University of Alberta

Galectin expression and effect of galectin hapten inhibitors on epithelial cell phenotype in the embryonic chick mesonephros.

by

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This thesis is dedicated to .....

.... John Murphy, my Dad, who would have been proud.

.... Marilyn Murphy, whose patience, understanding and support gave me the strength to finish this thing. Thanks Mom! You're the greatest.

ABSTRACT

Galectins are galactoside-binding animal lectins. In vertebrates, galectin proteins have been implicated in cell adhesion to polylactosamine-bearing glycoproteins of the extracellular matrix, such as laminin. Using immunofluorescence, Western blotting, and *in situ* hybridization I have described the localization of the chick 14 kDa and 16 kDa galectins during embryonic development of the mesonephric/gonadal complex. In the mesonephros, a temporary organ of excretion, the 16 kDa galectin (C16) is abundant in nephron basement membranes (BMs). C16 in BMs colocalizes with two potential glycoprotein ligands, laminin and fibronectin. To test the hypothesis that galectin-mediated laminin-epithelial cell interactions play a role in the maintenance of epithelial differentiation, an *in vitro* system for the culture of chick mesonephroi was developed. When compared with control saccharide-free or maltose-treated cultures, mesonephroi cultured with the galectin hapten inhibitors thiodigalactoside and lactose displayed several defects in the organization of epithelial tissue. These included a distorted tubule cell shape, pseudostratification of tubule epithelia, and an apparent disruption of glomerular podocyte adhesion to the GBM. Tubules expressing the epithelial-specific adhesion molecule E-cadherin also re-express vimentin, an intermediate filament typically restricted to mesenchymal cells. Also in hapten-treated cultures, the Na^+/K^-ATPase, a renal epithelial protein normally expressed at high levels only in the distal tubule, is expressed at low levels in most tubules. Re-expression of the mesenchymal protein vimentin by epithelial cells could indicate a reversion to the mesenchymal phenotype. Results suggest that galectin-BM glycoprotein interactions are important for the maintenance of the renal epithelial phenotype.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ATPase - adenosine triphosphatase
BM - basement membrane
BSA - bovine serum albumin
C14 - chick 14 kDa galectin
C16 - chick 16 kDa galectin
CRD - carbohydrate recognition domain
DAPI - diamidino-2-phenylindole
ECM - extracellular matrix
ER -endoplasmic reticulum
FN - fibronectin
Fuc - fucose
Gal - galactose
GalNAc - N-acetylgalactosamine
GBM - glomerular basement membrane
Glc - glucose
GlcNAc - N-acetylglucosamine
hnRNP - heterogenous ribonucleoprotein
IgG - immunoglobulin G
LAMP - lysosome-associated membrane protein
mAb - monoclonal antibody
Mac-2-BP - Mac-2 binding protein
MDCK - Madin-Darby canine kidney (cell line)
MEPBS - PBS with 4 mM β-mercaptoethanol
NCAM - neural cell adhesion molecule
Neu - neuraminic (sialic) acid
pAb - polyclonal antibody
PBS - phosphate buffered saline
PG - proteoglycan
RNA - ribonucleic acid
SDS-PAGE - sodium dodecylsulfate polyacrylamide gel electophoresis
TBS - Tris-buffered saline
TPBS - PBS with 0.1% Tween-20

INTRODUCTION

Embryonic development involves not only proliferation and differentiation of cells, but also cell-cell and cell-substratum adhesive interactions. Modulation of cell adhesion is crucial for such diverse processes as the segregation of different tissues into organs and the subsequent development of tissue architecture, axon pathfinding during development of the nervous system, development and activity of the immune system, and many others. In addition, binding of a cell-surface receptor to ligands on neighboring cells, or to non-cellular material such as that in the extracellular matrix, can influence cell proliferation, differentiation, and death. Thus, the cell-surface and extracellular matrix proteins that interact to mediate these effects have been, and continue to be, the focus of much study. Many of these molecules and their binding partners are glycosylated, and recently attention has been focused on the information, crucial to normal development and function, that can be encoded in glycoconjugate structures. Lectins are proteins that bind specifically to carbohydrate residues, and thus can interpret such "glycocodes" (Kasai & Hirabayashi, 1996). Galectins are a family of animal lectins that bind only β-galactose-containing carbohydrate structures; their biological role in nephrogenic differentiation in the mesonephros is the subject of this study. Because a lot of my work touches on the extracellular matrix, before I speak specifically about galectins I will give a general overview of the extracellular matrix.

EXTRACELLULAR MATRIX

Animal and plant tissues have two components: cells and their secreted non-cellular material; the latter is called extracellular matrix (ECM). ECM is a scaffolding which supports cells and provides a substrate for cell adhesion, thus maintaining tissue structure and function (MacDonald, 1988). In addition, the ECM plays several important roles during cell differentiation and organogenesis (MacDonald, 1988; Von der Mark, et al. , 1992).

Components of the extracellular matrix

Extracellular matrices consist chiefly of collagens, proteoglycans, and glycoproteins (Birk & Linsenmayer, 1994; Mosher, et al. , 1992). Collagens are the major component of many extracellular matrices. They may be either fibril-forming (interstitial; types I, II, III, etc.) or network-forming (basement membrane, among these, type IV) (Birk & Linsenmayer, 1994; Yurchenco, 1994). Both fibrillar and network-forming collagens self-assemble once they are secreted into the ECM (Kuhn, 1995). Collagen proteins have domains that bind other collagen molecules for self-assembly, as well as domains that interact with cell surface receptors and other ECM components (Kuhn, 1995; Veis & George, 1994). Thus collagens stabilize the ECM and facilitate adhesion of cells to the matrix.

Proteoglycans (PGs) are proteins with attached sulfated polysaccharide (glycosaminoglycan) moieties (Ruoslahti, 1988). In the

extracellular matrix, PGs perform several functions. They are typically highly hydrated and thus maintain a fluid environment in the ECM. PGs also interact with other ECM constituents and contribute to the tensile strength of a matrix. The charge-specific filtering activity of certain extracellular matrices, such as the glomerular basement membrane, is partly due to the charge density of PGs (Ruoslahti, 1988; Weber, 1992). Finally, PGs have binding sites for soluble growth factors and can maintain high local concentrations of those factors, mediating tissue growth and differentiation (Vogel, 1994; Ruoslahti, 1988). One of the most abundant PGs in extracellular matrices is the heparan sulfate proteoglycan perlecan (Yurchenco & O'Rear, 1994; Iozzo, 1994).

Glycoproteins are proteins with at least one covalently attached carbohydrate moiety (Blackshaw, et al. , 1995). Several different glycoproteins have been isolated from ECM including fibronectin, laminin, and nidogen. Fibronectin (FN) is a ubiquitous extracellular glycoprotein that forms fibrils in the ECM (Potts & Campbell, 1994). Unlike the interstitial collagen fibrils, fibronectin fibrils do not self-assemble; an interaction with specific cell-surface FN receptors is required to generate fibrils (MacDonald, 1988; Peters & Mosher, 1994). In addition to the domain that mediates binding to cell-surface FN-receptors, FN has binding sites for collagen, proteoglycans, and other matrix glycoproteins (Potts & Campbell, 1994; Peters & Mosher, 1994). The binding of FN to several constituents of the matrix, as well as to the cell surface, helps

stabilize the matrix around the cell. The laminins self-assemble into a stable network which is the major component of specialized ECM structures called basement membranes (Timpl, 1996; Timpl & Brown, 1994; Yurchenco, 1994). In addition to the homotypic binding to other laminin molecules for network formation, laminin binds heterotypically to perlecan, other ECM glycoproteins, and cell-surface receptors (Timpl & Brown, 1994). One of the glycoproteins bound by laminin is nidogen. Nidogen binds very tightly to laminin and less tightly to collagen type IV (Mayer & Timpl, 1994). Thus, independent networks formed by self-assembly of laminin and collagen IV can be crosslinked by nidogen, further stabilizing the basement membrane (Timpl, 1996; Mosher, et al. , 1992). Nidogen has also been shown to bind FN and perlecan.

Cellular receptors for the extracellular matrix

Most interactions between the cell surface and the ECM components collagen, FN and laminin are mediated by cell-surface receptors called integrins (Haas & Plow, 1994; Dedhar & Hannigan, 1996). Integrins are heterodimeric integral membrane proteins composed of an α and a β subunit. Different combinations of α and β subunits have different ligand specificities. For example, $\alpha_2\beta_1$ integrin binds collagen, laminin and FN. $\alpha_4\beta_7$ integrin binds only FN, while $\alpha_6\beta_1$ integrin binds only laminin (Haas & Plow, 1994). Binding of the appropriate ECM component to its integrin receptor activates intracellular signaling pathways which result in a variety of cellular responses (Dedhar & Hannigan, 1996; Von der Mark, et

al. , 1992). In addition to the integrin-family of ECM receptors, there are a number of less well characterized non-integrin receptors (Timpl & Brown, 1994). One such receptor, α-dystroglycan, links laminin and the proteoglycan agrin in the ECM to the actin cytoskeletal network inside the cell (Henry & Campbell, 1996). Given the complex and dynamic interactions between the ECM and cells within a tissue, it is not surprising that the physical characteristics and molecular constituents of ECM are often tissue specific.

Mesenchymal/embryonic matrices vs. basement membranes

Cells of mesenchymal and relatively undifferentiated embryonic tissues deposit ECM uniformly around the cell surface. In such tissues, cells are embedded in a randomly organized ECM (Ekblom, 1981b). Fibrillar components of this type of matrix include interstitial collagens and/or FN (MacDonald, 1988; Veis & George, 1994). FN, in particular, is a characteristic constituent of ECMs where tissue remodeling and reorganization is taking place, for example, during wound healing or organogenesis (MacDonald, 1988; Potts & Campbell, 1994). Development of mesenchymal cells into epithelial tissues such as the renal tubule, mammary gland, or skeletal muscle is accompanied by the disappearance of "interstitial" ECM and the formation of a basement membrane (Ekblom, 1981; Abrahamson & Leardkamolkarn, 1991).

Cells in epithelial tissues have functionally specialized domains of the cell surface and cytoplasm; they are polarized. ECM material is not

secreted randomly around the cell but is restricted to the basal surface, and consists of a continuous sheet of organized matrix material called a basement membrane. Basement membranes (BMs) always contain laminin, and often contain the network-forming collagen type IV. Collagen IV and laminin networks in BMs are cross-linked by nidogen. The heparan sulfate proteoglycan perlecan is also characteristic of BMs (Timpl, 1996; Yurchenco & O'Rear, 1994; Weber, 1992). In addition to providing physical support for epithelial cells, BMs provide cues for the maintenance of cell polarity (Eaton & Simons, 1995); they also form selectively permeable barriers which separate epithelial tissues from surrounding tissues (Yurchenco & O'Rear, 1994).

GALECTINS

In the last few decades, researchers have come to realize that biological information is not solely encoded in nucleic acid and amino acid sequences, but also in the structure and sequence of carbohydrate residues linked to proteins or lipids as glycoconjugates. The majority of these glycoconjugates are present as glycolipids or glycoproteins at the cell surface, or in the extracellular milieu as glycosylated proteins of the extracellular matrix. The extracellular location of glycoconjugates has suggested to researchers that these carbohydrate structures play a role in cell adhesion and cell signaling, providing machinery exists to decipher

these "glycocodes" (Sharon & Lis, 1989; Kasai & Hirabayashi, 1996). In fact, such machinery does exist, in the form of proteins called lectins.

Lectins recognize and bind specific carbohydrate residues. They were first isolated, in the late 1880's, as hemagglutinating factors from plant seeds (Sharon & Lis, 1987; Barondes, 1997). It was subsequently established that the hemagglutinating factors from different seeds were proteinaceous and that their activity could be selectively blocked by various simple sugars. The finding that some plant lectins were blood group-specific led to the concept that differing cell surface carbohydrates form the chemical basis for blood group specificity (Sharon & Lis, 1987).

Although plant lectins stimulate cell division in some animal cells, their effects, although interesting, are not directly relevant to animal cell function since plant lectins are exogenous factors (Sharon & Lis, 1987). In the early 1970's, the first animal lectins were discovered (Sharon & Lis, 1987; Barondes, 1997). Study of animal lectins, some of which do not have agglutinating activity, has led to acceptance of a broader definition of lectins: carbohydrate-binding proteins, other than antibodies, which do not have enzymatic activity (Barondes, 1988).

Five major families of animal lectins have been identified based on the structural and functional characteristics of their carbohydrate-recognition domains (CRDs) (Drickamer, 1988; Drickamer & Taylor, 1993; Hirabayashi, 1993; Gabius, 1997). Essential characteristics of each lectin

Table 1. Characteristics of the five major families of animal lectins.

Lectin Family	Identifying Feature	Carbohydrate Binding	Localization	Examples
C-type [1]	conserved CRD	variable; cation dependent	extracellular and integral membrane proteins	selectins asialoglycoprotein receptor
I-type [2]	immunoglobulin-like CRD	variable; cation independent	integral membrane proteins	sialoadhesin N-CAM
P-type [3]	conserved CRD	mannose-6-phosphate; cation dependent or independent	integral membrane proteins	mannose-6-phosphate receptors
pentraxins [3]	pentameric subunit arrangement	variable; cation dependent	extracellular (serum)	serum amyloid P C-reactive protein
galectins [3]	conserved CRD	β–galactosides; cation independent	extracellular/ intracellular	Galectin-1 Galectin 3 (CBP-35/Mac-2)

[1] From Hirabayashi, 1993; [2] From Powell & Varki, 1995; [3] From Gabius, 1997

family have been summarized in Table 1. **C-type lectins** require calcium for carbohydrate binding, and are found in serum, extracellular matrix, and cell membranes. Although each C-type lectin recognizes a specific carbohydrate residue, as a family of proteins these lectins bind to a diversity of carbohydrates and have been shown to have many functions. **I-type lectins** were recently identified as carbohydrate-binding members of the immunoglobulin superfamily (Powell & Varki, 1995). The immunoglobulin-like domains of I-type lectins form the CRD, which binds various carbohydrate residues in the absence of a divalent cation. I-type lectins are integral membrane proteins, preferentially expressed on

the plasma membrane, and their immunoglobulin-like CRD is extracellular. Another group of animal lectins, **P-type lectins**, are also integral membrane proteins. In contrast to the I-type lectins, however, P-type lectins are expressed in intracellular membranes such as the Golgi apparatus, the endoplasmic reticulum, and lysosomes (Gabius, 1997). P-type lectins have a conserved CRD which binds to mannose-6-phosphate moieties (Kishore, et. al, 1997). Some P-type lectins are cation independent, while others require a ligand-associated calcium ion for binding. A fourth lectin group, the **pentraxins**, are found in extracellular locations, especially in the serum (Gabius, 1997). The pentraxin CRD has not been well characterized but is known to bind various carbohydrate ligands in a cation-dependent fashion. The identifying feature of this lectin family is the formation of pentameric, disk-shaped multimers. Finally, **galectins** are lectins which do not require calcium ions for activity and recognize only β-galactoside-containing carbohydrate residues (Gabius, 1997; Leffler, 1997). Galectins are found both extra- and intracellularily and have been isolated from a wide range of organisms (reviewed by Cho & Cummings, 1997; Hirabayashi, et al. , 1997; Gitt, et al. , 1997; Magnaldo & Michel, 1997; Hadari, et al. , 1997; Müller, et al. , 1997; Vasta, et al. , 1997). This group of animal lectins is discussed in more detail.

Soluble galactoside-binding animal lectins: galectins

Galectins were first isolated and characterized as agglutinins whose activity could be inhibited by lactose, thiodigalactoside, and galactose

(Kobiler & Barondes, 1977; Levi & Teichberg, 1981). They were named lectins because of their sugar binding capacity. Unlike some other known animal lectins, the lactose-binding lectin was soluble, that is, could be extracted in an aqueous buffer containing lactose. Furthermore, agglutinating activity did not require the presence of calcium (Den & Malinzak, 1977; Leffler, 1997). Lactose- and galactose-binding lectins were subsequently isolated from several organisms and tissues and were found to have significant amino-acid sequence similarities (Figure 1) (Paroutaud, et al. , 1987). X-ray crystallography studies of the lactose-galectin and the N-acetyllactosamine-galectin complexes have shown that the side chains of certain highly conserved amino acids form hydrogen bonds with hydroxyl (-OH) groups in these sugars (Lobsanov, et al. , 1993; Liao, et al. , 1994). The tightest binding of the galectin was to the β-galactose residue, but hydrogen bonding with the glucose or N-acetylglucosamine moieties of lactose and N-acetyllactosamine played a role in complex formation. Thus, the affinity for β-galactoside-containing saccharides displayed by members of the galectin family is a result of both their primary structure i.e., amino acid sequence, and tertiary structure i.e., protein folding (Lobsanov & Rini, 1997). Proper orientation of amino acid side chains in the folded molecule is so important for carbohydrate binding activity that mutations in almost any section of the sequence, even in those amino acids not directly involved in ligand binding, can eliminate galectin activity (Abbott & Feizi, 1991).

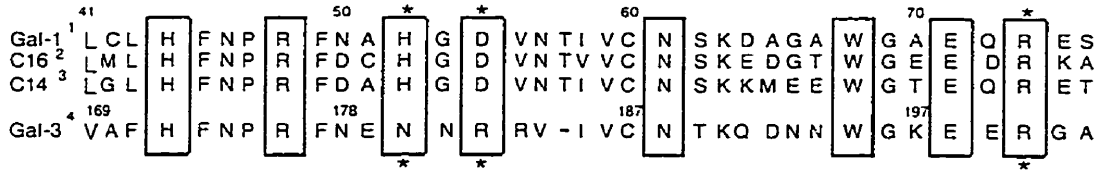


Figure 1. Sequence alignment of critical carbohydrate binding residues from bovine Galectin-1 (Gal-1), the chick 16- and 14 kDa galectins (C16 and C14 respectively), and murine Galectin-3 (Gal-3). Galectin residues forming hydrogen bonds with ligand hydroxyl groups, which are invariant in Type I CRDs (Gal-1, C-16, & C-14), are boxed. Residues which are variable in Type II galectin CRDs (Gal-3) are boxed and marked with an asterisk (*). [1] Abbott, et al. , 1989; [2] Sakakura, et al. , 1990; [3] Hirabayashi, et al. , 1987; [4] Jia & Wang, 1988.

Galectins can be grouped according to binding specificities and structure

Galectins have been divided into two broad groups based on fine specificity of carbohydrate binding, and this division has been correlated with conservation of specific residues in the galectin CRD (Ahmed & Vasta, 1994). Type I galectins have conserved CRDs in which His^{44}, Arg^{48}, His^{52}, Asp^{54}, Asn^{61}, Trp^{68}, Glu^{71}, and Arg^{73} are invariant (Figure 1), and they show more affinity for N-acetyllactosamine, and poly-N-acetyllactosamine, than for lactose (Table 2) (Ahmed & Vasta, 1994). Type II galectins have variable CRDs which can have substitutions for, or deletions of, His^{52}, Asp^{54} and Arg^{73} (Figure 1). Variance of those three residues results in marked differences in fine binding specificity (Table 2), with many Type II CRD-galectins showing particular affinity for complex saccharides like the tetrasaccharide GalNAcα1,3(Fucα1,2)-Galβ1,4Glc (Ahmed & Vasta, 1994). Complex saccharides of blood group antigens are often high affinity ligands for Type II galectins (Hughes, 1994).

Table 2. Effectiveness of saccharides in inhibiting hemagglutination by galectins with Type I and Type II CRDs. Inhibition of agglutination is directly correlated with binding affinity. (Adapted from Ahmed & Vasta, 1994).

Inhibitory Saccharides	All Type I CRDs	Representative Type II CRD (Gal-3)
most effective	N-acetyllactosamine (Galβ1,4GlcNAc)	GalNAcα1,3(Fucα1,2)Galβ1,4Glc
↓	lactose (Galβ1,4Glc)	N-acetyllactosamine
least effective	sialyllactose (NeuAcα2,3Galβ1,4Glc)	fucosyl lactose (Fucα1,2Galβ1,4Glc)

Based on the number and organization of CRDs and the presence or absence of other heterologous protein domains, three types of galectins have been identified: proto, chimeric, and tandem-repeat (Figure 2).

Proto-type galectins

Proto-type galectins have an apparent molecular weight of 14-16 K and consist of a single CRD, usually of Type I carbohydrate binding specificity, with little additional sequence (Figure 2). They are widely distributed in the animal kingdom (Table 3) among mammals, birds, amphibians, fish, the nematode *Caenorhabditis elegans* , and a marine sponge. Recently, two proto-type lectins were isolated from a mushroom, *Coprinus cinereus* (Cooper, et al. , 1997), which suggests that the galactoside-binding galectin CRD is an evolutionarily ancient cellular entity (Hirabayashi, 1997).

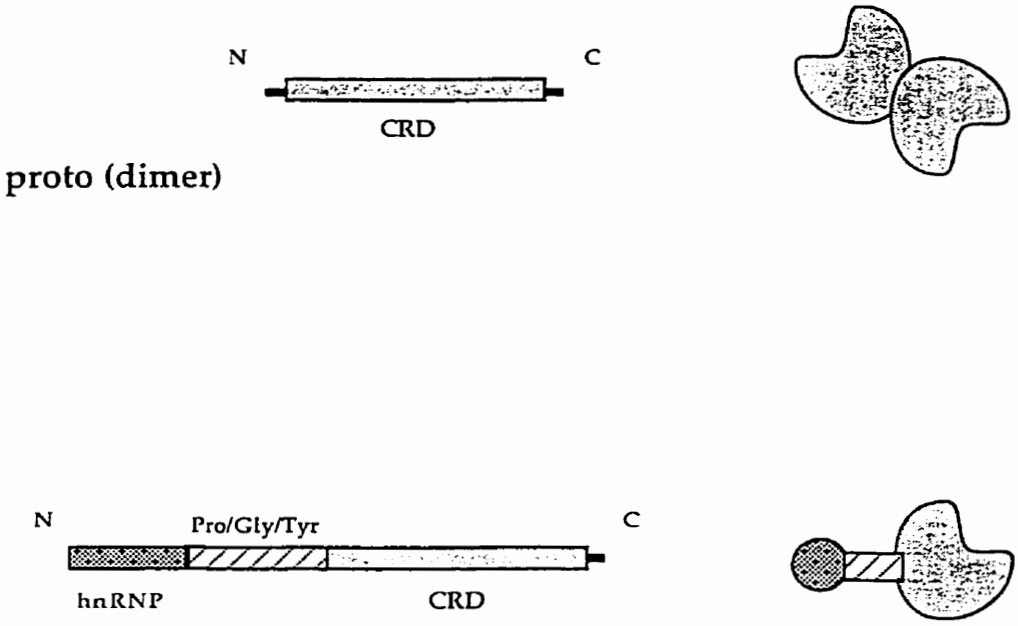


Figure 2. Structure and domain organization of the 3 types of galectins. Schematic of each type of galectin, shown as both a linear protein (left) and as a folded protein (after Barondes, 1988) (right). For the folded molecules , the carbohydrate binding site is represented by the cleft in the globular carbohydrate recognition domain (CRD). N, amino-terminus; C, carboxy terminus; hnRNP, sequence with homology to heterogenous ribonucleoprotein complex; Pro/Gly/Tyr, collagenase sensitive sequence rich in proline, glycine, and tyrosine amino acids.

Most proto-type galectins are capable of dimerization and exist in a monomer-dimer equilibrium (Cho & Cummings, 1997). The predominance of either the monomer or the dimer is characteristic of individual galectins. For example, under non-denaturing conditions, the chick 16 K galectin appears as a dimer (apparent MW = 31 K), while the chick 14 K galectin appears as a monomer (Beyer, 1980). The agglutinating activity of proto-type lectins is mediated by the dimeric form, since two carbohydrate binding sites are necessary to cross-link saccharide residues on adjacent cells (Cho & Cummings, 1997). X-ray crystal analysis of dimeric Galectin-1 in complex with N-acetyllactosamine indicates that the two subunits dimerize through interaction of their N and C terminal domains. These domains are presented on one side of the folded galectin protein, opposite the ligand binding site (Figure 2) (Liao, et al. , 1994). Site directed mutagenesis of Galectin-1 from Chinese hamster ovary (CHO) cells has confirmed that a few N-terminal residues are critical for dimerization (Cho & Cummings, 1996).

Chimera-type galectins

Chimeric galectins have an apparent molecular weight of 29-35 K, and consist of a C-terminal Type II galectin CRD and an N-terminal domain with sequence similarity to proteins of the heterogenous ribonucleoprotein (hnRNP) complex (Figure 2) (Jia & Wang, 1988). The hnRNP complex interacts with nucleic acids and regulates transport and processing of messenger RNA (mRNA) between the nucleus and

Table 3. Characteristics of galectins isolated from different sources.

Designation	Structural Type	Animal Source	Tissue Distribution	Apparent Mol. Wt. (SDS-PAGE)	CRD Type
Mammals					
Galectin-1	proto	human[1] calf[2] hamster[3] mouse[4]	placenta, lung liver, spleen, heart,thymus ovary adult muscle, blastocyst	14 K	Type I [17]
Galectin-2	proto	human[5]	liver	14.K	Type II [17]
Galectin-3	chimeric	human, mouse[6] human[7] human[8] mouse embryo[9] human[10] rat, dog[11]	liver, thymus, spleen, muscle breast carcinoma colon carcinoma notochord, bones, skin macrophages macrophages, epithelial tissues	29-32 K	Type II[17]
Galectin-4	tandem-repeat	rat[12] pig[13] human[8] mouse[14]	alimentary canal oral epithelium colon carcinoma alimentary canal	36 K	Type II[17]
Galectin-5	proto	rat[15]	erythrocytes	18 K	Type II[15]
Galectin-6	tandem-repeat	mouse[14]	alimentary canal	33-34 K	N/D
Galectin-7	proto	human[16]	skin keratinocytes	14.5 K	Type II[38]
Galectin-8	tandem-repeat	rat[17]	liver, lung, kidney, heart, skeletal muscle, spleen, brain	34 K	Type II[17]
Galectin-9	tandem-repeat	mouse, rat[18]	kidney, thymus, liver, heart, lung, muscle, intestine	36 K	N/D
Galectin-10 (Charcot-Leyden Crystal Protein)	proto	human[19]	eosinophil, basophil	17 K	N/D
Birds					
Chick 14K	proto	chicken[20] chick embryo[21] chick embryo[22]	intestine liver, intestine, pancreas, kidney yolk sac, neural tube, notochord, heart	14 K	Type I[17]

Table continues ....

Table 3. continued

Designation	Structural Type	Animal Source	Tissue Distribution	Apparent Mol. Wt. (SDS-PAGE)	CRD Type
Birds cont'd.					
Chick 16K	proto	chicken[20]	liver, muscle	16 K	Type I[37]
		chick embryo[21]	liver, intestine, cardiac muscle, kidney, brain		
		chick embryo[22]	yolk sac, germ cells, ECM surrounding organ primordia		
		chick embryo[23,24]	skeletal muscle		
		chick embryo[25]	mesonephros, metanephros		
Chick 30K	chimeric	chick embryo[26]	hypertrophic chondrocytes	30 K	Type II[26]
Amphibians					
Xenopus 16K	proto	*Xenopus laevis*[28]	skin	16 K	Type II[37]
Bufo 15K	proto	*Bufo arenarum* Hensel[29]	oocytes	15 K	N/D
Fish					
electrolectin	proto	electric eel[30]	electric organ	16.5 K	Type I[37]
congerin	proto	conger eel[31]	skin mucus	16 K	Type II[37]
Nematodes					
32K nematode lectin	tandem-repeat	*Caenorhabditis elegans*[32]	cuticle epithelium	32 K	Type II[37]
16K nematode lectin	proto	*Caenorhabditis elegans*[33]	N/D	16 K	Type II[33]
Sponges					
GCA-1 & -2	proto	*Geodia cydonium*[34,35]	extracellular (in sponge matrix)	15 K	Type II[37]
Fungi					
Cgl-I/II	proto	*Coprinus cinereus*[36]	fruiting bodies	16 K	Type II[36]

[1] Paratoud, et al., 1987; [2] Briles, et al., 1979; [3] Cho & Cummings, 1995; [4] Poirer & Robertson, 1993; [5] Gitt, et al., 1992; [6] Crittenden, et al., 1984; [7] Oda, et al., 1991; [8] Huflejt, et al., 1997; [9] Fowlis, et al., 1995; [10] Hughes, 1994; [11] Herrmann, et al., 1993; [12] Oda, et al., 1993; [13] Chiu, et al., 1994; [14] Gitt, et al., 1998; [15] Gitt et al., 1995; [16] Madsen, et al., 1995; [17] Hadari, et al., 1995; [18] Wada & Kanwar, 1997; [19] Leonidas, et al., 1995; [20] Beyer, et al., 1980; [21] Beyer & Barondes, 1982; [22] Zalik, et al., 1994; [23] Nowak, et al., 1977; [24] Den & Malinzak, 1977; [26] Nurminskaya & Linsenmayer, 1996; [27] Ozeki, et al., 1991; [28] Marschal, et al., 1992; [29] Ahmed, et al., 1996; [30] Levi & Teichberg, 1981; [31] Muramoto & Kamiya, 1992; [32] Hirabayashi, et al., 1992; [33] Hirabayashi, et al., 1996; [34] Pfeifer, et al., 1993; [35] Wagner-Hulsman, et al., 1996; [36] Cooper, et al., 1997; [37] Ahmed & Vasta, 1994, [38] Magnaldo & Michel, 1997..

cytoplasm. One stretch of the heterologous domain of the mammalian chimeric galectin, Gal-3, has a repeating motif rich in proline (Pro), glycine (Gly), and tyrosine (Tyr) residues which has been reported as a substrate for bacterial collagenases (Hermann, et al. , 1993). Galectin-3 is often found in the ECM and that of the rat lung copurifies with a 92 K protein with collagenase activity (Herrmann, et al. , 1993), suggesting a physical interaction between Gal-3 and the 92 K protein. In the absence of protease inhibitors, Gal-3 from the rat lung is slowly degraded. In addition, certain matrix proteases cleave the N-terminal domain of human Gal-3, leaving a 22 K C-terminal fragment with an intact and active CRD. (Ochieng, et al. , 1998). Some portion of the N-terminal sequence of these galectins has been implicated in dimerization which is critical for agglutinating activity (Massa, et al. , 1993; Kuklinski & Probstmeir, 1998)). Chimeric galectins have been isolated only from mammals and birds at this point (Table 3). The apparently more restricted distribution of chimeric lectins might reflect a more recent divergence within the galectin family.

Tandem-repeat-type galectins

Tandem-repeat galectins (32-36 K) consist of two galectin CRDs joined by a linking protein sequence (Figure 2). The first tandem-repeat galectin was isolated from the nematode *C. elegans*, but recently several mammalian tandem-repeat galectins have been characterized (Table 3). Tandem-repeat galectins are likely the result of gene duplication (Hirabayashi & Kasai, 1993).

The linking regions of different tandem-repeat galectins vary in length and sequence (Hirabayashi, et al. , 1992; Gitt, et al. , 1995) and are sensitive to protease enzymes (Lobsanov & Rini, 1997). All of the tandem-repeat galectins characterized so far have Type II CRDs (Table 3), and display a wide range of carbohydrate affinities. Adding to the functional flexibility of these galectins is the fact that the two CRDs within one tandem-repeat may be divergent and have different sugar-binding properties (Oda, et al. , 1993; Arata, et al. , 1997).

Galectins are proteins with intracellular characteristics and have an atypical secretion

Glycoconjugates are found predominantly outside the cell, either on the cell surface, in the extracellular matrix, or circulating in the serum (Hart, et al. , 1989). All galectin sequences examined so far have neither putative transmembrane segments nor signal sequences for secretion (Leffler, 1997). There is also evidence that lectins are synthesized on free cytoplasmic ribosomes (Wilson, et al. , 1989) and are not glycosylated (Hirabayashi & Kasai, 1993). Despite having such characteristics of intracellular proteins, galectins are often found extracellularily and appear to be secreted by a non-classical route that does not involve the ER-Golgi-vesicle system. Studies with cultured cell lines that secrete Galectin-3 have demonstrated that a number of drugs that block ER-Golgi-vesicular traffic have no inhibitory effect on secretion of the galectin (Sato, et al. , 1993; Lindstedt, et al. , 1993; Mehul & Hughes, 1997).

Immunohistochemical localization of Galectin-1 during its externalization from differentiating myoblasts revealed that cytosolic galectin first becomes concentrated in patches just beneath the plasma membrane (Cooper & Barondes, 1990). Subsequently the plasma membrane above a galectin patch evaginates and a galectin-containing vesicle pinches off the cell. Membrane evagination of this sort is called membrane blebbing and has recently been demonstrated for Galectin-3 (Mehul & Hughes, 1997). Results of these investigators, using fusion proteins, suggests that part of the N-terminal (non-CRD) domain of Gal-3 somehow directs the externalization. It is not known if N-terminal sequences from proto- and tandem-repeat galectins lacking the heterologous N-terminal domain of Gal-3 can support galectin release by blebbing.

Galectin receptors

Despite the prevalence of β-galactosylated molecules on the cell surface or in the extracellular milieu, galectins bind specifically to relatively few extracellular ligands. Both Type I and Type II galectins have very high affinity for linear polylactosamine chains (Merkle & Cummings, 1988). Polylactosamine consists of repeating units of lactosamine, $(\rightarrow 3Gal(\beta 1,4)GlcNAc\beta 1\rightarrow)_n$. It has been theorized that the increased affinity for polylactosamine, when compared to N-acetyllactosamine, may be due to simultaneous contact sites in the galectin CRD with more than one lactosamine unit within the polylactosamine chain (Cho &

Cummings, 1997). In many cases where galectins have been shown to modulate cell adhesion, galectin binding partners have been polylactosamine-bearing. The ECM glycoprotein laminin is heavily glycosylated with abundant polylactosamine side chains and is a very high affinity ligand for galectins (Zhou & Cummings, 1990; Castronovo, et al. , 1992; Ochieng & Warfield, 1995; Cooper, 1997). *In vitro*, Galectin-1 binds to human placental FN, another ECM glycoprotein (Ozeki, et al. , 1995). The chick 14 K galectin has been shown to bind to a polylactosamino-proteoglycan from chick skin (Oda & Kasai, 1984). Cell surface molecules which have been identified as galectin ligands also have (poly)lactosamine residues. In particular, LAMPs (lysosome associated membrane proteins), which cycle between lysosomes and the cell membrane, bear a large number of polylactosamine chains and are high affinity ligands for galectins (Do, et al. , 1990). Recently, heavily glycosylated transmembrane enzymes have been identified as ligands for Galectin-4 in the intestinal brush border (Danielsen & van Deurs, 1997). Glycosylated lipids in the plasma membrane can be bound by galectins, such as the β-lactosamine glycolipid implicated in cell-cell and cell-matrix adhesion of olfactory neurons (Mahanthappa, et al. , 1994). Galectins also display high affinity for polylactosamine serum proteins such as Mac-2 binding protein (Mac-2-BP) (Inohara, et al. , 1996).

Functional roles of galectins

Regulated expression of galectins during development suggests that these proteins play an important role in normal cellular function and development. There is evidence supporting a role for these proteins in the following cellular activities:

a) RNA Processing. Gal-3 and Gal-1 are present in the cell nucleus as well as the cytoplasm and extracellularly (Wang, et al. , 1992; Hubert, et al. , 1995; Vyakarnam, et al. , 1997; Patterson, et al. , 1997). A functional role for Galectin-3 in nuclear RNA splicing activity was suggested by the observation that saccharide ligands of the lectin (lactose, thiodigalactoside, and a blood group A tetrasaccharide) inhibits the processing of pre-mRNA to mRNA in HeLa cells (Wang, et al. , 1992). Dagher, et al. (1995) demonstrated that Gal-3 meets the standard identifying requirements for factors involved in pre-mRNA processing by the spliceosome, since depletion of Gal-3 from a cell-free nuclear extract abrogated splicing activity and addition of recombinant Gal-3 restored mRNA processing activity to the extract. Galectin-1 has also been implicated as a splicing factor, since it was shown that either Gal-1 or Gal-3 could restore splicing activity to a galectin-depleted extract (Vyakarnam, et al. , 1997). Thus Gal-1 and Gal-3 appear to be functionally redundant factors in mRNA processing.

b) Modulation of Cell Growth. Galectins have been shown to act as mitogens (Lipsick, et al. , 1980; Matsutani & Yamagata, 1982; Sanford & Harris-Hooker, 1990), to act as cytostatic factors (Wells & Mallucci, 1991),

and to have transforming effects (Yamoaka, et al. , 1996) on different cell types. Galectins have also been identified as factors capable of modulating immune function by regulating apoptosis of T-lymphocytes (Pace & Baum, 1997; Perillo, et al. , 1997; Rabinovich, et al. , 1997; Wada, et al. , 1997; Akahani, et al. , 1997b; Yang, et al. , 1996; Perillo, et al. , 1995).

c) Cell-cell and Cell-matrix Interactions. Galectins have been proposed to function as mediators of cell-cell and cell-matrix adhesion. The majority of galectins can function as bivalent molecules capable of cross-linking ligands. Indeed, galectins have been shown to form networks of cross-linked ligands in solution (Bourne, et al. , 1994; Brewer, et al. , 1997). Galectins could form links between glycoconjugate-bearing cellular receptors on adjacent cells, mediating cell-cell adhesion. These lectins could also link glycosylated cell surface molecules to glycoproteins and proteoglycans in the extracellular matrix, mediating cell-matrix adhesion.

Galectin-1 is synthesized at high levels by mouse myoblasts, which are the precursors of muscle cells. This lectin is not secreted until terminal myoblast differentiation begins (Cooper, et al. , 1991). Evidence indicates that this galectin might bind to polylactosamine chains on laminin and sterically block laminin/$\alpha_7\beta_1$ integrin interactions. As a consequence myoblasts lose adhesion to the ECM, stop migrating, and onset of myoblast fusion occurs (Cooper, 1997). Evidence indicates that Gal-1 disrupts binding of the integrin to laminin (Gu, et al. , 1994). Thus

in this system, disruption of cell-matrix adhesion by galectins may play a crucial role in the formation of skeletal muscle.

Galectin-1 is expressed in the neuroblasts and axons of the rat olfactory bulb (Mahanthappa, et al. , 1994) where it binds to a member of the laminin family in the ECM and to a β-lactosamine-containing glycolipid in the axon cell membrane. Both ligands colocalize with Gal-1 in this system (Mahanthappa, et al. , 1994). It has been suggested that Galectin-1 may be involved in axon fasciculation by cross-linking the cell surface glycolipid to laminin in the ECM. This suggestion is supported by studies of galectin null mutant mice (Poirer & Robertson, 1993) in which it was found that neurons expressing N-acetyllactosamine do not project to the correct termination sites in the dorsal surface of the caudal olfactory bulb (Puche, et al. , 1996).

Expression of galectins and their ligands are often upregulated in malignant cells compared to their untransformed counterparts (Sarafian, et al. , 1998; Inohara & Raz, 1995; Skrincosky, et al. , 1993; Castronovo, et al. , 1992), and increased galectin-ligand expression has been suggested to play a role in metastasis and invasion (Lotan, et al. , 1994; van den Brûle, 1995 a & b; Akahani, et al. , 1997a). Some tumour cell lines express Gal-3 and secrete Mac-2-BP (Inohara & Raz, 1995; Inohara, et al. , 1996). Since in isolated cells aggregation can be promoted by the addition of Mac-2-BP it has been suggested that Gal-3/Mac-2-BP interaction could promote embolus formation in blood vessels and allow invasion of surrounding

tissues. Cross-linking of cells to the ECM by divalent galectins requires a cell-surface ligand such as the LAMPs; surface expression of LAMPs is often increased in malignant cells (Sarafian, et al. , 1998). In some malignant cell lines, adhesion of cells to immobilized Galectin-1 is mediated by galectin-binding to lactosamine elements in cell-surface LAMPs (Skrincosky, et al. , 1993; Woynarowska, et al. , 1994).

The chick 14- and 16 K galectins are expressed throughout embryogenesis (Zalik, et al. , 1994; Guay & Zalik, 1994; Zalik, et al. 1990). During gastrulation both galectins are found in the cells migrating to form the primitive streak, a structure formed by the migration of superficial cells into the embryo (Zalik, et al. , 1990). Embryos cultured at the onset of gastrulation in the presence of galectin saccharide inhibitors or anti-16 K galectin antisera do not form a primitive streak and normal expansion of the embryo is inhibited (Jeeva & Zalik, 1996). Embryonic FN is heavily glycosylated and contains polylactosamines (Ozeki, et al. , 1995), and cells in the embryo express lactosamine-bearing molecules at their surfaces such as the HNK-1 epitope (Murumatsu, 1988). It has been suggested that the block in gastrulation could be due to the disruption of galectin-glycoprotein interactions with the consequent disruption of cell-cell and cell-ECM contacts.

One approach to study *in vivo* functions of proteins is to generate a null mutation in an organism. Null mice for the genes encoding Gal-1 and Gal-3 have been generated (Poirer & Robertson, 1993; Colnot, et al. ,

1997). These mice are viable and develop without any major abnormalities. A detailed examination of the olfactory nervous system, however, revealed defects in primary olfactory neuron outgrowth (Puche, et al. , 1996) in Gal-1 deficient mice. Double knockout mice lacking both Gal-1 and Gal-3 were described recently and these mice also appear phenotypically normal (Colnot, et al. , 1997). However, since at least 10 galectins have been reported in mammals whose expression patterns overlap considerably (Colnot et al. , 1997; Leffler, 1997) the possibility of functional redundancy and a compensation effect cannot be ruled out. Another consideration is that galectins may be most crucial for survival under stressed conditions. Galectin null mice were generated from extremely healthy laboratory stock and protected from pathogens, hunger, and other stresses during development. Galectin deficiency may not be critical under these conditions but could be extremely deleterious under stressed conditions (Leffler, 1997).

THE VERTEBRATE EXCRETORY SYSTEM

Since the excretory system of the chick embryo is my system of study, I will give a general overview of the function and organization of this system. Following that, I will briefly review the development of the excretory system in the chick embryo.

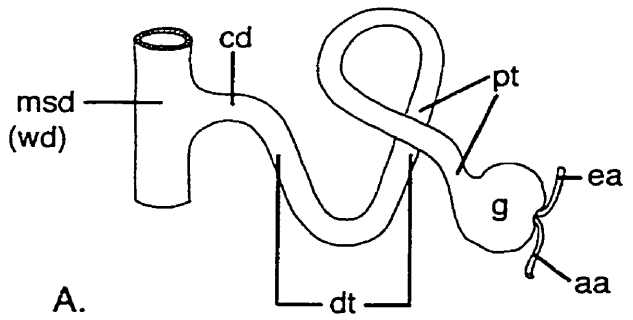
The function of the excretory system is to remove waste products from the blood, to regulate osmolarity of blood and body fluids, and to

control the acid-base balance of the blood (Bulger, 1983). In vertebrates, this function is performed by the kidney, whose functional unit is the nephron. The nephron has three basic components: the glomerulus, the secretory tubule composed of proximal, intermediate and distal segments, and the collecting ducts (Figure 3a). The glomerulus is the site of initial filtration of the blood during formation of the urine. After passing through the filtration barrier of the glomerulus, the filtrate is conducted through the secretory tubule where additional substances can be secreted into the glomerular ultrafiltrate, and where selective resorption of ions and water occurs. Finally, the collecting duct makes final adjustments on the pH and ionic concentration of the waste fluid and conducts it to a common duct; the latter leads eventually to the external opening of the excretory system which is the cloaca in birds.

The glomerulus

The glomerulus has two parts, the tuft and the capsule. The tuft contains capillaries and the various cell types and tissues involved in glomerular filtration, and is surrounded by the Bowman's capsule. (Figure 3, B and C) The lumen of the capsule is continuous with the lumen of the proximal secretory tubule. Blood enters the tuft via the afferent arteriole, and the rapid decrease in vessel diameter creates high pressure within the capillary, forcing much of the fluid through the filtration barrier. The

Figure 3. Structure of the vertebrate nephron, and detailed structure of the glomerulus. (A) Schematic of the mesonephric nephron (after Baird, et. al., 1994; Friebova-Zemanova & Goncharevskaya, 1982). (B) is a sagittal section through the glomerulus as depicted in (A). (C) is a cross-section through (B).
aa, afferent arteriole; bc, Bowman's capsule; bcbm, Bowman's capsule basement membrane; c, capillary; cd, collecting duct; dt, distal tubule; ea, efferent arteriole; en, endothelial cells; g, glomerulus; GBMen, endothelial glomerular basement membrane; GBMep, epithelial (podocyte) GBM; ma, mesangial cell; mm, mesangial matrix; msd (wd), mesonephric duct (Wolffian duct); p, podocyte; pe, pedicel (podocyte foot process); pt, proximal tubule; se, squamous epithelium; tbm, tubule basement membrane. A more complete description is found in the text.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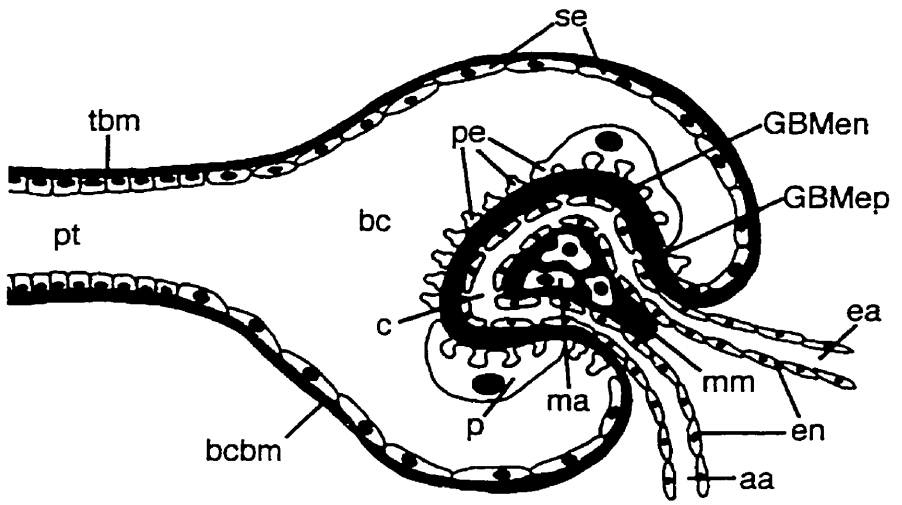
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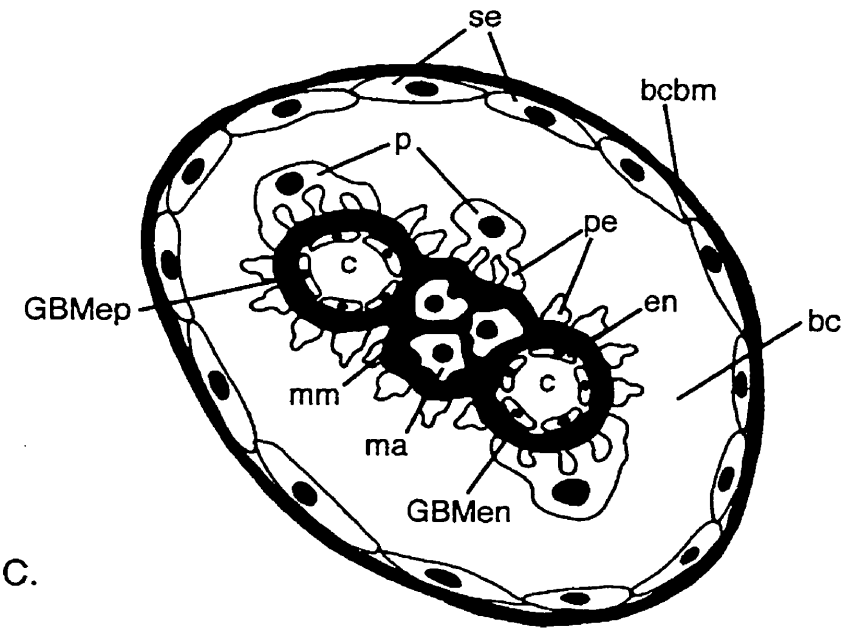
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filtration barrier retains macromolecules (proteins larger than albumin, approximately 70 kDa) but allows the passage of solutes of lower molecular weight (Mundel & Kriz, 1995). The resulting filtrate is collected in the lumen of the Bowman's capsule and flows into the proximal secretory tubule. Blood exits the tuft via the efferent arteriole. (Figure 3)

The most straightforward way to describe the components of the glomerulus in more detail is to begin from the capillary loop and work outwards towards the capsule (Figure 3, B and C). The endothelial cells which form the walls of the capillary are attached to each other in such a manner as to allow the existence of gaps, or fenestrations, between them (Gibley & Chang, 1967; Jones, 1977, Tiedemann & Egerer, 1984). Thus the fluid portion of the blood can pass through the gaps while the cellular components are retained. The capillary loop is completely surrounded by the endothelial glomerular BM and the mesangial matrix, a relatively fluid ECM, in which mesangial cells are embedded (Michael, et al. , 1980; Schlondorff, 1996; Gauer, et al. , 1997). The endothelial/mesangial matrix is secreted by the mesangial cells and by the endothelial cells of the capillary. Another, more rigidly organized, layer of ECM surrounds the endothelial/mesangial matrix. This ECM is produced by epithelial cells called podocytes which form the outermost layer of the glomerular tuft (Walker, 1973; Huang, 1979; Ekblom, 1981b; Sariola, 1984; Sariola, et al. , 1984 a & b). The endothelial/mesangial matrix and the epithelial matrix together comprise the glomerular basement membrane (GBM).

Macromolecules and other filtration residues retained in the GBM are cleared by the continual resorption of matrix material by the mesangial cells (Walker, 1972; Michael, et al. , 1980; Schlondorff, 1996). The podocytes surrounding the tuft are highly specialized epithelial cells with a very distinct cellular architecture (Mundel & Kriz, 1995). Podocyte cell bodies do not rest on the glomerular basement membrane, but rather extend several "foot processes" or pedicels that rest on and secrete the epithelial portion of the GBM. The foot processes of a given podocyte interdigitate with the foot processes from other podocytes to cover 90% of the GBM (Mundel & Kriz, 1995). The filtration slits between the interdigitating foot processes are spanned by an extracellular structure, of a tight junctional nature, called the slit diaphragm (Brown & Stow, 1996). The slit diaphragm has pores which retain macromolecules larger than 70 kDa and thus plays a major role in forming the glomerular filtration barrier.

The function of the Bowman's capsule surrounding the tuft is essentially just to collect the glomerular filtrate and funnel it into the proximal tubule. It is lined with a BM, which is continuous with the epithelial portion of the GBM as well as the BM lining the proximal tubule (Huang, 1979). The capsule BM is secreted by the epithelial cells lining the Bowman's capsule (Figure 3).

The proximal tubule

The proximal tubule, so-called because it is the portion of the secretory tubule closest to the glomerulus (Figure 3, A), is lined by

columnar epithelium with basal nuclei and an apical brush border (Bulger, 1983; Brown & Stow, 1996). In the metanephros, the proximal tubule is specialized for bulk resorption of fluid, ions, and low molecular weight proteins (Brown & Stow, 1996). Detailed functional analysis of the mesonephros has not been performed. However, given the high degree of morphological similarity between meso- and metanephros of various species (Tiedemann, 1979; Schiller & Tiedemann, 1981; Friebova-Zemanova, 1981; Tiedemann & Zaar, 1983) and the observed conservation of nephric structure and function among vertebrates, it is reasonable to conclude that the function of the mesonephric proximal tubule is equivalent to that of the metanephric tubule.

In the proximal tubule, the volume of the glomerular filtrate is rapidly reduced to about 20% of the original volume (Bulger, 1983). The large fluid volume received by the proximal tubule is reflected in its structure; the proximal tubule typically has a larger diameter than the distal tubule (Friebova-Zemanova, 1981), and the columnar epithelial cells are tall, forming thick tubule walls (Tiedemann & Zaar, 1983; Wettstein & Tiedemann, 1981). In addition, the absorptive surface area in the proximal tubule is greatly amplified by a dense apical brush border of microvilli present in the epithelial cell (Gibley, 1967; Tiedemann & Zaar, 1983; Bulger, 1983).

Absorption of water and ions by the proximal tubule is isotonic. That is, water and electrolytes are absorbed from the filtrate until the

filtrate osmolarity is equivalent to that of the blood circulating near the basal face of the proximal tubule epithelium (Huss & Marsh, 1975). Solutes that are actively absorbed through apical channels, transporters, and receptors are subsequently transported through the cytoplasm by transcytosis, and are finally released into the basolateral intercellular spaces. Water moves into the intercellular spaces due to osmosis, via a paracellular pathway where water passes around the epithelial cells and through the tight junction barrier (Brown & Stow, 1996; Huss & Marsh, 1975). Water can pass through the proximal tubule tight junctions because in this segment of the nephron, the tight junctions are "leaky" due to the presence of fewer junctional complexes with several discontinuities between complexes (Brown & Stow, 1996; Schiller & Tiedemann, 1981; Tiedemann & Zaar, 1983). The BM surrounding the proximal tubule is porous and allows resorption of fluid from the basolateral spaces into the bloodstream (Brown & Stow, 1996).

The distal tubule and collecting duct

In the mesonephros, after passing through the proximal tubule, the isotonic filtrate enters the distal tubule (Figure 3, A), and then the collecting duct. The metanephric nephron of mammals and some birds has a loop of Henle between the proximal and distal tubules, which is a highly specialized region of secretory tubule that functions in urinary concentration (Wettstein & Tiedemann, 1981). The distal tubule and the collecting duct are structurally and functionally similar so they are usually

considered together as the distal nephron (Bulger, 1983). As with the proximal tubule, comparison of the ultrastructure of meso- and metanephric distal nephron suggests similarity in function (Wettstein & Tiedemann, 1981; Tiedemann, 1985). In the distal nephron, the isotonic filtrate from the proximal tubule can be concentrated, that is, made hypertonic with respect to the blood, or diluted, that is, made hypotonic, in accordance with the physiological needs of the organism. The distal nephron is also the site of acid-base regulation (Bulger, 1983; Masden & Tisher, 1986).

The distal nephron is lined by two types of columnar epithelial cells: principal cells and intercalating cells. Intercalating cells are interspersed among the principal cells, and the ratio of principal to intercalating cells varies between the distal tubule and the collecting duct (Masden & Tisher, 1986; Wettstein & Tiedemann, 1981; Tiedemann, 1985). The principal cells selectively absorb or secrete solutes into the filtrate under hormonal direction (Masden & Tisher, 1986). In contrast to the proximal tubule, paracellular flow of water at the distal tubule and collecting duct is prevented by "tight" tight junctional complexes (Brown & Stow, 1996; Schiller & Tiedemann, 1981; Tiedemann, 1985). If necessary, water is actively secreted or absorbed through water transport channels (Brown & Stow, 1996). The intercalating cells are specialized to secrete protons and bicarbonate ions as a means of regulating the pH of body fluids. There are two types of intercalated cells, which differ in polarity.

The A cells secrete protons into the tubule lumen and bicarbonate into the renal interstitium; the B cells secrete bicarbonate ions into the filtrate and protons into the interstitium (Brown and Stow, 1996; Masden & Tisher, 1986). Urine exiting the collecting duct is conducted through a common duct, the Wolffian duct for the mesonephros, or the metanephric duct which leads eventually to the external opening of the excretory system.

DEVELOPMENT OF THE AVIAN EXCRETORY SYSTEM

The development of the avian excretory system involves the formation of three temporally and spatially distinct kidneys (Romanoff, 1960) (Figure 4). The first to develop, the pronephros, is nonfunctional and degenerates rapidly. The second, the mesonephros, functions through a large portion of embryonic life and contributes to the formation of the gonads and associated structures. In birds and mammals, the metanephros is the last to develop and remains as the functional kidney throughout adult life.

All three kidneys develop from "nephrogenic mesenchyme", which is simply a portion of the intermediate mesoderm that, given the appropriate inducing signal, has the capacity to form nephric structures. Inductive events are common throughout embryogenesis (Saxen, 1987), and are said to occur whenever one tissue is known to influence the development of a different separate tissue (Grobstein, 1956) In the case of the development of the vertebrate excretory system, the inducing tissue is

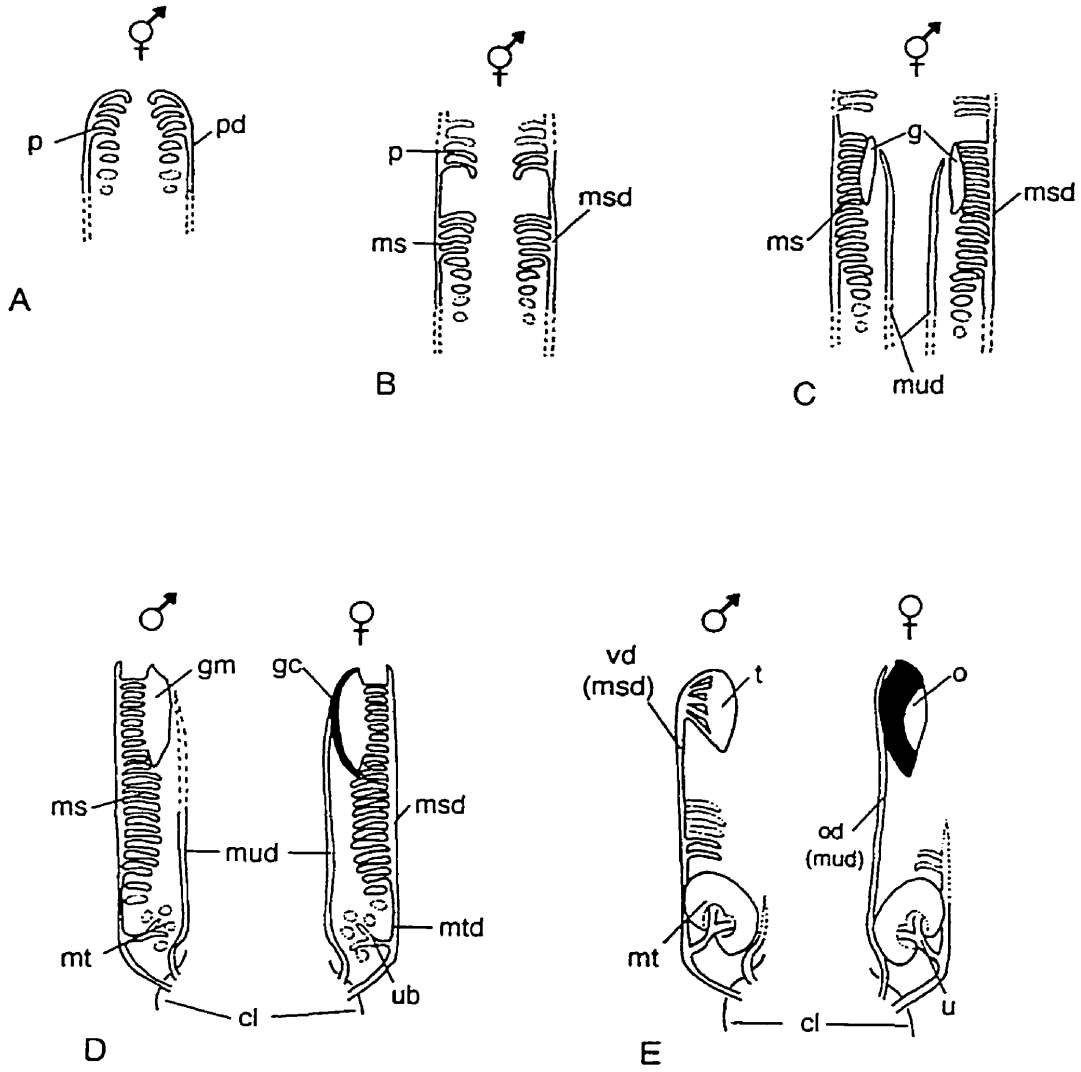


Figure 4. Development of the avian excretory system (after Saxen, 1987; Romanoff, 1960; Hamilton, 1952). (A) 20-40 hours of incubation. (B) 50-70 hrs. (C) 80-100 hrs. (D) 120 hrs (5 days). (E) Post-hatching (>21 days). Plates (A)-(C) are sexually indeterminate stages; in (D) and (E) male versus female development is indicated. cl, cloaca; g, gonad; gc, gonadal cortex; gm, gonadal medulla; ms, mesonephros; msd, mesonephric (Wolffian) duct; mt, metanephros; mtd, metanephric duct; mud, Müllerian duct; o, ovary; od, oviduct; p, pronephros; pd, pronephric duct; t, testis; u, ureter; ub, ureteric bud; vd, vas deferens. A description of the events here depicted can be found in the text.

an epithelial duct which develops from an anterior portion of the intermediate mesoderm and grows caudally until it connects to the cloaca (Figure 4). This duct is termed the pronephric duct or the mesonephric (Wolffian) duct, depending on its location in the embryo (Romanoff, 1960). The mechanism and consequences of induction in the nephros will be discussed in more detail below.

The pronephros

The pronephros is the first and most anterior organ of excretion to develop in the chick (Figure 4, A). Pronephric tubule primordia appear as early as 30 hours incubation (Romanoff, 1960), and once fully developed, consist of rudimentary glomeruli connected by tubules to the pronephric duct. Despite the presence of all necessary structures, the pronephros is believed to be nonfunctional in the chick embryo. It is not known if induction by the pronephric duct is required for development of the pronephros, but scanning electron microscopic studies have clearly demonstrated that the duct develops before pronephric tubules begin to form (Jarzem & Meier, 1987). In the chick embryo, the duct and tubules of the pronephros begin to degenerate after 50 hours of incubation. The pronephric glomeruli degenerate more slowly but are absent from the 8 to 9 day embryo (Romanoff, 1960).

The mesonephros

The mesonephros develops just posterior to the degenerating pronephros, in response to an inductive signal from the mesonephric or

Wolffian duct (Friebova, 1975; Saxen, 1987). Growth of the mesonephros is rapid and proceeds in an anterior to posterior direction, in conjunction with the caudal growth of the Wolffian duct (Figure 4, B). Rudimentary mesonephric tubules are first found in the embryo after about 50 hours of incubation, and excretory function begins at around the 5th day (Figure 4, C) of incubation (Romanoff, 1960; Friebova, 1975; Narbaitz & Kapal, 1986). Excretion by the mesonephros reaches a peak around the 11th day, after which time the mesonephros begins to degenerate in an anterior to posterior fashion (Morris, 1967). The time of cessation of function is a matter of debate, but in any case once the metanephros begins to function in earnest the mesonephros becomes redundant. In addition to being the primary organ of excretion during a significant portion of embryonic life, the mesonephros is important because the medulla or inner portion of the gonad develops from tissue of mesonephric origin (Figure 4, D) (Carlon, et al. , 1983) In addition, in male chicks the Wolffian duct persists and serves as the vas deferens of the testis (Figure 4, E) (Romanoff, 1960).

The metanephros

The metanephros develops in response to an inductive signal from a bud off the mesonephric duct (Romanoff, 1960; Saxen, 1987; Herzlinger, 1995). This ureteric bud grows laterally into the nephrogenic mesenchyme, where development of the metanephros begins (Figure 4, D). The first indications of metanephric development appear around the 4th day of incubation, but the definitive kidney does not begin to function

in the embryo until around the 12th day of incubation (Romanoff, 1960). For a period of several days the meso- and metanephroi function simultaneously, so that when the mesonephros degenerates the process of excretion is not interrupted. The metanephros continues to function throughout the adult life of the chicken (Figure 4, E).

DEVELOPMENT OF THE NEPHRON

The most striking feature of the development of the vertebrate kidney is the transformation of the dispersed cells of the nephrogenic mesenchyme into the highly organized structure of the nephron. Mesenchyme has been defined as embryonic tissue originating from the mesoderm (Blackshaw, et al. , 1995), but the term implies a tissue which is not highly differentiated. Mesenchymal cells are typically spindle-shaped (Herzlinger, 1995) and are embedded in randomly organized interstitial ECM (Ekblom, 1981b). Differentiated renal epithelial cells, in contrast, are cells with functionally specialized cell membrane and cytoplasmic domains which rest on a discrete ECM basement membrane (Sorokin & Ekblom, 1992). With the notable exceptions of the podocytes of the glomerular tuft, which are modified to perform a specialized role in a unique locale, and the squamous or flattened epithelial cells lining the Bowman's capsule, renal epithelial cells have a characteristic cuboidal shape. These cells form junctions with neighboring cells which creates a barrier between the lumen of the renal tubule and the interstial spaces

containing capillaries and blood vessels. The mesenchyme to epithelium transition and the subsequent formation of the nephron from the newly converted epithelial cells will be discussed below.

There is a scarcity of information regarding the causal factors involved in chick nephrogenesis. Due to the fact that a well established *in vitro* system exists, most studies on the cell biology of nephrogenesis have been performed in the developing mammalian metanephros of the mouse. The mammalian and the chick excretory organs, however, have the same tissue of origin and respond to an inductive signal from similar structures. In addition, their development appears morphologically similar (Saxen, 1987). It is therefore useful to discuss events occurring during the development of the mammalian metanephros, while noting cases where the chick mesonephros has been specifically investigated.

Nephrogenesis

Nephrogenesis begins with the condensation of induced nephrogenic mesenchyme into a tight ball (Ekblom, 1981; Saxen, 1987; Sorokin & Ekblom, 1992) (Figure 5, A and B). In the pro- and mesonephroi, mesenchymal condensates form lateral to the nephric duct, while in the metanephros condensates form around tips of the ingrowing ureteric bud. Shortly, the ball of mesenchymal cells elongates somewhat and becomes curved into the characteristic "comma shape" (Figure 5, C). The end of the comma- shape most lateral to the duct will be the proximal/glomerular end of the tubule. A slit develops in the proximal

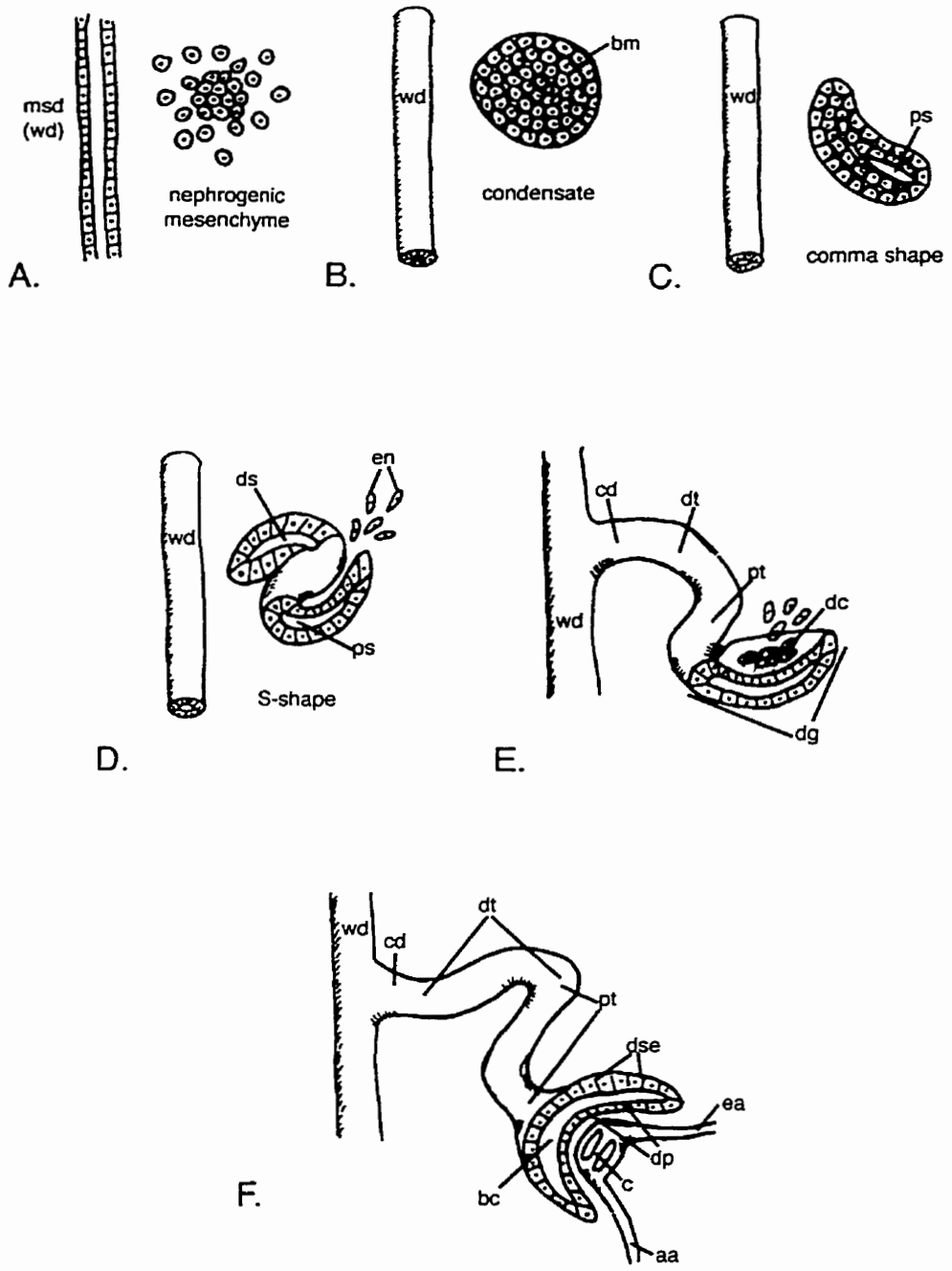


Figure 5. Development of the mesonephric nephron (adapted from Davies, 1996; Herzlinger, 1995; Saxen, 1987). aa, afferent arteriole; bm, basement membrane; c, capillary; cd, collecting duct; dc, developing capillary; dg, developing glomerulus; dp, developing podocyte epithelium; ds, distal slit; dsc, developing squamous epithelium; dt, distal tubule; ea, efferent arteriole; en, ingrowing endothelial cells; msd (wd), mesonephric duct (Wolffian duct); ps, proximal slit; pt, proximal tubule. A description of the events represented in this figure can be found in the text.

area as the surrounding cells begin to differentiate. Subsequently, a second slit forms in the opposite end of the comma, at the end closest to the nephric duct. By this point, the condensate has assumed an S-shape (Figure 5, D). Subsequently the distal end of the tubular S-shape joins the nephric duct or ureteric bud and thus forms the collecting system (Figure 5, E). Cells in the presumptive proximal and distal secretory tubules begin to differentiate as the distal and proximal slits of the S-shape join together to form a continuous lumen.

At this stage in the development of the nephron, the glomerulus takes shape as the proximal tip of the S-shape flattens and curls up around capillary endothelial cells (Saxen, 1987; Sorokin & Ekblom, 1992). The capillary loops of the glomerulus develop from endothelial cells that migrate into the developing glomerulus (Figure 5, D) (Ekblom, et. al, 1982). The glomerular tuft forms as the BM surrounding the proximal end of the S-shaped stucture becomes associated with the BM secreted by the developing capillary endothelium and the mesangial cells. The latter are also thought to be of endothelial origin (Ekblom, et.al., 1982; Sorokin & Ekblom, 1992). The tubular epithelial cells resting on the BM of the forming tuft differentiate into podocytes. The widening slit becomes the lumen of the Bowman's capsule which is continuous with the lumen of the developing proximal tubule. The capsule itself develops as the tubular epithelial cells across the lumen from the developing glomerular tuft flatten into the typical capsular squamous epithelium. (Figure 5, F)

Molecular aspects of differentiation

Induction by the nephric duct or ureteric bud involves a mesenchymal to epithelial transformation of the nephrogenic mesenchyme, and the first stage in nephrogenesis is the condensation of mesenchymal cells. In the mouse metanephros, levels of two proteins involved in cell adhesion, NCAM and syndecan 1, increase in the mesenchyme before cell aggregation into condensates (Davies, 1996).

As mesenchymal condensation proceeds, expression of ECM components is dramatically altered (Ekblom, 1981b; Baird, et al. , 1994). The secretion of interstitial-type collagens I and III decreases to undetectable levels, and expression of BM collagen IV begins. The basement membrane-specific ECM components laminin and perlecan also start to be expressed (Ekblom, 1981; Davies, 1996). These molecules are secreted around the condensates as the BM forms. Around this time, cells begin to produce $\alpha_6\beta_1$ integrin heterodimers. These are transmembrane receptors for the laminin α chain; these receptors allow cells to adhere to the laminin in the developing BM (Davies, 1996).

Differentiation into an epithelial cell type is reflected by the synthesis of epithelium-specific proteins by the cells in the condensate. Cells in the proximal end of the condensate, which are the presumptive podocytes, begin to differentiate first, and differentiation proceeds in a proximo-distal direction (Saxen 1987; Ekblom, 1981). During the later stage of condensation, aggregated cells begin to express E-cadherin, desmocollin,

and α-catenin, the first two being epithelial-specific (Davies, 1996). Shortly following the acquisition of these proteins, cells in the condensate polarize so that the basal domain faces the BM and the apical domain faces the lumen. Development of the full epithelial phenotype occurs when the intermediate filament cytoskeleton of the tubular cells changes from one containing vimentin intermediate filaments to one containing cytokeratins (Davies, 1996)

Subsequent to the transition from a mesenchymal to an epithelial character is the further differentiation of cells into podocytes, proximal tubule and distal tubule epithelial types. Regional specialization of the nephric epithelium is indicated by the acquisition of segment-specific molecular markers which reflect the specialized functions of the different nephron segments (Ekblom, 1981b). Again, this differentiation occurs in a proximo-distal sequence, with the unique character of the podocyte epithelium of the glomerulus becoming apparent first, followed by final differentiation of the cells of the proximal and then distal tubule. The final differentiation of the podocytes is unique because it involves a re-acquisition of some characteristics of mesenchymal cells (Sorokin & Ekblon, 1992). For example, podocytes express typical epithelial proteins such as components of the cell junctions called desmosomes, and α6 integrin. However, they also lack the epithelium-specific cytokeratins, and express vimentin and α_3 integrin; the latter proteins are characteristic of mesenchymal cells and typically absent from epithelia. Structurally,

podocytes form a cell sheet that is more organized than a mesenchyme but more "leaky" than tubular epithelium (Mundel & Kriz, 1995).

Characteristics of the inductive signal

In the embryo, the ureteric bud provides the inductive signal for the transition of the mesenchyme into an epithelium (Ekblom, 1981b; Saxen, 1987; Sorokin & Ekblom, 1992; Davies, 1996). *In vitro*, mesenchymes from other embryonic tissues can trigger this transition (Saxen, 1987, Sariola, et al. , 1989). It has been concluded that the presumptive renal mesenchyme has a "nephrogenic bias" and that the inducing signal consists of a stimulus to express the renal phenotype to which the mesenchyme has previously been committed. Transmission of the inductive signal appears to occur by short-range diffusion of soluble factors, interactions of cell membrane-associated or extracellular matrix-associated factors, or formation of intercellular channels (Saxen, 1987; Sariola, et al. 1989).

What is the inductive signal?

The signal that triggers nephrogenesis remains unknown, although current research indicates that members of the *Wnt* gene family are likely to be involved in the signaling pathway regulating nephrogenesis. Members of the *Wnt* gene family are closely related to the extensively studied *Drosophila* gene *wingless* (*wg*) (Nusse & Varmus, 1992; Klingensmith & Nusse, 1994), which functions as an extracellular signal

capable of influencing the development of adjacent cells (Klingensmith & Nusse, 1994).

Fibroblast cells expressing *Wnt*-1 can induce nephrogenesis in mouse metanephric mesenchyme (Herzlinger, et al. 1994), and inductive capacity of tissues which can induce nephrogenesis correlates with their expression of *Wnt*-1 (Herzlinger, et al. , 1994). However, *Wnt*-1 is not expresed in the ureteric bud. Given the high degree of structural similarity and apparent functional similarity of known *Wnt* genes, it is possible some member of the *Wnt* gene family is expressed in the ureteric bud and can signal the mesenchyme to differentiate into nephrons.

Condensation of mesenchyme and subsequent tubulogenesis occurs exclusively around the ureter tips. One *Wnt* gene, *Wnt*-11, is expressed in the tips of the branching ureter (Kispert, et. al, 1996), but does not induce nephrogenesis. It is likely that some as-yet-uncharacterized member of the *Wnt* family is the *in vivo* inductive signal for the formation of the nephron. Some chemicals such as lithium that induce mesenchyme to epithelium transition *in vitro* (Davies, 1996) activate the signaling pathway induced by Wnt (Hedgepeth, et al. , 1997), and several properties of Wnt proteins also make them likely candidates for the inductive signal.

Wnt genes encode secreted glycoproteins that are known to interact with components of the ECM and to modulate adhesiveness in Wnt-responsive cells. (Nusse & Varmus, 1992; Klingensmith & Nusse, 1994). Extracellular Wnt proteins are located at the cell surface or in association

with extracellular matrix material (Nusse & Varmus, 1992), which suggests that Wnt signaling would be short-range. Amino-acid sequences of Wnt proteins reveal several potential glycosylation sites (Nusse & Varmus, 1992; Klingensmith & Nusse, 1994), and it has been found that cells transfected with *Wnt*-1 secrete several Wnt-1 glycoforms which vary in the character and extent of glycosylation (Reichsman, et al. , 1996). Certain Wnt glycoforms expressed by a *Drosophila* cell line bind sulfated proteoglycans such as heparin or heparan-sulfate which contain N-acetyl-glucosamine and N-acetyl-iduronic acid residues; other glycoforms bind chondroitin-sulfate which contains N-acetyl-glucosamine and N-acetyl-galactosamine groups (Reichsman, et al. , 1996).

The role of Wnt in signaling cell fate is probably due to its effect on cell adhesiveness. Wingless protein (wg) in Drosophila is known to activate a signaling pathway which results in the accumulation of the armadillo protein. The vertebrate homologs of armadillo are β-catenin and plakoglobin which are the cytoplasmic proteins with which the membrane cell adhesion proteins cadherins interact (Nusse & Varmus, 1992; Hinck, et al. , 1994; Hedgepeth, et al. , 1997). One consequence of the accumulation of these proteins in the cytoplasm is the stabilization of the interaction between β-catenin and the cadherins. The latter also leads to reorganization of membrane-linked cytoskeletal proteins and to the activation of several intracellular signal cascades with multiple and

profound effects such as a functional reorganization of the cell, or cell differentiation (Nathke, et al. , 1995; Drubin & Nelson, 1996).

FUNCTIONS AND CHARACTERISTICS OF EPITHELIA

Since some of my thesis deals with changes in the epithelial phenotype, I will give a general overview of epithelial polarity. The epithelial cells lining the renal tubule form a barrier between two distinct environments: the filtrate/urine filled lumen and the pericellullar matrix which contains blood vessels. The osmoregulatory activity of the nephron is due to directional transport of solutes and water across this barrier. Controlled, vectorial transport is made possible by two specializations of epithelial cells (Griepp & Robbins, 1983). First, the cells are closely associated to each other via several types of junctional complexes. Second, epithelial cells have polarity and have structurally and functionally distinct plasma membrane domains. The free surface of the cell, which in the kidney tubule faces the lumen, is called the apical surface. The basolateral cell surface is the region of cell-cell or cell-substratum contact. Cell adhesion molecules mediating intracellular junctions are restricted to the lateral membrane, where cell-cell contacts occur (Wollner, et al. , 1992; Garrod, et al. , 1996; Nathke, et al. , 1994). Receptors for BM components and other molecules that participate in cell-substratum adhesion are localized to the basal cell surface (Borradori & Sonnenberg, 1996; Green & Jones, 1996; Salas, et al. , 1992; Ojakian & Schwimmer, 1994).

Polarized distribution of ion pumps and channels permits vectorial transport across the epithelial barrier (Le Gall, et al. , 1995; Shiel & Caplan, 1995; Brown & Stow, 1996). For example, in the distal tubule the intercalated cells regulate the pH of the bloodstream. There are two types of intercalated cells (Schuster, 1990; Madsen & Tisher, 1986). A-type cells, which secrete hydrogen ions into the lumen and bicarbonate ions (HCO3-) into the pericellular matrix, have a hydrogen ion adenosine triphosphatase (H+ATPase) pump in the apical membrane and a chloride/HCO3- pump in the basolateral membrane. B-type cells have a reversed polarity, with the Cl-/HCO3- pump in the apical membrane and the H+ATPase in the basolateral membrane, and thus secrete HCO3- into the tubule lumen. Another ion exchange pump, the sodium/potassium ATPase, is restricted to the basolateral surface of renal epithelia, where it functions in the resorption of sodium ions from the bloodstream (Marrs, et al. , 1993: Mays, et al. , 1995).

Epithelial junctions

Epithelial cells adhere to neighboring cells and to the BM with junctional complexes. Tight junctions, adherens junctions, and desmosomes link cells together and are located on the lateral cell surface. Hemidesmosomes and adhesion plaques link the basal surface of epithelial cells to the underlying BM (Borradori & Sonnenberg, 1996).

Tight junctions form the permeability barrier of epithelial cell sheets and function as the boundary between apical and basolateral

membrane domains (Furuse, et al. , 1993; Furuse, et al. , 1994). The plasma membranes of adjacent cells are very tightly apposed in the region of the tight junction, which forms a belt around the circumference of the cell just below the apical surface. Freeze-fracture electron micrographs of tight junctional complexes show distinct strands where the outer leaflets of the plasma membranes of adjacent cells appear to be fused (Brown & Stow, 1996). A "leaky" epithelium, such as in the proximal tubule of the nephron, has tight junctions with few and incomplete strands (Schiller & Tiedemann, 1981; Tiedemann & Zaar, 1983). In contrast, the collecting duct epithelium permits no paracellular flow of water or solutes and has tight junctions with several anastomosing strands (Schiller & Tiedemann, 1981; Tiedemann, 1985).

The strands of the tight junctional complex are thought to consist of a transmembrane protein called occludin (Brown & Stow, 1996; Furuse, et al. , 1993). Tight interactions between strands of occludin molecules embedded in the membranes of adjacent cells are proposed to form the paracellular seal. In addition, this seal creates a barrier to lateral diffusion of integral membrane proteins between the apical and basolateral domains (Brown & Stow, 1996; Furuse, et al. , 1994). Peripheral components of the tight junction are not integral membrane proteins but rather are thought to interact with occludin and link the tight junction strands to the underlying actin cytoskeletal elements (Brown & Stow, 1996). Peripheral components identified so far include: ZO-1 (zonula occludens 1), which

binds to occludin; ZO-2, which binds to ZO-1; and cingulin, which is associated with the ZO-1/ZO-2 complex by an unknown interaction (Furuse, et al. , 1994; Citi, et al. , 1988).

Adherens junctions are located on the lateral membrane below the apical/basolateral boundary imposed by the tight junctions. Cell-cell adhesion at adherens junctions is mediated by cadherins (Cowin & Burke, 1996), transmembrane glycoproteins that contribute to calcium-dependent cell adhesion via homotypic binding in a variety of tissues (Barth, et. al, 1997; Nathke, et al. , 1994). In epithelial cells, the predominant cadherin is E-cadherin (Ozawa & Kemler, 1992), which is linked to actin microfilaments via the intermediate proteins α, β, and γ-catenin (Ranscht, et al. , 1994; Nathke, et al. , 1994). γ-catenin is also known as plakoglobin. β-catenin and plakoglobin are components of signaling cascades that regulate cell function and differentiation (Barth, et al. , 1997).

Desmocollin and desmoglein are other cadherins that mediate desmosomal adhesion (Garrod, et al. , 1996; Koch & Franke, 1994). Desmosomal cadherins are linked to cytoskeletal elements of the intermediate filament family through the cytoplasmic accessory proteins desmoplakin and plakoglobin. (Barth, et al. , 1997, Garrod, et al. , 1996; Schmidt, et al. , 1994; Green & Jones, 1996). Plakoglobin forms complexes with both desmocollin and desmoglein and is believed to also function as a signal transduction molecule (Garrod, et al. , 1996).

Epithelial cells can be attached to the BM via hemidesmomes which link the ECM to the intermediate filament cytoskeleton (Garrod, 1993; Dowling, et al. , 1996; Green & Jones, 1996; Borradori & Sonnenberg, 1996). Two transmembrane components of hemidesmosomes have been identified. One is the bullous pemphigoid antigen 1 (BPAG-2/BP180) whose BM ligands have not been identified (Borradori & Sonnenberg, 1996; Green & Jones, 1996). The other transmembrane component of the hemidesmosome is $\alpha_6\beta_4$ integrin (Green & Jones, 1996; Spinardi, et al. , 1993) which binds to several laminin isoforms (Spinardi, et al. , 1995). Various accessory proteins bind to the cytoplasmic region of this integrin and link to intermediate filaments (Spinardi, et al. , 1995).

Simple epithelia (i.e./ one layered) such as that found in the renal tubule do not form intermediate filament-linked hemidesmosomes (Dowling, et al. , 1996). Adhesion to the BM in these tissues is mediated by adhesion plaques composed of integrins other than $\alpha_6\beta_4$ (Dedhar & Hannigan, 1996). Binding of integrin receptors to extracellular matrix components leads to clustering of integrins into adhesion plaques (Dedhar & Hannigan, 1996).

Maintenance of epithelial polarity

In order for harmonious nephrogenic differentiation to continue after the induction process has occurred, epithelial polarity per se must be maintained. Three general mechanisms contribute to the maintenance of epithelial polarity: 1) delivery of transmembrane and secreted proteins to

the appropriate membrane plasma membrane domain; 2) selective retention of the appropriate integral membrane proteins in membrane domains; and 3) prevention of lateral diffusion of transmembrane proteins between the apical and basolateral domains by the tight junction barrier (Le Gall, et al. , 1995).

Newly synthesized proteins as well as those "recycled" from the plasma membrane are sorted just before or immediately after leaving the Golgi apparatus (Odorizzi, et al. , 1996), and are segregated and packaged into specific vesicles destined for the apical or basolateral surface (Wandinger-Ness, et al. , 1990; Drubin & Nelson, 1996). These vesicles move along the fibers of cytoskeletal elements to the appropriate membrane domain (Le Gall, et al. , 1995; Brown & Stow, 1996; Mays, et al. , 1994). Secreted proteins sorted in this fashion include BM components (Natori, et al. , 1992; Svennevig, et al. , 1995). In addition, integral membrane proteins with a polarized distribution such as growth factor receptors (Crepaldi, et al. , 1994), channel proteins, and ion pumps (Brown & Stow, 1996) are often sorted into vesicles and delivered directly to the appropriate surface domain.

Proteins can also become polarized on the cell surface if their retention time is significantly longer when they are delivered to one membrane domain than the other. Many transmembrane proteins, such as the Na^+/K^+ ATPase, are stabilized in the appropriate membrane domain by interactions with components of adherens junctions, desmosomes,

adhesion plaques, and/or elements of the cortical cytoskeleton (Le Gall, et al. , 1995; Mays, et al. , 1995). Na^+/K^+ ATPase can be delivered to the cell surface randomly, but it is retained in the basolateral membrane because it is linked to the cortical cytoskeleton (Peters, et al. , 1995; Marrs, et al. , 1993).

The influence of extracellular matrix components on epithelial polarity

The establishment of epithelial cell polarity during development of the kidney has been examined *in vivo* as well as *in vitro*. Madin-Darby canine kidney (MDCK cell line) cells cultured under appropriate conditions become polarized and exhibit many of the characteristics of renal tubular epithelium, such as vectorial transport of ions and water (Leighton, et al. , 1970). In these cells, both cell-cell and cell-substratum adhesion is required to develop a fully polarized phenotype (Drubin & Nelson, 1996; Eaton & Simons, 1995). When MDCK cells are grown in suspension culture, they exhibit qualities of a renal neoplasm, namely uncontrolled growth and invasiveness when transplanted into an animal (Leighton, et al. , 1970). If MDCK cells in suspension culture are allowed to contact each other, they form small aggregates in which E-cadherin-mediated cell-cell adhesion is sufficient to define the lateral membrane domain, and basolateral proteins become restricted to areas of cell-cell contact in the aggregate (Drubin & Nelson, 1996).

It is generally accepted that cell-cell contacts establish the lateral domain of epithelial cells while cell-substratum contacts define the apico-basolateral axis, presumably by contact-induced orientation of cytoskeletal

elements (Drubin & Nelson, et al. , 1996; Eaton & Simons, 1995; Wang, et al. , 1994; Ojakian & Schwimmer, 1988; Vega-Salas, et al. , 1987). Furthermore, proper development of a polarized phenotype is dependent on the nature of the cell-substratum interaction. Extracellular matrix, including BM material, is laid down by cells within a tissue, and different cell types and tissues produce ECM with unique components and/or structural characteristics (Birk & Linsenmayer, 1994; Fitch & Linsenmayer, 1994; Ekblom, 1981b). In some cell lines, including rat kidney cells, the composition and organization of ECM, in turn, can affect cell morphology, gene expression, and polarity of cells contacting that matrix (Pienta, et al. , 1991; Van Adelsberg, et al. , 1994).

Cellular responses to ECM components are mediated by cell-membrane receptors, including integrins (von der Mark, et al. , 1992; Dedhar & Hannigan, 1996). After the onset of nephrogenic differentiation in the mouse developing renal tubule, the interaction of cellular receptors with the ECM glycoprotein laminin is crucial for cell polarization and tubule formation, and for the subsequent maintenance of the polarized phenotype (Ekblom, et al. , 1980; Ekblom, 1996; Durbeej & Ekblom, 1997).

Laminin is a heterotrimer composed of α, β, and γ chains (Timpl & Brown, 1994). During kidney development, β and γ laminin chains are produced throughout the uninduced mesenchyme as well as by developing epithelial cells. The laminin α chain, however, is produced only by induced cells and is found exclusively in the BM of developing

tubules (Klein, et al. , 1988; Durbeej & Ekblom, 1997). Antibodies to the cell binding sites of the α, β, and γ chains of laminin (Timpl & Brown, 1994; Durbeej & Ekblom, 1997) inhibit the development of polarity of kidney tubular cells in organ culture but not the condensation of induced mesenchyme, suggesting that this BM protein plays a role in the development of the epithelial phenotype (Klein, et al. , 1988). The $\alpha_6\beta_1$ integrin receptor, which binds to the carboxy terminal end of the cell binding domains of laminin (the E8 fragment), has been shown to be essential for epithelial polarization during nephrogenesis (Goodman, 1992). During the formation of nephric structures from induced mesenchyme, the spatial and temporal distribution of the α_6 integrin subunit correlates with that of the laminin α chain. Antibodies to the α_6 subunit that selectively block its binding to laminin E8 severely inhibit polarization in developing renal tubules (Sorokin, et. al, 1990).

Another laminin receptor, α-dystroglycan, has been implicated in epithelial morphogenesis. Alpha-dystroglycan binds to the E3 fragment of laminin (Ekblom, 1996) and is coexpressed with the laminin α chain during tubule formation (Durbeej, et al. , 1995). Antibodies that disrupt the binding of α-dystroglycan to laminin also inhibit development of renal tubules (Durbeej, et al. , 1995). The 37/67 kDa laminin receptor may also play a role in development of polarity. Treatment of MDCK cells plated on laminin with antibodies to this receptor results in missorting of an apical membrane marker (Salas, et al. , 1992). There is also evidence

that a cell-surface glycolipid, bearing the galactose-containing Forssman antigen, mediates cell-laminin interactions that are crucial for the maintenance of cell polarity in renal epithelial cells (Zinkl, et al. , 1996). It seems clear that laminin expression is crucial for development of epithelial polarity, and that laminin may be acting through several concurrent pathways to exert an effect on cellular phenotype.

STATEMENT OF PURPOSE

Because cellular interactions with laminin are so crucial for the development and maintenance of epithelial polarity, and because laminin is a high-affinity ligand for galectins, it is possible that galectins may affect cell polarization during development of nephric tubules and/or play a role in the maintenance of the polarized phenotype. In the present study I examined the expression of the chick 14 kDa and 16 kDa (hereafter indicated by C14 and C16, respectively) galectin proteins in the differentiating mesonephros of the chick embryo from 4 to 10 days of incubation. *In situ* hybridization revealed that message for the 16 kDa galectin was present in the 4 day mesonephros, indicating that mesonephric C16 was produced in that tissue. The expression of C16 protein in the mesonephros was confirmed by immunolocalization in tissue sections, and by Western blotting of tissue extracts. C16 appears in the basal laminae surrounding mesonephric tubules and in the glomerular basement membrane (GBM); two potential ligands, laminin

and FN, colocalize with the galectin. Since C16 was expressed in the basal lamina I investigated whether hapten inhibitors of the galectin could affect epithelial-mesenchymal transformation. To determine the possible functions of C16 in nephrogenic differentiation, chick mesonephroi were cultured in a defined medium in the presence of thiodigalactoside and lactose, hapten inhibitors of galectins. Interference with galectin function in this manner resulted in changes in the cellular architecture of the mesonephros and in a partial return of the tubular epithelial cells to a mesenchymal identity. This was reflected by the fact that the tubular epithelial cells, which normally express only epithelial-specific proteins, began to express mesenchyme-specific proteins simultaneously with epithelial proteins.

EXPERIMENTAL PROCEDURE

Immunohistochemistry of the chick mesonephric/gonadal complex

Fixation, Embedding, and Sectioning. Embryos from 4 to 10 days of incubation, or embryonic tissues, were washed briefly in phosphate buffered saline (PBS) containing 0.15 M NaCl and 5 mM $NaKHPO_4$ (pH 7.4) and immediately fixed in 1% acetic acid in 95% ethanol for 1 hour at room temperature. Fixed tissues were dehydrated in a graded ethanol series, transferred to xylene, and embedded in Paraplast X-Tra (Oxford Labware) under vacuum. Paraplast tissue blocks were sectioned at 7 μm with a Reichert-Jung 2040 Autocut rotary microtome. Paraplast tissue sections were placed onto glass slides coated with 2% 3-aminopropyl triethoxysilane in acetone, and stored at room temperature until used for histological staining with hematoxylin-eosin or for immunofluorescence analysis.

Immunofluorescense Staining. Sections were dewaxed with xylene and rehydrated with a graded alcohol series (100%, 95%, 70%, and 50% ethanol) and equilibrated in PBS (pH 7.4). Non-specific binding was blocked by incubating the tissue sections with 5% bovine serum albumin (BSA) and 5% normal goat serum in PBS, at room temperature, for one hour. To examine the expression pattern of the 16 kDa galectin and its putative ligands during the development of the mesonephric/gonadal

Table 4. Antisera used during the course of this study.

Antisera	Antigen	Host Animal (pAb or mAb)	Source
R10	chick liver 16 kDa galectin	rabbit (pAb)	Zalik *
D16	chick pectoral muscle 16 kDa galectin	rabbit (pAb)	Didier**
K14	chick 14 kDa galectin	rabbit (pAb)	Kasai‡
R701	chick liver E-cadherin	rabbit (pAb)	Gallin*
B3/D6	avian fibronectin	mouse (mAb)	DSHB+
31-2	avian laminin	mouse (mAb)	DSHB
H5	chicken vimentin	mouse (mAb)	DSHB
β4/B4	avian Na,K-ATPase	mouse (mAb)	DSHB
α-ACt	α-actinin	rabbit (pAb)	Sigma

* Zalik laboratory
** Gift from E. Didier, Universite de Clermont Blaise Pascal, Laboratoire d'Immunologie G, Aubiere Cedex, France.
‡ Gift from K. Kasai, Teikyo University, Department of Biological Chemistry, Kanagawa, Japan.
* Gift from W.J. Gallin, University of Alberta, Department of Biological Sciences, Edmonton, Canada
+ Developmental Studies Hybridoma Bank, Johns Hopkins University School of Medicine, Department of Pharmacology and Molecular Sciences, Balrtimore, USA.

complex, sections from 4 to 10 day embryos were incubated with one of the following primary polyclonal (pAb) or monoclonal (mAb) antisera: anti-16 kDa galectin pAb (D16), anti-avian laminin mAb (31-2) hybridoma supernatant, or anti-avian FN mAb (B3/D6) hybridoma supernatant. To establish the distribution of certain marker proteins to aid in the immunohistochemical analysis of the cultured mesonephros, sections from early developing (5 day) and fully developed (7 day) mesonephroi were also incubated with one of the following antisera: anti-E-cadherin

pAb (R701), anti-α-actinin (α-ACT) pAb, anti-vimentin mAb (H5) hybridoma supernatant, or anti-Na^+/K^+ ATPase mAb (β4/B4) hybridoma supernatant. Details of the antigen-specificity and source of these antisera are given in Table 4. Hybridoma supernatants containing monoclonal antisera (31-2, B3/D6, H5, and β4/B4) were used undiluted. All other antisera were diluted in PBS with 1% BSA. The D16 antiserum and the control sera, i.e. rabbit or mouse normal serum (Sigma), used as controls for polyclonal and monoclonal antisera, respectively, were applied at a 1:100 dilution. The R-701 and α-ACT antisera were diluted 1:250. Tissue sections were incubated with the primary antisera for 3 hours at room temperature, washed twice with PBS with 0.1% Tween-20 (TPBS), and washed once with PBS. For sections incubated with polyclonal primary antisera, the secondary antisera were either tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG or fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson Laboratories) diluted 1:100. For sections incubated with monoclonal primary antisera, the secondary antisera was FITC-conjugated goat anti-mouse IgG (Jackson Laboratories) diluted 1:50. Slides were incubated overnight at 4°C in the dark with the secondary antibodies and washed the following day with TPBS and PBS, as described previously. Subsequently, all sections were briefly incubated with 0.5 μg/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma) to stain nuclear DNA. After a final rinse in PBS, a Mowiol-based mounting medium containing anti-

fading agents (Zalik, et al. , 1990) was applied to the tissue sections. Fluorescence was viewed with a Zeiss Photomicroscope III. Photographs were taken using Kodak Ektachrome 400 color slide film.

In situ hybridization to detect galectin mRNA in the mesonephric/gonadal complex

The DNA clone 302 for the chicken 16 kDa galectin was a gift from Dr. Ken-ichi Kasai (Teikyo University, Kanagawa, Japan). Residues 250-356 corresponding to the least similar regions of the 16 and 14 kDa chicken galectins were generated using PCR and were cloned into the PCR II vector using the PCR Optimizer Kit (Invitrogen, San Diego, USA), according to the manufacturer's instructions. Using the DIG-RNA labeling kit (Boehringer Mannheim, Laval, QU Canada), the DIG-labeled RNA probe, recognizing a single RNA species of the correct size, was generated (Marsh, et. al., 1995)

Precautions were taken throughout the procedure to avoid contamination of any solutions with RNase. Embryos from four-day incubated eggs were isolated and fixed in 4% paraformaldehyde in ribonuclease (RNase)-free Pannett and Compton's saline (New, 1966). Following dehydration in dioxane, embryos were embedded in low melting point Paraplast (Oxford Labware). Five mm-thick sections were cut on a rotary microtome, then floated and fixed onto Poly-L-Lysine slides (Baxter). Sections were dewaxed, and rehydrated with a graded alcohol series before treatment with proteinase K to digest cellular proteins. Next,

slides were post-fixed in 4% formaldehyde and rinsed with diethyl pyrocarbonate (DEPC) water. Non-specific binding of probe was minimized by incubating slides in 0.25% acetic anhydride in 0.1M triethanolamine and briefly rinsing in DEPC water. Sections were then dehydrated through a graded alcohol series and allowed to air dry. The slides were incubated for 3-4 hours at 55°C in hybridization buffer (50% deionized formamide, 25% 20X PIPES, 10% 50X Denhardt's [Brahic & Ozden, 1992], 0.002% SDS, 2.5% 10mg/ml salmon sperm DNA, 2.5% herring sperm DNA, 2.5% yeast tRNA). Next, sections were incubated with the appropriate digoxigenin (DIG)-labeled probe at a concentration of 5 µg/ml in hybridization buffer overnight at 55°C. Following hybridization, slides were washed at room temperature in four 30 minute changes of 2X saturated sodium citrate (SSC) solution containing 300 mM NaCl and 30 mM sodium citrate. Next, slides were incubated for 1 hour in 0.1 SSC containing 15 mM NaCl and 1.5 mM sodium citrate, before a final 1 hour incubation in Buffer 1 (0.1M Tris pH 7.4 and 0.05M NaCl,). Non-specific adherence of anti-DIG antisera was minimized by blocking sections in Buffer 1 containing 5% BSA for one hour. Endogenous tissue alkaline phosphatase (AP) activity was inactivated by three 15 minute incubations in Buffer 1 with 1 mM levamisole. Slides were then incubated for two hours at room temperature with AP-conjugated anti-DIG Fab[1] fragments (Boehringer Mannheim) diluted 1:400 in Buffer 1 with 1% BSA. Following a brief wash in Buffer 1, sections were equilibrated in Buffer 3

(0.1M Tris, 0.05M MgCl, 0.1M NaCl, pH 9.5) before the color reaction was developed in Buffer 3 containing the substrates nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Boehringer Mannheim). Slides were washed repeatedly with distilled water to stop the developing reaction and were mounted with GelTol (Immunon).

Preparation of crude galectin extracts from chick embryo mesonephroi and gonads

The mesonephroi, right gonad, and left gonad were removed from 12 dozen 10 day chick embryos and transferred to ice-cold PBS (pH 7.2) in graduated cone-tipped plastic centrifuge tubes. The tissues were then centrifuged at 1000 x *g* for 1 minute to determine packed tissue volume, and the PBS was decanted and replaced with 10 volumes of ice-cold lectin extraction solution (LES). LES consists of PBS with 300 mM β-lactose, 4 mM β-mercaptoethanol, and 0.25 mM phenylmethylsulfonyl fluoride (PMSF). Tissues were homogenized in LES using a Polytron homogenizer (Brinkmann) and left shaking overnight at 4°C. Homogenates were subsequently centrifuged at 80 000 x *g* at 4°C, for 90 minutes. The resulting supernatants, hence called crude galectin extracts, were transferred to Spectrapor dialysis tubing (MW cut off 6000 - 8000). Lactose was removed by exhaustive dialysis against PBS containing 4 mM β-mercaptoethanol (MEPBS, pH 7.2), and extracts were concentrated to the original tissue volume on a bed of polyethylene glycol (PEG, MW 20,000). Total protein concentration was determined using the Pierce Micro BCA Protein Assay

Reagent Kit with bovine γ-globulin as the protein standard. When necessary, crude galectin extracts were further concentrated by acetone precipitation.

Partial purification of galectin from crude mesonephros extract:

Purification of Galectin Using an Affinity Column. Galactose-binding activity in the crude extract from 10 day chick mesonephros was further purified using column or batch affinity chromatography. APL-Sepharose was prepared by linking para-aminophenyl-β-D-galactoside (APL, Sigma) to Sepharose 6B (Pharmacia) beads and poured into a 10 ml column attached to an LKB 2111 Multirac pump and fraction collector. Protein levels in the fractions were detected by a Uvicord S 2138 Monitor (OD_{280}) and recorded on an LKB 2210 Potentiometric Recorder. One ml of crude extract from 10 day chick mesonephroi was applied to the column and washed with MEPBS. Subsequently, the column was washed with 300 mM sucrose in PBS (pH 7.2), to remove non-specifically bound proteins. Following a wash with 10 volumes (100 ml) of MEPBS, galectins were eluted by application of a 300 mM lactose in PBS solution.

Purification of Galectin by a Batch Method. Crude galectin extracts from 10 day mesonephros were incubated with α-lactose immobilized on 6% beaded agarose (Sigma). Purification was carried out in 10 ml polypropylene snap-cap tubes using 1 ml of α-lactose beads. The lactose-conjugated beads were thoroughly washed in MEPBS (pH 7.2) and pelleted by light centrifugation at 700 x *g* for 1 minute. One ml of crude

mesonephros extract was diluted in 3 ml of MEPBS and applied to the beads, which were left shaking at 4°C overnight. After light centrifugation, the supernatant was removed. The beads were then washed 3 times with 4 ml of MEPBS, followed by overnight incubation with 4 ml of 300 mM sucrose in PBS (pH 7.2). The sucrose-eluted material was removed by centrifugation and the beads were washed as before. Four ml of 300 mM lactose in PBS were added to the beads which were incubated overnight at 4°C with shaking. The lactose-eluted material was removed by centrifugation and possible residual galectin was removed from the beads by washing 3 time with MEPBS, as above. All supernatant fractions, including MEPBS washes, were saved for analysis by dot immunoblotting.

Immunodot Analysis of Fractions Obtained During Galectin Purification. To identify galectin-containing fractions from the affinity purification procedures, all fractions were subjected to immunoblot analysis using a polyclonal antiserum raised against 16 kDa galectin from chick liver (R10). Two microlitre samples of each fraction were removed and dotted onto Trans-Blot nitrocellulose membrane (Biorad, 2 μm pore size) and allowed to dry. Controls consisted of 2 μl dots of MEPBS, bovine fetuin (2 mg/ml solution), and egg albumin (2 mg/ml solution). Membranes with dot blots were washed overnight in Tris-buffered saline (TBS) containing 50 mM Tris-HCl and 0.15 M NaCl, pH 7.5, to remove residual lactose or sucrose. Non-specific binding was blocked by overnight

incubation, at 4°C, with 5% electrophoresis-grade BSA (Sigma A 2153) in TBS. To detect galectin, dot blots were incubated overnight at 4°C with the R10 antiserum diluted 1:1000 in TBS with 1% BSA, washed two times with TBS containing 0.05% Tween-20 (Biorad) (TTBS), and three times with TBS. Subsequently, blots were incubated for 3 hours at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Biorad) diluted 1:3000 in TBS containing 1% BSA, and then washed with TTBS and TBS, as above. Galectin-containing dots were detected by the HRP color reaction using 4-chloro-1-napthol (Biorad) as described by Hawkes et al. (1982).

Fractions exhibiting galectin immunoreactivity were pooled and concentrated to a volume of 1 ml as described previously. Total protein concentration in each partially purified galectin extract was determined as described previously. When necessary, these extracts were further concentrated by acetone precipitation.

SDS-PAGE of crude and partially purified galectin extracts

Crude and partially purified galectin extracts were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). For reference purposes, a crude galectin extract previously prepared from 15 day yolk sac was run concurrently. One mm-thick polyacrylamide-gradient (10%-18%) gels were prepared using the Biorad Mini-Protean II apparatus. Protein concentration in the samples was normalized to 2.3 μg/μl for the crude extracts and 0.3 μg/μl for the pure extracts and samples

were suspended in SDS sample buffer (50 mM dithiothreitol, 0.07 M Tris-HCl, 5% SDS, 22% glycerol, 30% urea, and 0.01% bromophenol blue), pH 6.8. Broad-range protein molecular weight standards (Biorad) were diluted 1:20 in sample buffer, according to manufacturers directions. Proteins were reduced in the SDS sample buffer by heating at 95°C for 5 minutes before application to the gel. Gels were run, in a buffer containing 25 mM Tris, 0.2 M glycine, and 0.2% SDS, at 100 mV constant voltage until the dye front passed through the stacking gel. Once proteins entered the separating gradient gel, the voltage was increased to 200 mV until the dye front reached the bottom of the gel. Proteins were visualized by staining the gels with Coomassie R-250 dye (Biorad).

Immunoblot analysis of extracts

Immediately after SDS-PAGE, gels were placed in Transfer Buffer containing 25 mM Tris, 0.2 M glycine, and 20% methanol and allowed to equilibrate for 30 minutes. Gels were assembled into the Biorad Mini Protean II Western transfer apparatus which was placed in ice. Electrophoretic transfer to nitrocellulose was performed for 2-3 hours at a constant amperage of 200 mA. After transfer, protein standards were visualized on the nitrocellulose membrane by staining with Ponceau Quick Red (Sigma).

Immunoreactive (galectin-containing) bands were detected on the nitrocellulose membrane by immunoblot analysis. Several thin strips were cut from each lane so that different antibodies could be used for the

analysis. Immunostaining and detection of immunoreactive bands on the blot strips was performed as described above for dot blot staining. The primary antisera used were: R10, a polyclonal antiserum (pAb) raised in rabbit against 16 kDa galectin from chick liver; D16, a pAb to 16 kDa galectin of chick pectoral muscle, and control (normal) rabbit serum. All sera were diluted 1:1000 in TBS with 1% BSA.

Culture of isolated mesonephroi in a defined medium

Five-day incubated eggs were swabbed with 70% ethanol and allowed to dry in in a positive-pressure air flow hood. Embryos were removed aseptically into filter-sterilized Tyrode's saline (0.8% NaCl, 0.02% KCl, 0.02% $CaCl_2$, 0.01% $MgCl \cdot 6H_2O$, 0.005% $NaH_2PO_4 \cdot 2H_2O$, 0.1% $NaHCO_3$, and 0.1% glucose), pH 7.2, and staged according to Hamburger and Hamilton (1951). Mesonephroi from stage 25-26 embryos were dissected out and removed to a drop of Basic Medium in a sterile tissue-culture treated plastic petri dish on ice. Basic Medium is a 1:1 mixture of Ham's F12 and Dulbecco's Minimum Essential Medium (DMEM) with 1 mM glutamine, 1 mg/L insulin, 1 µg/L selenium, 50 µg/ml conalbumin, 50 µg/ml gentamycin, and 50 µg/ml polymyxin-B-sulfate (all from Sigma). Using a tungsten needle, the ends of the mesonephroi were removed and discarded. The central portion of each organ, approximately 1.5 mm long and less than 1 mm in diameter, was transferred to the top of the membrane filter (polycarbonate membrane with 4 µm pore size) in a Costar Transwell (Corning) tissue culture plate (wells 6.5 mm diameter).

After the drop of medium surrounding the mesonephros had soaked into the suspended membrane filter, test medium was added to the well underneath the filter. Test media consisted of Basic Medium with 50 mM maltose, lactose, or thiodigalactoside (TDG), or Basic Medium alone. The Transwell plates were incubated at 37°C with 5% CO_2 and high humidity, in a water-jacketed incubator (Forma Scientific model 3157). After 24 hours, approximately half of the medium below the membrane filter was removed and replaced with the same volume of the same fresh medium. After 48 hours the membrane support with the attached tissue was removed and fixed first with 1% acetic acid in 95% ethanol for 30 minutes at room temperature, and subsequently overnight at -20°C. Fixed tissues were embedded in Paraplast as described previously. Sections were cut and used for immunofluorescence staining, as described above, or were stained with hematoxylin and eosin to observe tissue architecture.

RESULTS

Immunolocalization of 14 kDa and 16 kDa galectin in the chick mesonephric/gonadal complex.

In 4 day embryos, both C14 and C16 galectin proteins are expressed in the primordial germ cells (PGCs), which are in the process of colonizing the genital ridge (GR) (Fig. 6, A and B). In the 7 day embryo, after the PGCs have established themselves in the GR, PGCs cease to express the C14 and C16 galectin proteins; at this stage both galectins are found only in what appears to be ECM material of the gonad. The most intense staining for both galectins is found at the boundary between the gonadal cortex and medulla (Fig. 6, C and D). In subsequent development PGCs start to reexpress the 16 kDa galectin protein, as evidenced by the fact that in 10 day embryos PGCs have regained a diffuse cytoplasmic stain for the 16 kDa galectin (Fig. 6, F). The 14 kDa galectin protein in 10 day PGCs is not detectable by immunofluorescent staining (Fig. 6, E). Both galectins persist in the gonadal ECM.

Both galectin proteins are expressed already in the 4 day chick mesonephros (Fig. 7, A and B). Staining for C14 is predominantly cytoplasmic (Fig. 7, A) and this largely intracellular distribution persists through 10 days of incubation (Fig. 7, C and E). Immunofluorescence for C16 in the mesonephros of the 4 day embryos is largely cytoplasmic, and often is abundant in the basal cytoplasm. Galectin accumulations can be seen in many cells, giving the staining a granular appearance (Figs. 7, B;

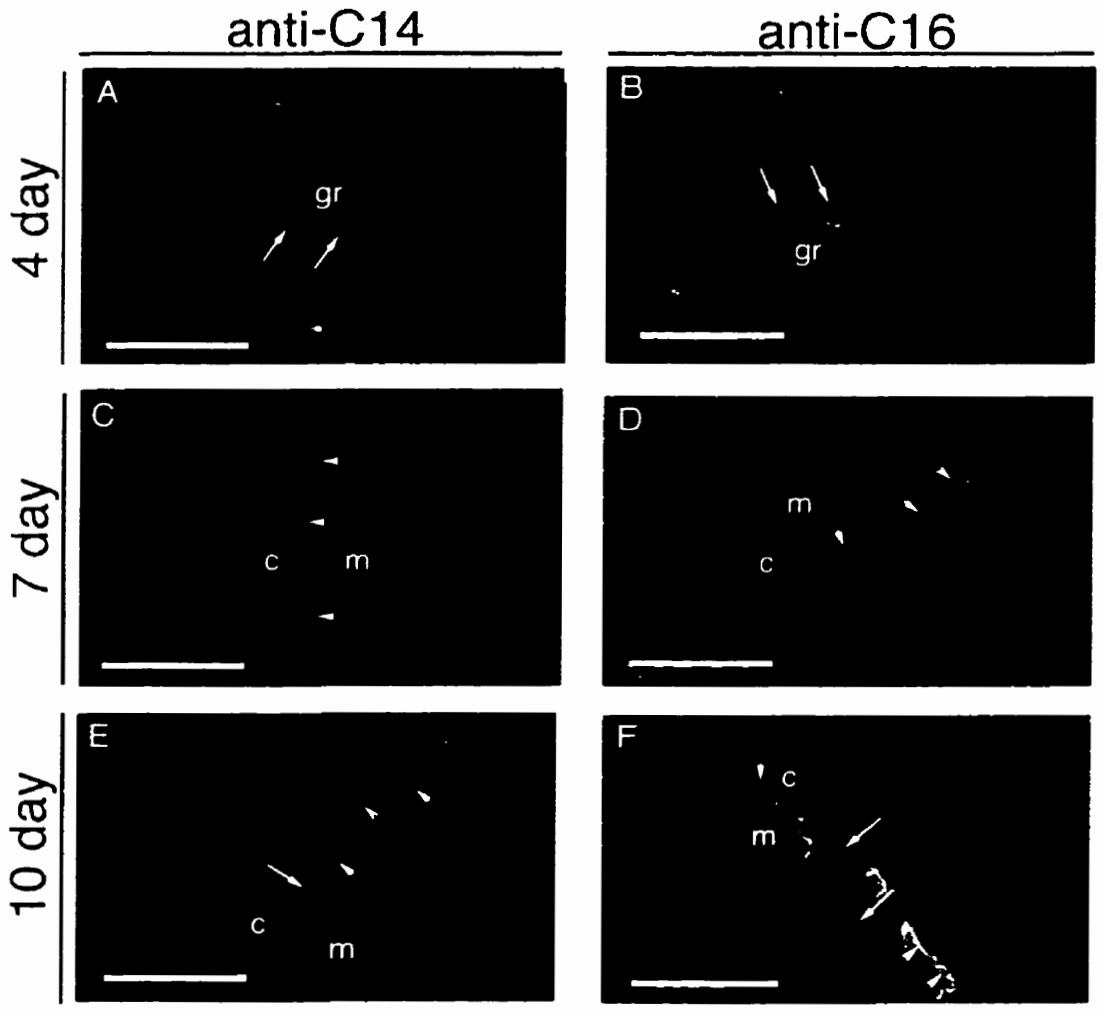


Figure 6. Immunofluorescence staining of C14 and C16 in the developing gonad. The anti-C14 antiserum K14 and anti-C16 antiserum D16 were used to detect these galectin proteins in the gonad of 4-10 day chick embryos. In the 4 day embryo (A,B) in the genital ridge (gr), which is the presumptive gonad, both C14 (A) and C16 (B) galectins are present in the cytoplasm of primordial germ cells (arrows) colonizing the genital ridge. By 7 days of incubation (C,D), galectin staining has disappeared from the primordial germ cells, which are located in the gonadal cortex (c). The 14 kDa galectin (C) can be seen in matrix material (arrowheads) at the boundary between cortex and medulla (m). C16 is also seen in this boundary ECM, and additionally is found in ECM in the medulla (D). In the 10 day gonad (E,F), C14 (E) is abundant in the ECM at the cortex/medulla boundary and is also seen in medullary ECM. The 16 kDa galectin (F) remains in the medullary ECM and is particularly abundant in the boundary ECM. In plate F, the C16-immunoreactive material in the upper right is dorsal mesentery. Scale bars = 50 μm.

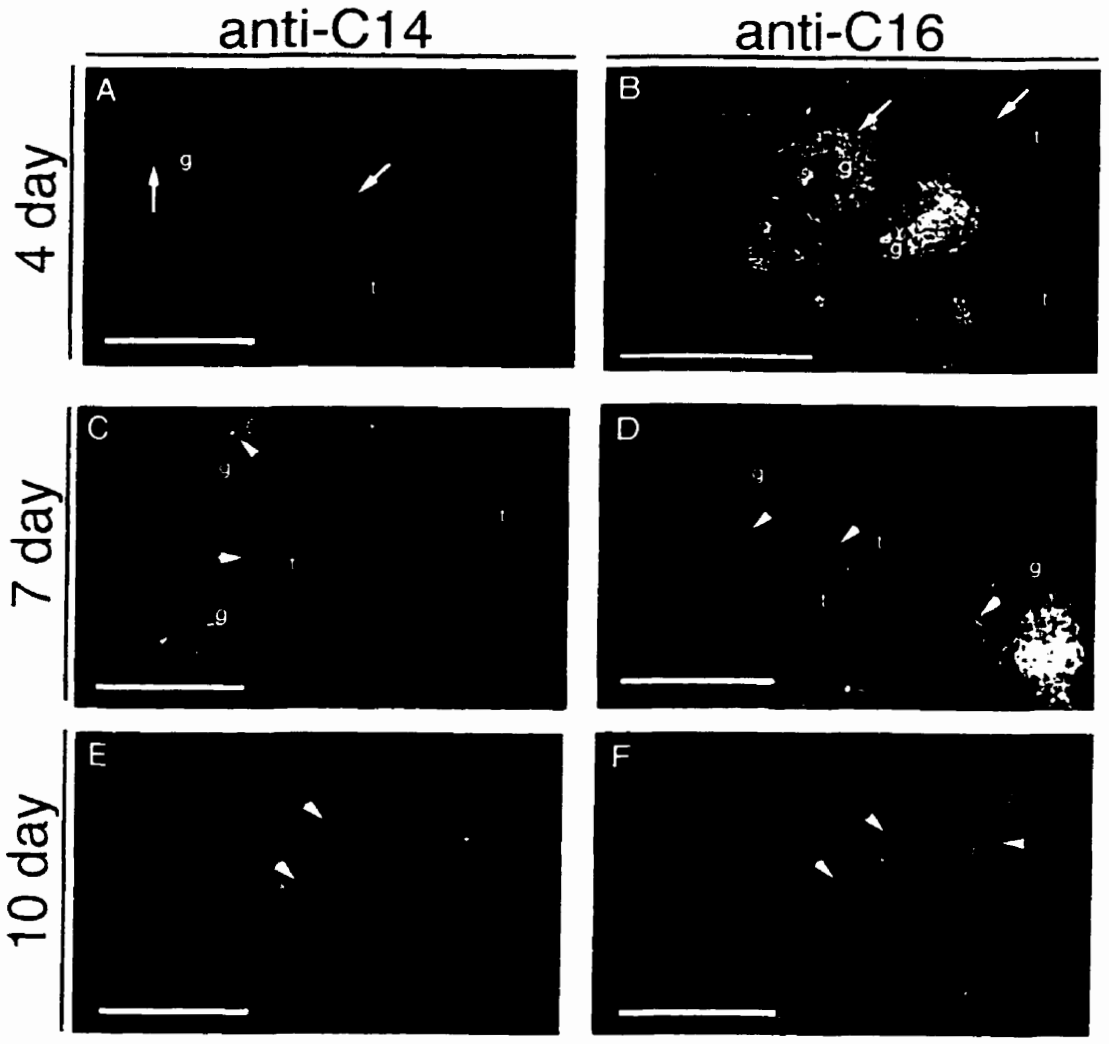


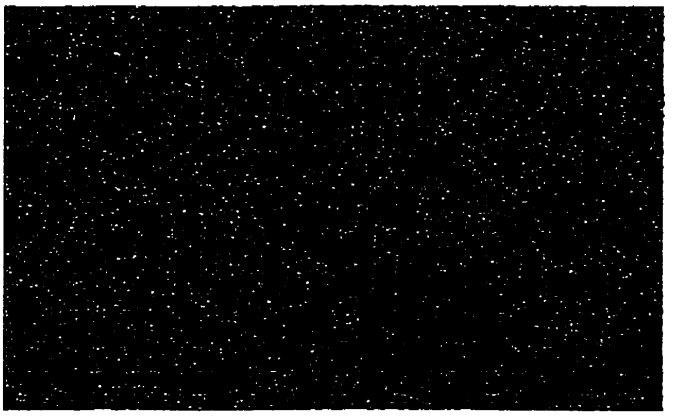
Figure 7. Immunofluorescence staining of C14 and C16 in the developing mesonephros. The anti-C14 antiserum K14 and anti-C16 antiserum D16 were used to detect these galectin proteins in the mesonephros of 4-10 day chick embryos. In the 4 day mesonephros (A,B), C14-immunoreactive material (A) is present in condensates (arrows) in the basal cytoplasm of tubule (t) cells, and in the glomeruli (g). At the same stage (B), C16-rich condensates (arrows) are abundant in the glomeruli (g). Cytoplasmic condensates are also present in the basal cytoplasm of tubule cells in the 4 day mesonephros. Yellow fluorescence in the glomeruli is due to autofluorescence of hemoglobin in erythrocytes present in the glomerular capillaries. By 7 days of incubation (C,D) C14 (C)is present in the cytoplasm of tubule cells and what appear to be podocytes in the glomerulus. The tubule basal laminae and GBM do not contain C14 galectin protein. In contrast, at 7 days C16 (D) is concentrated in tubule and glomerular basement membranes (arrowheads) and cytoplasmic immunoreactivity is faint. Cytoplasmic staining for C14 persists in tubule cells of the 10 day mesonephros (E). At this stage, C16 (F) is abundant in tubule basal laminae (arrowheads) and is not detected in tubule cell cytoplasm. Scale bars = 50 μm.

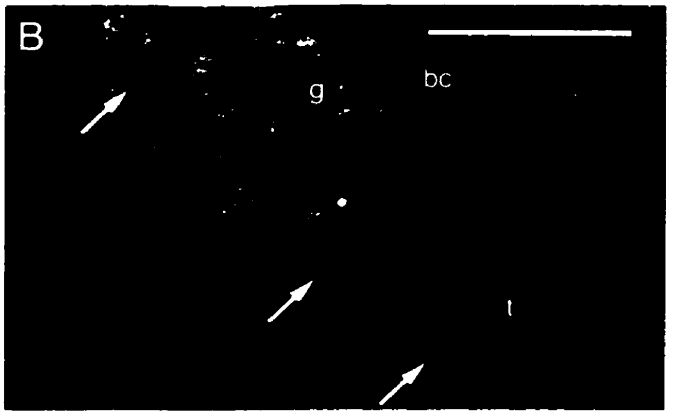
and 8, B). In particular, the podocytes of the developing glomeruli are intensely stained with the anti-16 kDa galectin antiserum (Fig. 7, B). However, as development proceeds, the 16 kDa galectin accumulates in the basement membranes surrounding mesonephric tubules and in the glomerular basement membrane of the 7 day embryonic mesonephros (Fig. 7, D). By 10 days of incubation, there is intense C16 immunoreactivity in the basal laminae of the tubules (Fig 7, F) and in the GBM (Fig. 12, B).

Detection of C16 mRNA in 4 day embryonic mesonephros

It has been suggested (Didier, et al. , 1988) that 16 kDa galectin present in the embryonic chick mesonephros is produced elsewhere in the embryo and travels through the blood until it becomes trapped in the GBM and tubule basement membranes. In the present study, *in situ* hybridization with a probe to the 16 kDa galectin mRNA transcript was used to determine if the C16 galectin in the mesonephric basement membranes was synthesized by the mesonephric tissue. In the 4 day embryo, C16 galectin message was found in the Wolffian duct, developing tubules, and developing glomeruli of the mesonephros (Fig. 8, A) suggesting that this galectin is, in fact, produced by the mesonephros itself. Close examination of the immunolocalization of C16 protein in 4 day mesonephros (Fig 8, B) reveals that this lectin is found in granular cytoplasmic inclusions in the podocytes, in the squamous epithelial cells lining the Bowman's capsule, and in the tubule cells.

Figure 8. Detection of C16 mRNA and immunofluorescence staining of C16-rich cytoplasmic condensates in the 4 day mesonephros. (A) Detection of the C16 message in distinct tissues of the 4 day chick embryo by *in situ* hybridization. C16 mRNA is present in the Wolffian duct (wd), in the developing glomeruli (g) and tubules (t) of the mesonephros, in the genital ridge (gr) and in the notochord (n). For comparison, C16 RNA was not detected in the dorsal mesentery (dm), dorsal aorta (da) and the surrounding mesenchyme. In (B), C16 immunoreactivity is present in cytoplasmic condensates (arrows) in podoctyes of the glomerular tuft, which is within the Bowman's capsule (bc). C16-rich condensates can also been seen in the basal cytoplasm of tubule cells (arrow). (C) is the same section as (B), stained with DAPI to visualize nuclei. Scale bar in (A) = 200 μm; in (B) and (C) = 50 μm.

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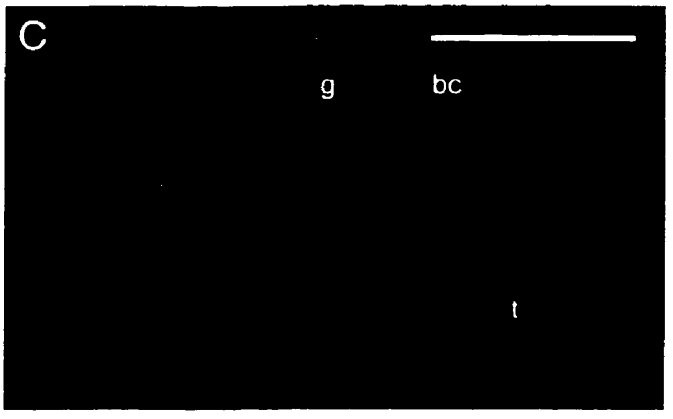
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SDS-PAGE and Western blot analysis of crude galectin extracts

Lactose-extracts of chick 10 day embryonic left gonad, right gonad, and mesonephros were separated by SDS-PAGE (Fig. 9, A) and transferred to nitrocellulose for immunoblot analysis using two anti-C16 polyclonal antisera, R10 and D16 (Table 4). For reference purposes, a similar extract from 5 day yolk sac, a rich source of both C16 and C14, was used as a positive control.

Analysis by SDS-PAGE of crude lactose-extracts from gonad and mesonephros (Fig. 9, A) reveals that the protein spectra of these extracts differ. In particular, a number of proteins with apparent molecular mass between 45- and 98 K are seen in the gonad/mesonephric extracts but are not seen in the yolk sac. Immunoblot analysis of nitrocellose transfers of SDS-PAGE-separated proteins from the same extracts was performed to confirm the presence of C16 (Fig. 9, B). The R10 antisera, raised against C16 from chick liver, recognizes a band with an apparent molecular mass of approximately 16 K in extracts from combined right and left gonad, mesonephros, and yolk sac, indicating the presence of C16 in those extracts. This antiserum is known to cross-react with the C14 galectin to some degree (Zalik, et al. , 1990) Thus the faint bands at 14 K in the immunoblot strips prepared from gonad and mesonephros extracts likely indicate the presence of C14 in those extracts. In the yolk sac extract R10, besides reacting with the 14 kDa galectin, also detects a protein of slightly

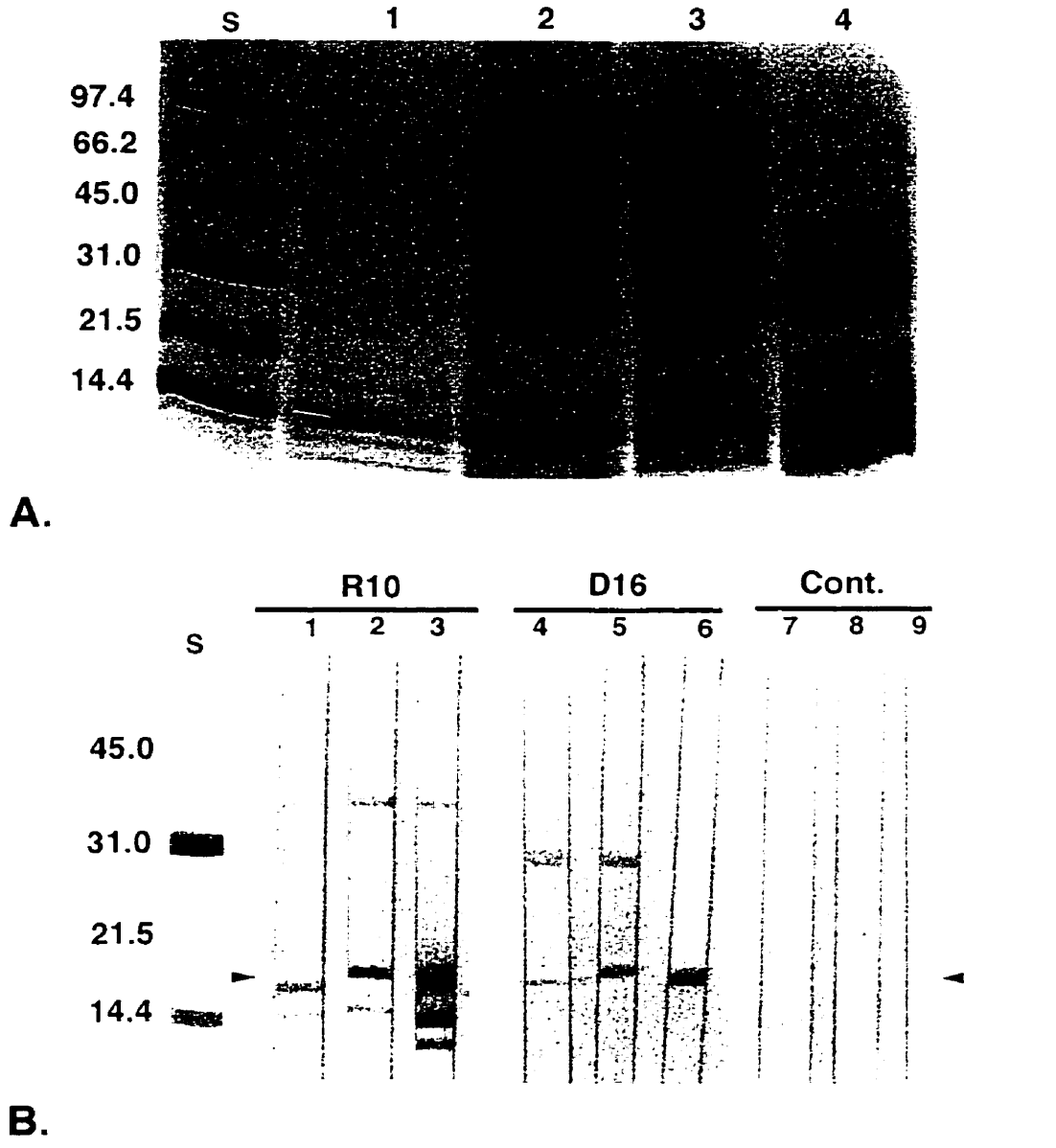
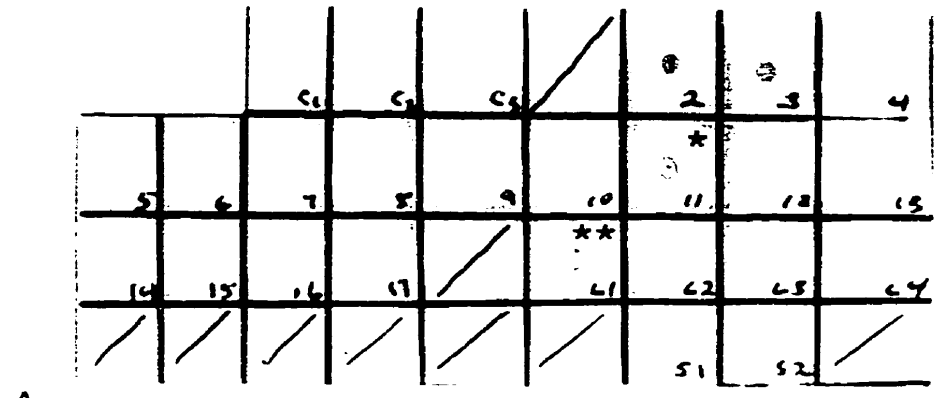


Figure 9. SDS-PAGE and immunoblot analysis of crude galectin extracts from left and right gonad, and mesonephros of 10 day chick embryos. In (A) crude extracts from 10 day left gonad (lane 1), right gonad (lane 2) and mesonephros (lane 3) were separated by SDS-PAGE and the resulting gel was stained to reveal proteins. For comparative purposes, a crude extract from 5 day chick yolk sac (lane 4) was also used. Protein molecular weight standards (in kDa) are in lane S. In (B) SDS-PAGE separated crude extracts were transferred to nitrocellulose and immunoblotted with the anti-C16 antisera R10 (lanes 1-3) and D16 (lanes 4-6). Normal rabbit serum (lanes 7-9) was used as a control. Lanes 1, 4 and 6 are a mixture of crude extracts from 10 day left and right gonad. Lanes 2, 5 and 8 are a crude extract from 10 day mesonephros. Lanes 3, 6 and 9 are an extract from 5 day yolk sac. Protein molecular weight standards (in kDa) are in lane S. Arrowheads indicate approximately 16 kDa on the blot. The slight difference in the location of the C16 immunoreactive band in the gonad and mesonephros are due to "smiling" in the gel (such curvature can be seen in A). The gonad extract was loaded in the centre of the gel while the others were more peripheral.

lower apparent molecular weight; this has been suggested to be an isoform of C14 (Guay & Zalik, 1994). The D16 antiserum, which was used for immunohistochemical studies, recognizes a single 16 K band in nitrocellulose transfers of extracts prepared from gonad, mesonephros, and yolk sac. It does not appear to cross-react with the 14 kDa galectin. In these transfers R10 and D16 react weakly with bands present at approximately 40 and 30 K respectively. Such reactivity has been observed before in galectin preparations (Guay, 1993) and may represent higher-order oligomers of the galectins or as-yet-uncharacterized proteins with some immunological identity with the galectins. Normal rabbit sera, used as a control, did not bind to any proteins in the extracts (Fig. 9, B).

Concentration of 16 kDa galectin in crude extracts of the mesonephros by batch and column affinity chromatography.

SDS-PAGE and immunoblot analysis of the crude galectin extract (above) demonstrated that the anti-16 kDa galectin antisera recognized a protein of the appropriate apparent molecular weight. We wished also to demonstrate that this protein had lactose/galactose-binding activity and thus was in fact a galectin. Lactose-affinity chromatography was used to concentrate lactose-binding proteins from crude galectin extracts of the mesonephros of the 10 day chick embryo. Two methods, batch and column affinity chromatography, were used. The presence of galectin in the different fractions was detected with R10 using dot blot analysis. A representative dot blot is shown in Fig. 10 (A). Dots marked C_1, C_2, and C_3



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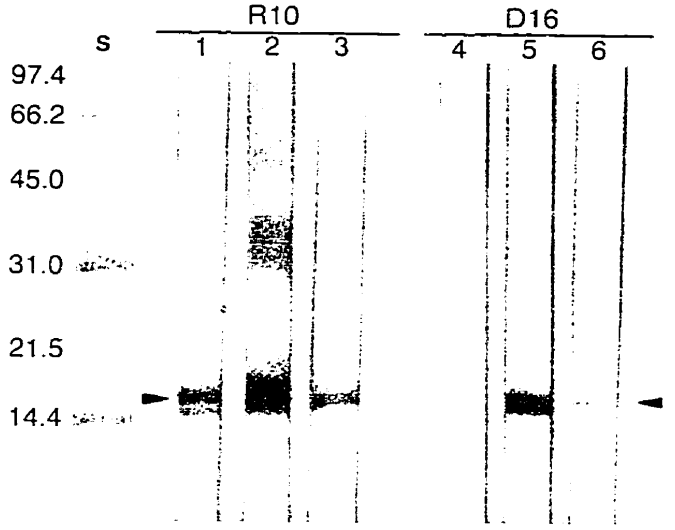


B.

Figure 10. Immunodot analysis of fractions obtained during partial purification of galectin in crude extracts from 10 day chick mesonephros and subsequent immunoblot analysis of those partially purified extracts after SDS-PAGE. (A) Lactose-binding proteins in crude galectin extract from 10 day mesonephros were concentrated by two affinity chromatography protocols. Dots C_1, C_2 and C_3 are controls consisting of MEPBS, bovine fetuin and ovalbumin, respectively. Dots 2-17 are fractions from batch affinity chromatography; fraction #11 (*) was reserved for SDS-PAGE and Western blotting analysis. Dots L_1-L_4 and S_1-S_2 are from column affinity chromatography. S_1-S_2 were sucrose-eluted fractions while L_1-L_4 were eluted with lactose. Fraction L_1 (**) was reserved for further analysis. (B) Partially purified extracts #11 and L_1, and crude galectin extract from 10 day chick mesonephros, were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the anti-C16 antisera R10 (lanes 1-3) and D16 (lanes 4-6). Lanes 1 and 4 are 10 day crude mesonephric extract. Lanes 2 and 5 are from fraction L_1; lanes 3 and 6 are from fraction #11. Protein molecular weight standards (in kDa) are shown in lane S.

are controls consisting of MEPBS, bovine fetuin, and egg albumin, respectively. Dots marked with numbers 2-17 are fractions obtained from batch affinity chromatography. In these batch fractions, galectin immunoreactivity was found in fractions 2-8, obtained after washing the beads with MEPBS and 300 mM sucrose solution. Galectin immunoreactivity in these fractions indicates that some mesonephros galectin was bound non-specifically to the derivatized beads, and could be removed by washing with MEPBS and sucrose. The antiserum did not detect galectin in fractions 9 and 10, indicating that non-specifically bound galectin had been cleared from the beads by that time. The immunoreactive fraction number 11 (Fig. 10, A; marked with *) is galectin specifically eluted from the beads by application of lactose. This fraction was reserved for subsequent analysis by SDS-PAGE and Western immunoblotting.

The results of the partial purification of the galectins using column lactose-affinity chromatography are shown in fractions L_1-L_4 and S_1-S_2 in Figure 10 (A). Dots labeled S_1 and S_2 are fractions eluted after the application of sucrose to remove non-specifially bound proteins, while dots L_1-L_4 are fractions obtained after elution with lactose. Fraction L_1 (**) was reserved for analysis by SDS-PAGE and immunoblotting.

To confirm the presence of the 16 kDa galectin, immunoreactive fractions from column (L_1) and batch affinity chromatography (#11) (Fig 10, A), as well as a sample of crude lactose-extract from 10 day

mesonephros, were separated by SDS-PAGE and transferred to nitrocellulose for Western blot analysis using the R10 and D16 antisera (Fig. 10, B). The R10 antiserum, which is very sensitive for immunoblotting, recognized a band of approximately 16 K (apparent molecular weight) in crude mesonephros (strip 1) and both affinity-concentrated extracts (strips 2 and 3). The D16 antiserum, which is less sensitive than R10 for immunoblotting, did not detect the 16 kDa galectin in this crude extract (strip 4) but did react with 16 K species in the concentrated samples (strips 5 and 6). Since total protein loaded in each lane was equalized, these results indicate that the proportion of C16 was enriched considerably in fraction L_1, and to a lesser extent in fraction #11. This indicates that the proteins detected in the Western blot and in immunohistochemical studies have lactose-binding activity and are indeed galectins.

Colocalization of C16 and two potential ligands in the mesonephros.

Fibronectin and laminin have been suggested as endogenous galectin ligands (Cooper, 1997; Ozeki, et al. , 1995). To determine the localizaton of these matrix glycoproteins in the mesonephric/gonadal complex, sections from 4-10 day embryos were immunostained with the anti-C16 galectin polyclonal antiserum D16 as well as with monoclonal antisera to avian fibronectin (B3/D6) and avian laminin (31-2) (Table 4).

In the 6 day embryo, gonadal expression of the C16 galectin protein is present in matrix material in the medulla and is also seen in the ECM

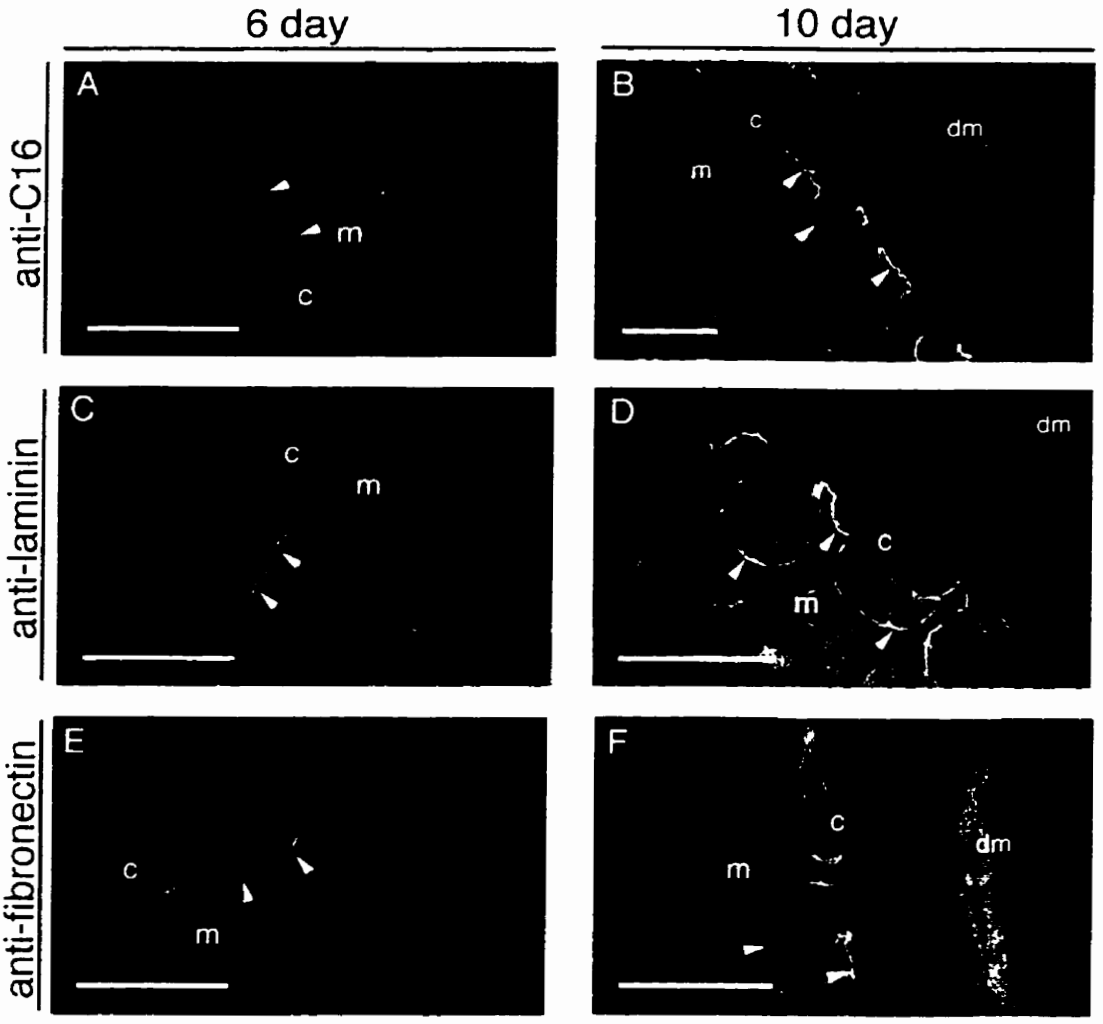


Figure 11. Immunofluorescence staining of C16 galectin and the potential galectin ligands laminin and fibronectin in the developing gonad of 6 and 10 day chick embryos. The antisera D16, 31-2, and B3/D6 were used to detect C16, laminin, and fibronectin, respectively. In the 6 day gonad (A, C and E), C16 (A) is present in matrix material throughout the medulla (m) and at the boundary (arrowheads) between cortex (c) and medulla. At this stage, laminin (C) and fibronectin (E) are also located in ECM at the cortex/medulla boundary. By 10 days of incubation (B, D and F), C16 protein (B) is abundant in the medullary and boundary ECM, where laminin (D) is also located. Fibronectin (F) is prominent in the ECM at the cortex/medulla boundary while a small amount of fluorescence is present in the medulla. (dm, dorsal mesentery) Scale bars = 50 μm.

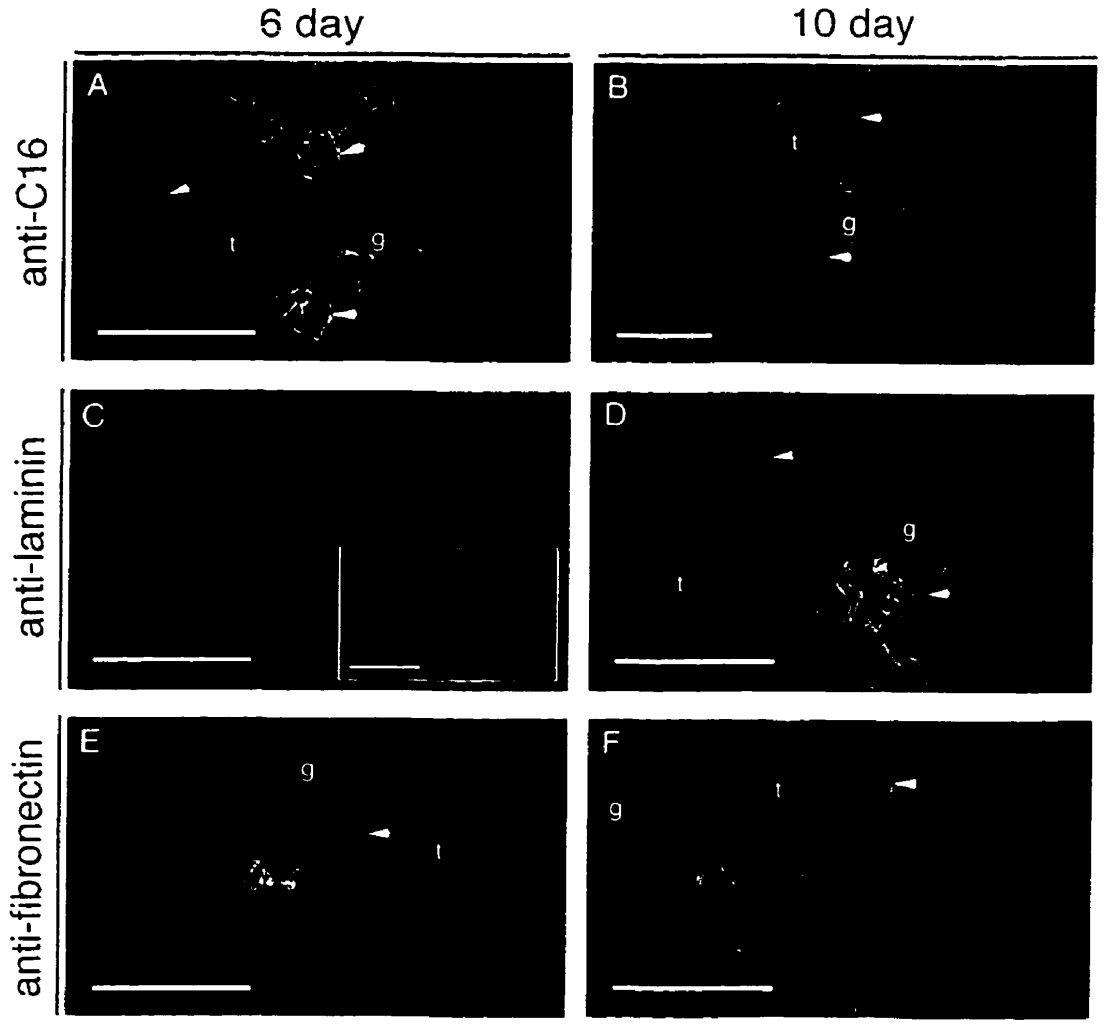


Figure 12. Immunofluorescence staining of C16 galectin and the potential galectin ligands laminin and fibronectin in the developing mesonephros of 6 and 10 day chick embryos. The antisera D16, 31-2, and B3/D6 were used to detect C16, laminin, and fibronectin, respectively. In the 6 day embryo (A, C and E), C16 (A) is abundant in basal laminae (arrowheads) of mesonephric tubules (t) and in the GBM (arrowheads) of the glomerulus (g). (C) is a timed exposure of 6 day mesonephric tissue stained with anti-laminin antisera. The photograph was exposed for the same length of time as (D), which was immunostained in the same experiment. The inset in (C) is DAPI staining to visualize nuclei in that section. Fibronectin immunoreactivity (E) is present in tubule basal laminae (arrowhead) and in the centre of the glomeruar tuft, in matrix material which is assumed to be mesangial matrix. At 10 days (B, D and F), D16 (B) and laminin (D) are localized in the tubule basal laminae and GBM (arrowheads). Fibronectin (F) persists in tubule basal laminae (arrowhead) and in glomerular matrix material; in addition some is present in tubule cell cytoplasm. (g, glomerulus; t, tubule) Scale bars = 50 μm.

found at the boundary between cortex and medulla (Fig 11, A). At this stage of development laminin and fibronectin have the same localization as the C16 galectin at the medulla/cortex boundary (Fig 11, C and E). As development proceeds the spatial distribution of C16, laminin, and fibronectin in the gonad does not change. By 10 days of incubation, there is intense immunoreactivity for all three proteins at the boundary between cortex and medulla (Fig 11, B, D and F)), and less intense staining for C16, laminin, and fibronectin in the medulla. The 16 kDa galectin and fibronectin can also be seen in the dorsal mesentery at this stage of development.

In the 6 and 10 day mesonephros, C16 is found in the basement membrane surrounding the tubules and in the GBM (Fig. 12, A and B). In the mesonephros of the 6 day embryo, fibronectin is present both in the apical cytoplasm of tubule cells and in the basal lamina of tubules and glomeruli (Fig. 12, E). By 10 days of mesonephros development, the cytoplasmic content of fibronectin has increased and some remains in the basement membranes (Fig. 12, F). It was not possible to detect the presence of laminin in the 6 day mesonephros. A photograph of laminin immunofluorescence in the 6 day mesonephros, with the film exposed for the same length of time as required to photograph 10 day laminin immunofluorescence in the mesonephros, is shown in Figure 12 (C); it appears black. Detectable laminin immunostaining appears in the mesonephros around 7 days of incubation (not shown) and by 10 days this

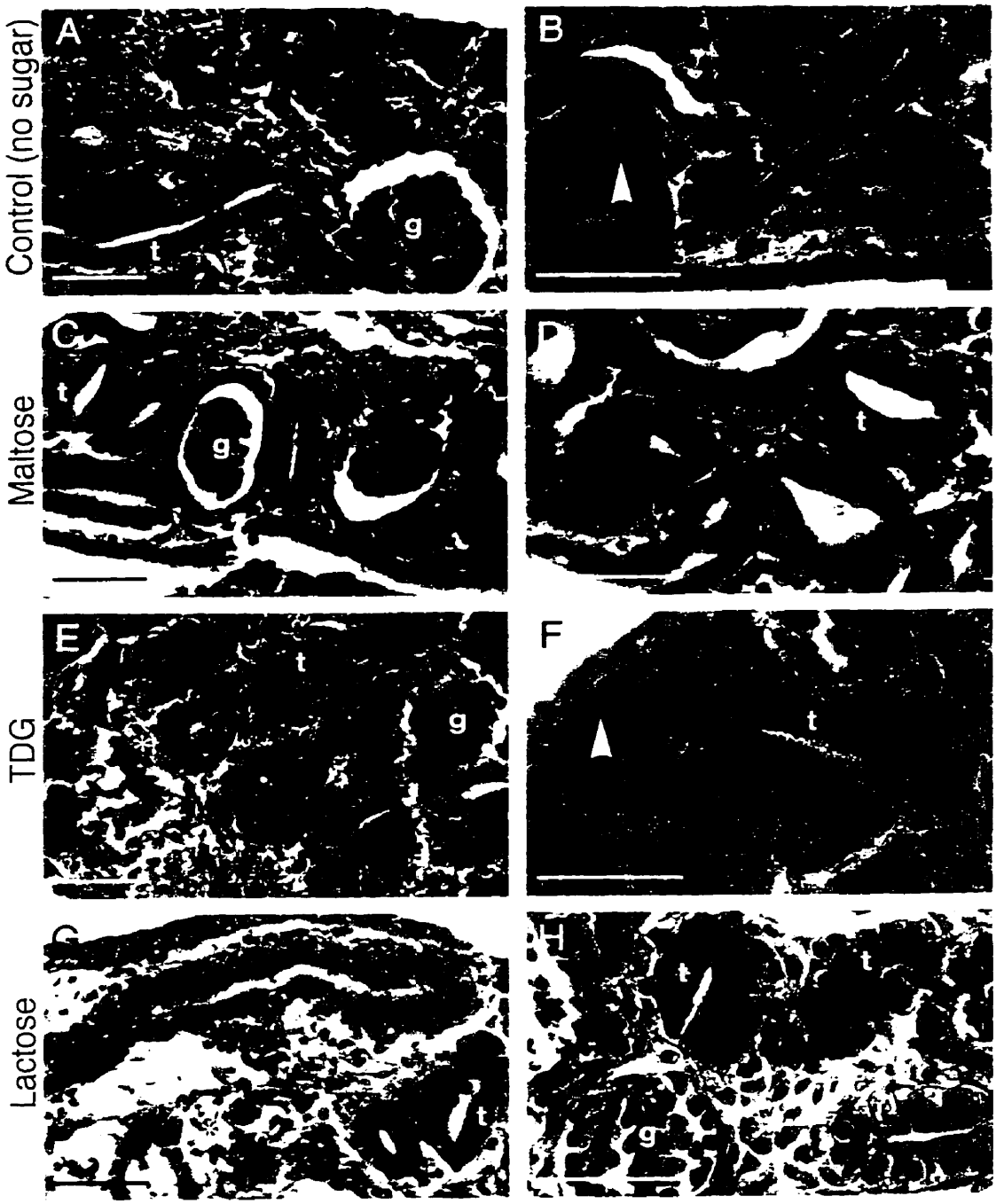
protein is found exclusively in tubule basal laminae and GBM (Fig. 12, D), and has the same localization as the 16 kDa galectin (Fig. 12, B).

Histology of the mesonephros under culture conditions

In order to test the effect of galectin hapten-inhibitors in the maintenance of mesonephric epithelial differentiation, an *in vitro* system for the culture of the chick mesonephros in a defined medium had to be developed. Isolated mesonephroi from stages 25-26 (Hamburger & Hamilton, 1951), or approximately 5 days of incubation, were cultured in media with various components. It was necessary to assay tissue viability in each of these culture conditions, so tissues were fixed and processed for histological staining with hematoxylin and eosin to reveal tissue architecture. A defined medium containing a high concentration of conalbumin sustained mesonephric tissue for up to 3 days of culture with a reasonable preservation of tissue morphology. Tissues cultured for 48 hours in this medium are shown in Figure 13 (A and B). In such tissues, nephric tubules and glomeruli appeared similar to those *in vivo*. Tubule cells are cuboidal and typically have basal nuclei. Glomerular tufts are compact, and the Bowman's capsule is well-defined. In addition, this medium supported continued cell division as evidenced by the presence of mitotic figures (Fig. 13, B, arrowhead).

Next, mesonephric tissue was cultured in the high-conalbumin medium with the addition of maltose, TDG, or lactose. Addition of

Figure 13. General histological appearance of mesonephros cultured for 48 hours in the absence of added sugars or in the presence of maltose, TDG or lactose. Sections from cultured mesonephroi were stained with hematoxylin and eosin to show tissue architecture. Mesonephric tissue cultured without added sugar (A, B) or in the presence of maltose (C, D) maintained its tissue architecture as demonstrated by the general organization of the glomeruli (g) and the mesonephric tubules (t). Tubule cells are cuboidal and nuclei are generally restricted to the basal cytoplasm. A mitotic figure is shown in B (arrowhead). In contrast, tissue cultured in the presence of TDG (E, F) or lactose (G, H) has a "disorganized" appearance. More specifically, many tubules are not clearly demarcated from the surrounding mesenchyme. Tubule cells are misshapen and often appear to have elongated into the lumen, obstructing the latter. Tubule epithelium has largely become pseudostratified; that is, nuclei are not restricted to basal cytoplasm. In addition, in cultures with hapten inhibitors of the galectins areas of cell death can be seen (*). This cell death is not due to generalized toxicity of those hapten inhibitors because cell division continues (arrowhead in F indicates a cell in metaphase). Scale bars = 50 μm.


Control (no sugar)
Maltose
TDG
Lactose
A
B
C
D
E
F
G
H
t
g

maltose to the medium (Fig. 13, C and D) did not result in any noticeable changes in tissue architecture when those tissues are compared to cultures without added saccharides (Fig. 13, A and B). Mesonephric tissue cultured in the presence of the galectin hapten-inhibitors TDG (Fig. 13, E and F) or lactose (Fig. 13, G and H) display a general "disorganization" of tissue architecture. Many nephric tubules in such cultures have lost the lumen. Tubule cells, in general, are not cuboidal in shape as is characteristic for renal epithelial cells. In addition, tubule cell nuclei are often displaced from the basal cytoplasm; this effect is termed pseudostratification. Finally, in cultures with TDG and lactose, areas of cell death which were not observed in control or maltose-treated cultures, are present (Fig. 13, E and G, *). In other areas of those same tissues, mitotic figures can be seen (Fig 13, F, arrowhead), indicating that TDG and lactose are not inherently cytotoxic.

The presence of hapten inhibitors of galectins had a distinct effect on the structural organization of the glomerular tuft. In control tissues cultured without added sugars (Fig. 14, A) or with the addition of maltose (Fig. 14, B), the glomerular tuft is compact. Within the tuft, darkly stained podocyte cell bodies can be seen in close association with the non-cellular material of the GBM. The cytoplasmic protrusions of podocyte foot processes cannot be distinguished from the GBM in these photographs. In TDG- (Fig. 14, C) and lactose-treated (Fig. 14, D) cultures, many glomeruli appear to have been disrupted by those galectin inhibitors. In these

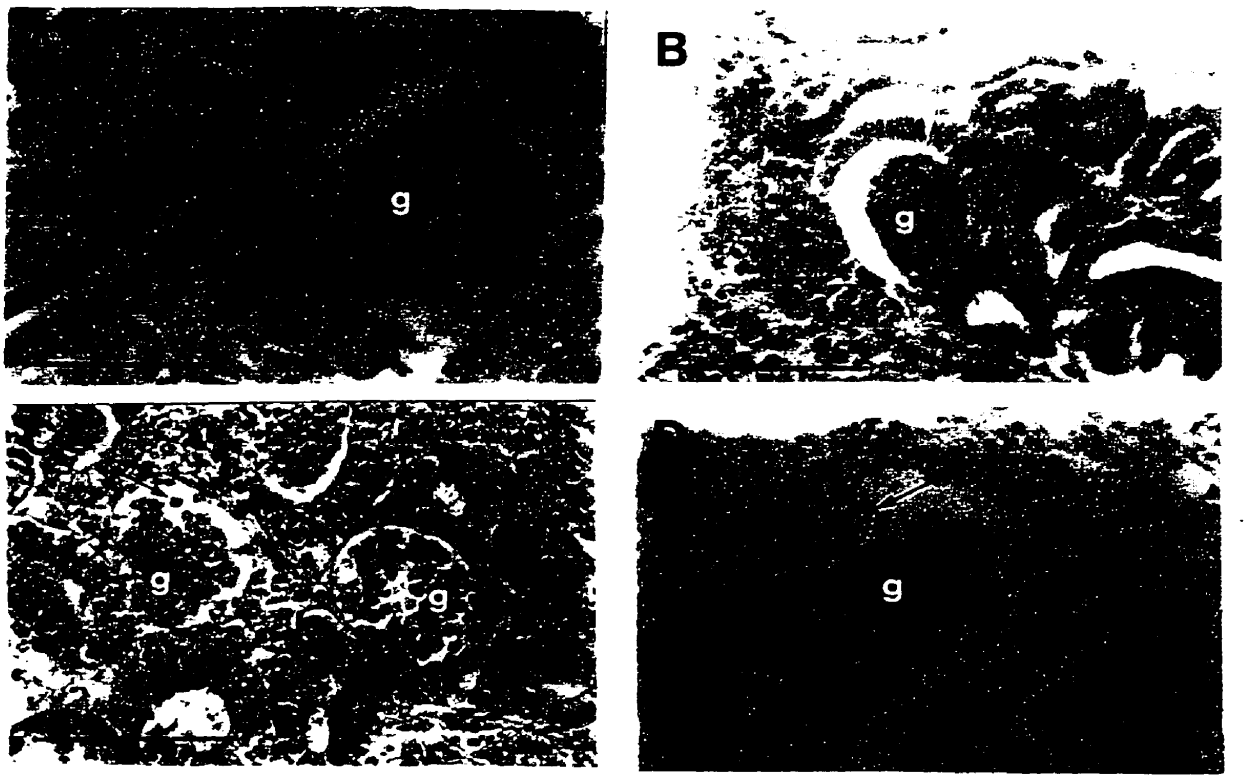


Figure 14. Histological appearance of glomeruli in mesonephric tissue cultured for 48 hours in the absence of added sugars or in the presence of maltose, TDG or lactose. Sections from cultured mesonephroi were stained with hematoxylin and eosin to show tissue architecture. Glomeruli in tissues cultures without added sugar (A) or in the presence of maltose (B) are shown. Note that the glomerular tuft is compact and that the podoctyes are closely associated with the GBM. In contrast, many glomeruli in tissues cultured with TDG (C) or lactose (D) have a disrupted appearance. Namely, the glomerular tuft is not compact as in control cultures (A and B) and podoctyes can be seen that appear to have lifted off of the GBM (arrows). (g, glomerulus; t, tubule) Scale bars = 50 μm.

disrupted glomeruli, the glomerular tuft has a ragged appearance, and in many cases podocytes can be seen that have no visible connection to the GBM (Fig. 14, C and P, arrows).

Localization of basement membrane proteins in cultures of mesonephric tissue

To test the effect of sugar hapten inhibitors in mesonephros differentiation, nine different mesonephric explants, from 3 separate culture experiments, were reserved for immunofluorescent staining using a variety of antisera to basement membrane proteins and, later, to marker molecules. In control, sugar free, cultures, or in cultures incubated in the presence of maltose, the basement membrane localization of the 16 kDa galectin, fibronectin, and laminin was not noticeably different from that of uncultured embryos (not shown). Although the application of lactose or TDG to the cultured mesonephros was hypothesized to disrupt cell adhesion to the basal laminae, C16, fibronectin, and laminin were found in ECM material, particularily in basal laminae and the GBM.

In cultures without added sugar or with the addition of maltose, staining of the GBM for C16, laminin and fibronectin typically appeared as sharp "lines" of acellular material between rows of podocytes; representative GBM (arrowhead) immunoreactivity for C16 is shown in Figure 15 (A). In cultures treated with TDG (Fig. 15, C to F) or lactose (not shown), immunoaccessible material in the GBM appears as an amorphous

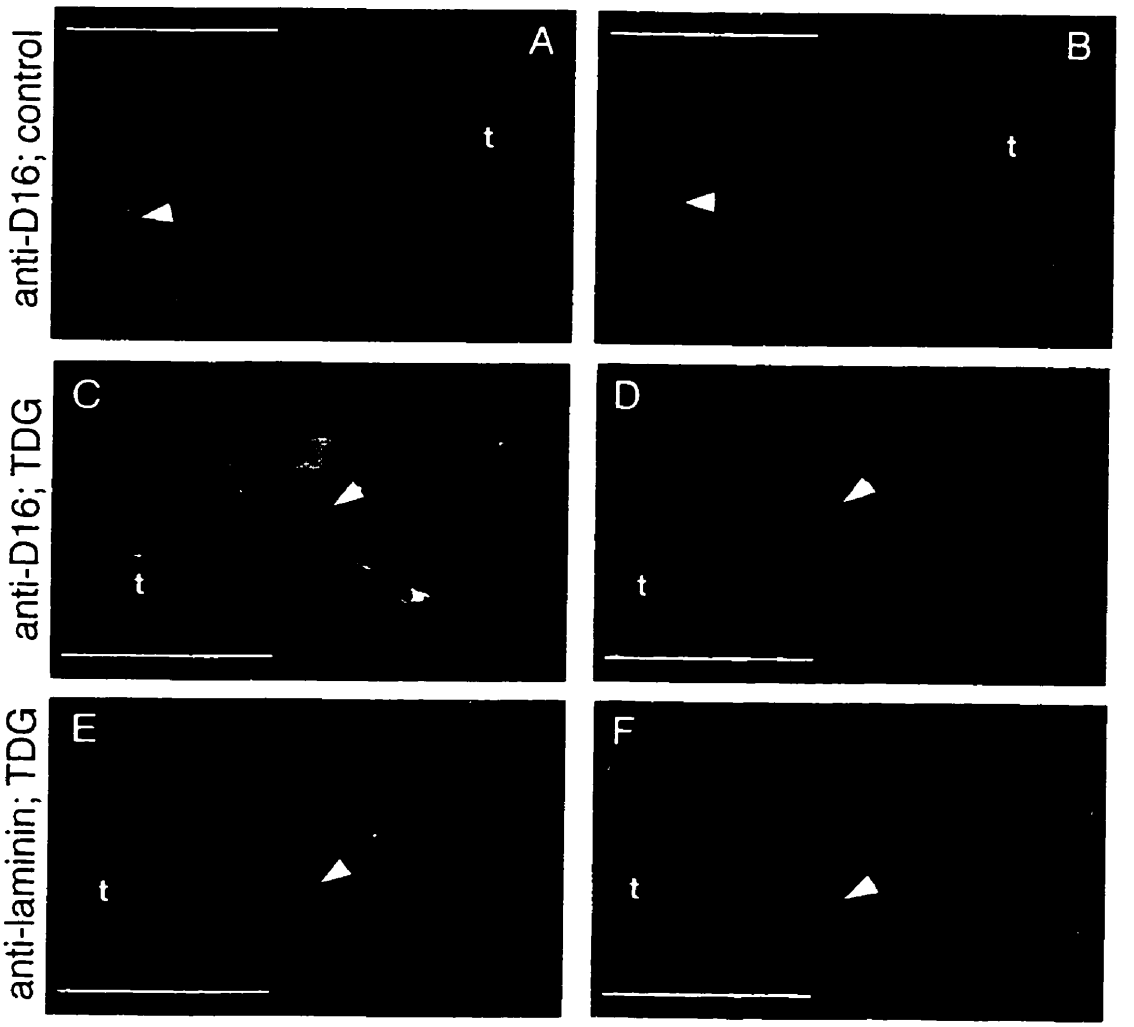


Figure 15. Immunofluorescence staining of C16 and laminin in the GBM of cultured mesonephric tissue. The antisera D16 and 31-2 were used to detect C16 and laminin, respectively. DAPI was used to visualize nuclei (B, D and F). In (A), which shows a control sugar-free culture stained for D16, the typical appearance of the GBM (arrowhead) in control cultures is shown. In (B) the acellular space (arrowhead) occupied by the GBM can be seen. Note that staining for C16 is restricted to a sharp thick line in the centre of the acelluar space; presumably only the cut edge of the GBM is accessible to antisera, as the majority of GBM surface area is covered by podocyte foot processes. In TDG-treated (C-E) cultures, the GBM appears as an amorphous aggregate when stained for C16 (C). Comparing (C) with (D), note that C16-immunoreactive material, some of which is out of the plane of focus, appears to fill a larger acellular space (D, arrowhead). This suggests that more GBM surface area is accessible to the antisera in cultures treated with galectin inhibitors than in control cultures. A similar appearance of the GBM ispresent in lactose-treated cultures. (E) is a serial section with (C), showing laminin immunoreactivity in the GBM. Again, some laminin staining is below the plane of focus, indicating that more GBM material is accessible to the antisera. Scale bars = 50 μm.

aggregate that largely fills the acellular space. This effect could be due to the exposure of more GBM surface area due to the retraction of podocyte foot processes.

In vivo expression of molecular markers of mesonephros differentiation

Changes in histological appearance are valid criteria to determine the effect of galectin hapten inhibitors on mesonephric differentiation. However, it was important to have additional criteria to determine whether the above incubations affected specifically the epithelial or mesenchymal phenotype of mesonephric cells. To that end, a search for molecular markers was undertaken. I tested a number of antisera, on sections from 5 to 7 day embryos, that would allow for the detection of proteins characteristic of epithelia. These included components of cell-cell junctional complexes (desmocollin, desmoplakin, and zonula occludens-1), cellular receptors for the ECM (integrin a_6 subunit, syndecan), epithelial-type intermediate filaments (keratin, pan-cytokeratin), and a cytoskeletal/ion pump linking protein called ankyrin (kidney-specific ankyrin A3). When used for immunostaining, under conditions where anti-C16, anti-laminin, and anti-fibronectin antisera stained well, none of these antisera detected proteins in the mesonephric epithelia. This was not suprising since many of the antisera were raised to mammalian proteins and may not recognize avian proteins. However, antisera to avian E-cadherin (R701), chicken vimentin (H5), avian Na/K-ATPase

Table 5. Localization of marker molecules in 5 and 7 day embryonic mesonephros.

Marker	Day	Tubule Cells	Glomerular Podocytes	Mesenchymal Cells
E-cadherin	5	cytoplasm; some concentrated on cell membranes	not present	not present
	7	basolateral cell membranes	not present	not present
vimentin	5	basal cytoplasm (faint)	cytoplasm (intense)	cytoplasm (intense)
	7	not present	cytoplasm (intense)	cytoplasm (intense)
α–actinin	5	cytoplasm (faint in some tubules, intense in others)	cytoplasm	cytoplasm
	7	cytoplasm (accumulations in apical cytoplasm of putative proximal tubule cells)	cytoplasm	cytoplasm
Na^+/K^+ ATPase	5	basolateral membrane of cells of some tubules	not present	not present
	7	basolateral membranes of distal tubule cells (intense)	not present	not present

(β4/B4), and α-actinin (α-act) detected these proteins. The use of these antisera allowed for studies on changes in expression pattern of those proteins during development of the epithelial phenotype in the chick mesonephros (Figs. 16 and 17). A summary of the localization of these proteins is given in Table 5.

E-cadherin is the epithelial-specific cadherin and is restricted to epithelial cells. In contrast, the intermediate filament vimentin is expressed by mesenchymal cells and is absent in epithelial cells, which

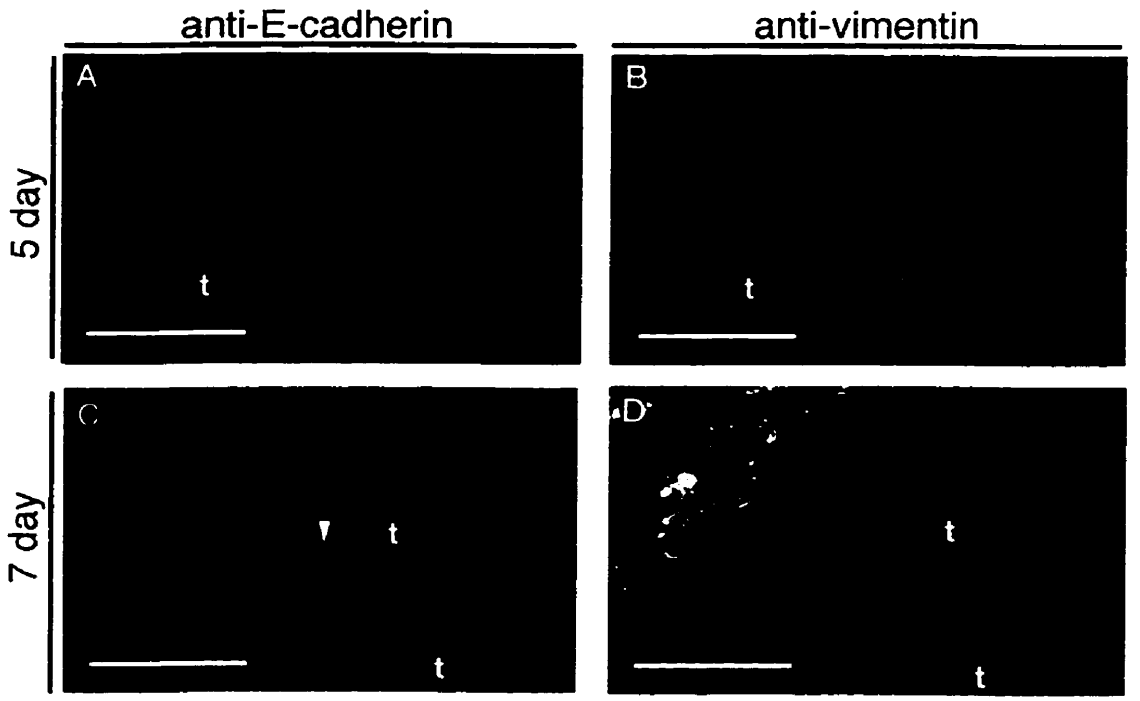


Figure 16. Double-staining immunofluorescence detection of E-cadherin and vimentin proteins in sections of mesonephros from 5 and 7 day embryos. The antisera R701 and H5 were used to detect E-cadherin and vimentin, respectively. In the 5 day mesonephros (A and B), E-cadherin (A) is present as a diffuse cytoplasmic stain with some concentration in the membrane of tubule (t) cells. In the same section, vimentin protein (B) is found in the cytoplasm of tubule cells, and in mesenchymal cells of the nephric interstitium. By 7 days (C and D), E-cadherin (C) is abundant in the lateral membrane of tubule cells (arrowhead). In the same section, vimentin (D) is restricted to mesenchymal tissue and is no longer seen in tubule cells. The vimentin-rich structure in the upper left of (D) is dorsal mesentery. Scale bars = 50 μm.

instead express keratin in their intermediate filaments. Thus the pattern of E-cadherin and vimentin expression may be used as a gauge of the state of differentiation in the mesonephros. In the 5 day mesonephros, the epithelial marker E-cadherin is present in developing tubules both in the cytoplasm and on basolateral and apical cell surfaces (Fig. 16, A). E-cadherin protein is not expressed by the podocytes of the glomerulus (not shown). By 7 days, E-cadherin is restricted largely to lateral cell surfaces of mesonephric tubules (Fig. 16, C). Vimentin is expressed intracellularily in mesenchyme, in the developing tubule cells (Fig. 16, B), and in the presumptive podocytes of the 5 day mesonephros (not shown). In the 7 day mesonephros, vimentin is restricted to mesenchyme (Fig. 16, D) and glomerular podocytes (not shown). Vimentin is not found in tubule cells which have a discrete basolateral distribution of E-cadherin, that is, in those tubules whose epithelial cells are fully differentiated.

The Na/K-ATPase is reportedly found predominantly on the basolateral cell surface of distal tubule and collecting duct cells of the mammalian kidney (Tiedemann, 1983; Madsen & Tisher, 1986). In the chick mesonephros, at 5 days of incubation, weak Na/K-ATPase immunoreactivity is seen on the basolateral surface of many tubules (Fig. 17, B). By 7 days of incubation only a subset of tubules express Na/K-ATPase, and stain very intensely along the basolateral cell surface (Fig. 17, D); these tubules were assumed to be distal tubules because of the presence of the ATPase. Alpha-actinin is an actin-binding protein (Brown & Stow,

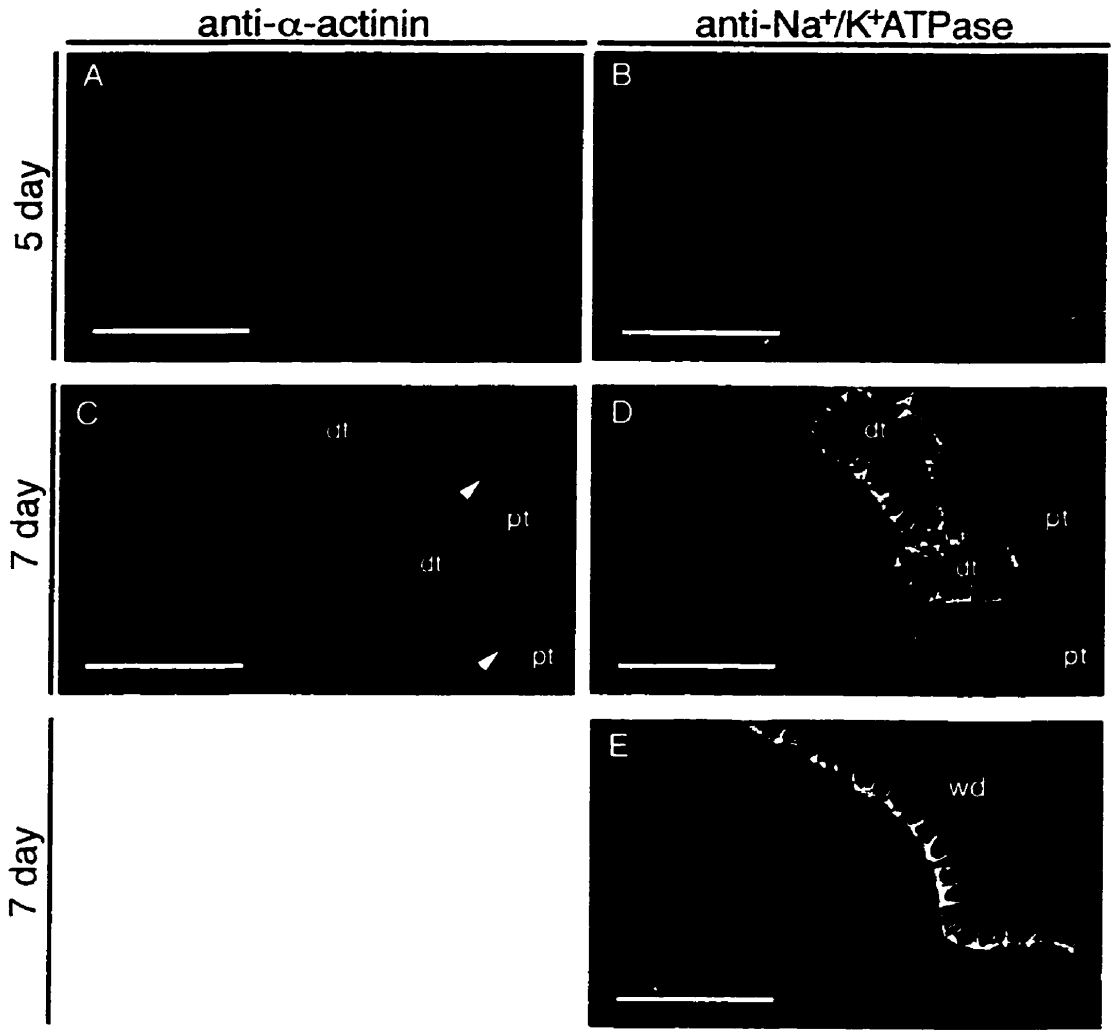


Figure 17. Double-staining immunofluorescence detection of α-actinin and Na^+/K^+ ATPase proteins in sections of the embryonic mesonephros. The antisera α-ACT and β4/B4 were used to detect α-actinin and Na^+/K^+ ATPase, respectively, in the mesonephroi of 5 and 7 day chick embryos. In the 5 day mesonephros (A and B), α-actinin (A) is found in the cytoplasm of all tubules, but is abundant in a subset of those tubules. In the same section, faint Na^+/K^+ ATPase immunoreactivity (B) is seen in the basolateral membrane of some tubules. Note that tubules rich in α-actinin in (A) are not Na^+/K^+ ATPase-positive in (B). At 7 days of incubation (C and D), staining for α-actinin (C) persists in all tubules; a subset of tubules have an apical concentration of this protein (arrowheads). In the same section, intense Na^+/K^+ ATPase staining (D) is found in a subset of tubules which are assumed to be distal tubules (dt). Note that Na^+/K^+ ATPase-positive tubules do not have an apical accumulation of α-actinin and thus tubules with such an apical concentration of the latter protein are assumed to be proximal tubules (pt). In (E), a cross-section of the 7 day mesonephros is shown. The non-symmetrical distribution of Na^+/K^+ ATPase among the cells of the Wolffian duct (wd) is evident. The medial face of this duct (left), adjacent to the secretory tubules of the mesonephros, is Na^+/K^+ ATPase-positive; in the more lateral face of the Wolffian duct (upper right) Na^+/K^+ ATPase is not detectable. Scale bar = 50 μm.

1996) whose distribution, I felt, might change along with the cytoskeletal reorganization that takes place during epithelial differentiation (Mays, et al. , 1994; Bacallao, 1995). In the 5 day mesonephros, α-actinin was found in most mesonephric tubules with a diffuse intracellular distribution; some tubules stained very intensely for this protein (Fig. 17, A). In the 7 day mesonephros, most tubules retained a diffuse cytoplasmic distribution of α-actinin, but a subset of tubules had accumulations of this protein in the apical cytoplasm (Fig. 17, C). Tubules with an apical concentration of α-actinin were never Na/K-ATPase-positive, suggesting that, in the chick mesonephros, apical accumulation of this actin-binding protein might be characteristic of proximal tubule cells. Since Na/K-ATPase is found in distal and collecting tubules (Tiedemann, 1983; Madsen & Tisher, 1986), this enzyme and α-actinin could be considered markers of nephron segmentation into proximal and distal tubule elements.

An interesting feature of the expression pattern of Na/K-ATPase was noted. The Wolffian duct, which is part of the collecting system in the mesonephros, had a non-symmetrical distribution of ATPase (Fig. 17, E). In cross section the medial face of the duct, that is, the side of the duct adjacent to the mesonephric interstitium, was always Na/K-ATPase-positive. The outer face of the Wolffian duct, next to the connective tissue that encapsulates the mesonephros, did not have ATPase immunoreactivity.

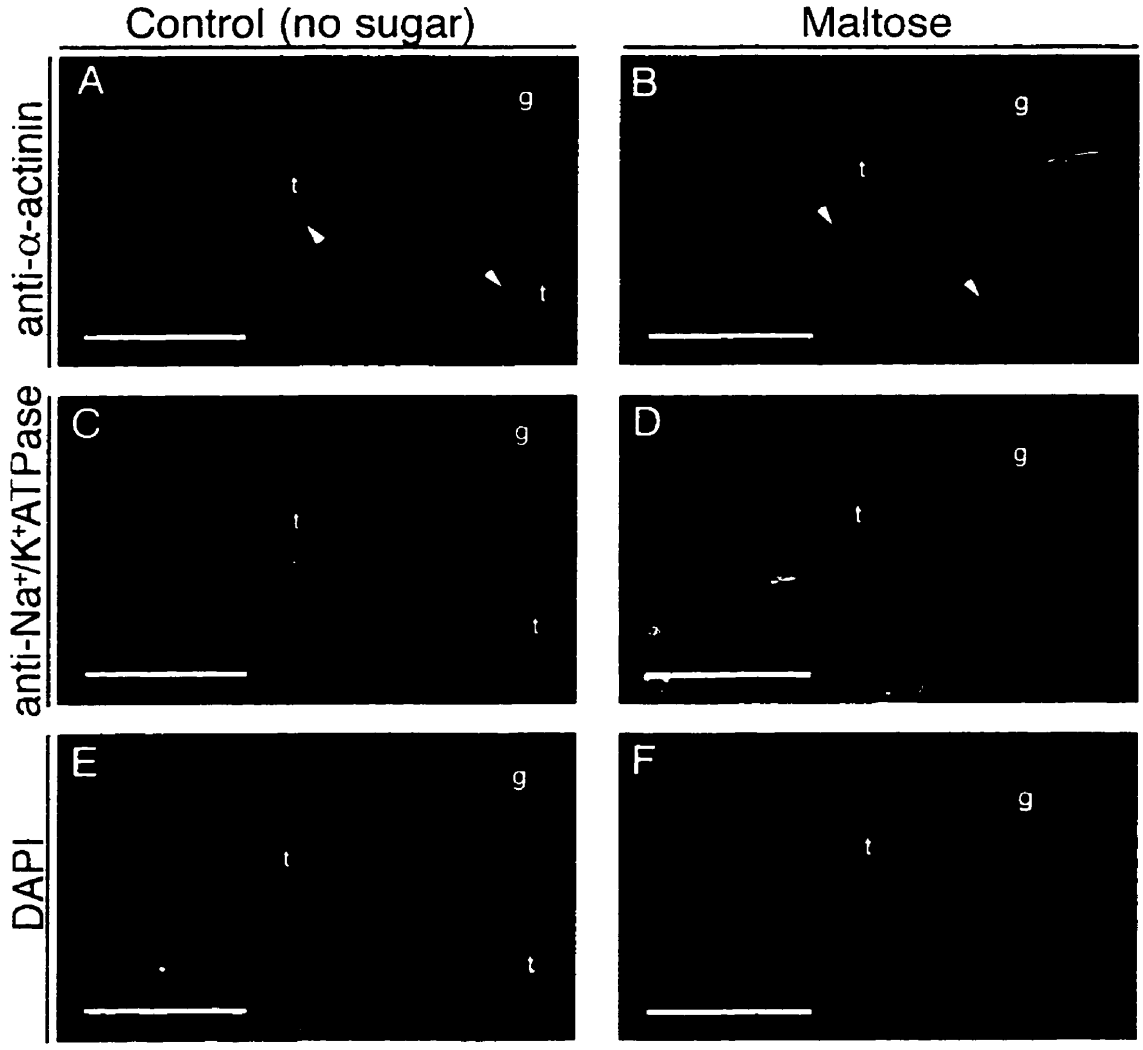


Figure 18. Double-staining immunofluorescence detection of α-actinin and Na^+/K^+ ATPase in mesonephric tissue cultured for 48 hours in the absence of added sugar and in the presence of maltose. The antisera α-ACT and β4/B4 were used to detect α-actinin and Na^+/K^+ ATPase, respectively. DAPI was used to visualize cell nuclei (E,F). In both control (A) and maltose (B) cultures, cytoplasmic α-actinin immunoreactivity is seen throughout the mesonephric tissue and is now concentrated in the basal cytoplasm (arrowheads) of tubules. In the same sections, Na^+/K^+ ATPase (C and D) is present in basolateral membranes of tubule cells in both control (C) and maltose (D) cultures. Scale bars = 50 μm.

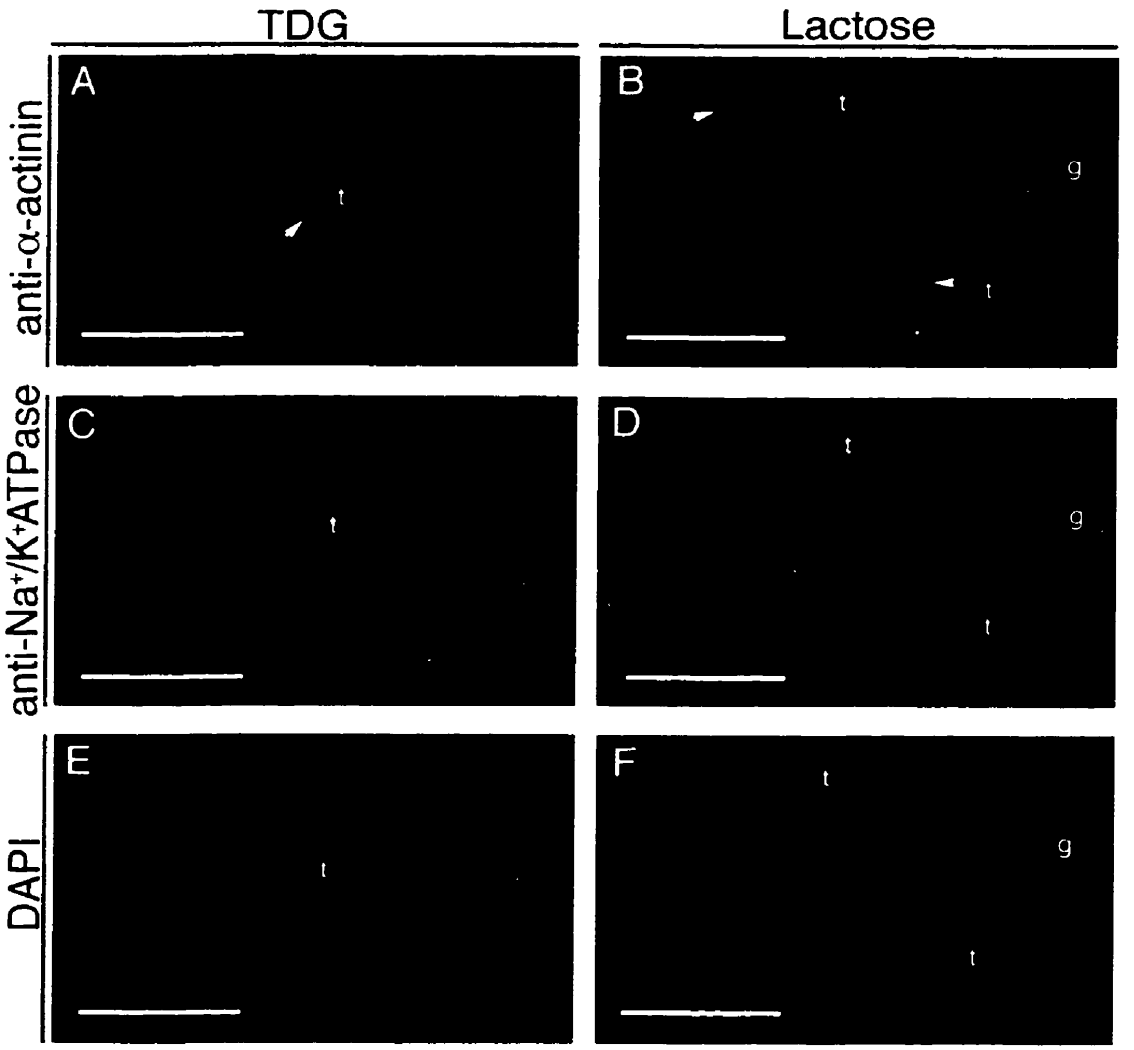


Figure 19. Double-staining immunofluorescence detection of α-actinin and Na^+/K^+ ATPase in mesonephric tissue cultured for 48 hours in the presence of TDG and lactose, hapten inhibitors of galectins. The antisera α-ACT and β4/B4 were used to detect α-actinin and Na^+/K^+ ATPase, respectively, in 48-hour cultured mesonephroi. DAPI was used to visualize cell nuclei (E,F). In both TDG- (A) and lactose-treated (B) cultures, cytoplasmic α-actinin immunoreactivity is seen throughout the mesonephric tissue and is concentrated in the basal cytoplasm (arrowheads) of tubules. In the same sections, Na^+/K^+ ATPase (C and D) is present in tubule cell membranes in both TDG- (C) and lactose-treated (D) cultures. (g, glomerulus; t, tubule) Scale bars = 50 μm.

Expression of marker molecules in cultured mesonephroi

In the cultured mesonephros, regardless of treatment, α-actinin was concentrated in the extreme basal cytoplasm of tubule cells (Fig. 18, A and B; Fig. 19, A and B), in contrast to the diffuse cytoplasmic or apical cytoplasmic stain observed in the mesonephros *in vivo* (Fig. 17, A and C). This suggests a generalized effect of the culture conditions on the distribution of α-actinin. Basolateral distribution of Na^+/K^+ATPase on the surface of tubule cells was not affected by the culture conditions (Fig. 18, C and D; Fig. 19. C and D). In control cultures without added sugar or with maltose, only a subset of the tubules were strongly Na^+/K^+ATPase - positive (Fig. 18, C and D), consistent with the localized distribution of the ATPase in distal tubules of the uncultured 7 day mesonephros (Fig. 17, D). In cultures treated with the galectin-inhibitors TDG or lactose (Fig. 19, C and D), many tubules had a weak but positive staining for Na^+/K^+ATPase, a situation found in the 5 day mesonephros *in vivo* (Fig. 17, B).

The pattern of E-cadherin and vimentin expression in control cultures was similar to that in uncultured 7 day mesonephros (Fig. 16, C and D). Namely, E-cadherin was expressed on the basolateral surfaces of mesonephric tubules (Fig. 20, A and B), and vimentin expression was restricted to those tissues that did not express E-cadherin (Fig. 20, C and D). In contrast, in TDG- and lactose-treated cultures, E-cadherin and vimentin often colocalized (Fig. 21, A to D), a pattern of localization seen in cells of the *in vivo* 5 day mesonephros (Fig. 16,A and B). In the cultured organs treated with lactose and TDG, many E-cadherin-positive structures also expressed vimentin.

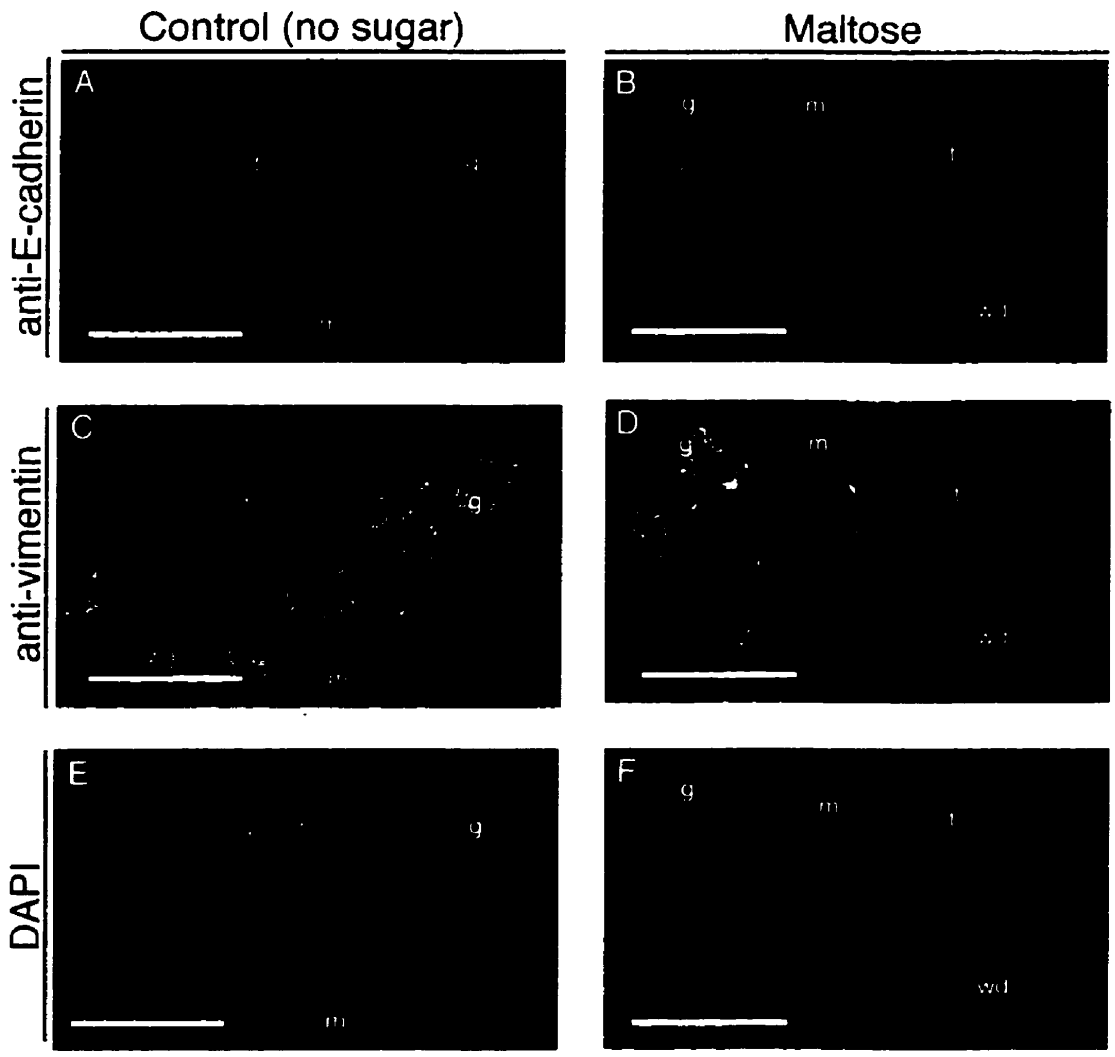


Figure 20. Double-staining immunofluorescence detection of E-cadherin and vimentin in mesonephric tissue cultured for 48 hours in the absence of added sugar or in the presence of maltose. The antisera R701 and H5 were used to detect E-cadherin and vimentin, respectively. DAPI was used to visualize cell nuclei (E,F). In both control (no sugar) (A) and maltose-treated (B) cultured tissue, E-cadherin is localized to the cell membranes of mesonephric tubule (t) cells. In the same sections, vimentin (C and D) is abundant in glomeruli (g) and is also present in mesenchymal tissue (m). Note that E-cadherin-positive tubules in (A) and (B) do not express vimentin protein (C and D). Yellow fluorescence in glomeruli is due to erythrocyte autofluorescence. Scale bars = 50 μm.

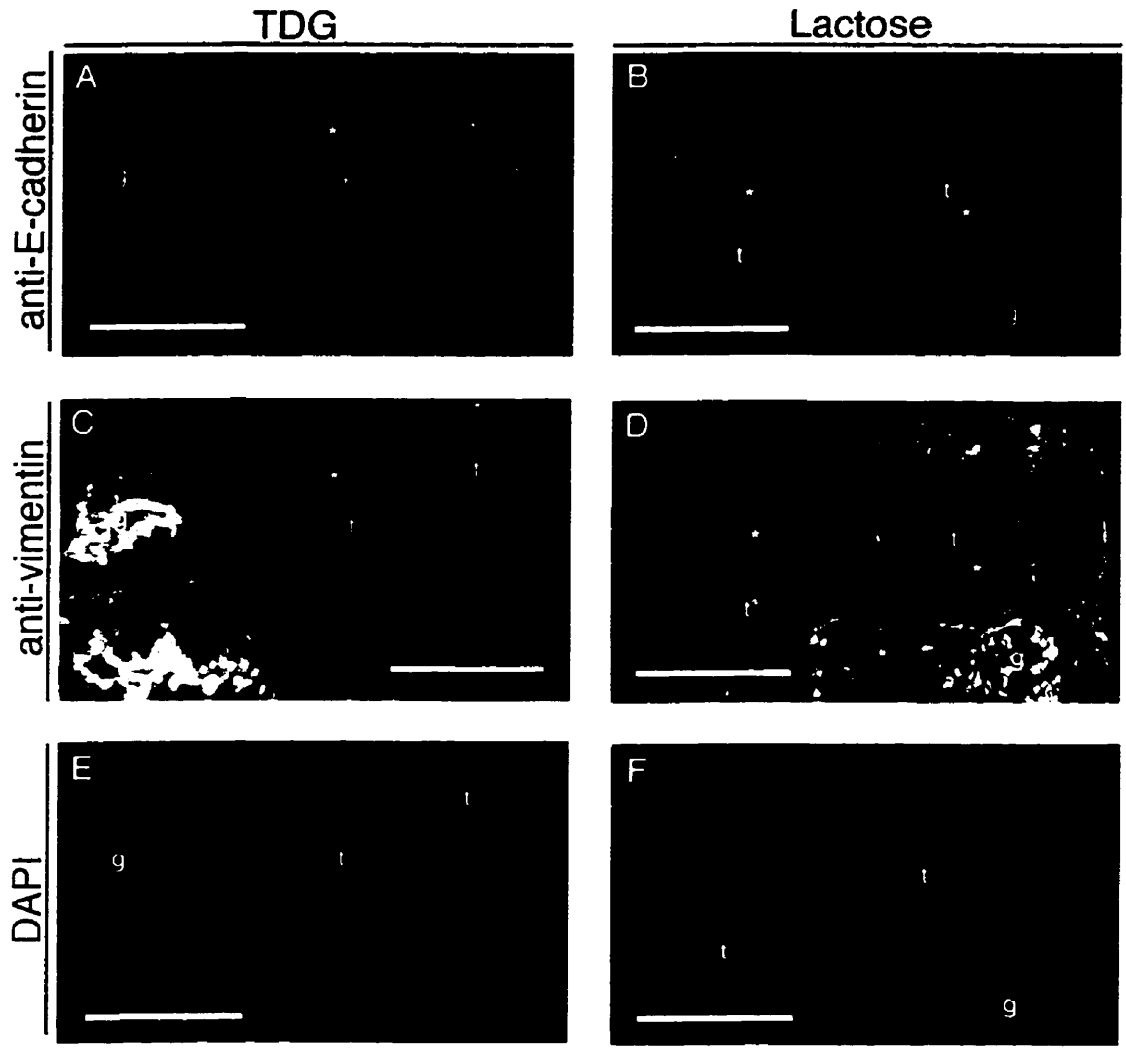


Figure 21. Double-staining immunofluorescence detection of E-cadherin and vimentin in mesonephric tissue cultured for 48 hours in the presence of TDG and lactose, hapten inhibitors of galectins. The antisera R701 and H5 were used to detect E-cadherin and vimentin, respectively. DAPI was used to visualize cell nuclei (E,F). In both TDG- (A) and lactose-treated (B) cultures, E-cadherin protein is found in cell membranes of tubules, and in disorganized structures that are assumed to have been tubules at the beginning of culture. In the same sections, vimentin is seen in glomeruli and in mesenchymal tissue (C, D); in addition vimentin is present in many E-cadherin positive tubules (*). Yellow fluorescence in glomeruli is due to erythrocyte autofluorescence in the glomerular capillaries. Scale bars = 50 μm.

DISCUSSION

Localization of galectin and its potential ligands in the mesonephric/gonadal complex

Earlier in my work, while investigating galectin expression in the primordial germ cells of the gonad, I also examined galectin expression in the associated structure, the mesonephros. I found that the 16 kDa galectin, C16, is abundant in the tubule basal laminae and in the glomerular basement membranes (GBM). Basement membrane localization of C16 in the chick mesonephros after 7 days of incubation has been reported before, by Didier, et al. (1988). These investigators were not able to detect galectin activity in the medium where dissociated cells from 13 day embryonic mesonephros had been cultured. They suggested that this lectin is produced elsewhere in the embryo and released into the circulation, to be eventually sequestered in the mesonephric basement membranes. I observed that at earlier stages of mesonephric development, that is in the 4 day mesonephros, most 16 kDa galectin is found not in the ECM but in the cytoplasm of tubule cells and glomerular podocytes. *In situ* hybridization revealed that C16 mRNA is present in cells of the Wolffian duct, developing glomeruli and developing tubules of 4 day embryos; this indicates that the RNA message for the synthesis of the 16 kDa galectin is present in these cells. Immunofluorescence examination of the mesonephros at this stage revealed that intracellular galectin is concentrated in small cytoplasmic patches or granules. A

similar organization of galectins into condensates in the submembranous cytoplasm has been reported during the early steps of galectin externalization (Cooper & Barondes, 1990; Mehul & Hughes, 1997). It is probable that the C16 present in the mesonephric basement membranes is released via these cytoplasmic granules. Galectin-rich cytoplasmic granules are abundant only before 5 days of incubation; after this time, few if any of these granules are seen in tubule cells. This observation, coupled with the finding by Didier, et al. , (1988) that cells from 13 day mesonephros do not release galectin, suggests that most of the basement membrane galectin is released, en masse, from the tubule cells and podocytes in the early stages of mesonephric development. If this is the case, the release of galectin would coincide with the onset of laminin production by mesonephric tissue (Ekblom, 1981 a & b). The C16 released in this manner into the tubule basement membranes could play a role in the final steps of differentiation into epithelium.

As the mesonephros develops intracellular 16 kDa galectin disappears from tubule cells, presumably as a result of externalization; however it persists in glomerular podocytes. From 5 days onwards in the mesonephric podocytes C16 is present in a diffuse, cytoplasmic localization but can also be seen in isolated condensates. The continued expression, and presumably continued externalization, of C16 by glomerular podocytes may be due to the need to replace basement

membrane galectin which is probably constantly being consumed by mesangial cells during GBM turnover (Walker, 1973).

In the basement membranes of the mesonephros, the 16 kDa galectin colocalizes with laminin and FN. Laminin has been shown to be a high affinity ligand for galectins, including proto-type galectins such as the C16 (Cooper, 1997). Embryonic FN, which is heavily glycosylated and has polylactosamine side chains, has also been shown to be a ligand for galectins (Ozeki, et al. , 1995). Thus in the developing mesonephric basement membrane the most probable ligands for C16 are laminin and/or FN.

Although both the 14 kDa and 16 kDa galectins are found in the ECM of the chick gonad, a profusion of cytoplasmic galectin-rich "granules" is never seen in this organ. It is possible that the mode of galectin externalization differs between the gonad and mesonephros, and that this may reflect a different functional role of these galectins in each of these organs.

Characteristics of the mesonephros cultured in a defined medium

In order to study what roles galectin may play in the development of the chick mesonephros, it was necessary to develop culture conditions to maintain this tissue in a defined medium. Such a system would allow the selective inhibition of galectins with hapten inhibitors, without the confounding effects of serum glycoproteins. Only morphological and ultrastructural information is available for the embryonic chick

mesonephros. As a consequence a defined culture system for the chick mesonephros had to be developed. The membrane filter support system, which I employed, is a common technique used for culture of organ explants (Hardman, et al. , 1990; Schultheiss, et al. , 1995). Attempts to culture the mesonephros in the presence of fetal calf serum, and in a defined medium that supports growth and differentiation of the mouse metanephros (Woolf, et al. , 1995), resulted in death of the mesonephric explants within 24 hours of culture. After numerous trial cultures with culture media of different compositions, a defined medium was developed which contained a high concentration of conalbumin (the chick transferrin). Transferrin is an iron-transport molecule that is essential for *in vitro* organogenesis of the metanephros (Ekblom, et al. , 1981). This medium supports mesonephric explants in culture for up to 3 days, with reasonably good preservation of tissue structure. In control sugar-free cultures, as well as in those containing maltose, thiodigalactoside, and lactose, dividing cells were present. The presence of mitotic figures in cultured tissues is an indication that the tissue is healthy and that the culture conditions are supportive of growth.

Molecular markers for mesonephric tissue.

In order to assess changes in cellular phenotypes in the cultured mesonephros, I needed to find marker molecules for its epithelial and mesenchymal cellular components. The marker proteins used in the present studies were the following: E-cadherin, an epithelial-specific

component of adherens junctions; vimentin, an intermediate filament characteristic of mesenchymal cells; Na^+/K^+ATPase, an ion exchange pump found on the basolateral surface of distal tubule epithelium; and α-actinin, an actin-binding protein that has an apical localization in what appear to be proximal tubule cells. These molecular markers were used to determine whether different experimental treatments had an effect on phenotypic expression in the cells of the cultured chick mesonephros.

When control saccharide-free cultures were immunostained with antisera to Na^+/K^+ATPase, E-cadherin and vimentin, the localization of these markers was similar to that in uncultured mesonephros from 7 day chick embryos. The distribution of α-actinin, however, was markedly changed. In the cultured mesonephros this protein was concentrated in the basal cytoplasm. Such a change in distribution of an actin-binding protein suggests that, in the tubular cells of the mesonephros, the organization of the actin cytoskeleton is affected by the culture conditions. Proximal tubule cells characteristically have a brush border consisting of microvilli supported by bundles of actin, and α-actinin is an actin-cross-linking protein (Brown & Stow, 1996). It is possible that the apical concentration of α-actinin in putative proximal tubules is due to the association of α-actinin with the actin at the base anchors of the microvillar axes. If this is the case, the redistribution of α-actinin to the basal cytoplasm in culture may be due to the loss of apical microvilli, which could be a response to a relative uniform environment in culture.

Since in culture there is no difference between the contents of the tubule lumen and the nephric interstitium, there is no need for increased absorptive surface area on the apical cell surface, and the apical microvilli would not be required. Nevertheless distribution of E-cadherin, which is linked to the actin cytoskeleton via catenins (Hitt & Luna, 1994), and the Na^+/K^+ATPase, which is retained in the basolateral membrane through interactions with the cytoskeleton (Le Gall, et al. , 1995; Mays, et al. , 1995), were unaffected by culture. This suggests that in the mesonephric tubule cells only limited aspects of actin cytoskeletal organization were affected by the culture conditions. The expression pattern of vimentin in the cultured mesonephros did not differ from that of this embryonic tissue *in vivo*.

Effects of hapten inhibitors of the galectins on cultured mesonephroi

TDG and lactose are galectin-hapten inhibitors that compete with polylactosamine moieties on laminin and other glycoproteins for the galectin CRD binding site. Maltose was used as a control since galectins do not bind this sugar. Lactose and TDG would inhibit any galectin in this system, not just C16. Although my results indicate that C14 is a negligible component of mesonephric basement membranes, the possibility that these sugars would inhibit galectins other than C16 in the ECM cannot be ruled out. In the cartilage of the developing chick embryo, a 30 kDa galectin has been reported (Nurminskaya & Linsenmayer, 1996). The presence of the latter lectin in the chick mesonephros has not been

investigated. However, if a similar situation as in mammalian tissues occurs in the chick mesonephros, it is likely that the chick 30 kDa galectin would be released apically from renal cells into the tubule lumen (Lindstedt, et al. , 1993; Mehul & Hughes, 1997; Winyard, et al. , 1997) and therefore would not be deposited in the basement membranes where C16 predominates. Regardless of which other galectins may be affected, the presence of TDG and lactose in culture would certainly inhibit binding of C16 to its ligands. When compared to either control sugar-free or maltose-treated mesonephric cultures, mesonephric tissues maintained in the presence of galectin hapten inhibitors exhibited specific effects. There is a strong possiblity that these changes are due to a disturbance of galectin function.

a) Programmed Cell Death. When stained with hematoxylin and eosin, the cultured mesonephros treated with TDG and lactose show areas of cell death. This probably does not indicate a generalized cytotoxic effect of those sugars, since mitotic figures indicating continuing cell division were present in regions of the cultures adjacent to areas of cell death. In control cultures, comparable areas of cell death were not seen. More specific techniques such as TUNEL (TdT-mediated dUTP-biotin nick end labeling) (Gavrieli, et. al., 1992) must be used to determine if the cell death observed in cultured mesonephric tissue treated with galectin hapten inhibitors is indeed programmed cell death (apoptosis) or is necrotic cell

death. TUNEL allows for the *in situ* detection of terminal 3′ ends of DNA fragments characteristic of apoptotic cell death.

Disturbance of galectin-binding to its receptors could affect the events involved in the modulation of programmed cell death, or apoptosis, in the mesonephros. In other systems, galectins have been shown to regulate apoptotic events (Perillo, et al. , 1997; Rabinovich, et al. , 1997; Wada, et al. , 1997; Akahani, et al. , 1997b; Yang, et al. , 1996; Perillo, et al. , 1995). In the chick the mesonephros eventually degenerates as the metanephros develops and takes over the excretory and osmoregulatory activity of the mesonephros. Since it is likely that programmed cell death plays a role in the degeneration of the mesonephros, galectins could be involved in the modulation of this event. One of the ways galectins could affect cell survival in the degenerating mesonephros is by modulating integrin-mediated cell-matrix adhesion. Appropriate integrin-ligand interactions can rescue epithelial cells from apoptosis due to loss of cell adhesion; such adhesion-dependent cell death pathways have been collectively termed anoisis (Frisch, et. al., 1996).

b) Adhesion to the GBM. Another effect of the disturbance of galectin function by hapten inhibitors is the partial detachment of glomerular podocytes from their ECM. At the developmental stages used in the present experiments the uncultured mesonephros, as well as cultures maintained in the absence of sugar or the presence of maltose, have an abundant C16 distribution in the GBM. Podocytes also retain a

diffuse cytoplasmic distribution of this galectin, as opposed to tubule cells which, from 5 days of incubation onwards, have little or no intracellular C16. *In vivo* and presumably *in vitro*, the foot processes of the glomerular podocytes cover 90% of the GBM, and these processes are the only points of contact between podocytes and the GBM (Mundel & Kriz, 1995). Immunofluorescence for FN, laminin, and C16 reveals the presence of these proteins in the GBM between podocyte cell bodies and capillary spaces; *in vivo* and in control sugar-free and maltose-treated cultures, the immunostained GBM appears as a sharp line. This suggests that because of the coverage by podocyte foot processes, only the cut edge of the GBM is accessible to those antisera. In cultures with hapten inhibitors FN, laminin and C16 immunofluorescence in the GBM is visible as an aggregate of non-cellular material surrounded by podocytes, suggesting that more antigenic material in the GBM is accesible to the antisera. This effect could be due to retraction of podocyte foot processes subsequent to those processes losing adhesion to the GBM. Hematoxylin and eosin staining confirms that treatment with TDG or lactose results in a loss of structure in the glomerular tuft. Specifically, podocytes appear to have "lifted off" the GBM so that more GBM is exposed, and in some cases podocytes that have no physical connection to the GBM can be seen.

Podocyte foot processes are known to express $\alpha_3\beta_1$ integrin, which binds laminin, FN and collagen; in addition they express $\alpha_6\beta_1$ integrin which binds only laminin (Mundel & Kriz, 1995; Davies, 1996; Martins-

Green & Bissel, 1995). These integrins presumably facilitate binding of podocyte foot processes to those glycoproteins in the GBM. The loss of contact between podocytes and the GBM induced by galectin inhibitors suggests that galectins could play a role in adhesion of podocytes to this basement membrane. Histochemical studies with plant lectins have revealed that in the meso- and metanephros the podocyte cell surface, including the basal surface of the foot process, express abundant galactosaminyl-glycoconjugates (Holthöfer, et al. , 1988; Gheri, et al. , 1990; Gheri, et al. , 1993). The detachment of the podocytes from the GBM could be the result of interference by TDG and lactose with the cross-linking of cell-surface galactosides to ECM glycoproteins such as laminin and FN.

c) Modification of Cellular Phenotype. A third effect observed in mesonephric tissue cultured with TDG and lactose is a disruption of tubule architecture. This was evident by the fact that often tubule cells lost their cuboidal shape, the tubule cell nuclei were not restricted to the basal cytoplasm, and when compared to controls the tubule lumen was often obliterated. Also when compared with control cultures, TDG- and lactose-treated explants showed an overall "disorganization" suggestive of a change in the differentiated state of the tubular epithelial cells.

The most striking effect of these galectin hapten inhibitors on cultured mesonephroi is the re-expression of vimentin intermediate filaments in tubular epithelial cells expressing E-cadherin. Nephrogenesis can be considered to be a three-step process. First, there is an inductive

event and subsequent compaction of nephrogenic mesenchyme. This is followed by the differentiation of mesenchymal cells into an epithelium with the concurrent expression of E-cadherin (Davies, 1996). Finally, epithelial cells differentiate further into the segment-specific phenotypes. In the mammalian metanephros, and presumably in the meta- and mesonephros of other species, final differentiation into podocytes involves a partial return to mesenchymal phenotype as evidenced by loss of E-cadherin and the re-expression of vimentin intermediate filaments (Mundel & Kriz, 1995).

In the 5 day mesonephros *in vivo*, tubules often co-express E-cadherin and vimentin; however, in the 7 day mesonephros, which is comparable to explants from the 5 day mesonephros cultured for 2 days as in this study, vimentin is restricted to mesenchymal tissues and tubule cells expressing the epithelial marker E-cadherin are always vimentin-negative. This localization was also observed in control explants cultured in the absence of sugar or the presence of maltose. The concurrent expression of vimentin and E-cadherin in the tubular-epithelial cells of cultures treated with galectin hapten inhibitors suggests that the cells are dedifferentiating or re-acquiring features of mesenchyme.

Cellular interactions with laminin, in particular with the laminin α chain, are crucial for differentiation and moreover are required for the maintenance of the renal epithelial phenotype (Klein, et al. , 1988; Drubin & Nelson, 1996). Modulation of cellular binding to laminin by galectins

could in principle affect: a) the differentiation into epithelium; b) the segment-specific specialization of epithelial phenotypes of the nephron; and c) the maintenance of the epithelial phenotypes. Tubule cells that are re-expressing vimentin could either be dedifferentiating into mesenchyme, or differentiating further into podocytes which typically express vimentin but lack E-cadherin. Based on the short-term culture conditions of the present experiments, it cannot be determined whether the cells expressing vimentin have stopped producing E-cadherin. The presence of E-cadherin in those tubules could be due to an insufficient time avilable for removal of E-cadherin from the cell membrane. In addition, although the histological localization and the subcellular distribution of the Na^+/K^+ATPase was not markedly different between control and hapten-treated cultures, qualitatively it appeared that in hapten-treated cultures the percentage of tubules with Na^+/K^+ATPase-expressing cells had increased. This suggests an effect on nephron segmentation.

Regardless of whether TDG and lactose cause dedifferentiation into mesenchyme, further differentiation into podocyte-like cells, or modifications in the phenotypic segmentation of the nephron, these results strongly suggest that galectins are important for the maintenance of the renal phenotype. Experiments using younger tissues would be required to determine if galectins are necessary for initial development of renal epithelium.

The requirement for cell-laminin interactions for the development and the maintenance of renal epithelia is well established (Timpl & Brown, 1994; Durbeej & Ekblom, 1997). Given the high affinity of galectins for laminin and the colocalization of C16 and laminin in tubule basement membranes, it is reasonable to suggest that the effects of TDG and lactose on tubule differentiation are due to a disruption of galectin-laminin interactions. Several cellular laminin receptors have been implicated in epithelial differentiation including $\alpha_6\beta_1$ integrin (Sorokin, et al. , 1990; Goodman, 1992), the 37/67 kDa laminin/elastin receptor (Salas, et al. , 1992), and α-dystroglycan (Durbeej, et al. , 1995). Theoretically, by binding to laminin and sterically blocking association of this glycoprotein with its receptors, galectins in the mesonephric basement membranes could modulate cell-matrix adhesion. During development of mouse skeletal muscle, steric inhibition of integrin-laminin binding by Galectin-1 has been identified as a key factor in the terminal differentiation of myoblasts (Cooper, et al. , 1991; Gu, et al. , 1994). If galectin in the mesonephric basement membranes functions in a similar fashion, by disrupting receptor-laminin binding, then galectins would likely be acting as a negative factor in differentiation of the renal epithelium. As demonstrated in the myoblast system, adding excess galectin ligands in this case would alleviate steric inhibition and increase the binding of laminin to cellular receptors, which would likely have a stabilizing

influence on differentiation. Such a scenario is not supported by the results of this study.

Another possibility is that in the mesonephric basement membranes, dimerized galectins are cross-linking laminin to a galactosylated cell-surface receptor. If occupation of this receptor by a galectin is required for maintenance of the epithelial phenotype, then galectin hapten inhibitors would disrupt cellular phenotype, as was seen in this study. If mesonephric basement membrane galectins function in this manner, then what is the nature of the galactosylated cell surface receptor? A good candidate for such a receptor is a galactosylated lipid. Glycolipids can have polarized distributions in the membrane of epithelial cells. Kidney-derived (MDCK) epithelial cells accumulate galactosyl-ceramide glycosphingolipids in the basolateral membrane domain (van der Bijl, et al. , 1996). In the same MDCK cells, glucosyl-ceramide glycosphingolipids are randomly distributed in the cell membrane. Interaction of laminin with a cell-surface glycosphingolipid bearing the Forssman pentasaccharide (GalNAc-[α1-3]-GalNAc-[β1,4]-Gal-[α1,4]-Gal-[β1,4]-Glc-[β1,1]-ceramide) has been implicated in the development of the epithelial phenotype (Zinkl, et al. , 1996). Antibodies to this glycolipid inhibit MDCK cell adhesion to laminin and prevent the development and maintenance of cell polarity. A Forssman saccharide, putatively conjugated to a lipid moiety (Siddiqui & Hakamori, 1971), is expressed on the surface of chick mesonephric cells (Szulman, 1975), and antibodies to

this saccharide antigen prevent reformation of tubules in cultures of dissociated chick mesonephric cells.

The nature of the interaction between the Forssman saccharide and laminin has not been determined. However, laminin could be linked to the Forssman glycolipid through a dimerized galectin cross-link. The Forssman disaccharide (GalNAc-[α1,3]-GalNAc)) has been identified as the natural ligand for the *Geodia cydonium* galectin (Hanisch, et al. , 1996). Further experiments are necessary to determine if the chick 16 kDa galectin binds to a Forssman glycolipid on the surface of chick mesonephric cells, and if that glycolipid is involved in the development and maintenance of renal epithelial cell polarity.

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