation fluid and embedded in polyethylene glycol 1500. Sections were cut and transferred onto Digene silanated slides and immunostained using rabbit antiserum to bpRBP followed by goat anti-rabbit IgG labeled with 1 nm gold. Gold particles were then enlarged by silver enhancement, viewed and photographed under epipolarization illumination microscopy. Epithelial cells of all three membranes (i.e. amniotic ectoderm, chorionic trophoctoderm, and allantoic endoderm) were immunoreactive whilst underlying mesodermal cells were not. These data, together with our previous observation that these three placental membranes synthesize and secrete retinol-binding protein indicate that epithelial cells that line the amnion, chorion and allantois are the major sources of this protein. The presence of retinol binding protein in placental membranes and their fluids may be indicative of an important role for retinol in placental differentiation and development.

CHAPTER 2

INTRODUCTION

It has been recognized for over half a century that vitamin A (retinol) is essential for normal fetal growth, development (Evans, 1928; Wolbach and Howe, 1925; Thompson et al. 1964; O'Toole et al. 1974) and for the maintenance of pregnancy (Moore, 1957; Howell et al. 1963). However, until recently the mechanism of vitamin A action in these processes have been largely unknown. Retinoids, a family of vitamin A metabolites and analogs (Lotan, 1980; Dawson et al. 1985; Sporn and Roberts, 1984) are now recognized to modulate differentiation and growth of several cell types (Sherman, 1986; Harper, 1988; Keeble and Maden, 1989; Elias, 1987) and influence production of many cell surface molecules, extracellular matrix and cytoskeleton components (Fuchs and Green, 1981; Strickland et al. 1980; Yuspa et al. 1982). Retinoic acid (RA), a natural metabolite of retinol, has been identified as a morphogen in the induction of pattern formation in the chick limb bud (Tickle, et al. 1975; Maden, 1982; Eichele et al. 1985) and nervous system in the mouse (Wagner et al. 1990) and chick (Maden, et al. 1991).

Retinol is transported in plasma complexed to retinol-binding protein. RBP is the only known plasma transport

protein that delivers retinol from its source (mainly liver) to target cells (Soprano et al. 1987). Within the cell, the mechanism of vitamin A action has been proposed to involve the interaction with distinct retinoid binding proteins in the cytosol (Chytil and Ong, 1984) and nuclear receptors (Petrovich et al. 1987; Giguere et al. 1987).

Recently, we have demonstrated synthesis and secretion of RBP by bovine extraembryonic membranes (Liu et al. 1990). The protein was a major low molecular weight acidic protein (LMWAP) synthesized by amnion, chorion and allantois in organ culture and a major component of amniotic and allantoic sac fluids (Liu et al. 1990). Sequence analysis of the first 43 NH₂-terminal amino acids demonstrated complete homology with bovine plasma RBP (Berni et al. 1990.) and > 90% homology with human and rabbit plasma RBP (Liu et al. 1990). The object of the present study was to identify the cellular source of this protein, which we refer to as bovine (b) placental (p) RBP (Liu et al. 1990) in amnion, chorion, and allantois by immunohistochemical localization.

CHAPTER 3

MATERIALS AND METHODS

Production of Anti-bpRBP Serum

Purification of bpRBP and preparation of an antiserum against purified bpRBP raised in a rabbit were described by Liu et al. (1990).

Tissue Sample Preparation

Bovine extra-embryonic membranes (chorion, allantois, and amnion) from a 35 cm fetus were fixed in Bouin fixation fluid (EM Diagnostic System, Inc., Gibbstown, NJ) for 30 min at room temperature and washed thoroughly with 70% ethanol.

After rehydration in phosphate buffered saline (PBS), pH 7.3, membranes were treated with a permeabilization solution containing 1% Surfact-Amps X-100 (Pierce Chemical Co., Rockford, IL) and 1% NH_4Cl in PBS for 10 min at room temperature. After a brief wash with distilled water, samples were infiltrated with increasing concentrations (25%, 50%, 75%) of polyethylene glycol 1500 (PEG) (Aldrich Chemical Co. Inc., Milwaukee, WI) in water at 55°C for 20 min at each concentration. Samples were then infiltrated with molten PEG 1500 at 55°C (3 changes, 20 min each) and embedded in molten PEG 1500. Samples were allowed to cool to room temperature to form blocks which were further hardened at 4°C for 15 min. Sections (5 μm) were cut with a

microtome (American Optical Co., Buffalo, NY) equipped with an Accu-Edge microtome blade (Miles Laboratories Inc., Elkhart, IN). Sections were transferred to Digene silanated slides (Digene Diagnostic Inc., Silver Spring, MD) according to the minute agarose block blotting method (Gao and Godkin, 1991).

Immunohistochemistry

Immunohistochemistry was performed according to the method reported previously (Gao and Godkin, 1991). Briefly, it consisted of the following steps:

- (a) Sections were first permeabilized with 1% Surfact Amps X-100 and 1% NH_4Cl in PBS for 15 min at room temperature.
- (b) Sections were treated with a PBS based blocking medium containing 1% bovine serum albumin (BSA), 5% normal goat serum (NGS) and 0.1% cold water fish gelatin (Sigma Chemical Co., St. Louis, MO) for 45 min at room temperature.
- (c) Sections were incubated in a medium similar to blocking medium as in (b) but with 1% NGS. Concentrations of first antibodies added to the incubation medium were as follows: anti-bpRBP 1:100, 1:300, and 1:800; preimmune serum (negative control) 1:100; rabbit anti-human keratin. (Accurate Chemical and Scientific Corp. Westbury,

NY) 1:1000.

- (d) Sections were then washed with PBS, incubated in a solution of 1:30 1 nm gold labeled goat anti-rabbit IgG (Janssen Life Sciences Products, Olen, Belgium), washed with PBS and fixed with 2% glutaraldehyde (EM grade, Polysciences Inc., Warrington, PA) in PBS. Silver enhancement procedures were according to manufacturer's directions using a freshly prepared IntenSE mixture (Janssen Life Sciences Products, Olen Belgium).
- (e) Finally, sections were stained with Ehrlich hematoxylin and eosin Y (H and E stain) and mounted with Visio-Bond light sensitive and instantly curable resin (ESPE-Premier Sales Corp., Norristown, PA) according to the method of Gao and Peng (1987).

Epipolarization microscopy

Slides were viewed under an Olympus BH-2 epi-illumination fluorescence microscope equipped with BH₂-DM BIGS block which was specially designed for epipolarization detection of silver enhanced colloidal gold particles at the light microscopic level (Olympus Optical Co. Ltd., Tokyo, Japan, New York Branch).

Either transmission light or a combination of

epipolarization illumination and transmission light or epifluorescence was used. Photographs were taken using Kodak Ektachrome 160 Tungsten 35 mm color slide films. A green filter (IF550) was inserted in the transmission (TM) light path and combined with epipolarization (EP) illumination as compensation for color temperature for photography. In some cases, both EP and TM light were used to visualize tissue profile and fluorescence-like antigenic sites simultaneously.

CHAPTER 4

RESULTS

The fixation and PEG embedding method described provided excellent morphological preservation of cell structure as well as a high degree of sensitivity for antigen detection by immunostaining (Fig. 1-10). Chorion consisted of an apical monolayer of cells that appeared as a pseudo-multi-cellular layer due to nuclei arranged at different distances from the basement substratum (Fig. 1). Nuclei of chorionic epithelium (Fig. 1) were larger than nuclei from underlying mesoderm and more spherical in shape. Figure 2 illustrates the pattern of keratin immunostaining in chorionic epithelium with H & E counterstaining. The brown reaction product localized cytokeratin primarily at the apical and basolateral compartments. Rod-like branches of keratin filaments, deeply rooted basolaterally, were often connected to apical filaments. In contrast, bpRBP antigenic sites in chorionic epithelium were clustered in the apical region of these cells and diffused basolaterally (Fig. 6). No immunostaining was observed when pre-immune rabbit serum was applied as a control in place of the anti-bpRBP (Fig. 5). Figure 7 is a higher magnification of chorionic epithelium immunostained for bpRBP and counterstained with H & E. Immunostaining of bpRBP was seen

Figure. 1. Bovine chorionic membrane stained with hematoxylin and eosin (H & E). AC, apical cytosol; Ne, nuclei of epithelial cells; Nm nuclei of mesenchymal cells. x 330. Bar, 0.05 mm.

Figure 2. Keratin immunostain in bovine chorionic membrane. Rabbit anti-keratin 1:1000, goat anti-rabbit IgG labeled with 1 nm colloidal gold (IgG-Au), Silver enhancement (IGSS) and counterstained with H & E. Transmission light (TM) x 330.

Figure 3. Localization of bpRBP in bovine allantois. Anti-bpRBP 1:300, IgG-Au 1:30, IGSS, H & E. Photograph was taken with epipolarization illumination (EP). M, mesenchymal cells; E, epithelium. SLD, glass slide x 264. Bar, 0.05 mm.

Figure 4. The same area as in Figure 3 showing overall nonspecific fluorescence in tissue with an FITC filter x 264.

Figure 5. Chorionic membrane immunostained with pre-immune rabbit serum (1:100) followed by IgG-Au (1:30), IGSS, H & E (as a negative control for Figure 6). TM plus EP illumination x 264.

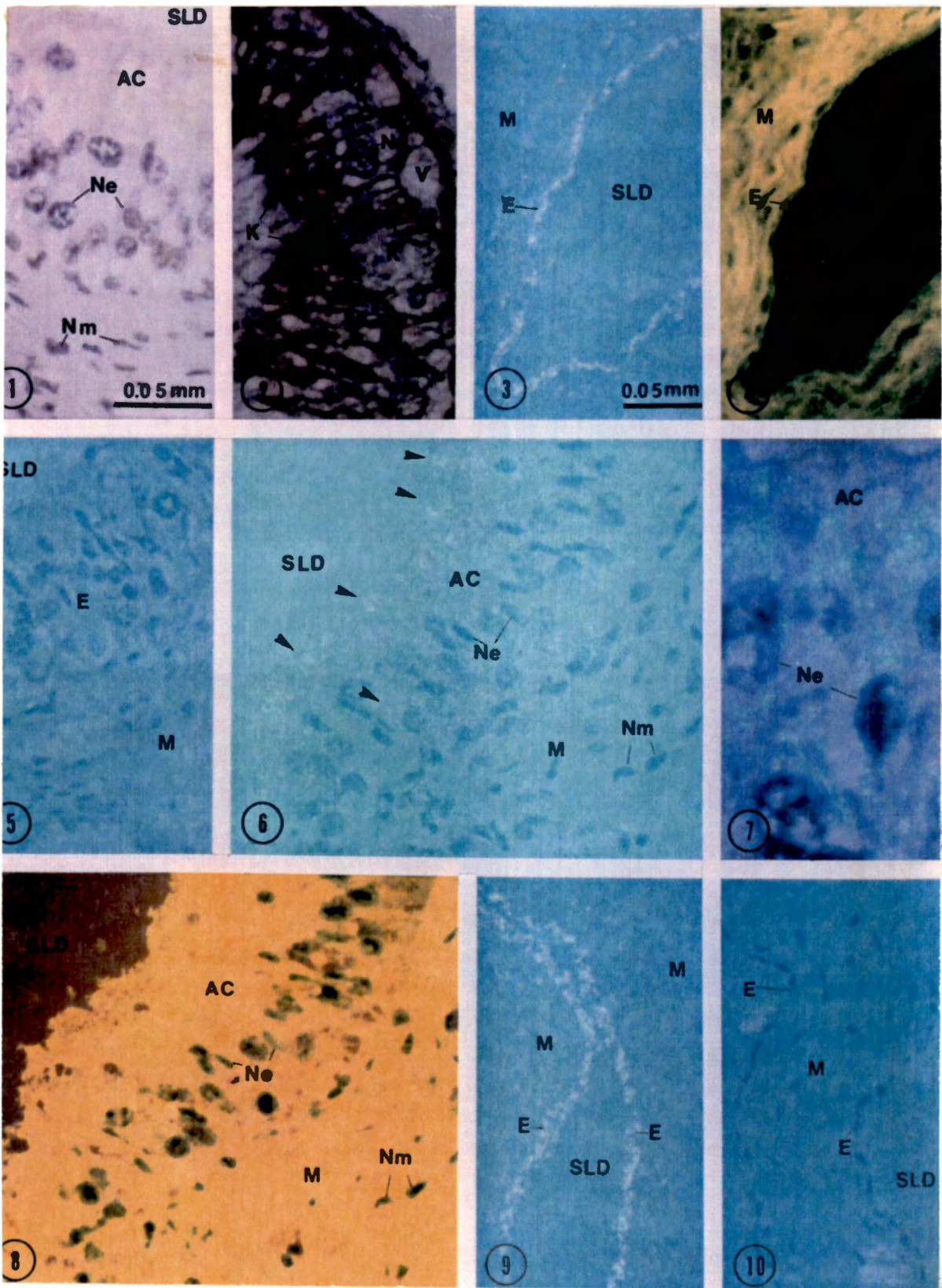
Figure 6. Localization of bpRBP in bovine chorionic membrane. Anti-bpRBP 1:100, IgG-Au 1:100, IGSS, H & E. TM plus EP illumination. AC, apical part of epithelial cells; Nm, nuclei of mesenchymal cells; SLD, glass slide; arrowheads point to sites of bpRBP x 330.

Figure 7. Distribution of bpRBP antigenic sites in apical region of chorionic epithelial cells. Anti bpRBP 1:100, IgG-Au 1:30, IGSS and H & E. High magnification (x 990) with TM plus EP. Note antigenic sites (bright blue-green spots) in apical cytosol (AC) but not nuclei (Ne).

Figure 8. Same area as shown in Figure 6. Photographed with an FITC filter to show tissue profile with nonspecific fluorescence x 330.

Figure 9. Immunostain of bpRBP antigenic sites in bovine amniotic membrane. Anti-bpRBP 1:800, IgG-Au 1:100, IGSS and with EP illumination. x 264.

Figure 10. Negative control for Figure 3. Allantois pre-immune rabbit serum 1:300, IgG-Au 1:30, IGSS with TM plus EP illumination x 264.



predominantly in the apical region of cells with a declining gradient of staining basolaterally. Nuclei and vacuoles were unstained. Figure 8 is a photograph of the same field illustrated in Figure 6 but without epipolarization illumination and with an FITC filter to demonstrate non-specific fluorescence. Comparison of Figures 6 and 8 demonstrates the specific immunostaining detected with the BH₂-DM BIGS filter.

Immunolocalization of bpRBP in allantois is illustrated in Figure 3. The entire epithelial lining of this membrane was heavily stained with the exception of nuclei. Some diffuse staining was observed in the underlying mesoderm. For comparison, Figure 10 shows allantoic membrane treated with pre-immune rabbit serum in place of anti-bpRBP serum. No immunostaining was apparent. Figure 4 is the same microscopic field as illustrated in Figure 3 but without epipolarization illumination and with an FITC filter. The high degree of non-specific fluorescence in tissue is apparent.

The presence of bpRBP in amnion is illustrated in Figure 9. As observed in allantois, localization of the protein was restricted predominantly in the epithelial lining of this membrane. Underlying mesoderm expressed diffuse staining. Control sections (not shown) were totally negative for immuno-staining.

CHAPTER 5

DISCUSSION

It is well established that retinol is essential for normal fetal development and pregnancy maintenance (Evans and Bishop, 1922; Evans, 1928; Mason, 1935; Thompson et al. 1964; O'Toole et al. 1974). In animals fed retinol-deficient diets, fetal development and pregnancy has been maintained with retinyl ester but not retinoic acid (RA) supplementation (Howell et al. 1964). This may result from the lack of a mechanism to transport RA to and across the placenta. Retinol and retinyl esters are interconvertable. Retinoic acid is a metabolite of retinol but cannot be converted to retinol. Retinol binding protein is the only known transport protein for retinol in plasma. It forms a 1:1 complex with retinol and this complex generally is bound non-covalently to transthyretin (reviewed in Sherman, 1986). Liver serves as a major site of RBP synthesis, however, several extrahepatic tissue sites of RBP and/or RBP mRNA synthesis have recently been identified (Soprano et al. 1986). Identification of RBP and/or its mRNA in placental tissue of the rat (Soprano et al. 1986), cow (Liu et al. 1990) pig (Harney et al. 1990), sheep (Liu et al. 1991) and goat (Liu and Godkin, unpublished observation) suggest that the placenta may play an important role in regulating

vitamin A storage, transport and metabolism during pregnancy.

Recently, we have demonstrated that RBP is synthesized by and released from bovine allantois and chorion in organ culture (Liu et al. 1990). The protein was purified and shown to bind retinol. It has a molecular weight of 23,000 $\pm$ 500 Daltons as determined by SDS-PAGE under reducing conditions; it is not glycosylated (Godkin et al. 1988) and is composed of at least three isotypes of identical molecular weight with pIs in the range of 5.6-6.1. The first 43 NH₂-terminal amino acids sequenced were shown to be identical to those of bovine plasma RBP (Berni et al. 1990) and > 90% homologous to human and rabbit RBP (Liu et al. 1990). Results from the present study identified and localized bpRBP in the epithelial cells lining the amnion, allantois and chorion. The protein was localized primarily in the apical regions of these cells. Based on these findings and our previous demonstration that these placental membrane synthesize and release the protein, we suggest that bpRBP is synthesized and secreted by epithelia of the chorion, allantois, and amnion.

Although it is clear that one function of RBP is the transport of retinol, defining a role for retinol in reproductive processes is problematic due, in part, to its pleiotropic and synergistic effects. Retinoids have been

implicated in differentiation (Sherman, 1986; Harper, 1988; Keeble and Maden, 1988); morphogenesis (Elias, 1987; Eichele et al. 1985, Tickle et al, 1982); protein glycosylation (DeLuca, 1977, Shapiro and Mott, 1981) production of cell surface, cytoskeleton and extracellular matrix molecules (Jetten, 1981; Brindkerhoff and Harris, 1981; Fuchs and Green 1981; Strickland et al. 1980) as well as expression of some growth factors (Jetten, 1981; Rizzino and Bowen-Pone, 1985) and their receptor (Mummery et al. 1990; Mercola et al. 1990; Rees et al. 1979). Expression of peptide growth factors have been demonstrated in the early embryo and implicated in development (reviewed in Nilson-Hamilton, 1990; Simmens and Simmens, 1991). The RBP producing chorionic epithelium is the trophoctoderm which is intimately associated with uterine epithelium and the site of fetal-maternal attachment (placentation) and nutritive exchange (for review of placental development see Stevens, 1975). RBP is apparently released from the trophoctoderm toward the uterine epithelium where it may act in a paracrine manner. Amnion develops from folding of the chorion around the embryonic disc with chorionic ectoderm (trophoctoderm) on the inside and mesoderm on the outside. Our results suggest that RBP is secreted from ectoderm into the amniotic fluid that surrounds the developing fetus. The allantois is an outgrowth of the hindgut composed of

endoderm on the inside covered with vascular mesoderm which comes in opposition to and eventually fuses with chorionic mesoderm. Production of bpRBP is from the endoderm. Fluids of both the amniotic and allantoic sacs contain high concentrations of RBP. Given the importance of Vitamin A as a potential morphogen and differentiation factor, regulation of its transport and storage appears to be an important placental function mediated through RBP production.

LIST OF REFERENCES

LIST OF REFERENCES

- Berni, R., M. Stoppini, M. C. Zapponi, M. L. Meloni, M. L. Monaco, H. L. Monaco, and G. Zanotti. 1990. The bovine plasma retinol-binding protein: amino acid sequence, interaction with transthyretin, crystallization and preliminary x-ray data. *Eur. J. Biochem.* 192:507-513.
- Brinckerhoff, C. E. and E. D. Jr. Harris. 1981. Modulation by retinoic acid and corticosteroids of collagenase production by rabbit synovial fibroblasts treated with phorbol myristate acetate or poly (ethylene glycol). *Biochim. Biophys. Acta* 677:424-432.
- Chytil, F. and D. E. Ong. 1984. Cellular retinoid-binding proteins. In: *The Retinoids* (ed. M.D.) Sporn D.S., Roberts, A.B. and Goodman, D.S. pp 89-123, Academic Press, New York.
- Dawson, M. I., P. D. Hobbs, R. Chan, K. Derdzinski, C. T. Helmes, W. Chao, E. Meichenry, and L. J. Schiff. 1985. Conformational restrictions of the retinoid skeleton. In *Retinoids, differentiation and disease*. Ciba Foundation Symposium; 113, pp 6-55. Pitman Publishing Ltd, London.
- DeLuca L. M. 1977. The direct involvement of vitamin A in glycosyl transfer reactions of mammalian membranes. *Vit. Horm.* 35:1-57.

- Eichele, G., C. Tickle, and B. M. Alberts. 1985. Studies on the mechanism of retinoid-induced pattern duplications in the early chick limb bud: Temporal and spatial aspects. *J. Cell Biol.* 101:1913-1920.
- Elias, P. M. 1987. Retinoid effects on the epidermis. *Dermatologica*, 175 Suppl 1 (6):28-36.
- Evans, H. M. and K. S. Bishop. 1922. On an invariable and characteristic disturbance of the reproductive function in animals reared on a diet poor in fat soluble vitamin A. *Anat. Rec.* 23:17.
- Evans, H. M. 1928. The effects of inadequate vitamin A on sexual physiology of the female. *J. Biol. Chem.* 77:651-654.
- Fuchs, E. and H. Green. 1981. Regulation of terminal differentiation of cultured human keratinocytes by vitamin A. *Cell* 25:617-625.
- Gao, K. X. and J. D. Godkin. 1991. A new method for transfer of polyethylene glycol embedded tissue sections to silanated slides for immunocytochemistry. *J. Histochem. Cytochem.* (in press).
- Gao, K. X. and B. Peng. 1987. VisioBond as an embedding medium for light and electron microscopy. *J. Cell Biol.* 105 (4, pt 2):226a.

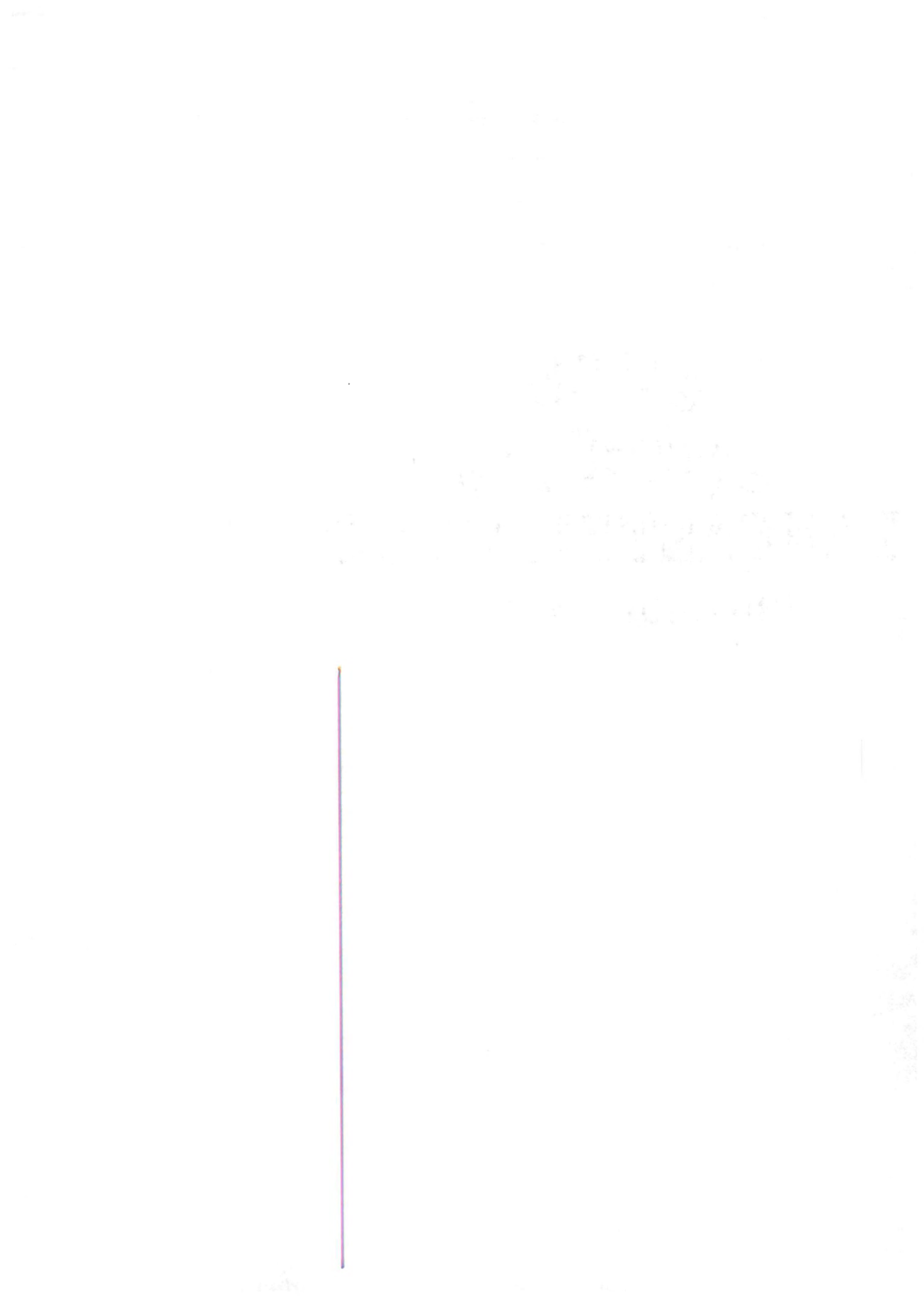
- Giguere, V., E. S. Ong, P. Segui, and R. M. Evans. 1987
Identification of a receptor for the morphogen retinoic acid. *Nature* 330:624-629.
- Godkin, J. D., B. J. Lifsey, and G. A. and Baumbach, G. A.
1988. Characterization of protein production by bovine chorionic and allantoic membranes. *Biol. Reprod.* 39:195-204.
- Harney, J. P., M. A. Mirando, L. C. Smith, and F. W. Bazer.
1990. Retinol-binding protein: a major secretory product of the pig conceptus. *Biol. Reprod.* 42:523-532.
- Harper, R. A. 1988. Specificity in the synergism between retinoic acid and EGF on the growth of adult human skin fibroblasts. *Exptl. Cell Res.*, 178(2):254-263.
- Howell, J.M., J. M. Thompson, and G. A. J. Pit. 1963.
Histology of the lesions produced in the reproductive tract of animals fed a diet deficient in vitamin A alcohol but containing vitamin A acid. *J. Reprod. Fert.* 5:159-167.
- Jetten, A. M. 1981. Action of retinoids and phorbol esters cell growth and the binding of epidermal growth factor. *Ann. N.Y. Acad. Sci.* 359:200-216.
- Keeble, S. and M. Maden, M. 1989. The relationship among retinoid structure, affinity for retinoic acid binding protein and ability to respecify pattern in the regenerating limb. *Dev. Biol.* 132:26-34.

- Liu, K. H., G. A. Baumbach, P. M. Gillevet, and J. D. Godkin. 1990. Purification and characterization of bovine placental retinol-binding protein. *Endocrinology*, 127:2696-2704.
- Liu, K. H., R. G. Brewton, G. A. Baumbach, and J. D. Godkin. 1991. Characterization of protein production by ovine placental membranes: identification of a placental retinol-binding protein. *Endocrinol.* (in press).
- Lotan, R. 1980. Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. *Biochim. Biophys. Acta* 605, 33-91.
- Maden, M., 1982. Vitamin A and pattern formation in the regenerating limb. *Nature* 295:672-675.
- Maden, M., P. Hunt, U. Eriksson, A. Kuroiwa, R. Krumlauf, and D. Summerbell. 1991. Retinoic acid binding-protein, rhombomeres and the neural crest. *Development* 111:35-44.
- Mason, K.E. 1935. Fetal death, prolonged gestation and difficult parturition in the rat as a result of vitamin A deficiency. *Am. J. Anat.* 57:303-349.
- Mercola, M., C. Wang, J. Kelly, C. Brownlee, J. Jackson, L.Grusby, C. Stiles, and D. Bowen-Pope. 1990. Selective expression of PDGF A and its receptor during early mouse embryogenesis. *Develop. Biol.* 138:114-122.

- Moore, T. 1957. Vitamin A. Elsevier, Co. New York. pp. 237-262.
- Mummery, C. L., H. Slager, W. Kruiger, A. Feigen, E. Freund, I. Koornneef, and A. J. M. Vanden Eijnden-Van Raaij. 1990. Expression of transforming growth factor B₂ during the differentiation of murine embryo carcinoma and embryonic stem cells. *Devel. Bio.* 138:114-122.
- Nilsen-Hamilton, M. 1990. Growth factor signaling in early mammalian development. In *Growth Factors in Mammalian Development*. (ed. S. Hegner), pp. 135-166. CRC Press, Inc. Boca Raton.
- O'Toole, B. A., R. Fradkin, J. Warkany, J. D. Wilson, and G. V. Mann. 1974. Vitamin A deficiency and reproduction in rhesus monkeys. *J. Nutr.* 104:1513-1524.
- Petrovich, M., N. J. Brand, A. Krust, and P. Chambon. 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* 330:444-450.
- Rees, A. R., E. D. Adamson, and C. F. Graham. 1979. Epidermal growth factor receptors increase during the differentiation of embryonal carcinoma cells. *Nature* 281:309-311.
- Rizzino, A. and D. F. Bowen-Pone. 1985. Production of PDGF-like factors by embryonal carcinoma cells and response to PDGF by endoderm like cells. *Dev. Biol.* 110:115-22.

- Shapiro, S. and D. J. Mott. 1981. Modulation of glycosaminoglycans by retinoids. *Ann. NY Acad Sci.* 359:366-321.
- Sherman, M. I. 1986. How do retinoids promote differentiation? In: *Retinoids and Cell Differentiation* (ed. M.I. Sherman) pp 161-186. CRC Press, Inc. Boca Raton, Florida.
- Simmen, F. A. and R. C. M. Simmen. 1991. Peptide growth factors and proto-oncogenes in mammalian conceptus development. *Biol. Reprod.* 44:1-5.
- Soprano, D. R., M. L. Wyatt, J. L. Dixon, K. L. Soprano, and D. S. Goodman. 1987. Retinol binding protein synthesis and secretion by the rat visceral yolk sac. *J. Biol. Chem.* 263:2934-2938.
- Sporn, M. B. and A. B. Roberts. 1984. Biological methods of analysis and assay of retinoids. In: *The Retinoids*, M. B. Sporn, A.B. Roberts, and D. S. Goodman (eds.). vol 1, pp 235-279. Academic Press, Orlando.
- Stevens, D. H. 1975. *Comparative Placentation: Essays in Structure and Function*. London: Academic Press.
- Strickland, S., K. K. Smith, and K. R. Marotti. 1980. Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. *Cell* 21:347-355.

- Tickle, C., D. Summerbell, and L. Wolpert. 1975. Positional signalling and specification of digits in chick limb morphogenesis. *Nature* 254:199-202.
- Thompson, S. Y., J. M. Howell, and G. A. J. Pitt. 1964. Vitamin A and reproduction in rats. *Proc. R. Soc. Land.* 159:510-535.
- Wolbach, S. B., and P.R. Howe. 1933. Tissue changes following deprivation of fat soluble vitamin A. *J. Exp. Med.* 57:689-722.
- Yuspa, S. H., T. Ben, and P. Steinert. 1982. Retinoic acid induces transglutaminase activity but inhibits cornification of cultured epidermal cells. *J. Biol. Chem.* 257:9906-9908.



**PART 4: CHARACTERIZATION OF PROTEIN PRODUCTION BY OVINE
PLACENTAL MEMBRANES: IDENTIFICATION OF A
PLACENTAL RETINOL-BINDING PROTEIN**

CHAPTER 1

ABSTRACT

Ovine chorion, allantois and amnion from Days 23, 26, 28, 35, 45, 53, 62 and 72 and yolk sac from Day 23 of pregnancy were isolated by dissection and cultured for 24 h in modified minimum essential medium in the presence of [³⁵S]methionine to characterize in vitro synthesis and release of proteins. Proteins synthesized and released into medium were analyzed by two-dimensional polyacrylamide gel electrophoresis and fluorography. Patterns of protein production by these isolated membranes remained relatively unchanged from Days 23 through 72 with the exception of the products of yolk sac which regresses by Day 35 of pregnancy. In general, chorion was the source of a number of basic-to-neutral proteins, allantois and amnion were the sources of more acidic proteins and yolk sac was the source of serum-like proteins. A major low mol wt acidic protein (LMWAP), D₄, was produced by all membranes and present in fetal membrane fluids. Protein D₄ consisted of three isoelectric variants (pI 5.3-6.1) with identical mol wts (23,000 ± 800) and was shown to react immunologically with anti-bovine placental retinol-binding protein (RBP) serum. Hence, protein D₄ is a putative ovine placental RBP. The present study is the first to characterize and compare protein

production by isolated ovine chorionic, allantoic, amniotic and yolk sac membranes from Day 23 through mid-pregnancy and identify the major LMWAP produced by each membrane as a placental RBP.

CHAPTER 2

INTRODUCTION

In domestic ruminants, secretory proteins produced by the developing conceptus are believed to mediate some fetomaternal interactions which are essential for the establishment and maintenance of pregnancy. Proteins produced in vitro by intact conceptuses of the sheep (Godkin et al., 1982; 1984a; 1984b; Master et al., 1982), cow (Bartol et al., 1985; Godkin et al., 1988), and goat (Gnatek et al., 1989) have been characterized. It was observed that patterns of protein production appeared to be associated with different stages of conceptus development. During the periattachment period, ovine (o), bovine (b) and caprine (c) conceptuses secreted an interferon- α like protein, called trophoblast protein-1 (TP-1), transiently between the second and fourth week of pregnancy. These proteins have been implicated as an embryonic signal for maternal recognition of pregnancy (Godkin et al., 1984b; Gnatek et al., 1989; Helmer et al., 1989; Vallet et al., 1988). Numerous other proteins are produced by conceptuses during this period of development and one, a high molecular weight glycoprotein, appears to be common to all these species (Masters et al., 1982) and has been characterized as a lactosaminoglycan in the cow (Newton et al., 1988) and

sheep (Newton, Hansen, 1988).

As gestation advances, patterns of protein production by the conceptus become more complex. Dramatic changes in the pattern of conceptus protein synthesis occur at about Day 24-29 in cows (Godkin et al., 1988; Godkin et al., 1988) and Day 21-23 in sheep (Godkin et al., 1982) and in goats (Gnatek et al., 1989). These changes are coincident with the development of the allantois, its subsequent fusion with chorion and the attachment of the chorion to the uterine endometrium (reviewed in ref. 13). Production of oTP-1, bTP-1 and cTP-1 apparently diminishes over this time period and synthesis of another discrete low mol wt acidic protein (LMWAP) , designated 3B₃ in bovine (Godkin et al., 1988; Godkin et al., 1988) becomes prominent. Protein 3B₃ is produced by bovine placental membranes from at least Day 22 through Day 90 of pregnancy and is a major component of fetal membrane fluids (Godkin et al., 1988; Godkin et al., 1988; Liu et al., 1990). Recently, 3B₃ has been isolated and identified as a bovine placental retinol-binding protein (bpRBP) with a high affinity for retinol. Its mol wt, microheterogeneity, and the first 43 N-terminal amino acid sequence were similar to human serum RBP (Liu et al., 1990).

Here, we report the identification of an ovine placental retinol-binding protein (opRBP) synthesized by isolated chorionic, allantoic, amniotic and yolk sac

membranes. In addition, we have characterized and compared the patterns of protein synthesis by these membranes between Days 23 and 72 of pregnancy.

CHAPTER 3

MATERIALS AND METHODS

Materials

Supplies for tissue culture and electrophoresis were as described previously (Godkin et al., 1988; Godkin et al., 1988). Halothane was from Fort Dodge Laboratories, Fort Dodge, CO. Immunoblot Assay Kit was from Bio-Rad Laboratories, Richmond, CA. All other supplies for polyacrylamide gel electrophoresis (PAGE), protein blotting and immune complex precipitation were as described previously (Lifsey et al., 1989). L-[³⁵S]-methionine (1134 Ci/mmol) was obtained from New England Nuclear. Rabbit antiserum against bprBP was produced previously (Liu et al., 1990).

Animals

Adult crossbred ewes were checked twice daily for estrus with vasectomized rams. Estrous ewes (estrus = Day 0) were mated by intact rams. Pregnant ewes were anesthetized with halothane administered via a closed circuit anesthesia unit. Reproductive tracts were collected surgically via midventral laparotomy on Days 23, 26, 28, 35, 45, 53, 62 and 72 of pregnancy as described previously (1) and transported to the laboratory on ice within 10 min after surgery.

In Vitro Culture of Yolk Sac, Chorion, Allantois and Amnion

Intact conceptuses were dissected from uteri under sterile conditions in a laminar flow tissue culture hood. Samples of allantoic and amniotic fluids were aspirated with a minimum of contamination using sterile syringes fitted with 18-gauge needles. Intercotyledonary chorionic, allantoic, amniotic and yolk sac tissues were carefully dissected and cultured as described by Godkin et al. (Godkin et al., 1988) and utilized by Liu et al. (Liu et al., 1990). Proteins synthesized in cultures were metabolically labeled with [³⁵S]-methionine. Specific proteins were identified by two dimensional (2D)-PAGE and immunoprecipitation studies (see below).

Two-dimensional PAGE

Two-dimensional PAGE was performed according to the method of Roberts et al. (Roberts et al., 1984). After electrophoresis, Coomassie Brilliant Blue R-250-stained gels were photographed and fluorographs were prepared as described by Chamberlain (Chamberlain, 1979). Rabbit muscle phosphorylase b, bovine serum albumin, hen egg white albumin, bovine carbonic anhydrase, soybean trypsin inhibitor, and hen egg white lysozyme were used as protein molecular weight standards.

Immunoprecipitation of Putative opRBP

One milliliter aliquots of culture medium from Day 35 sheep chorionic, allantoic and amniotic membranes (³⁵S-labeled) were incubated with anti-bpRBP or preimmune rabbit serum. Immune complexes were absorbed onto Protein A-Sepharose as described by Lifsey et al. (Lifsey et al., 1989) and analyzed by 2D-PAGE and fluorography.

Immunoblot Analysis of Sheep Allantoic and Amniotic Fluid Proteins

Aliquots (60 ug) of unfractionated Day 53 sheep allantoic and amniotic fluid proteins separated by 2D-PAGE. One set of gels was stained with Coomassie Brilliant Blue and photographed. Another set was electrophoretically transferred to nitrocellulose membranes. Blots were incubated in anti-bpRBP serum or preimmune rabbit serum at a dilution of 1:1000. Bound antibody was visualized using a 1:2000 dilution of goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate according to the Immunoblot™ (GAR-HRP) Assay Kit instructions. Protein concentrations were determined by the method of Lowry et al. (Lowry et al., 1951), with bovine serum albumin as the standard.

CHAPTER 4

RESULTS

Analysis of Yolk Sac, Chorionic, Allantoic and Amniotic Proteins by 2D-PAGE

For clarity in describing the protein distribution patterns produced by ovine chorion, allantois and amnion between Days 23 and 72 of pregnancy, we classified the proteins which showed only one spot or a series of spots with characteristic location, molecular weight (Mr) or pI into four groups (A-D) along the mol wt gradient (Fig. 1). The approximate mol wt ranges were as follows: Group A, > 100,000; B, 45,000-97,000; C, 31,000-45,000; D, 14,000-31,000.

Within a membrane, patterns of protein production remained consistent over all time periods examined (Days 23-72). Because of this result, we have shown just one representative fluorogram from each membrane. Intensities of images from an individual protein on X-ray films showed slight variation between animals from the same and different days. Some isolated membrane preparations showed contaminating proteins that were clearly a product of another membrane. For example, some Day 23 chorionic and allantoic membranes showed proteins clearly identified as products of yolk sac (Fig. 2) while other chorionic and

Figure 1. Fluorograms of two-dimensional gels for analysis of ^{35}S -methionine-labeled secretory proteins from Day 35 chorion (A) , allantois (B) and amnion (C). 200,000 cpm were loaded onto each gel and fluorographs were exposed to dried gels for 7 days. Analysis of immunoprecipitates of Panels A, B, C with anti-bpRBP serum by 2D-PAGE and fluorography are shown in Panels D, E, F respectively. Exposure time of Panels D, E, F was weeks.

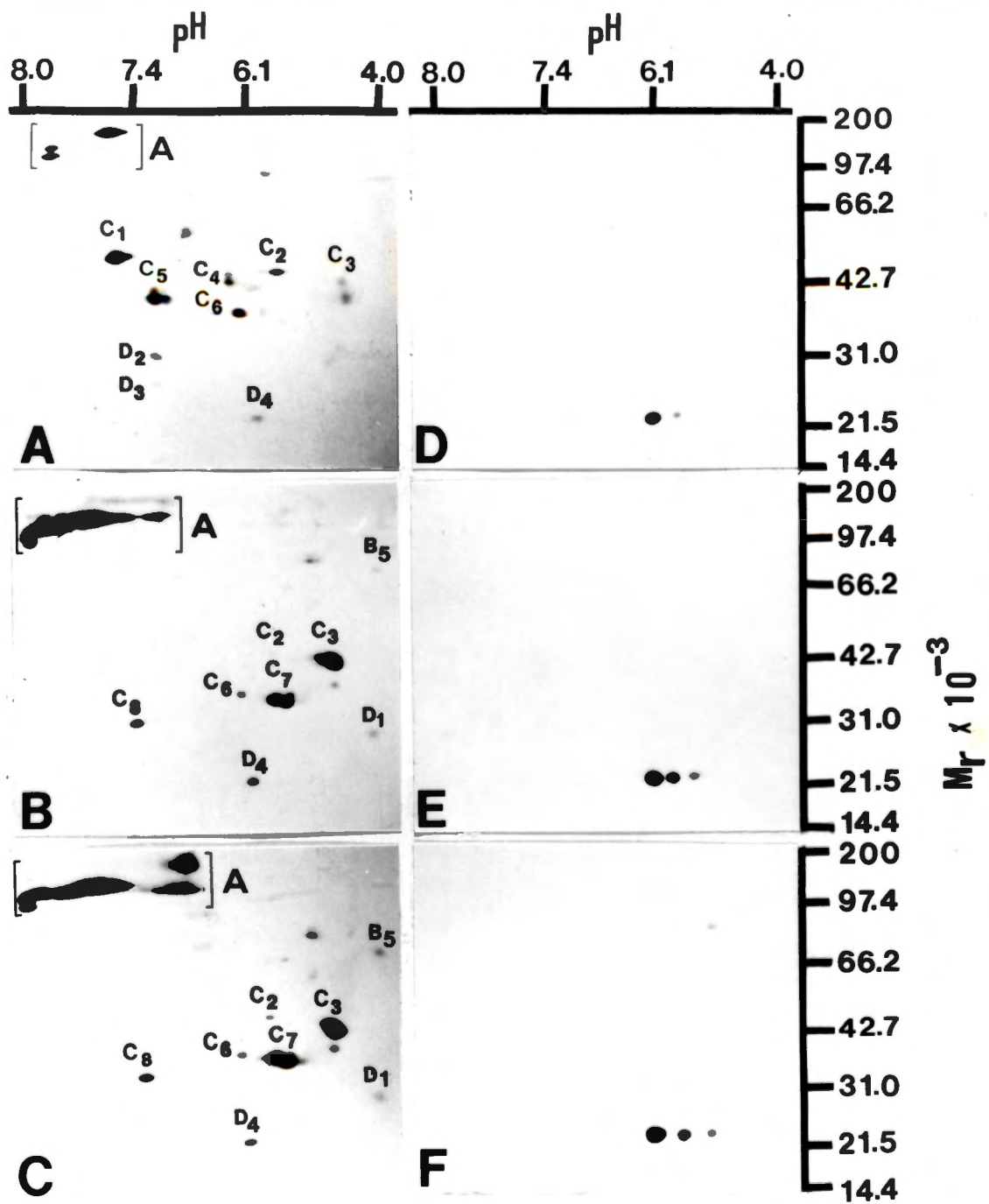
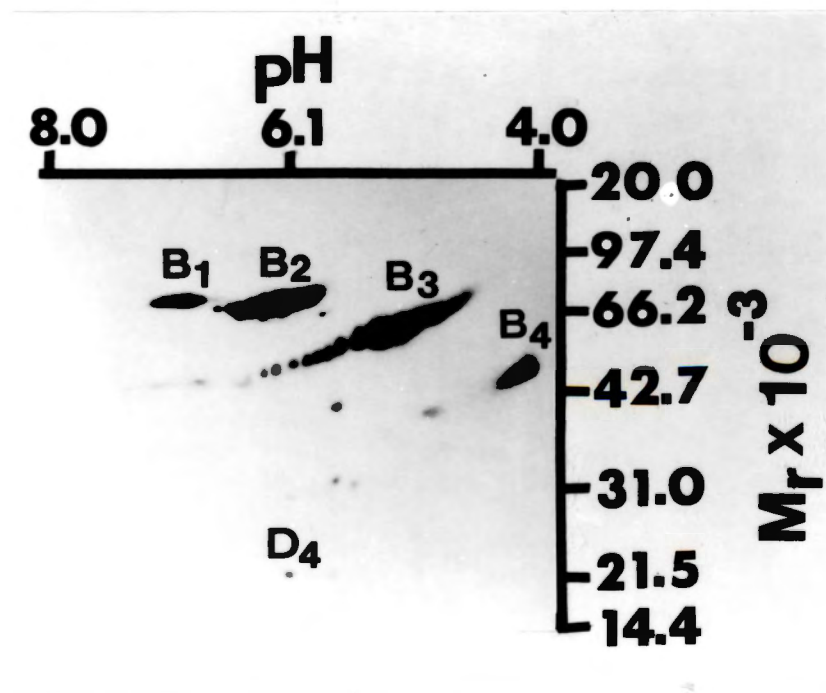


Figure 2. Fluorogram of two-dimensional gel for analysis of ³⁵S-methionine-labeled proteins synthesized and released by yolk sac isolated from a Day 23 conceptus. Sample preparation was identical to that for placental membranes described in Figure 1.



allantoic membrane preparations did not. For this reason we examined at least two replicates each from three different animals at each time point (except yolk sac) before determining which membrane synthesized which protein (Table 1). Yolk sac, which undergoes rapid regression between Day 23 and 30 was prepared at Day 23. Chorion (Fig. 1A), allantois (Fig. 1B) and amnion (Fig. 1C) all synthesized high mol wt basic proteins (Group A). This group consisted of at least five proteins with mol wt greater than 100,000 spanning a pI range from 6.6 to 8.0. Yolk sac was the major source of at least four proteins ($B_1 - B_4$) in the mol wt range of 45,000 to 80,000 (Fig. 2). Each of these proteins consisted of numerous isoelectric species and exhibited electrophoretic mobilities and spatial arrangement on gels nearly identical to proteins present in fetal serum. In addition, yolk sac produced a LMWAP, D_4 (Mr 23,000, pI 6.1), synthesized by the other placental membranes.

Chorion alone synthesized and secreted C_1 (Mr 45,000, pI 7.5-7.7), C_4 (Mr 43,000, pI 6.4), C_5 which existed as two isoforms of identical mol wt (Mr 40,000, pI 6.8-7.2), D_2 (Mr 29,000, pI 7.1) and D_3 (Mr 25,000, pI 7.1) as major products. Analysis of chorio-allantois tissue proteins (not shown) revealed that C_2 was a major synthetic cellular product with electrophoretic mobilities similar to actin. Chorion was the most fragile of the placental membranes and

Table 1. Protein identified in yolk sac (YS), chorionic (C), allantoic (AL) and amniotic (AM) membrane cultures^a

Protein	Mr x 10 ^{-3b}	pI	source
Group A	> 100	6.6-8.0	C, AL, AM
B ₁	75	7.6-6.9	YS
B ₂	73-80	6.8-5.7	YS
B ₃	45-80	6.5-4.6	YS
B ₄	45-54	3.8-4.0	YS
B ₅	75	4.0	AL, AM
C ₁	45	7.5-7.7	C
C ₃	43	5.0	(C) ^c AL, AM
C ₄	43	6.4	C
C ₅	40	6.8-7.2	C
C ₆	38	6.2	C, AL, AM
C ₇	35-38	5.2-6.0	AL, AM
C ₈	32	7.2	AL, AM
D ₁	30	4.0	AL, AM
D ₂	29	7.1	C
D ₃	25	7.1	C
D ₄ (RBP)	23	5.3-6.1	YS, C, AL, AM

^aIdentification of proteins was based on examination of three fluorograms from different extraembryonic membranes from the same day.

^bApparent mol wt ($M_r \times 10^{-3}$) was estimated for each protein based on its relative mobility compared to protein standards.

^cPresence of C₃ in C probably due to AL contamination.

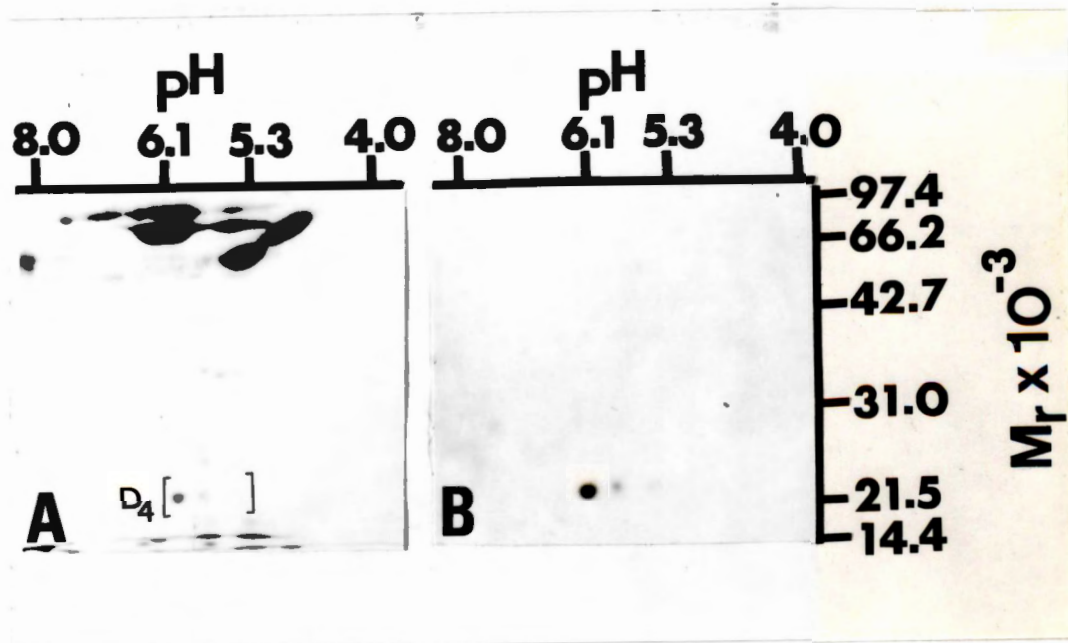
released the greatest quantity of cellular debris during culture. Hence it is reasonable that actin would be found in the culture medium from this membrane. Similar to allantois and amnion, chorion produced C₆ (Mr 38,000, pI 6.2) and D₄, a major LMWAP.

Surprisingly, patterns of proteins synthesized and secreted by allantois and amnion were identical. With the exception of C₆ and D₄, the proteins produced by amnion and allantois were distinct from those produced by yolk sac or chorion. Very acidic proteins B₅ (Mr 75,000, pI 4.0) and D₁ (Mr 30,000, pI 4.0) were produced in relatively modest concentrations by amnion and allantois. Group C₇ was a major product of both membranes and consisted of three to five polypeptides with slightly different mol wt (35,000-38,000) and pIs (5.2-6.0). This group may be composed of several different proteins rather than isoforms of a single protein. Protein C₃ (Mr 43,000, pI 5.0) consistently produced the largest and darkest image on X-ray film suggesting that it is a major product of these membranes. Accordingly, this protein appeared as a light image on fluorograms from chorion preparations. This was probably due to allantois tissue contamination. The basic protein C₈ (Mr 32,000, pI 7.2) was another specific product of allantois and amnion. Each of these membranes synthesized D₄ as the major LMWAP product.

Immunological Identification of D₄ as an opRBP

Immune-complex precipitation with anti-bpRBP, followed by 2D-PAGE and fluorography was used to identify protein D₄ as an opRBP synthesized by chorion (Fig. 1D), allantois (Fig. 1E) amnion (Fig. 1F) and yolk sac (not shown). The protein consisted of three isoelectric variants (pI, 5.3-6.1) with identical mol wt of $23,000 \pm 800$ (mean $\pm$ SD, n=6). The least acidic of the isoforms was the most abundant while the most acidic isoform was least abundant. When preimmune serum was used as a negative control, fluorograms were blank (not shown). Proteins from Day 53 ovine allantoic and amniotic fluids were submitted to 2D-PAGE and Western blot analysis using the anti-bpRBP serum (Fig. 3A, only allantoic fluid proteins shown). Positive reactions were observed for a group of three polypeptides with identical mol wt of 23,000 and pI's ranging from 5.3-6.1 (Fig. 3B). Similar to the results of immune precipitation, the two less acidic isotypes produced stronger reactions. No reaction was observed with preimmune serum (not shown).

Figure 3. Ovine allantoic fluid proteins (60 ug) analyzed by two-dimensional polyacrylamide gel electrophoresis (A) and Western blot of Panel A using 1:1000 dilution of anti-bovine placental retinol-binding protein antiserum. Positions of the immunoreactive polypeptides are indicated by brackets. The bpRBP antibody detected three acidic antigens with identical mol wt of 23,000. Note that serum-like proteins are present at upper portion of Panel A.



CHAPTER 5

DISCUSSION

Earlier studies demonstrated that dramatic alterations in the electrophoretic pattern of proteins synthesized by intact ovine conceptuses occurred between Days 21 and 23 of gestation (Godkin et al., 1982). A major alteration was the cessation of production of the protein referred to as protein X which was later named oTP, (Godkin et al., 1984a) and the appearance of a different, more basic protein as the major low mol wt product. These alterations coincided temporally with developmental changes in the placental membranes. Notably, the allantois had become a prominent structure, filling and contacting the chorion. The amnion was fully formed at this time and the yolk sac had reached peak development and would soon undergo steady regression as its nutritive role was taken over by the rapidly expanding allantois. In the present study we have characterized and compared protein production by isolated chorionic, allantoic, amniotic and yolk sac membranes from Day 23 through mid-pregnancy and identified the major LMWAP produced by each membrane as a placental RBP.

Isolated yolk sac produced a group of at least 4 proteins (B_1 - B_4) in the mol wt range of 45,000-80,000 as major products. These proteins had electrophoretic

mobilities, pIs, and spatial arrangement on gels identical to proteins present in fetal bovine serum. The yolk sac and fetal liver are generally believed to be the source of fetal serum proteins (Gitlin, Gitlin, 1975). Production of serum-like proteins in porcine and bovine conceptus cultures (Godkin et al., 1988; Godkin et al., 1982), mouse blastocyst cultures (Nieder et al., 1987) and porcine and ovine extraembryonic cell cultures (Godkin et al., 1985) has been reported previously. Based on the tentative identification of serum proteins in these previous studies, B₁-B₄ correspond to transferrin, α -fetoprotein, α 1-antitrypsin and α 1-acid glycoprotein, respectively. We acknowledge that immunological or amino acid sequence information is necessary before identity of these proteins can be ascertained. All of these proteins have been identified immunologically in porcine fetal serum, allantoic and amniotic fluids (McKenna, 1984). Occasionally, we detected protein B₁-B₄ in cultures of chorion, allantois and amnion preparations up to but not after Day 35. We suggest these results were due to yolk sac contamination and that the reason for their complete absence after Day 35 was due to yolk sac regression.

Chorion, allantois and amnion all synthesized high mol wt basic proteins at all time points examined. Production of high mol wt glycoprotein in vitro by pre-attachment stage

pig, sheep (Masters et al., 1982; Godkin et al., 1982), cow (Masters et al., 1982; Bartol et al., 1985) and goat (Gnatek et al., 1989) conceptuses has been reported. The protein complex produced by cow and sheep conceptuses has been suggested to contain lactosaminoglycans (Newton et al., 1988; Newton, Hansen, 1988) that have immunosuppressive activity (Murray et al., 1987). Crouch et al. (1978) showed that a high mol wt glycoprotein identified as a fibronectin was synthesized and secreted by amniotic fluid cells in culture. It is possible but not confirmed that the high mol wt proteins identified in the present study are similar to those observed previously. Chorion produced a number of proteins that were not present in the culture medium from the other membranes and their functions are yet to be determined. These included C₁, C₄, C₅, D₂ and D₃. In addition, chorion produced the LMWAP, D₄, that has been identified as a placental RBP. Patterns of protein synthesis by chorion, allantois and amnion remained unchanged from Days 23 through 72.

One surprising observation was that allantois and amnion produced identical proteins. The allantois is an outgrowth of the hind gut composed of an inner layer of endoderm and an outer layer of vascular mesoderm that comes in apposition to and eventually fuses with chorionic mesoderm (reviewed in ref Stevens, 1975). Amnion develops

from folding of the developing chorion around the embryonic disc and is composed of an inner layer of ectoderm and an outer layer of mesoderm. Hence allantois and amnion are not composed of identical cell types. However, ultrastructural studies have shown that the epithelia of these membranes are very similar morphologically (Tiedemann, 1982). Functions attributed to the allantois have included providing a blood vascular network to the placenta for respiration and nutrition (Perry, 1981), forcing the chorioallantois into close apposition to the endometrium for attachment and nutritive exchange by increasing the fluid volume (Eley et al., 1978), and providing a reservoir for urinary waste (Zobundzija, 1968). Functional roles attributed to the amniotic fluid include protection of the fetus, lubrication of the birth canal during birth and as a nutritional source for the fetus (Gitlin et al., 1972). In addition to its more passive functions, the amniotic membrane plays an active role in water and ion metabolism (Barnes, 1976). Proteins present in bovine (Okana et al., 1977) and porcine (McKenna, 1984) allantoic and amniotic fluids have been identified as being similar to fetal plasma proteins and most appeared to be distributed between plasma and fluids by passive diffusion. The fact that both membranes produced identical proteins may indicate a commonality in function.

Ovine chorion, allantois, amnion and yolk sac all

synthesized a LMWAP (D₄) that we have identified immunologically as a placental RBP. The antiserum used for identification was raised against RBP that was purified from bovine allantois culture medium (Liu et al., 1990). Bovine placental RBP binds retinol and has complete amino acid sequence homology with bovine plasma RBP (Berni et al., 1990) over the first 41 N-terminal amino acids and greater than 90% homology with human and rabbit plasma RBP (Liu et al., 1990). Both opRBP and bpRBP consist of three isotypes with identical mol wt of 23,000 as determined by 2D-PAGE under reducing conditions. Preliminary immunocytochemical results demonstrated that opRBP was localized in the ectodermal cells lining the amnion, endodermal cells lining the allantois and trophoctodermal cells of the chorion (Liu and Godkin, unpublished data). In addition, opRBP was identified by Western blot analysis as a major LMWAP present in ovine amniotic and allantoic fluids. Taken together, these results suggest that RBP is a product of sheep placental membranes and is released into the allantoic and amniotic fluids. Furthermore, we have identified a protein from goat conceptus cultures that cross reacts with our bpRBP antiserum (Liu and Godkin unpublished data). Harney et al. (1990) identified RBP synthesized and released into medium of Day 10-15 porcine conceptus cultures. Together these results indicate a common, local mechanism for the

transport of retinol in conceptuses of large domestic farm animals.

Retinol-binding protein is the only known transport protein in serum that delivers the bioactive alcohol form of vitamin A (retinol) from the liver to target cells (Smith and Goodman, 1971). Cellular uptake of retinol is mediated by a specific membrane receptor that recognizes RBP (Heller, 1975; Rask and Peterson, 1976; Sivaprasadarao and Findlay, 1988). Within the target cell, retinol and/or its metabolite, retinoic acid (RA), interact with distinct cellular retinoid binding proteins (CRBP and CRABP, respectively) (Chytil and Ong, 1984) that have been shown to transfer their ligands to isolated nuclei (Takase et al., 1979; 1986). Recently, nuclear RA receptors (RARs) have been identified. RARs belong to the superfamily of steroid-thyroid hormone receptors that are believed to control some cell functions by direct regulation of gene expression (de The et al., 1987; Giguere et al., 1987; Petkovich et al., 1987; Krust et al., 1989). The identification of RBP production by placental membranes indicates local control of vitamin A metabolism in the developing embryos.

The essential requirement for vitamin A in reproductive processes was first identified nearly 70 years ago (Evans, 1922) and confirmed in several species over the following half-century (Evans, 1928; Mason, 1935; O'Toole et al.,

1974; Thompson et al., 1964). Retinoids are now recognized to play central roles in development (Sherman, 1986). Retinoic acid is the only unambiguously identified morphogen in vertebrates. In the chick limb bud, RA is believed to be the the endogenous morphogen that specifies anteroposterior axis pattern formation (Maden, 1982; Tickle et al., 1982; Thaller and Eichele, 1987). In the mouse embryo, RA is believed to be involved in the morphogenesis of the nervous system (Wagner et al., 1990). Although retinoids are potent modulators of cell growth and differentiation (Sherman, 1986), their mechanism(s) of action have been largely unknown. However, recent studies suggest that some of their actions are mediated through the ability of retinoids to induce the expression of some peptide growth factors and their receptors (Jetten, 1980; Mercola et al., 1990; Mummery et al., 1990). Retinoids modulate the expression of transforming growth factor- β (TGF- β) and the epidermal growth factor/TGF- α receptor in certain embryonic derived cell lines (Jetten, 1980; Mummery et al., 1990). Retinoids and TGF- β both exhibit growth promoting and growth inhibitory effects, depending on the target tissues and both influence extracellular matrix formation (Sherman, 1986; Nilsen-Hamilton, 1990). TGF- α , TGF- β (Wilkinson et al., 1990) and RBP (Liu et al., 1990) have been identified in bovine allantoic and amniotic fluids. Bovine trophoblastic

cell-conditioned medium has both TGF- α and TGF- β activity (Munson et al., 1990). Moreover, the trophoblast synthesizes RBP (Liu et al., 1990). Recently, RBP, TGF- α and TGF- β have been immunolocalized in ovine and bovine trophectoderm and uterine epithelium (Munson et al., 1990; Dore and Godkin, unpublished observations). Together, these data may indicate a functional link between retinoids and growth factors in ruminant placental, embryonic and uterine development.

In summary, patterns of protein synthesis by isolated placental membranes remained consistent from Days 23 through midpregnancy with the exception of the products of yolk sac which regresses by Day 35 of pregnancy. It appears that once the chorioallantois is established there are few qualitative changes in protein synthesis by these membranes. Surprisingly, allantois and amnion produced identical proteins, suggesting that these membranes may have functions more similar than thought previously. Importantly, all membranes synthesized RBP as a major product and exhibited the protein in the membrane fluids. Given the importance of vitamin A as a potential morphogen and differentiation factor, regulation of its storage and transport appears to be an important placental function.

LIST OF REFERENCES

LIST OF REFERENCES

- Barnes R. J. 1976. Water and mineral exchange between maternal and fetal fluids. In: Beard RW and Nathanielsz PW (eds) Fetal Physiology and Medicine. WB Saunders, London, pp 194-214.
- Bartol F. F., R. M. Roberts, F. W. Bazer, G.S Lewis, J. D. Godkin, and W. W. Thatcher. 1985. Characterization of proteins produced in vitro by periattacment bovine conceptuses. Biol. Reprod. 32:681-693.
- Berni R., M. Stoppini, M. C. Zapponi, M. L. Meloni, H. L. Monaco, G. Zanotti. 1990. The bovine plasma retinol-binding protein: amino acid sequence, interaction with transthyretin, crystallization and preliminary X-ray data. Eur. J. Biochem. 192:507-513.
- Chamberlain J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water soluble fluor, sodium salicylate. Anal. Biochem. 98:132-135.
- Chytil F., and D. E. Ong. 1984. Cellular retinoid-binding proteins. In: M. B. Sporn, A. B. Roberts, and D. S Goodman (eds.) The Retinoids. pp 89-123. Academic Press, New York.

- Crouch E. D., G. Balian, K. Holbrook, D. Duksin, and P. Bornstein. 1978. Amniotic fluid fibronectin: characterization and synthesis by cells in culture. *J. Cell Biol.* 78:701-715.
- de The H., A. Marchio A., P. Tiollais, and A. Dejean. 1987 A novel steroid thyroid hormone receptor-related gene inappropriately expressed in human hepatocellular carcinoma. *Nature* 330:667-670.
- Eley R. M., W. W. Thatcher, F. W. Bazer, C. J. wilcox, R. B. Becker, H. H. Head, R. W. Adkinson. 1978. Development of the conceptus in the bovine. *J. Dairy Sci.* 61:467-473.
- Evans H. M. 1928. The effects of inadequate vitamin A on sexual physiology of the female. *J. Biol. Chem.* 77:651-654.
- Evans H. M., and K. S. Bishop. 1922. On an invariable and characteristic disturbance of the reproductive function in animals reared on a diet poor in fat soluble vitamin A. *Anat. Rec.* 23:17.
- Giguere V., E. S. Ong, P. Segui, and R. M. Evans. 1987. Identification of a receptor for the morphogen retinoic acid. *Nature* 330:624-629.

- Gitlin D., and J. D. Gitlin. 1975. Fetal and neonatal development of human plasma proteins. In: Putnam FW (ed) The Plasma Proteins: Structure, Function and Genetic Control, Academic Press, New York, vol. 2:263-321.
- Gitlin D, J. Kumate, C. Morales, L. Noriegan, N. Arevala. 1972. The turnover of amniotic fluid protein in the human conceptus. Am. J. Obstet. Gynec. 113:632-645.
- Gnatek G. G., L. D. Smith, R. T. Duby, J. D. Godkin. 1989. Maternal recognition of pregnancy in the goat: effects of conceptus removal on interestrus intervals and characterization of conceptus protein production during early pregnancy. Biol. Reprod. 42:655-663.
- Godkin J. D., F. W. Bazer, and R. M. Roberts. 1985. Protein production by cultures established from Day 14-16 sheep and pig conceptuses. J. Reprod. Fertil. 74:377-382.
- Godkin J. D., F. W. Bazer, G. S. Lewis, R. D. Geisert, R. M. Roberts. 1982. Synthesis and release of polypeptides by pig conceptuses during the period of blastocyst elongation and attachment. Biol. Reprod. 27:977-987.
- Godkin J. D, F. W. Bazer, J. Moffatt, F. Sessions, R. M. Roberts. 1982. Purification and properties of a major low molecular weight protein released by the trophoblast of sheep blastocysts at day 13-21. J. Reprod. Fertil. 65:141-150.

- Godkin J. D., F. W. Bazer, and R. M. Roberts. 1984. Ovine trophoblast protein-1, an early secreted blastocyst protein, binds specifically to uterine endometrium and affects protein synthesis. *Endocrinology* 114:120-130.
- Godkin J. D., F. W. Bazer, W. W. Thatcher, R. M. Roberts. 1984. Proteins released by Day 15-16 conceptuses prolong luteal maintenance when introduced into the uterine lumen of cyclic ewes. *J. Reprod. Fertil.* 71:57-64.
- Godkin J. D., B. J. Lifsey, and G. A. Baumbach. 1988. Characterization of protein production by bovine chorionic and allantoic membranes. *Biol. Reprod.* 39:195-204.
- Godkin J. D., B. J. Lifsey, and B. E. Gillespie. 1988. Characterization of bovine conceptus proteins produced during the peri- and postattachment periods of early pregnancy. *Biol. Reprod.* 38:703-711.
- Greenstein J. S., R. W. Murray, and R. C. Foley. 1958. Observations on the morphogenesis and histochemistry of the bovine preattachment placenta between 16 and 33 days of gestation. *Anat. Rec.* 132:321-341.
- Harney J. P., M. A. Mirando, L. C. Smith, and F. W. Bazer. 1990. Retinol-binding protein: a major secretory product of the pig conceptus. *Biol. Reprod.* 42:523-532.

- Heller, J. 1975. Interactions of plasma retinol-binding protein with its receptor: specific binding of bovine and human retinol-binding protein to pigment epithelium cells from bovine eyes. *J. Biol. Chem.* 250:3613-3619.
- Helmer S. D., P. J. Hansen, W. W. Thatcher, J. W. Johnson, and F. W. Bazer. 1989. Intrauterine infusion of highly enriched bovine trophoblast protein-1 complex exerts an antiluteolytic effect to extend corpus luteum lifespan in cyclic cattle. *J. Reprod. Fertil.* 87:89-101.
- Jetten A. M. 1980. Retinoids specifically enhance the number of epidermal growth factor receptors. *Nature* 284:626-629.
- Krust A., P. Kastner, M. Petkovich, A. Zelent A, and P. Chambon. 1989. A third human retinoic acid receptor, hRAR-gamma. *Proc. Natl. Acad. Sci.* 86:5310-5314.
- Lifsey B. J., G. A. Baumbach, and J. D. Godkin. 1989. Isolation, characterization and immunocytochemical localization of bovine trophoblast protein-1. *Biol. Reprod.* 40:343-352.
- Liu K. H., G. A. Baumbach, P. M. Gillevet, and J. D. Godkin. 1990. Purification and characterization of bovine placental retinol-binding protein. *Endocrinology* 127:2696-2704.

Lowry O. H., N. C. Rosebrough, A. L. Farr, R. J. Randall.

1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Maden M. 1982. Vitamin A and pattern formation in the regenerating limb. Nature 295:672-675.

Masters R. A., R. M. Roberts, G. S. Lewis, W. W. Thatcher, F. W. Bazer, J. D. Godkin. 1982. High molecular weight glycoproteins released by expanding, preattachment sheep, pig and cow blastocysts in culture. J. Reprod. Fertil. 66:571-583.

Mason K. E. 1935. Fetal death, prolonged gestation and difficult parturition in the rat as a result of vitamin A deficiency. Am. J. Anat. 57:303-34934.

McKenna P. 1984. Proteins in the amniotic and allantoic fluids Comp. Biochem. Physiol. 78:663-66757.

Mercola M., C. Wang, J. Kelly, C. Brownlee, L. Jackson-Grusby, C. Stiles, D. Bowen-Pope. 1990. Selective expression of PDGF A and its receptor during early mouse embryogenesis. Dev. Biol. 138:114-122.

Mummery C. L, H. Slager, W. Kruijer, A. Feijen, E. Freund, I. Koornneef, A. J. M. Van Den Eijnden-Van Raaij. 1990. Expression of transforming growth factor β_2 during the differentiation of murine embryonal carcinoma and embryonic stem cells. Dev. Biol. 137:161-170.

Munson L., J. E. Wilkinson, and V. F. Boltz. 1990.

Transforming growth factors are potential regulators of placental development. Program of the 19th Annual Meeting of Cell Biochem, Los Angels, California, 1990, p 80 (Abstract)

Murray M. K., E. C. Segerson, P. J. Hansen, F. W. Bazer, and R. M. Roberts. 1987. Suppression of lymphocyte activation by a high molecular weight glycoprotein released from preimplantation ovine and porcine conceptuses. Am. J. Reprod. Immunol. Microbiol. 14:38-44.

Newton G. R., and P. J. Hansen. 1988. Secretion of a lactosaminoglycan- containing glycoprotein by peri-implantation sheep conceptuses. Biochem. Biophys. Acta. 996:56-64.

Newton G. R., P. J. Hansen, and B. G. Low. 1988. Characterization of a high molecular weight glycoprotein secreted by the peri-implantation bovine conceptus. Biol. Reprod. 39:553-560.

Nieder G. L., H. M. Weitlauf, and M. Suda-Hartman. 1987. Synthesis and secretion of stage-specific proteins by periimplantation mouse embryos. Biol. Reprod. 36:687-699.

- Nilsen-Hamilton M. 1990. Growth factor signaling in early mammalian development. In: Hegner S (ed.) Growth Factors in Mammalian Development. pp 135-166. CRC Press, Inc. Boca Raton, Florida.
- Okana A., Y. Shioya, T. Obata, and F. Riichi. 1977. Specific protein in bovine allantoic fluid and amniotic fluid. Jap. J. Zootech. Sci. 49:276-282.
- O'Toole, B. A., R. Fradkin, J. Warkany, J. G. Wilson, and G. V. Mann. 1974. Vitamin A deficiency and reproduction in rhesus monkeys. J. Nutr. 104:1513-1524.
- Perry J. S. 1981. The mammalian fetal membranes. J. Reprod. Fertil. 62:321-335.
- Petkovich M., N. J. Brand, A. Krust, and P. Chambon. 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 330:444-450.
- Rask L., and P. A. Peterson. 1976. In vitro uptake of vitamin A from the retinol-binding plasma protein to mucosal epithelial cells from the monkey's small intestine. J. Biol. Chem. 251:6360-6366.
- Roberts R. M., G. A. Baumbach, W. C. Buhi, F. B. Denny, L. A. Fitzgerald, S. F. Babelyn, and M. N. Horst. 1984. Analysis of membrane polypeptides by two-dimensional polyacrylamide gel electrophoresis. In: J. C. Venter, and L. C. Harrison (eds.), Molecular and Chemical Characterization of Membrane Receptors. pp 61-113, Liss, New York.

- Sherman M. I. 1986. How do retinoids promote differentiation. In: M. I. Sherman (ed.) Retinoids and Cell Differentiation, pp 161-186, CRC Press, Inc. Boca Raton, Florida.
- Sivaprasadarao A, J. B. C. Findlay. 1988. The interaction of retinol-binding protein with its plasma membrane receptor. Biochem. J. 255:561-569.
- Smith J. E, and D. S. Goodman. 1971. The effects of diseases of the liver, thyroid and kidneys on the transport of vitamin A in human plasma. J. Clin. Invest. 50:2426-2436.
- Stevens D. H. 1975. Comparative Placentation: Essays in Structure and Function. Academic Press, London.
- Takase S., D. E. Ong, and F. Chytil. 1979. Cellular retinol-binding protein allows specific interaction of retinol with the nucleus in vitro. Proc. Natl. Acad. Sci. 76:2204-2208.
- Takase S., D. E. Ong, F. Chytil. 1986. Transfer of retinoic acid from its complex with cellular retinoic acid-binding protein to the nucleus. Archs. Biochem. Biophys. 247:328-334.
- Thaller C., and G. Eichele. 1987. Identification and spatial distribution of retinoids in the developing chick limb bud. Nature 327:625-628.

- Thompson S. Y., J. M. Howell, G. A. J. Pitt. 1964. Vitamin A and reproduction in rats. *Proc. R. Soc. Lond.* 159:510-535.
- Tickle C., B. Alberts, L. Wolpert, and J. Lee. 1982. Local application of retinoic acid to the limb bud mimics the action of the polarizing region. *Nature* 196:564-566.
- Tiedemann K. 1982. The bovine allantoic and amniotic epithelia: SEM and TEM studies. *Anat. Embryol.* 163:403-416.
- Vallet J. R, F. W. Bazer, M. F. V. Fliss, and W. W. Thatcher. 1988. Effects of ovine conceptus secretory proteins and purified ovine trophoblast protein-1 on interestrus interval and plasma concentrations of prostaglandin F, E and 13,14-dihydro-15-keto prostaglandin $F_{2\alpha}$ in cyclic ewes. *J. Reprod. Fertil.* 84:493-504.
- Wagner M., C. Thaller, T. Jessell T, and G. Eichele. 1990. Polarizing activity and retinoid synthesis in the floor plate of the neural tube. *Nature* 345:819-822.
- Wilkinson J. E., L. Munson, A. Wilhite, and P. Massey, Transforming growth factors in amniotic and allantoic fluids. Program of the 74th Annual Meeting of FASEB, Washington DC, 1990, p A631 (Abstract).

**PART 5: PURIFICATION AND IMMUNOLocalIZATION OF OVINE
PLACENTAL RETINOL-BINDING PROTEIN**

CHAPTER 1

ABSTRACT

A retinol-binding protein (RBP), synthesized and secreted by ovine allantois in vitro, was purified from culture medium. The protein consisted of three isoelectric variants (pI 5.3-6.1) of identical molecular weight of about 23,000 as determined by two-dimensional polyacrylamide gel electrophoresis under reducing conditions. Thirty-one of the first 34 N-terminal amino acids of the purified protein were sequenced and shown to have complete homology with bovine placental and bovine plasma RBP. The UV absorption spectrum and fluorescence excitation and emission spectra of the purified ovine placental RBP indicated the presence of bound retinol. Metabolic labelling studies demonstrated that the protein was synthesized by placental membranes. Using antiserum to bovine placental RBP, ovine placental RBP was immunolocalized in trophectoderm of 13 day old blastocysts and trophectodermal cells of the chorion, endodermal cells lining the allantois and ectodermal cells lining the amnion of 23 day old conceptuses. Results from this study suggest that ovine placental membrane epithelia synthesize and secrete RBP. Transport, storage, and metabolism of retinol mediated by placental RBP may be essential for normal embryonic development during pregnancy.

CHAPTER 2

INTRODUCTION

Vitamin A is essential for reproductive processes including normal embryonic development and maintenance of pregnancy (reviewed by Wolf, 1984). Vitamin A is transported as a lipid alcohol (retinol), bound to a specific transport protein, retinol binding protein (RBP). In blood RBP delivers retinol stored in the liver to target cells (reviewed by Goodman, 1984). The molecular mechanism(s) of action of vitamin A are not fully understood. In some target cells, cellular uptake of retinol is dependent on a membrane receptor that recognizes RBP (Otonello et al. 1987; Sivaprasadarao and Findlay, 1988). Effects of vitamin A have been proposed to be mediated by distinct cellular retinoid-binding proteins (Chytil and Ong, 1984) and retinoic acid nuclear receptors (de The et al. 1987; Giguere et al. 1987; Petkovich et al. 1987; Krust et al. 1989).

Although the liver is the major site of plasma RBP synthesis (Smith and Goodman, 1971), extrahepatic sites of RBP mRNA and/or RBP synthesis have been identified. These include the rat yolk sac (Soprano et al. 1987; Makover et al. 1989), preimplantation pig conceptuses (Harney et al. 1990) and pig endometrium (Adams et al. 1981; Clawitter et

al. 1990). Recently, we purified and identified a RBP synthesized and secreted by bovine chorion and allantois. The protein binds retinol and is present in allantoic and amniotic fluids. The bovine placental RBP (bpRBP) has similar molecular weight, microheterogeneity, and N-terminal amino acid sequence to human plasma RBP (Liu et al. 1990). We have recently performed electrophoretic characterization of protein production by isolated ovine chorion, allantois, amnion and yolk sac. A protein, designated D₄, was demonstrated to be a product of all extraembryonic membranes and identified as a putative ovine placental RBP (opRBP) by immunoprecipitation with antiserum to bpRBP (Liu et al. 1991).

In the present study, we have purified the protein D₄ by bpRBP antibody affinity chromatography, identified 31 of the first 34 N-terminal amino acids, investigated its interaction with retinol and immunolocalized the protein in specific cells of the extraembryonic membranes. Evidence presented here demonstrates that protein D₄ is indeed a RBP which is synthesized by the extraembryonic membranes of sheep conceptuses.

CHAPTER 3

MATERIALS AND METHODS

Materials

Supplies for tissue culture and polyacrylamide gel electrophoresis (PAGE) were as described by Godkin et al. (1988a; 1988b). Halothane was from Fort Dodge Laboratories, Fort Dodge, CO. Protein A-Sepharose CL-4B was from Sigma Chemical Co., St. Louis, MO. Ag/Ab Immobilization Kit was from Pierce, IL. Q-Sepharose and Sephadex G-75 chromatography media were obtained from Pharmacia LKB Biotechnology, Inc, Piscataway, NJ. Vectastain ABC peroxidase anti-rabbit IgG immunohistochemical staining kit was from Vector Laboratories, Burlingame, CA. L-[³⁵S]-methionine (1134 Ci/mmol) and L-[2,4,5-³H]-Leucine (53 Ci/mmol) was purchased from New England Nuclear, Boston, MA.

Animals

Adult crossbred ewes were checked twice daily for estrus with vasectomized rams. Estrous ewes (estrus = Day 0) were mated by intact rams. Pregnant ewes were anesthetized with halothane and the reproductive tracts were collected via midventral laparotomy on Days 13 (n=3), 23 (n=3), 35 (n=3) and 45 (n=3) of pregnancy as described by Godkin et al. (1982) and transported to the laboratory on ice after surgery.

In Vitro Culture of Allantoic Membranes

Placental tissues (allantois, chorion, and amnion) collected on Days 35 and 45 of pregnancy were carefully dissected and collected separately under sterile conditions with the aid of a stereo zoom microscope in a laminar flow tissue culture hood. Allantoic tissues were cultured in a modified Minimum Essential Medium (GIBCO, Laboratories, Grand Island, NY), in the presence of [^3H]-leucine or [^{35}S]-methionine as described Godkin et al. (1988a) and utilized by Liu et al. (1990).

Protein Electrophoresis

One-dimensional (1D)-PAGE and two dimensional (2D)-PAGE were performed according to the method of Laemmli (1970) and Roberts et al. (1984), respectively. After electrophoresis, proteins were visualized by Coomassie Brilliant Blue R-250-staining. Fluorographs were prepared as described by Chamberlain (1979). Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Rabbit muscle phosphorylase b, bovine serum albumin, hen egg white albumin, bovine carbonic anhydrase, soybean trypsin inhibitor, and hen egg white lysozyme were used as protein molecular weight standards.

Purification of Protein D₄

Medium from allantoic membrane cultures containing metabolically labelled ([^3H]-leucine) protein, was pooled

(approximately 500 ml), concentrated, dialyzed, and fractionated by Q-Sepharose anion-exchange and Sephadex G-75 column chromatography as described by Liu et al. (1990). Fractions containing protein D₄ were pooled, and concentrated. Samples were then applied to a 2 ml column of activated agarose which had been covalently coupled with 2 mg of anti-bpRBP IgG isolated by Protein A-Sepharose CL-4B column chromatography (1 X 2 cm) according to manufacturer's directions. After applying the sample, the immunoaffinity column was washed with 10 mM Tris-HCl, pH 7.6 to remove unbound proteins. Bound material was eluted with 20 ml of 0.1 M glycine, pH 2.8. The eluate was neutralized by addition of 50 ul of 1 M of Tris-HCl, pH 11, and dialyzed against 10 mM Tris-HCl buffer, pH 7.6 for 4 hours and stored at -20 C.

Protein Sequence Determination

Gas-liquid phase Edman degradation of purified protein D₄ (1 nmole) was carried out using an Applied Biosystems 473A sequencer coupled with a model 120A phenylthiohydantoin HPLC analyzer (Protein/DNA Molecular Biology Core Facility, University of Tennessee). Cysteines were not reduced and alkylated and, thus, could not be identified. The Genetics Computer Group program of the University of Wisconsin (Devereux et al. 1984) was used to search the National Biomedical Research Foundation-Protein Identification

Research database for proteins with amino acid sequence homologies.

Fluorescence Detection of Bound Retinol

The fluorescence of retinol in 1 ml fractions from chromatographies on Q-Sepharose, Sephadex G-75, and immunoaffinity columns, described above, was measured on a Perkin-Elmer fluorescence spectrophotometer (Norwalk, CT) at an excitation wavelength of 335 nm and at an emission wavelength of 465 nm. Spectra analysis of purified protein D₄ was performed to determine the excitation and emission maxima of bound material (retinol) in D₄.

Immunocytochemical Localization of D₄

Ovine conceptus tissues at Day 13 of pregnancy and chorioallantoic and amniotic tissues at Day 23 of pregnancy were prepared for immunocytochemical study as described in Gao and Godkin (1991). Detection of D₄ in tissues examined was performed according to the Vectastain ABC horseradish peroxidase anti-rabbit IgG immunohistochemical staining Kit instructions.

CHAPTER 4

RESULTS

Two dimensional-PAGE of Synthesized Protein

Figure 1 (left) shows the electrophoretic pattern of [³⁵S]-methionine labeled protein synthesized and released by Day 45 allantois. Protein D₄ appeared to consist of two major isoelectric variants (pI 5.7-6.1) of identical molecular weight of about 23,000. Overexposing the fluorogram demonstrated the presence of a third, more acidic (pI 5.3) isoform (not shown).

Purification of Protein D₄

Approximately 500 ml of allantoic membrane culture medium containing [³H]-leucine labeled proteins were pooled, concentrated, and submitted to Q-Sepharose column chromatography. A predominant retinol fluorescence peak was eluted by the salt gradient (Fig. 2) which contained a large amount of high and low molecular weight proteins determined by 1D-PAGE (not shown). Most of the retinol fluorescence was associated with molecular weight fractions between 17,000 and 26,000 determined by Sephadex G-75 column chromatography (Fig. 3). The fluorescent fractions (tubes 41-45) were pooled, dialyzed, concentrated and then applied to the immunoaffinity column. A typical elution profile is shown in Fig. 4. A single fluorescent peak was eluted

Figure 1. Two-dimensional polyacrylamide gel electrophoresis analyses of medium proteins from Day 45 allantois cultures (left) and purified placental RBP (right). 200,000 cpm of radiolabeled medium proteins were separated by 2D-PAGE. The resulting fluorograph was generated by exposure of X-ray film to the dried gel for 2 weeks (left). Coomassie Brilliant Blue-stained 2D-PAGE gel (10%) containing 20 ug of immunoaffinity purified protein D₄ (right).

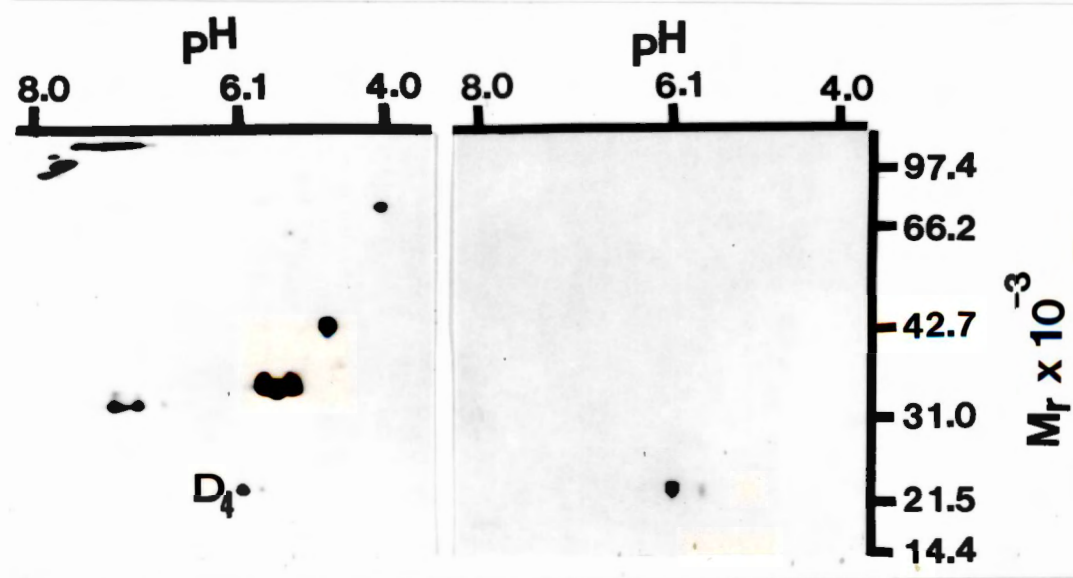


Figure 2. Q-Sepharose ion exchange chromatography of proteins released into culture medium by ovine allantois at 35-45 days of pregnancy. Bound proteins were eluted with a 0.0 to 0.6 M gradient of NaCl (---). Fractions were monitored for both protein and retinol. Retinol-containing fractions 22-40 (indicated by double-headed arrows) were pooled.

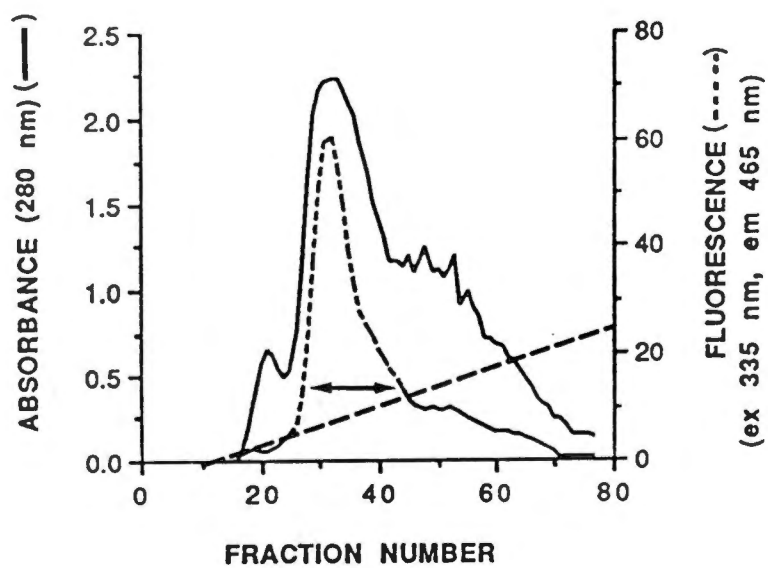


Figure 3. Sephadex G-75 gel chromatography of retinol-containing fractions from Figure 2. Pooled retinol-containing fractions from Q-Sepharose chromatography were dialysed, concentrated and applied to a column (1.7 x 100 cm) of Sephadex G-75. Radioactivity in fractions was monitored for identification of metabolically labelled proteins. Retinol-containing fractions were pooled as indicated by bracket. Arrows indicate the elution positions of the molecular weight markers (1) transferrin (78,000), (2) ovalbumin (43,000), (3) chymotrypsinogen (26,000) and myoglobin (16,900).

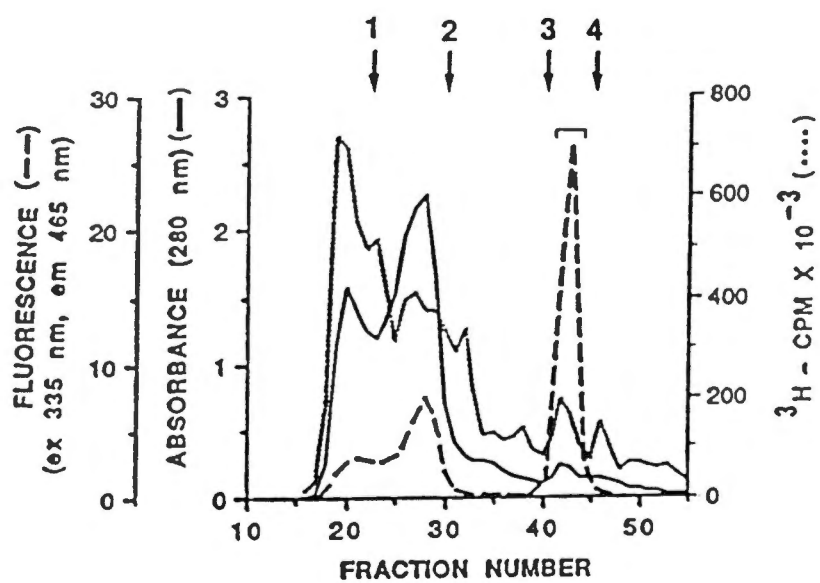
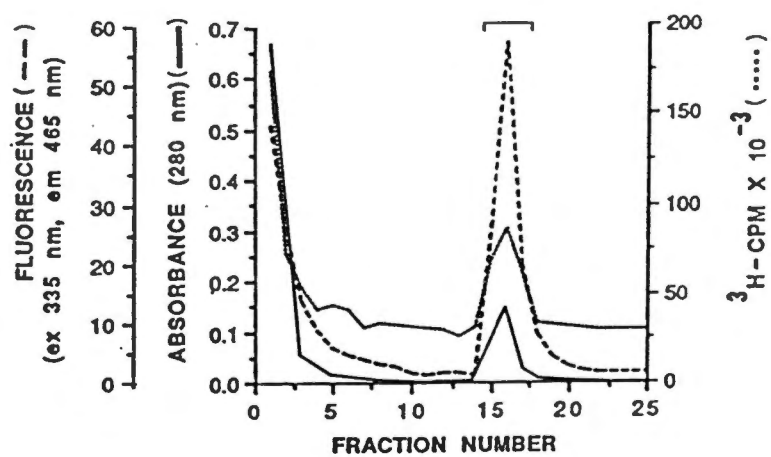


Figure 4. Purification of protein D₄ (opRBP) by immunoaffinity chromatography. The retinol-containing material from Sephadex G-75 column chromatography was applied to the column. The column was washed with equilibration buffer and protein eluted with 20 ml of 0.1 M glycine, pH 2.8. Fractions containing retinol (bracket) were pooled and dialyzed for further analysis.



coincident with the radiolabeled and protein absorbance peaks. Two-dimensional PAGE analysis of 20 ug of the affinity purified protein showed 2 major isoforms (pI 5.7-6.1) with identical molecular weight of about 23,000 (Fig. 1, right). In addition, one very faint isoform (pI 5.3) was present, but barely discernable by Coomassie Brilliant Blue-staining. Generally, 120-150 ug of purified protein was obtained from 500 ml of sheep allantois culture medium by the three-step chromatography procedure.

N-terminal amino acid sequence of purified D₄

Table 1 shows the first 34 residues of the N-terminal amino acid sequence of purified D₄. Three cycles (4, 8 and 24) did not contain an identifiable amino acid residue. Cysteines could not be identified since the sample was not reduced and alkylated before sequencing. Cycle 4 may represent a cysteine residue. Thirty-one of the first 34 sequenced amino acids were identical to bpRBP (Liu et al. 1990) and bovine plasma RBP (Berni et al. 1990). An alanine/serine interchange at position 21 was the only observed difference between opRBP and human plasma RBP (Rask et al. 1979) as well as porcine conceptus RBP (Harney et al. 1990).

Presence of retinol in opRBP (D₄)

As with bpRBP (Liu et al. 1990) purified opRBP exhibited two absorbance peaks with maxima at 280 nm

Table 1. N-terminal amino acid sequences of ovine and bovine placental RBP (opRBP and bpRBP), pig conceptus RBP (pcRBP), bovine and human plasma RBP (BPRBP and HPRBP).

	Amino acid position						
Protein	5	10	15	20	25	30	34
opRBP	ERD-R	VS-FR	VKENF	DKARF	AGT-Y	AMAKK	DPEG
bpRBP	ERD-R	VS-FR	VKENF	DKARF	AGTWY	AMAK-	DPEG
pcRBP	ERDCR	VSSFR	VKENF	DKARF	SGTWY	AM-KK	-PE
BPRBP	ERDCR	VSSFR	VKENF	DKARF	AGTWY	AMAKK	DPEG
HPRBP	ERDCR	VSSFR	VKENF	DKARF	SGTWY	AMAKK	DPEG

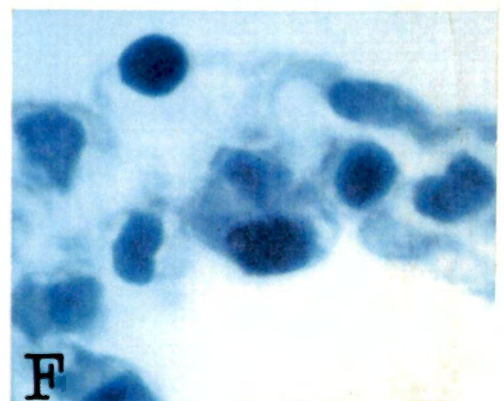
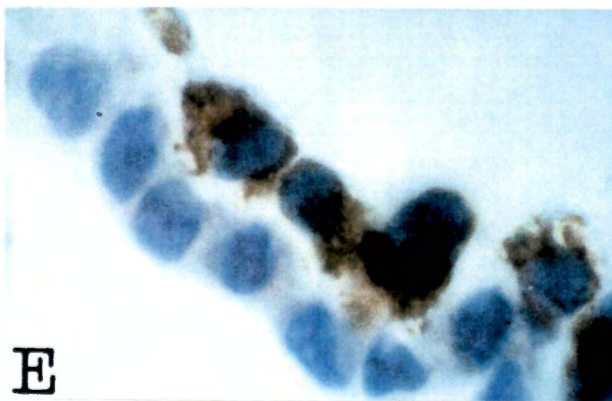
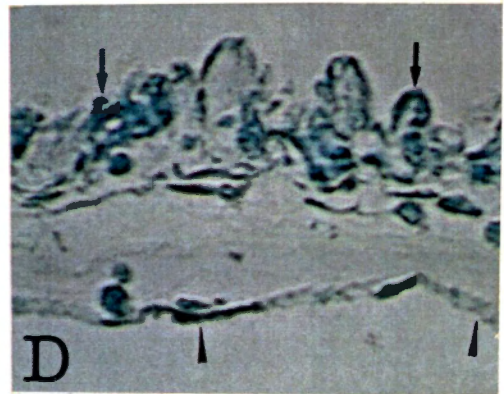
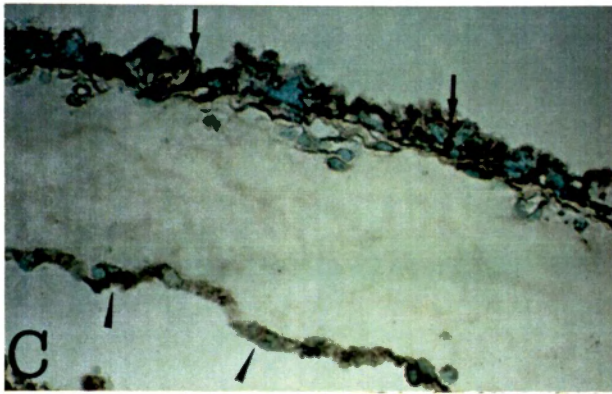
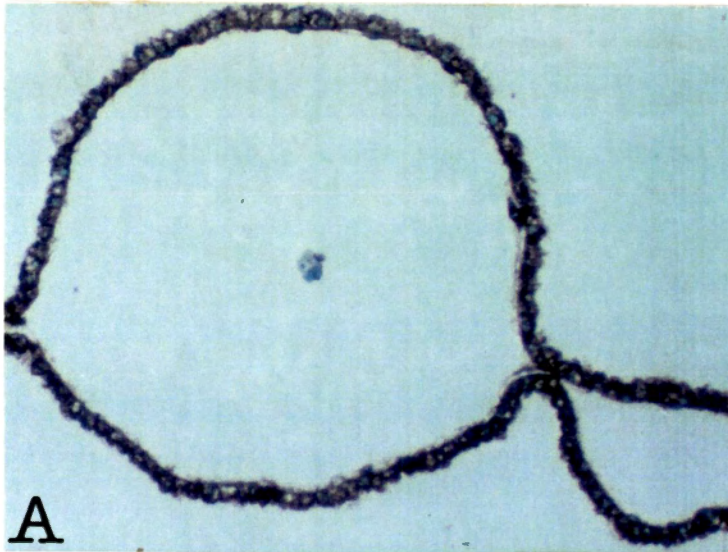
The dashes indicate unidentified amino acid residues.

(protein) and 330 nm (retinol) in the ultraviolet spectrum. The absorption ratio (330 nm/280 nm) was 0.28. In addition, the fluorescence spectrum of purified opRBP displayed an excitation maximum at 335 nm and emission maximum at 465 nm. These results indicated the presence of bound retinol in protein D₄.

Immunocytochemistry localization of opRBP (D₄)

Conceptus tissues from Days 13 and 23 of pregnancy immunostained with anti-bpRBP serum or nonimmune serum (controls) are shown in Fig. 5. Intensive immunostaining for RBP was present in Day 13 trophectoderm (Fig. 5A). Immunoreactive RBP was also detected in trophectodermal cells of the chorion (Fig. 5C, arrows), endodermal cells lining the allantois (Fig. 5C, arrowheads), and ectodermal cells lining the amnion (Fig. 5E). No staining was observed in respective sections treated with nonimmune serum (Fig. 5B, 5D and 5F).

Figure 5. Photomicrographs of ovine conceptus tissues immunostained for identification of RBP. A. Day 13 blastocyst; C. chorioallantois (Day 23); E. amnion (Day 23) immunostained with anti-bpRBP serum (1:300) using the peroxidase-antiperoxidase method. Negative controls of Panels A, C and E using preimmune serum on an adjacent tissue section are shown in Panels B, D and F respectively. Panel A x150; B x120; C x280; D x420; E x1200 and F x1300.



CHAPTER 5

DISCUSSION

A RBP, synthesized by ovine allantois in vitro, was purified from medium by a three-step procedure with immunoaffinity chromatography as the final step. The purified protein consisted of two major and one minor isoelectric variants (pI 5.1-6.3) with identical molecular weight of about 23,000. The protein was identified as a RBP by demonstration of crossreactivity with anti-bpRBP serum, its ability to bind retinol and N-terminal amino acid sequencing. Thirty-one of the first 34 N-terminal amino acids of the protein showed complete homology with bovine placental (Liu et al. 1990) and plasma RBP (Berni et al. 1990) and extensive homology with porcine conceptus (Harney et al. 1990) and human plasma RBP (Rask et al. 1979). The fluorescence maxima of excitation and of emission of the purified protein were 335 nm and 465 nm, respectively. These spectral properties are unique for retinol (Muto and Goodman, 1972; Peterson and Berggard, 1971) and indicate the presence of retinol bound to opRBP. The molar ratio of RBP to retinol in human serum is 1:1 (Heller and Horwitz, 1973) and spectra analysis (A330/A280) is generally in the range of 0.8-1.05 (Raz et al. 1970; Heller, 1975). In the present study, the ratio of A330/A280 was 0.28. The low ratio may

have been due to loss of retinol during purification procedures as suggested by Peterson (1971). Alternatively, the possibility exists that the extraembryonic membranes secrete RBP in the apo-form.

Results of the immunocytochemical study presented here demonstrate RBP in trophoctoderm of the conceptus at the time of blastocyst expansion and maternal recognition of pregnancy (Day 13). In addition, we have demonstrated uterine synthesis of RBP by immune precipitation of metabolically labelled RBP from uterine endometrial explants obtained on Day 15 of pregnancy (Liu and Godkin unpublished data). Furthermore, Harney et al. (1990) demonstrated RBP production by preimplantation pig conceptus. These data indicate that RBP and its ligand, retinol, may play an important function in very early conceptus development. The observations that RBP is produced by and localized in bovine (Liu et al. 1990; Gao et al. 1991) and ovine (Liu et al. 1991, this study) placental membranes and their fluids through at least midpregnancy indicate that the placental membranes play an important role in the transport, storage and metabolism of retinol throughout much of pregnancy.

Retinol-binding protein is the only retinol-specific binding protein present in serum that delivers retinol from the liver to target cells (reviewed by Goodman, 1984). The fetus is dependent on placental transfer of maternal retinol

for growth and development (reviewed by Wolf, 1984). Furthermore, Donoghue et al. (1982) reported that retinol transfer was bidirectional between fetal lambs and their mothers during pregnancy. High levels of retinoids introduced into embryos are teratogenic (reviewed by Nanda et al. 1977); therefore, control of retinol supply is essential for normal fetal growth and development. The mechanism of maternal retinol transfer to the fetus is not clear but our studies indicate that it may involve RBP of uterine and placental origin, at least up to mid-gestation.

Retinoids are potent regulators of early embryonic development (reviewed by Wolf, 1984). Retinoic acid (RA), an active metabolite of retinol, may be the morphogen that specifies axial information in developing and regenerating chick limbs (Tickle et al. 1975; Maden, 1982; 1983; Ludolph et al. 1990). In addition, RA is believed to be involved in the establishment of pattern formation of the nervous system in the mouse (Wagner et al. 1990) and chick embryos (Maden et al. 1991). The ability of retinoids to regulate expression of homeobox genes (Deschamps et al. 1987; LaRosa and Gudas, 1988; Mavilio et al., 1988; Simeone et al. 1990) and genes encoding proteins and proteases of the extracellular matrix (Brinckerhoff and Harris, 1981; Yuspa et al., 1982; Vasios et al. 1989) indicate that retinoids may play important roles during early morphogenesis stages

of vertebrate development. The mechanism(s) by which retinoids exert their physiological roles are not well understood. Retinoids have been shown to have specific effects on the regulation of expression of transforming growth factor β (TGF β) and the receptor for epidermal growth factor/TGF α in certain embryonic cell lines (Rees et al., 1979; Jetten, 1980; Mummery et al. 1990). Retinoids and TGF β both exhibit growth promoting and growth inhibitory effects, depending on the target tissues (Nilsen-Hamilton, 1990). Recent studies have indicated that TGF β may play important developmental roles during mouse embryogenesis (Millan et al., 1991; Schmid et al., 1991). These studies suggest some of effects of retinoids on embryonic growth, differentiation, and morphogenesis may be closely related with peptide growth factors and their receptors. TGF α , TGF β (Wilkinson et al. 1990) and RBP (Liu et al. 1990) have been identified in bovine allantoic and amniotic fluids. Bovine trophoblastic cell-conditioned medium has both TGF α and TGF β activity (Munson, 1990). Moreover, the trophoblast synthesizes RBP (Liu et al. 1990). Recently, RBP, TGF α and TGF β have been immunolocalized in ovine and bovine trophectoderm and uterine epithelium (this study; Munson et al., 1990; Dore, Gao, Wilkinson and Godkin, unpublished observations). Together, these data may indicate a functional link between retinoids and growth

factors in ruminant placental, embryonic and uterine development.

In summary, RBP has been shown to be a product of the early conceptus and placental membranes in swine (Harney et al. 1990), cattle (Liu et al. 1990), sheep (this study) and goats (Liu and Godkin, unpublished data). In addition, RBP is produced by the uterine endometrium of swine (Adams et al. 1981; Clawitter et al. 1990), cattle and sheep (Liu and Godkin, unpublished data). Together, these data illustrate clearly that an important function of the fetal-placental-maternal unit is transport, storage and metabolism of retinol in large domestic animals.

LIST OF REFERENCES

LIST OF REFERENCES

- Adams, K. L., F. W. Bazer, and R. M. Roberts. 1981. Progesterone induced secretion of a retinol-binding protein in the pig uterus. *J. Reprod. Fert.* 62:39-47.
- Berni, R., M. Stoppini, M. C. Zapponi, M. L. Meloni, H. L. Monaco, and Zanotti, G. 1990. The bovine plasma retinol-binding protein: amino acid sequence, interaction with transthyretin, crystallization and preliminary X-ray data. *Eur. J. Biochem.* 192:507-513.
- Brinckerhoff, C. E. and E. D. Harris, Jr. 1981. Modulation by retinoic acid and corticosteroids of collagenase production by rabbit synovial fibroblasts treated with phorbol myristate acetate or poly(ethylene glycol). *Biochem. Biophys. Acta.* 677:424-432.
- Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water soluble fluor, sodium salicylate. *Anal. Biochem.* 98: 132-135.
- Chytil, F. and D. E. Ong. 1984. Cellular retinoid-binding proteins. In *The Retinoids*, vol. 2 (ed. M. B. Sporn, A. B. Roberts, and D. S. Goodman), pp 89-123. New York: Academic Press.

- Clawitter, J., W. E. Trout, M. G. Burke, S. Araghi, and R. M. Roberts. 1990. A novel family of progesterone induced, retinol binding proteins from uterine secretions of the pig. *J. Biol. Chem.* 265:3248-3255.
- de The, H., A. Marchio, P. Tiollais, and A. Dejean. 1987. A novel steroid thyroid hormone receptor-related gene inappropriately expressed in human hepatocellular carcinoma. *Nature* 330:667-670.
- Deschamps, J., R. DeLaaf, L. Joosen, F. Meijlink, and O. Destree. 1987. Abundant expression of homeobox genes in mouse embryonal carcinoma cells correlates with chemically induced differentiation. *Proc. natn. Acad. Sci. U.S.A.* 84, 1304-1308.
- Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acid Res.* 12:387-395.
- Donoghue, S., D. W. Richardson, D. Sklan, and D. S. Kronfeld. 1982. Placental transport of retinol in sheep. *J. Nut.* 112:2197-2203.
- Gao, K. and J. D. Godkin. 1991. A new method for transfer of polyethylene glycol-embedded tissue sections to silanated slides for immunocytochemistry. *J. Histochem. Cytochem.* 39:537-540.

Gao, K., K. H. Liu, and J. D. Godkin. 1991.

Immunohistochemical localization of bovine retinol-binding protein. Submitted to J. Cell. Sci.

Giguere, V., E. S. Ong, P. Segui, and R. M. Evans. 1987.

Identification of a receptor for the morphogen retinoic acid. *Nature* 330:624-629.

Godkin, J. D., F. W. Bazer, J. Moffatt, F. Sessions, and R.

M. Roberts. 1982. Purification and properties of a major low molecular weight protein released by the trophoblast of sheep blastocysts at day 13-21. *J. Reprod. Fertil.* 65:141-150.

Godkin, J. D., B. J. Lifsey, and G. A. Baumbach. 1988a.

Characterization of protein production by bovine chorionic and allantoic membranes. *Biol. Reprod.* 39: 195-204.

Godkin, J. D., B. J. Lifsey, and B. E. Gillespie. 1988b.

Characterization of bovine conceptus proteins produced during the peri- and postattachment periods of early pregnancy. *Biol. Reprod.* 38:703-711.

Goodman, D. S. 1984. Plasma retinol-binding protein. In *The Retinoids*, vol. 2 (ed. M. B. Sporn, A. B. Roberts, and D. S. Goodman), pp 42-88. New York: Academic Press.

- Harney, J. P., M. A. Mirando, L. C. Smith, and F. W. Bazer. 1990. Retinol-binding protein: a major secretory product of the pig conceptus. *Biol. Reprod.* 42:523-532.
- Heller, J. 1975. Characterization of bovine plasma retinol-binding protein and evidence for lack of binding between it and other bovine plasma proteins. *J. Biol. Chem.* 250:6549-6554.
- Heller, J. and J. Horwitz. 1973. Conformational changes following interaction between retinol isomers and human retinol-binding protein and between the retinol-binding protein and prealbumin. *J. Biol. Chem.* 248:6308-6316.
- Jetten, A. M. 1980. Retinoids specifically enhance the number of epidermal growth factor receptors. *Nature* 284:626-629.
- Krust, A., P. Kastner, M. Petkovich, A. Zelent, and P. Chambon. 1989. A third human retinoic acid receptor, hRAR-gamma. *Proc. Natl. Acad. Sci. U.S.A.* 86:5310-5314.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

- LaRosa G. J. and L. J. Gudas. 1988. Early retinoic acid induced F9 teratocarcinoma stem cell gene ERA-a: alternate splicing creates transcripts for a homeobox containing protein and one lacking the homeobox. *Molec. Cell. Biol.* 8:3906-3917.
- Liu, K. H., G. A. Baumbach, P. M. Gillevet, and J. D. Godkin, 1990. Purification and characterization of bovine placental retinol-binding protein. *Endocrinology* 127,2696-2704.
- Liu, K. H., R. G. Brewton, G. A. Baumbach, and J. D. Godkin. 1991. Characterization of protein production by ovine placental membranes: identification of a placental retinol-binding protein. *Endocrinology* (in press).
- Lowry, O. H., N. C. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Ludolph, D. C., J. A. Cameron, and D. L. Stocum. 1990. The effect of retinoic acid on positional memory in the dorsoventral axis of regenerating axolotl limbs. *Devl Biol.* 140:41-52.
- Maden, M. 1982. Vitamin A and pattern formation in the regenerating limb. *Nature* 295:672-675.
- Maden, M. 1983. The effect of vitamin A on limb regeneration in *Rana temporaria*. *Devl Biol.* 98:409-416.

- Maden, M., P. Hunt, U. Eriksson, A. Kuroiwa, R. Krumlauf, and D. Summerbell. 1991. Retinoic acid-binding protein, rhombomeres and the neural crest. *Development* 111:35-44.
- Makover, A., D. R. Soprano, M. L. Wyatt, and D. S. Goodman, 1989. An in situ-hybridization study of the localization of retinol-binding protein and transthyretin messenger RNAs during fetal development. *Differentiation* 40:17-25.
- Mavilio, F., A. Simeone, A., E. Boncinelli, and P. W. Andrews. 1988. Activation of four homebox gene clusters in human embryonal carcinoma cells induced to differentiate by retinoic acid. *Differentiation* 37:73-79.
- Millan, F. A., F. Denhez, P. Kondaiah, and R. J. Akhurst. 1991. Embryonic gene expression patterns of TGF β 1, β 2 and β 3 suggest different developmental functions in vivo. *Development* 111,131-144.
- Mummery, C. L., H. Slager, W. Kruijer, A. Feijen, E. Freund, I. Koornneef, A. J. M. Van Den Eijnden-Van Raaij. 1990. Expression of transforming growth factor β 2 during the differentiation of murine embryonal carcinoma and embryonic stem cells. *Devl. Biol.* 137:161-170.

Munson, L., J. E. Wilkinson, and V. F. Boltz. 1990.

Transforming growth factors are potential regulators of placental development. J. Cell Biochem. Supplement 14E, 80.

Muto, Y. and D. S. Goodman. 1972. Vitamin A transport in rat plasma. Isolation and characterization of retinol-binding protein. J. Biol. Chem. 247:2533-2541.

Nanda, R., D. L. May, and S. Lite. 1977. The role of amniotic fluid on the in vitro palatal fusion of normal and vitamin A treated rat fetuses. Arch. Oral. Biol. 22:613-618.

Nilsen-Hamilton, M. 1990. Growth factor signaling in early mammalian development. In Growth Factors in Mammalian Development. (ed. S. Heyner), pp. 135-166. Boca Ratan: CRC Press, Inc.

Otonello, S., S. Petrucci, and G. Maraini. 1987. Vitamin A uptake from retinol-binding protein in a cell-free system from pigment epithelial cells of bovine retina. J. Biol. Chem. 262:3975-3981.

Peterson, P. A. 1971. Studies on the interaction between prealbumin, retinol-binding protein and vitamin A. J. Biol. Chem. 246:44-49.

Peterson, P. A. and I. Berggard, 1971. Isolation and properties of a human retinol-transporting protein. J. Biol. Chem. 246: 25-33.

- Petkovich, M., N. J. Brand, A. Krust, and P. Chambon. 1987.
A human retinoic acid receptor which belongs to the
family of nuclear receptors. *Nature* 330:444-450.
- Rask, L., H. Anundi, P. A. Peterson, 1979. The primary
structure of the human retinol-binding protein. *FEBS
Lett* 104:55-58.
- Raz, A., T. Shiratori, and D. S. Goodman, 1970. Studies on
the protein-protein and protein-ligand interactions
involved in retinol transport in plasma. *J. Biol. Chem.*
245:1903-1912.
- Rees, A. R., E. D. Adamson, and C. F. Graham. 1979.
Epidermal growth factor receptors increase during the
differentiation of embryonal carcinoma cells. *Nature*
281:309-311.
- Roberts, R. M., G. A. Baumbach, W. C. Buhi, F. B. Denny, L.
A. Fitzgerald, S. F. Babelyn, and M. N. Horst. 1984.
Analysis of membrane polypeptides by two-dimensional
polyacrylamide gel electrophoresis. In *Molecular and
Chemical Characterization of Membrane Receptors*, (eds.
J. C. Ventor, and L. C. Harrison), pp 61-113. New York:
Liss. Inc.
- Schmid, P., D. Cox, G. Bilbe, R. Maier, and G. K. McMaster,
1991. Differential expression of TGF β 1, β 2 and β 3
genes during mouse embryogenesis. *Development* 111:117-
130.

- Simeone, A., A. Acampora, L. Arcioni, P. W. Andrews, E. Boncinelli, and F. Mavilio, 1990. Sequential activation of human HOX2 homeobox genes by retinoic acid in embryonal carcinoma cells. *Nature* 346:763-766.
- Sivaprasadarao, A. and L. B. C. Findlay. 1988. The interaction of retinol-binding protein with its plasma-membrane receptor. *Biochem. J.* 255:561-569.
- Smith, J. E. and D. S. Goodman. 1971. The effects of diseases of the liver, thyroid and kidneys on the transport of vitamin A in human plasma. *J. Clin. Invest.* 50:2426-2436.
- Soprano, D. R., Wyatt, M. L., Dixon, J. L., Soprano, K. L. and Goodman, D. S. 1987. Retinol-binding protein synthesis and secretion by the rat visceral yolk sac. *J. Biol. Chem.* 263, 2934-2938.
- Tickle, C., D. Summerbell, and L. Wolpert, 1975. Positional signalling and specification of digits in chick limb morphogenesis. *Nature* 254:199-202.
- Vasios, G. W., J. D. Gold, M. Petkovich, P. Chambon, and L. J. Gudas. 1989. A retinoic acid-responsive element is present in the 5'-flanking region of the laminin B1 gene. *Proc. natn. Acad. Sci. U.S.A.* 86:9099-9103.
- Wagner, M., C. Thaller, T. Jessell, and G. Eichele. 1990. Polarizing activity and retinoid synthesis in the floor plate of the neural tube. *Nature* 345:891-822.

- Wilkinson, J. E., L. Munson, A. Wilhite, and P. Massey.
1990. Transforming growth factors in amniotic and
allantoic fluids. FASEB J. Supplement 3, A631.
- Wolf, G. 1984. Multiple functions of vitamin A. Physiol.
Rev. 64:873-937.
- Yuspa, S. H., T. Ben, and P. Steinert. 1982. Retinoic acid
induces transglutaminase activity but inhibits
cornification of cultured epidermal cells. J. Biol.
Chem. 257:9906-9908.

PART 6: GENERAL DISCUSSION

CHAPTER 1

GENERAL DISCUSSION OF PARTS 2 - 5

In domestic ruminants, secretory proteins produced by the developing conceptus are believed to mediate some feto-maternal interactions which are essential for the establishment and maintenance of pregnancy. Proteins produced in vitro by intact conceptuses of the sheep (Godkin et al., 1982), cow (Bartol et al., 1985; Godkin et al., 1988b) and isolated extraembryonic membranes (allantois and chorion) of the cow (Godkin et al., 1988a) during early pregnancy has been characterized by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and fluorography. Dramatic alterations in the electrophoretic patterns of protein synthesis occurred between Days 24-29 in bovine (Godkin et al., 1988a; 1988b) and 21-23 in ovine (Godkin et al., 1982). A major alteration was the cessation of production of the ovine trophoblast protein-1 (oTP-1) (Godkin et al., 1982) and bovine TP-1 (Godkin et al., 1988a; 1988b) and the appearance of a different, more basic protein as the major low molecular weight protein (LMWP). These alterations coincided temporally with developmental changes in the placental membranes. Notably, the allantois had become a prominent structure, filling and contacting the chorion. The amnion was fully formed at this time and the yolk sac had reached peak development and would soon undergo

steady regression as its nutritive role was taken over by the rapidly expanding allantois (Greenstein et al., 1958). In the present study we have characterized and compared protein production by isolated ovine chorionic, allantoic, amniotic and yolk sac membranes from Day 23 through mid-pregnancy. Furthermore, we identified the major LMWP produced by bovine and ovine extraembryonic membranes as a placental retinol-binding protein (RBP).

As with bovine extraembryonic membranes (Godkin et al., 1988a), ovine extraembryonic membranes were active in protein synthesis. Patterns of protein production by isolated ovine extraembryonic membranes remained relatively unchanged from early postattachment through midpregnancy with the exception of the products of yolk sac which regresses by Day 35 of pregnancy. In general, chorion was the source of a number of basic-to-neutral proteins, allantois and amnion were the sources of more acidic proteins and yolk sac was the source of serum-like proteins.

Importantly, a LMWP, designated 3B₃ in bovine (Godkin et al., 1988a) and D₄ in ovine (this study) was produced by all extraembryonic membranes and present in fetal membrane fluids. In the present study, the protein synthesized and secreted by bovine and ovine allantois in vitro was purified from culture medium. Thirty-one of the first 34 N-terminal amino acids of the purified protein from both sources were sequenced and shown to have complete homology with bovine

plasma RBP (Berni et al., 1990) and extensive homology with porcine conceptus (Harney et al. 1990) and human RBP (Rask et al., 1979). The UV absorption spectrum and fluorescence excitation and emission spectra of the purified 3B₃ and D₄ indicated the presence of bound retinol. These results demonstrated that proteins 3B₃ and D₄ are bovine (b) and ovine (o) placental retinol-binding protein (pRBP), respectively. Both bpRBP and opRBP consisted of three isoelectric variants (pI 5.3-6.1) with identical molecular weight ($23,000 \pm 800$) when analyzed by 2D-PAGE.

Rabbit antiserum was raised against purified bovine placental RBP. Monospecificity of the antiserum was demonstrated by selective immune precipitation of the protein from bovine placental membrane culture medium and Western blot analysis of allantoic fluid proteins. The anti-bpRBP antiserum immunologically crossreacts with opRBP as determined by immunoprecipitation of opRBP from isolated ovine chorion, allantois and amnion culture medium. Ovine placental RBP was also detected in allantoic and amniotic fluids by immunoblotting. These metabolic labelling, and immunoprecipitation studies demonstrated that RBP was secreted by bovine and ovine extraembryonic membranes and released into the allantoic and amniotic fluids.

Results of the immunocytochemical study presented in this study demonstrate RBP in trophoctodermal cells of the chorion, endodermal cells lining the allantois and

ectodermal cells lining the amnion of bovine and ovine. Furthermore, we have identified a protein from goat conceptus cultures that cross reacts with our bpRBP antiserum (Liu and Godkin unpublished data). Harney et al. (1990) demonstrated RBP production by preimplantation pig conceptus. In addition, we have demonstrated uterine synthesis of RBP by immune precipitation of metabolically labelled RBP from uterine endometrial explants obtained on Day 15 of pregnancy (Liu and Godkin unpublished data). Together these results indicate a common, local mechanism for the transport of retinol in conceptuses of large domestic farm animals.

Retinol-binding protein is the only known transport protein in serum that delivers the bioactive alcohol form of vitamin A (retinol) from the liver to target cells (Smith and Goodman, 1971). Cellular uptake of retinol is mediated by a specific membrane receptor that recognizes RBP (Heller, 1975; Rask and Peterson, 1976; Sivaprasadarao and Findlay, 1988). Within the target cell, retinol and/or its metabolite, retinoic acid (RA), interact with distinct cellular retinoid binding proteins (CRBP and CRABP, respectively) (Chytil and Ong, 1984) that have been shown to transfer their ligands to isolated nuclei (Takase et al., 1979). Recently, nuclear RA receptors (RARs) have been identified. RARs belong to the superfamily of steroid-thyroid hormone receptors that are believed to control some

cell functions by direct regulation of gene expression (de The et al., 1987; Giguere et al., 1987; Petkovich et al., 1987; Krust et al., 1989). The identification of RBP production by placental membranes indicates local control of vitamin A metabolism in the developing embryos.

Retinoids are potent regulators of early embryonic development (reviewed by Wolf, 1984). Retinoic acid (RA) may be the morphogen that specifies axial information in developing and regenerating chick limbs (Tickle et al. 1975; Maden, 1982; 1983; Ludolph et al. 1990). In addition, RA is believed to be involved in the establishment of pattern formation of the nervous system in the mouse (Wagner et al. 1990) and chick embryos (Maden et al. 1991). The ability of retinoids to regulate expression of homeobox genes (Deschamps et al. 1987; Mavilio et al., 1988; Simeone et al. 1990) and genes encoding proteins and proteases of the extracellular matrix (Brinckerhoff and Harris, 1981; Yuspa et al., 1982; Vasios et al. 1989) indicate that retinoids may play important roles during early morphogenesis stages of vertebrate development.

The mechanism(s) by which retinoids exert their physiological roles are not well understood. Retinoids have been shown to have specific effects on the regulation of expression of transforming growth factor β (TGF β) and the receptor for epidermal growth factor/TGF α in certain embryonic cell lines (Rees et al., 1979; Jetten, 1980;

Mummery et al. 1990). Retinoids and TGF β both exhibit growth promoting and growth inhibitory effects, depending on the target tissues (Nilsen-Hamilton, 1990). Recent studies have indicated that TGF β may play important developmental roles during mouse embryogenesis (Millan et al., 1991; Schmid et al., 1991). These studies suggest some of the effects of retinoids on embryonic growth, differentiation, and morphogenesis may be closely related with peptide growth factors and their receptors. Many processes occur during the time of placental RBP secretion including: outgrowth of chorion, allantois and amnion; placentation; growth and differentiation of embryo as well as growth of the uterus and placentome formation. Therefore, retinoids may play an important function in controlling these processes to establish and maintain a successful pregnancy.

LIST OF REFERENCES

LIST OF REFERENCES

- Bartol F. F., R. M. Roberts, F. W. Bazer, G.S Lewis, J. D. Godkin, and W. W. Thatcher. 1985. Characterization of proteins produced in vitro by periattacment bovine conceptuses. *Biol. Reprod.* 32:681-693.
- Berni R., M. Stoppini, M. C. Zapponi, M. L. Meloni, H. L. Monaco, G. Zanotti. 1990. The bovine plasma retinol-binding protein: amino acid sequence, interaction with transthyretin, crystallization and preliminary X-ray data. *Eur. J. Biochem.* 192:507-513.
- Brinckerhoff, C. E. and E. D. Harris, Jr. 1981. Modulation by retinoic acid and corticosteroids of collagenase production by rabbit synovial fibroblasts treated with phorbol myristate acetate or poly(ethylene glycol). *Biochem. Biophys. Acta.* 677:424-432.
- Chytil F., and D. E. Ong. 1984. Cellular retinoid-binding proteins. In: Sporn MB, Roberts AB, Goodman DS (eds) *The Retinoids*. pp 89-123. Academic Press, New York.
- de The H., A. Marchio A., P. Tiollais, and A. Dejean. 1987 A novel steroid thyroid hormone receptor-related gene inappropriately expressed in human hepatocellular carcinoma. *Nature* 330:667-670.

- Deschamps, J., R. DeLaaf, L. Joosen, F. Meijlink, and O. Destree. 1987. Abundant expression of homeobox genes in mouse embryonal carcinoma cells correlates with chemically induced differentiation. *Proc. natn. Acad. Sci. U.S.A.* 84, 1304-1308.
- Giguere V., E. S. Ong, P. Segui, and R. M. Evans. 1987. Identification of a receptor for the morphogen retinoic acid. *Nature* 330:624-629.
- Godkin J. D, F. W. Bazer, J. Moffatt, F. Sessions, R. M. Roberts. 1982. Purification and properties of a major low molecular weight protein released by the trophoblast of sheep blastocysts at day 13-21. *J. Reprod. Fertil.* 65:141-150.
- Godkin J. D., B. J. Lifsey, and G. A. Baumbach. 1988a. Characterization of protein production by bovine chorionic and allantoic membranes. *Biol. Reprod.* 39:195-204.
- Godkin J. D, B. J. Lifsey, and B. E. Gillespie. 1988b. Characterization of bovine conceptus proteins produced during the peri- and postattachment periods of early pregnancy. *Biol. Reprod.* 38:703-711.
- Greenstein J. S., R. W. Murray, and R. C. Foley. 1958. Observations on the morphogenesis and histochemistry of the bovine preattachment placenta between 16 and 33 days of gestation. *Anat. Rec.* 132:321-341.

- Harney J. P., M. A. Mirando, L. C. Smith, and F. W. Bazer. 1990. Retinol-binding protein: a major secretory product of the pig conceptus. *Biol. Reprod.* 42:523-532.
- Heller, J. 1975. Interactions of plasma retinol-binding protein with its receptor: specific binding of bovine and human retinol-binding protein to pigment epithelium cells from bovine eyes. *J. Biol. Chem.* 250:3613-3619.
- Jetten, A. M. 1980. Retinoids specifically enhance the number of epidermal growth factor receptors. *Nature* 284:626-629.
- Krust A., P. Kastner, M. Petkovich, A. Zelent A, and P. Chambon. 1989. A third human retinoic acid receptor, hRAR-gamma. *Proc. Natl. Acad. Sci.* 86:5310-5314.
- Ludolph, D. C., J. A. Cameron, and D. L. Stocum. 1990. The effect of retinoic acid on positional memory in the dorsoventral axis of regenerating axolotl limbs. *Devol. Biol.* 140:41-52.
- Maden, M. 1982. Vitamin A and pattern formation in the regenerating limb. *Nature* 295:672-675.
- Maden, M. 1983. The effect of vitamin A on limb regeneration in *Rana temporaria*. *Devol Biol.* 98:409-416.
- Maden, M., P. Hunt, U. Eriksson, A. Kuroiwa, R. Krumlauf, and D. Summerbell. 1991. Retinoic acid-binding protein, rhombomeres and the neural crest. *Development* 111:35-44.

- Mavilio, F., A. Simeone, A., E. Boncinelli, and P. W. Andrews. 1988. Activation of four homeobox gene clusters in human embryonal carcinoma cells induced to differentiate by retinoic acid. *Differentiation* 37:73-79.
- Millan, F. A., F. Denhez, P. Kondaiah, and R. J. Akhurst. 1991. Embryonic gene expression patterns of TGF β 1, β 2 and β 3 suggest different developmental functions in vivo. *Development* 111,131-144.
- Mummery C. L, H. Slager, W. Kruijer, A. Feijen, E. Freund, I. Koornneef, A. J. M. Van Den Eijnden-Van Raaij. 1990. Expression of transforming growth factor β ₂ during the differentiation of murine embryonal carcinoma and embryonic stem cells. *Dev. Biol.* 137:161-170.
- Nilsen-Hamilton M. 1990. Growth factor signaling in early mammalian development. In: Hegner S (ed) *Growth Factors in Mammalian Development*. CRC Press, Inc. Boca Raton, Florida, pp 135-166.
- Petkovich M., N. J. Brand, A. Krust, and P. Chambon. 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* 330:444-450.
- Rask, L., H. Anundi, P. A. Peterson, 1979. The primary structure of the human retinol-binding protein. *FEBS Lett* 104:55-58.

- Rask L., and P. A. Peterson. 1976. In vitro uptake of vitamin A from the retinol-binding plasma protein to mucosal epithelial cells from the monkey's small intestine. *J. Biol. Chem.* 251:6360-6366.
- Rees, A. R., E. D. Adamson, and C. F. Graham. 1979. Epidermal growth factor receptors increase during the differentiation of embryonal carcinoma cells. *Nature* 281:309-311.
- Schmid, P., D. Cox, G. Bilbe, R. Maier, and G. K. McMaster, 1991. Differential expression of TGF β 1, β 2 and β 3 genes during mouse embryogenesis. *Development* 111:117-130.
- Simeone, A., A. Acampora, L. Arcioni, P. W. Andrews, E. Boncinelli, and F. Mavilio, 1990. Sequential activation of human HOX2 homeobox genes by retinoic acid in embryonal carcinoma cells. *Nature* 346:763-766.
- Sivaprasadarao A, J. B. C. Findlay. 1988. The interaction of retinol-binding protein with its plasma membrane receptor. *Biochem. J.* 255:561-569.
- Smith J. E, and D. S. Goodman. 1971. The effects of diseases of the liver, thyroid and kidneys on the transport of vitamin A in human plasma. *J. Clin. Invest.* 50:2426-2436.

- Takase S., D. E. Ong, and F. Chytil. 1979. Cellular retinol-binding protein allows specific interaction of retinol with the nucleus in vitro. *Proc. Natl. Acad. Sci.* 76:2204-2208.
- Tickle, C., D. Summerbell, and L. Wolpert, 1975. Positional signalling and specification of digits in chick limb morphogenesis. *Nature* 254:199-202.
- Vasios, G. W., J. D. Gold, M. Petkovich, P. Chambon, and L. J. Gudas. 1989. A retinoic acid-responsive element is present in the 5'-flanking region of the laminin B1 gene. *Proc. natn. Acad. Sci. U.S.A.* 86:9099-9103.
- Wagner, M., C. Thaller, T. Jessell, and G. Eichele. 1990. Polarizing activity and retinoid synthesis in the floor plate of the neural tube. *Nature* 345:891-822.
- Wolf, G. 1984. Multiple functions of vitamin A. *Physiol. Rev.* 64: 873-937.
- Yuspa, S. H., T. Ben, and P. Steinert. 1982. Retinoic acid induces transglutaminase activity but inhibits cornification of cultured epidermal cells. *J. Biol. Chem.* 257:9906-9908.

PART 7: GENERAL CONCLUSIONS

CHAPTER 1

SUMMARY OF PARTS 2 - 5

Vitamin A (retinol) is essential for severe reproductive processes including fetal and placental growth, development and pregnancy maintenance. Retinoids and growth factors may play important developmental roles in embryonic differentiation and morphogenesis in vertebrate. This study demonstrated that retinol-binding protein (RBP) is synthesized and secreted by bovine and ovine placental membranes. In addition, RBP is a product of the uterine endometrium of cattle and sheep (Liu and Godkin unpublished data). These data illustrate clearly that an important function of the fetal-placental-maternal unit is transport, storage and metabolism of retinol in large domestic animals.

Importantly, RBP, TGF α and TGF β have been immunolocalized in ovine and bovine trophoctoderm and uterine epithelium (this study; Dore, Gao, Wilkinson and Godkin, unpublished observations). These data may indicate a functional link between retinoids and growth factors in ruminant placental, embryonic and uterine development.

VITA

Kaung-Huei (Arthur) Liu was born in Taiwan on November 27, 1958. He is the son of Mr. and Mrs. Chen C. Liu, the eldest of three children. He graduated in 1981 from the National Pingtung Institute of Agriculture in the field of Veterinary Medicine. Upon graduation from college, he spent two years in the Chinese Army as a second lieutenant. He then took a job working at a safari park as a veterinarian for one and a half years. On July 16, 1986, Mr. Liu and Miss Rou C. (Shirley) Lee were married. He received a Master of Science degree in Animal Science from Angelo State University in December, 1986.

He was admitted to the Graduate School of The University of Tennessee, Knoxville in September, 1987 on a research assistantship. In August, 1991, he was awarded a Doctor of Philosophy degree with a major in Animal Science.

11/12