Int. J. Dev. Biol. 35: 485-489 (1991)

Short Contribution

Immunohistochemical localization of bovine placental retinol-binding protein

KUIXIONG GAO, KAUNG H. LIU and JAMES D. GODKIN*

Department of Animal Science, The University of Tennessee, Knoxville, USA

ABSTRACT An immunogold staining method was used in combination with epipolarization microscopic detection to demonstrate the presence of bovine placental retinol-binding protein in bovine extraembryonic membranes. Amnion, chorion and allantois were fixed in Bouin fixation fluid and embedded in polyethylene glycol 1500. Sections (5 mm) were cut and transferred onto Digene silanated slides and immunostained using rabbit antiserum raised against bovine placental retinol-binding protein followed by goat anti-rabbit IgG labeled with 1 nm gold. Gold particles after silver enhancement were viewed and photographed under epipolarization microscopy. Epithelial cells of all three membranes (*i.e.* amniotic ectoderm, chorionic trophectoderm, and allantoic endoderm) were immunoreactive, while mesodermal cells, collagen, and blood 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 lining 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.

KEY WORDS: *retinol-binding protein, PEG, placenta and immunohistochemistry*

It has been recognized for over half a century that vitamin A (retinol) is essential for normal fetal growth and development (Evans, 1928; Wolbach and Howe, 1933; Thompson *et al.*, 1964; O'Toole *et al.*, 1974) as well as for the maintenance of pregnancy (Moore, 1957; Howell *et al.*, 1963). However, until recently the mechanism of action caused by vitamin A in these processes has been largely unknown. Retinoids, a family of vitamin A metabolites and analogs (Lotan, 1980; Sporn and Roberts, 1984; Dawson *et al.*, 1985) were known to modulate differentiation and growth of several cell types (Sherman, 1986; Elias, 1987; Harper, 1988; Keeble and Maden, 1989) and influence production of many cell surface molecules, extracellular matrix and cytoskeleton components (De Luca, 1977; Strickland *et al.*, 1980; Fuchs and Green, 1981; Jetten, 1981; Shapiro and Mott, 1981; Yuspa *et al.*, 1982). Retinoids also influence expression of some growth factors (Jetten, 1981; Rizzino and Bowen-Pone, 1985) and their receptors (Rees *et al.*, 1979; Mercola *et al.*, 1990; Mummery *et al.*, 1990). Retinoic acid, a natural metabolite of retinol, has been identified as a morphogen in induction of pattern formation in the chick limb bud (Tickle, *et al.*, 1975; Maden, 1982; Eichele *et al.*, 1985). Retinol is transported in plasma complexed to retinol-binding protein (RBP). RBP is the only known plasma transport protein that delivers retinol from its source (mainly liver) to target cells (Smith and Goodman, 1971). 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 (Giguere *et al.*, 1987; Petrovich *et al.*, 1987).

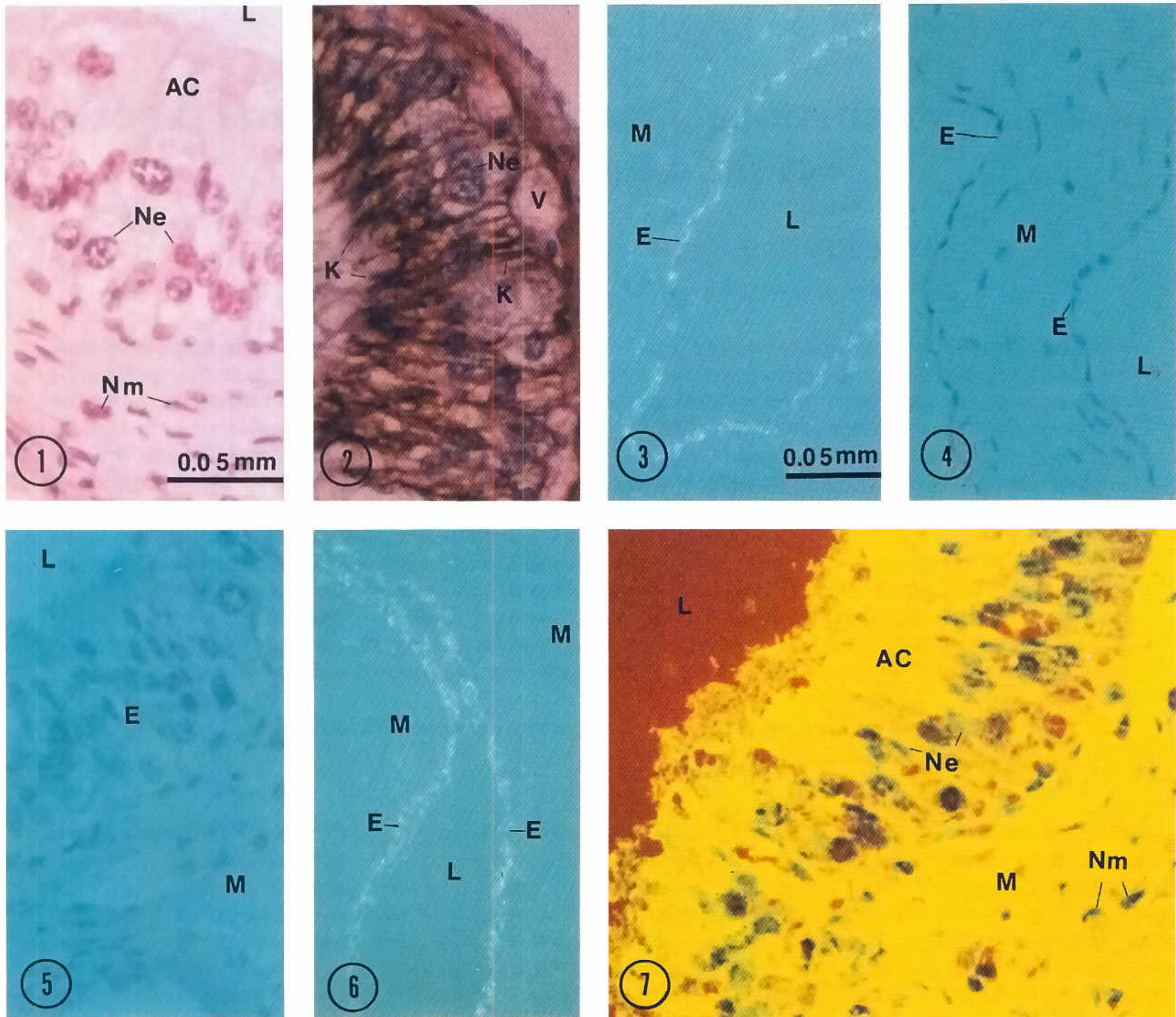
Recently, we have demonstrated the synthesis and secretion of RBP by bovine extraembryonic membranes (Liu *et al.*, 1990). The protein has been identified as a major low molecular weight acidic protein (LMWAP) synthesized by amnion, chorion and allantois in organ culture and a major component of amniotic and allantoic sac fluids (Liu *et al.*, 1990). It is not glycosylated and composed of three isotypes (Godkin *et al.*, 1988). Sequence analysis of the first 43 NH_2-terminal amino acids of the protein demonstrated that they were identical to those of bovine plasma RBP (Berni *et al.*, 1990.) and >90% homologous with human and rabbit plasma RBP (Liu *et al.*, 1990). The objective of the present study was to identify the cellular source of this protein, which we refer to as bovine placental RBP, or, bpRBP (Liu *et al.*, 1990), in amnion, chorion, and allantois.

Polyethylene glycol 1500 (PEG) was used as a water soluble embedding medium. PEG embedded tissue sections were transferred onto silanated slides for immunohistochemistry (Gao and

Abbreviations used in this paper: RBP, retinol-binding protein; bpRBP, bovine placental retinol-binding protein; LMWAP, low molecular weight acidic protein; PBS, phosphate buffered saline; PEG, polyethylene glycol; BSA, bovine serum albumin; NGS, normal goat serum; H&E, hematoxylin and eosin; TM, transmission (light or illumination); EP, epipolarization (illumination); IGSS, immunogold silver stain (enhancement).

***Address for reprints**: 201 McCord Hall, Animal Science, University of Tennessee, Knoxville, TN 37901, USA. FAX: (615) 974-7448

0214-6282/91/$03.00
© UBC Press
Printed in Spain



Figs. 1-7. Histochemical and immunohistochemical staining of bovine extraembryonic membranes. (1) *Bovine chorionic membrane. Tissue was fixed in Bouin's fixative solution and embedded in PEG 1500. 5μm section was stained with hematoxylin and eosin (H&E). AC, apical cytosol; Ne, nuclei of epithelial cells; Nm, nucleus of mesenchymal cells; L, lumen (x330, Bar 0.05 mm).* **(2)** *Keratin immunostain in chorionic membrane. Rabbit anti-keratin 1:1000, goat anti-rabbit IgG labeled with 1 nm colloidal gold (IgG-Au) 1:30, silver enhancement (IGSS) and counterstained with H&E, observed with transmission light (TM), x330. K, keratin filament; Ne, nucleus of epithelial cell; v, vacuol. Transmission light (TM) x330.* **(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; L, lumen. x264. Bar 0.05 mm.* **(4)** *Allantois treated with pre-immune serum (1:300 as negative control for Fig. 3). IgG-Au, 1:30; IGSS. Photograph was taken with TM and EP. x264.* **(5).** *Chorionic membrane. Immunostained with pre-immune rabbit serum (1:100) followed by IgG-Au (1:30), H&E (as a negative control for Fig. 8). E, epithelium; L, lumen; M, mesenchyme; TM plus EP. x264.* **(6)** *Immunostain of bpRBP antigenic sites in bovine amniotic membrane. Anti bpRBP 1:800, IgG-Au 1:100, IGSS and observed with EP. E, epithelium; L, lumen; M, mesenchyme. x264.* **(7)** *Nonspecific fluorescence in chorionic epithelium. Photographed with FITC filter. Comparison of Figs. 7 and 8 demonstrates the specific immunostaining detected only with the BH_2-DM BIGS filter (Fig. 8)*

Godkin, 1991). The method provided excellent morphological preservation of cell structure as well as good sensitivity for antigen detection by immunostaining (Figs. 1-8).

Chorion consisted of an apical monolayer of cells that appeared as a pseudostratified epithelium due to nuclei arranged at different distances from the basement substratum (Fig. 1). Fig. 1 is a micrograph of normal bovine chorionic membrane stained with hematoxylin and eosin (H&E). Nuclei of chorionic epithelial cells (Ne) are larger and more spherical than nuclei of underlying mesenchymal cells (Nm). Fig. 2 illustrates the pattern of keratin immunostaining in chorionic membrane with H&E counterstaining. The brown reaction product localized cytokeratin primarily at the apical and basolateral compartments. Rod-like branches of vertical 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

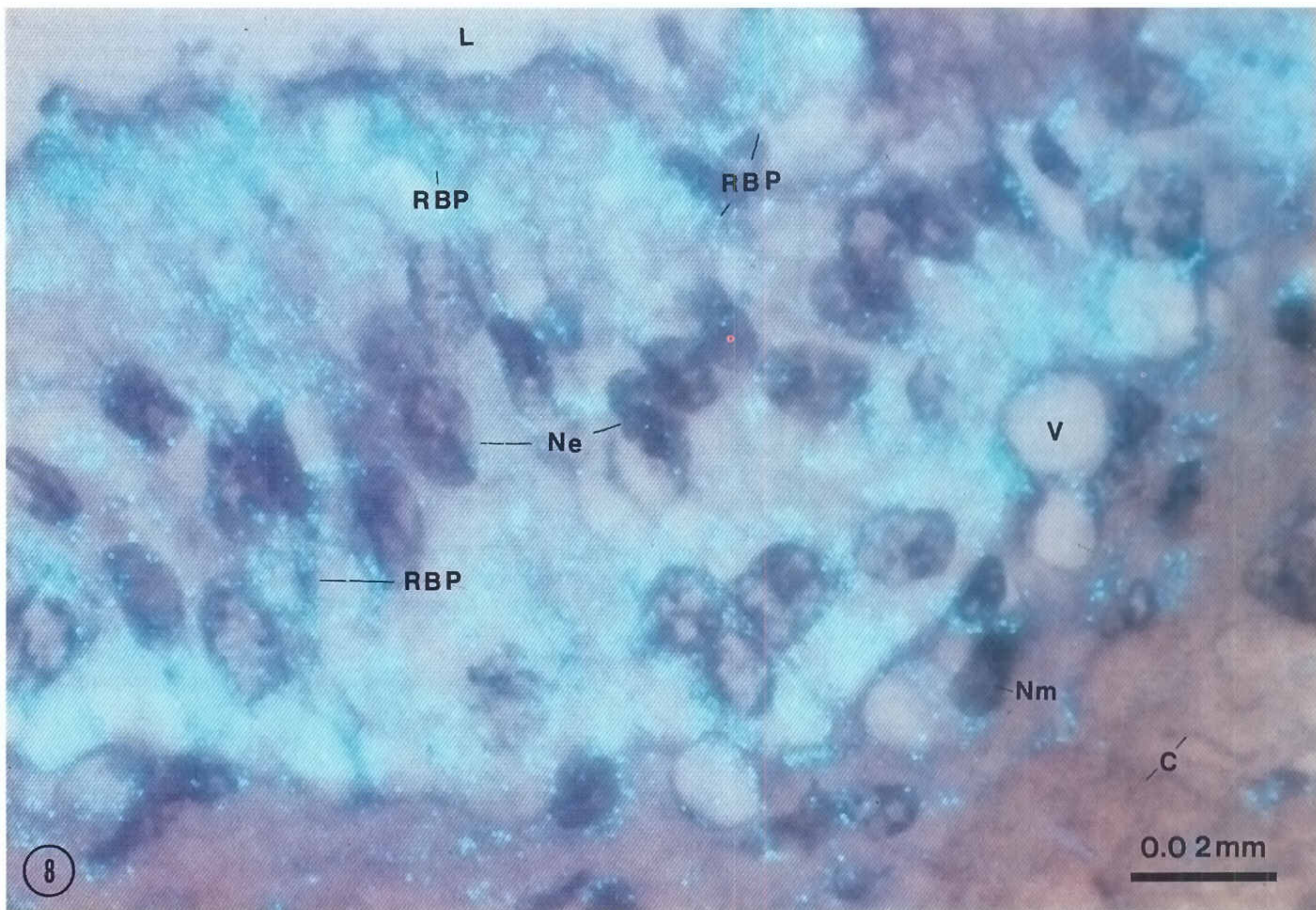


Fig. 8. Localization of bpRBP in bovine chorionic membrane. *Anti-bpRBP 1:100, IgG-Au 1:30, IGSS, H&E. TM plus EP illumination. RBP, antigenic sites of bpRBP; Ne, nuclei of epithelial cells; Nm, nuclei of mesodermal cells; C, collagen fibers; V, vacuoles. x900.*

cells and diffused basolaterally (Fig. 8). Nuclei and vacuoles were not stained. No immunostaining was observed when pre-immune rabbit serum was applied in place of the anti-bpRBP (Fig. 5). Fig. 7 is a photograph of chorionic epithelium after both bpRBP immunostain and H&E counterstain but without epipolarization illumination and observed with an FITC filter to show non-specific fluorescence in tissue. Comparison of Figs. 7 and 8 demonstrates the specific immunostaining (immunogold enhanced by silver stain) detected with the BH_2-DM BIGS filter (Fig. 8).

Immunolocalization of bpRBP antigenic sites in allantois is illustrated in Fig. 3. The entire epithelial lining of this membrane was intensely stained with the exception of nuclei. Some diffuse staining was observed in the underlying mesoderm. For comparison, Fig. 4 shows allantoic membrane treated with pre-immune rabbit serum (1:100) in place of anti-bpRBP serum followed with IgG-Au (1:30) and IGSS and viewed with TM and EP (x264). No immunostaining was apparent.

The presence of bpRBP in amnion is illustrated in Fig. 6. Similar to the observation in allantois, localization of the bpRBP in amnion was restricted predominantly to the epithelial lining of this membrane. Control sections (not shown) were totally negative for immunostaining.

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 (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 observations) suggest that the placenta may play an important role in regulating vitamin A storage, transport and metabolism during pregnancy.

Results from the present study identified and localized bpRBP in the epithelial cells lining the amnion, allantois and chorion. The bpRBP was sometimes more concentrated in the apical regions of these cells. Based on these findings and our previous demonstration (Liu *et al.*, 1990; Gao and Godkin, 1991) that these placental membranes synthesize and release the protein, we suggest that bpRBP is synthesized and secreted by epithelia of the chorion, allantois, and amnion.

The RBP producing chorionic epithelium is the trophectoderm 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 trophectoderm 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 (trophectoderm) 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 hind gut 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.

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

Experimental Procedures

Production of anti-bpRBP serum

Purification of bpRBP, production of rabbit-anti-bpRBP and characterization of the antiserum were as previously described by Liu *et al.* (1990).

Tissue sample preparation

Bovine extraembryonic membranes (chorion, allantois, and amnion) from a 35 cm fetus (135 days of pregnancy) 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 mm) 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 keratin wide spectrum screening. (Accurate Chemical and Scientific Corp. Westbury, NY) 1:1000.
(d) Sections were then washed with PBS, incubated in diluted (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 mixture of IntenSE^{tm} (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-Premer 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 BH_2-DM BIGS filter set 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 TM or a combination of EP and TM 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 TM light path and combined with 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.

Acknowledgments
This work was supported with USDA grant 88-3742-4012.

References

BERNI, R., STOPPINI, M., ZAPPONI, M.C., MELONI, M.L., MONACO, M.L., MONACO, H.L. 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.

CHYTIL, F. and ONG, D.E. (1984). Cellular retinoid-binding proteins. In T*he Retinoids* (Eds. M.D. Sporn, D.S., Roberts, A.B. and Goodman, D.S.). Academic Press, New York, pp. 89-123.

DAWSON, M.I., HOBBS, P.D., CHAN, R., DERDZINSKI, K., HELMES, C.T., CHAO, W., MEICHENRY, E. and SCHIFF, L.J. (1985). Conformational restrictions of the retinoid skeleton. In *Retinoids, Differentiation and Disease.* Ciba Foundation Symposium 113. Pitman Publishing Ltd, London, pp. 6-55.

De LUCA, L.M. (1977). The direct involvement of vitamin A in glycosyl transfer reactions of mammalian membranes. *Vit. Horm. 35:* 1-57.

EICHELE, G. TICKLE, C. and ALBERTS, B.M. (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. (1928). The effects of inadequate vitamin A on sexual physiology of the female. *J. Biol. Chem. 77:* 651-654.

FUCHS, E. and GREEN, H. (1981). Regulation of terminal differentiation of cultured human keratinocytes by vitamin A. *Cell 25:* 617-625.

GAO, K.X. and GODKIN, J.D. (1991). A new method for transfer of polyethylene glycol embedded tissue sections to silanated slides for immunocytochemistry. *J. Histochem. Cytochem. 34:* 517-520.

GAO, K.X. and PENG, B. (1987). VisioBond as an embedding medium for light and electron microscopy. *J. Cell Biol. 105 (Suppl. 4):* 226a.

GIGUERE, V., ONG, E.S., SEGUI, P. and EVANS, R.M. (1987). Identification of a receptor for the morphogen retinoic acid. *Nature 330:* 624-629.

GODKIN, J.D., LIFSEY, B.J. and BAUMBACH, G.A. (1988). Characterization of protein production by bovine chorionic and allantoic membranes. *Biol. Reprod. 39:* 195-204.

HARNEY, J.P., MIRANDO, M.A., SMITH, L.C. and BAZER, F.W. (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. *Exp. Cell Res. 178 (2):* 254-263.

HOWELL, J.M., THOMPSON, J.N. and PIT, G.A.J. (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 on cell growth and the binding of epidermal growth factor. *Ann. NY Acad. Sci. 359:* 200-216.

KEEBLE, S. and 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., BAUMBACH, G.A., GILLEVET, P.M. and GODKIN, J.D. (1990). Purification and characterization of bovine placental retinol-binding protein. *Endocrinology 127:* 2696-2704.

LIU, K.H., BREWTON, R.G., BAUMBACH, G.A. and GODKIN, J.D. (1991). Characterization of protein production by ovine placental membranes: identification of a placental retinol-binding protein. *Endocrinology 129:* 126-132.

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.

MERCOLA, M., WANG, C., KELLY, J., BROWNLEE, C., JACKSON, GRUSBY, L., STILES, C. and BOWEN-POPE, D. (1990). Selective expression of PDGF A and its receptor during early mouse embryogenesis. *Dev. Biol. 138:* 114-122.

MOORE, T. (Ed.) (1957). *Vitamin A.* Elsevier, Co. New York. pp. 237-262.

MUMMERY, C.L., SLAGER, H., KRUIGER, W. FEIGEN, A., FREUND, E., KOORNNEEF, I., and VANDEN EIJNDEN-VAN RAAIG, A.J.M. (1990). Expression of transforming growth factor B_2 during the differentiation of murine embryo carcinoma and embryonic stem cells. *Dev. Biol. 138:* 114-122.

O'TOOLE, B.A., FRADKIN, R., WARKANY, J. WILSON, J.G. and MANN, G.V. (1974). Vitamin A deficiency and reproduction in rhesus monkeys. *J. Nutr. 104:* 1513-1524.

PETROVICH, M., BRAND, N.J., KRUST, A. and CHAMBON, P. (1987). A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature 330:* 444-450.

REES, A.R., ADAMSON, E.D. and GRAHAM, C.F. (1979). Epidermal growth factor receptors increase during the differentiation of embryonal carinoma cells. *Nature 281:* 309-311.

RIZZINO, A. and BOWEN-PONE, D.F. (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 MOTT, D.J. (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). CRC Press, Inc. Boca Raton, Florida, pp 161-186.

SOPRANO, D.R., WYATT, M.L., DIXON, J.L., SOPRANO, K.L. and GOODMAN, D.S. (1986). Retinol binding protein synthesis and secretion by the rat visceral yolksac. *J. Biol. Chem. 263:* 2934-2938.

SMITH, J.E. and GOODMAN, D.D. (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.

SPORN, M.B. and ROBERTS, A.B. (1984). Biological methods of analysis and assay of retinoids. In *The Retinoids* Vol. 1 (Eds. M.B. Sporn, A.B. Roberts and D.S. Goodman). Academic Press, Orlando, pp. 235- 279.

STEVENS, D.H. (Ed.) (1975). *Comparative Placentation: Essays in Structure and Function.* Academic Press, London, pp. 1-99.

STRICKLAND, S., SMITH, K.K. and MAROTTI, K.R. (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., SUMMERBELL, D. and WOLPERT, L. (1975). Positional signalling and specification of digits in chick limb morphogenesis. *Nature 254:* 199-202.

THOMPSON, S.Y., HOWELL, J.M. and PITT, G.A.J. (1964). Vitamin A and reproduction in rats. *Proc. R. Soc. Lond. 159:* 510-535.

WOLBACH, S.B. and HOWE, P.R. (1933). Tissue changes following deprivation of fat soluble vitamin A. *J. Exp. Med. 57:* 689-722.

YUSPA, S.H., BEN, T. and STEINERT, P. (1982). Retinoic acid induces transglutaminase activity but inhibits cornification of cultured epidermal cells. *J. Biol. Chem. 257:* 9906-9908.

Accepted for publication: September 1991