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Original Article

Expression of the galactose-binding lectins during the formation of organ primordia in the chick embryo

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ABSTRACT Early chick embryos contain two ß-galactoside-binding lectins of 16 kDa and 14 kDa. Using several antisera to these proteins, we have studied lectin expression at embryonic stages when the segregation and early differentiation of organ primordia are taking place. With antisera to the 16 kDa lectin that display similar immunoreactivity in immunoblot analysis, we show that these antisera exhibit varying immunoreactivity in embryo sections. One antiserum reacts preferentially with a matrix form of lectin while another detects mainly a cellular form of this protein. During early development, galactoside-binding lectins of the matrix type are expressed in the vitelline membrane, the outer and inner limiting membranes of the neural tube, the surface of the notochord and the coelomic surface of the cardiac rudiments. The cellular form of the lectin occurs in the intracellular yolk of early embryos, in the primordial germ cells, the myocardium, in the early myotome, and in a cohort of cells which are presumed to belong to the neural crest. Our results indicate that, although all of the antisera recognize the intracellular lectin of the extraembryonic endoderm, some antisera to the 16 kDa lectin exhibit preferential reactivity with different lectin isoforms. The extracellular matrix form of lectin is transiently expressed during early development at the stages when the segregation of organ primordia is occurring. It's expression could be related to the acquisition of polarity in developing epithelia. Results also suggest that various versions of the same protein may perform distinct developmental roles in the embryo.

KEY WORDS: *lectin, galactose, chick embryogenesis*

Introduction

In early embryogenesis, the segregation of the embryonic cell layers and the organization of the organ primordia are crucial developmental events which involve cell-cell interactions and cell-matrix interactions in which carbohydrate-bearing molecules at the cell surface and the extracellular matrix are thought to play important roles (Sanders, 1989; Shur, 1989; Fenderson *et al.*, 1990). Recent evidence indicates that carbohydrate-binding proteins or lectins also play a role in the mediation of cell interactions during fertilization (Urch, 1991; Hedrick and Nishihara, 1991), development (Barondes, 1986, 1988; Regan *et al.*, 1986; Levi and Teichberg, 1989; Oda *et al.*, 1989; Zalik, 1991), as well as differentiation and tumor formation in the adult organism (Joubert *et al.*, 1989; Lehman *et al.*, 1990; Lotan, 1992).

Based on sequence homology of their carbohydrate recognition domains, most animal lectins studied so far are thought to belong to two main families; the C-type and the S -type (Drickhamer, 1988). The C-type lectins are, in general, integral membrane proteins that require calcium or other divalent cations for activity. Among these are the selectins or LEC-CAMs involved in lymphocyte homing and adhesion to the endothelial cells (Rosen, 1989; Springer, 1990). The S-type or S-Lac lectins are galactose-binding proteins that are thiol dependent and bind compounds with terminal lactosaminyl-like groups. These lectins lack a signal sequence and are located in the nucleus, the cytoplasm, the cell surface and the extracellular matrix (Barondes, 1986; Harrison, 1991a,b; Wang *et al.*, 1991; Lotan, 1992). In mammals, the most studied of the latter proteins are the galactose binding lectins of 14 kDa, 29 to 35 kDa, and 69 kDa. During myoblast differentiation the 14 kDa lectin,

Abbreviations used in this paper: Apo VLDL II, apoprotein of the very low density lipoproteins; Anti,I-14, antiserum to the 14kDa galactose-binding lectin from adult chick intestine; Anti,L-16, antiserum to the 16kDa galactose-binding lectin from adult chicken liver; Anti,M-16, antiserum to the 16kDa galactose-binding lectin from embryonic chick pectoral muscle; Anti,S-14, antiserum to the 14kDa galactose-binding lectin from embryonic chick skin; BSA, bovine serum albumin; DABCO, 2,4, diazabicyclo (2,2,2, octane); DAPI, 4,6, Diamidino-2-phenylindole; EDTA, Ethylenediaminotetraacetic acid; PCS, Pannett and Compton's saline; PEG, polyethylene glycol; PBS, phosphate buffered saline; PAS, Protein A Sepharose; SDS-PAGE Sodium dodecylsulphate polyacrylamide gel electrophoresis; TRIS, (hydroxymethy) aminomethane.

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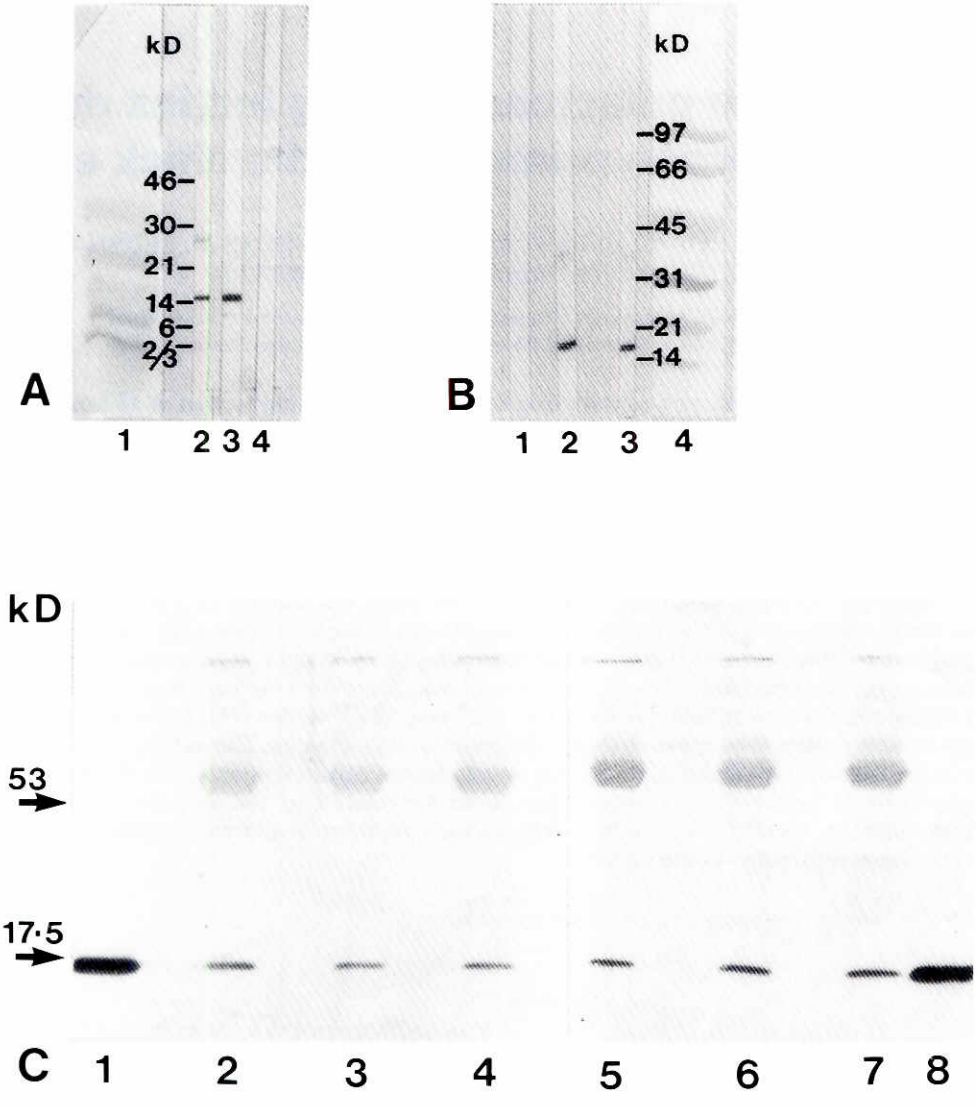

Fig. 1. Immunoblot analysis and immunoprecipitation studies on the anti,16-KDa lectin antisera used in the immunolocalization studies. (A) *Crude lectin extracts from chick blastoderms at stages 5 to 8. Lane 1, molecular weight standards, lane 2 strip reacted with anti,M-16, lane 3, reacted with anti,L-16, lane 4 shows the corresponding preimmune serum of the antiserum shown in 3.* **(B)** *Immunoblot analysis of a purified lectin preparation from chick blastoderms reacted with; 1 preimmune serum, 2 anti,L-16, 3 anti,M-16, lane 4 shows migration of molecular weight standards.* **(C)** *Immunoblot analysis of material immunoprecipitated by anti,M-16 kDa antiserum in crude extracts of embryos at different developmental stages. Immunoprecipitates were obtained after treatment of crude lectin extracts with anti,M-16 and protein A-Sepharose. Lanes 1 and 8 show the affinity purified 16 kDa lectin from pectoral muscle; lane 2, stage 5 embryos; lane 3, stage 8 embryos; lane 4, stage 14 embryos; lane 5, stage 16 embryos; lane 6, stage 19 embryos; lane 7, stage 23 embryos. Arrows point to the 16 kDa lectin and to a band migrating at around 55 kDa, the latter corresponds to the rabbit IgG of the immune complex visualized by the peroxidase conjugated second antibody. Standards in* **(A)** *(Rainbow, prestained Amersham) are: Insulin-a (2.3 kDa), insulin-b (3.4 kDa), aprotinin (6.5 kDa), lysozyme (14.3 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (30 kDa), Ovalbumin (46 kDa). Standards in* **(B)** *(Biorad) are: Phosphorylase B (97.4kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa). Some of the pre-stained standards vary slightly in molecular weight when compared to the non stained proteins.*

which is predominantly intracellular, is externalized via a non-conventional mechanism involving the budding and extrusion of lectin-packed areas of the cytoplasm (Cooper and Barondes, 1990; Harrison and Wilson, 1992). This lectin accumulates in the extracellular matrix and it binds to laminin and glycosylated forms of fibronectin (Cooper *et al.*, 1991; Sato and Hughes, 1992; Massa *et al.*, 1993; Zhou and Cummings, 1993). It has been suggested that this protein may be involved in the detachment of cells from the extracellular matrix during myoblast fusion (Cooper *et al.*, 1991). The 29 to 35 kDa lectin is an IgE-binding protein that has recently been shown to bind laminin and has been suggested to be involved in cell matrix interactions (Laing *et al.*, 1989; Mercurio, 1990; Woo *et al.*, 1991). The 67 kDa lectin was first reported as the elastin receptor of the mammalian lung (Hinek *et al.*, 1988), and has

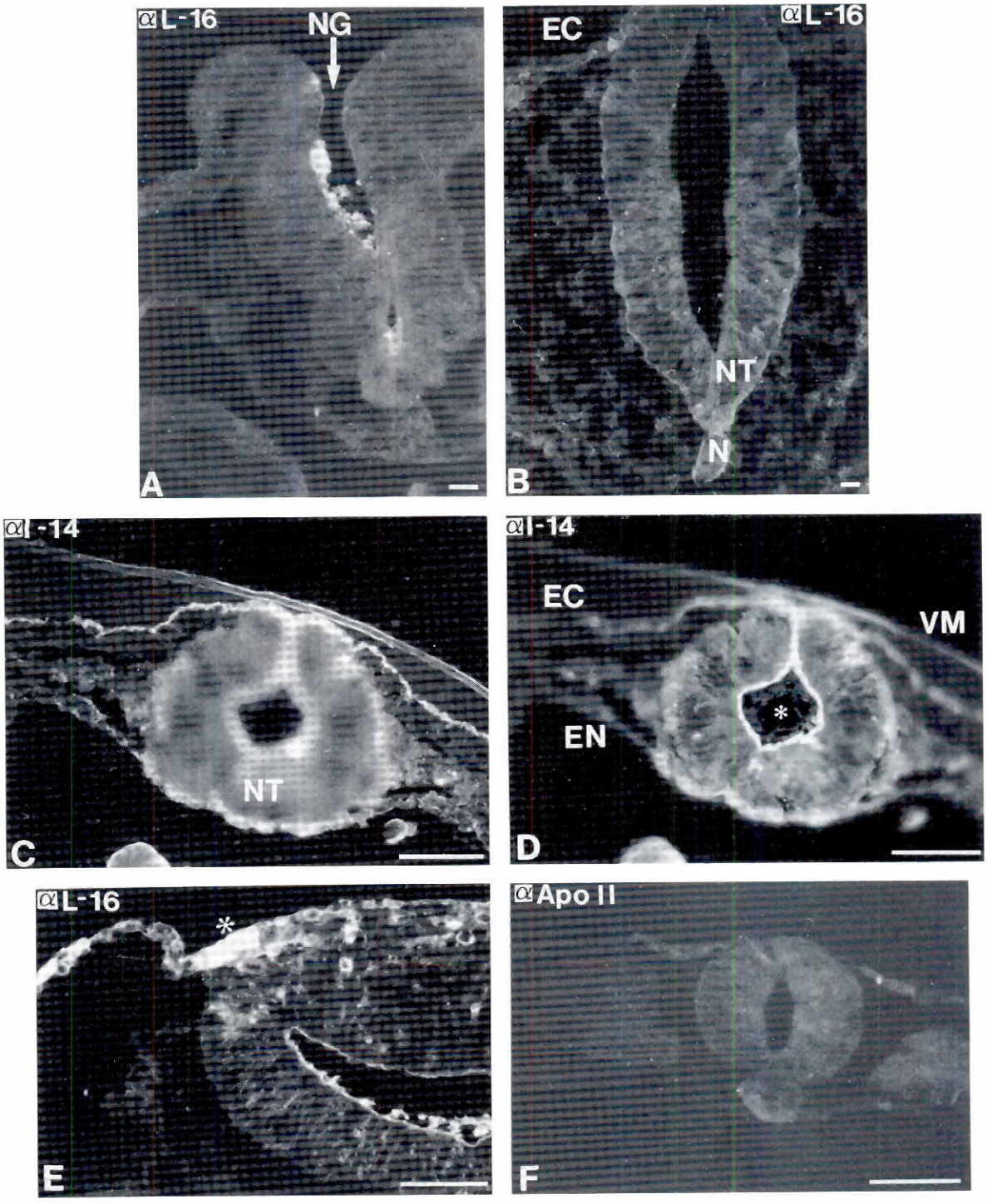

Fig. 2. Immunoreactivity of sections of early embryos with different anti-lectin antisera. (A) *Section of a stage 5-6 embryo;* **(C to E)** *are from stage 8 embryos;* **(B and F)** *sections stage of 11 embryos. Sections through different regions of the neural groove have been reacted with anti,L-16* **(A,B and E)** *anti,I-14* **(C and D)** *and anti, Apo VLDL II* **(F)**. *In* **A** *and* **E**, *the anti,L-16 antiserum stains amorphous material in the apical region of the invaginating neural tube as well as the internal limiting membrane. In* **E**, *some cells of the ectoderm overlying the neural tube stain intensely with the anti,L-16. Material reacting with anti,L-16 is also present at the basal surface of the neural tube and around the notochord* **(B)**. **(C and D)** *display the same section at different focal planes showing that the anti,L-14 stains the dorsal surface of the epiblast and the inner limiting membrane of the neural tube. This antiserum reacts with amorphous extracellular material of the neural tube (*), and stains the vitelline membrane.* **(F)** *the antiserum to Apo VLDL II did not stain any of the tissues within the embryo. (EC) ectoderm, (EN) endoderm, (NT) neural tube, (VM) vitelline membrane. Bars, A and B: 20 μM; C to F: 50 μM. The antiserum used in the sections is shown at the upper region of the photograph; the letter α is used for anti.*

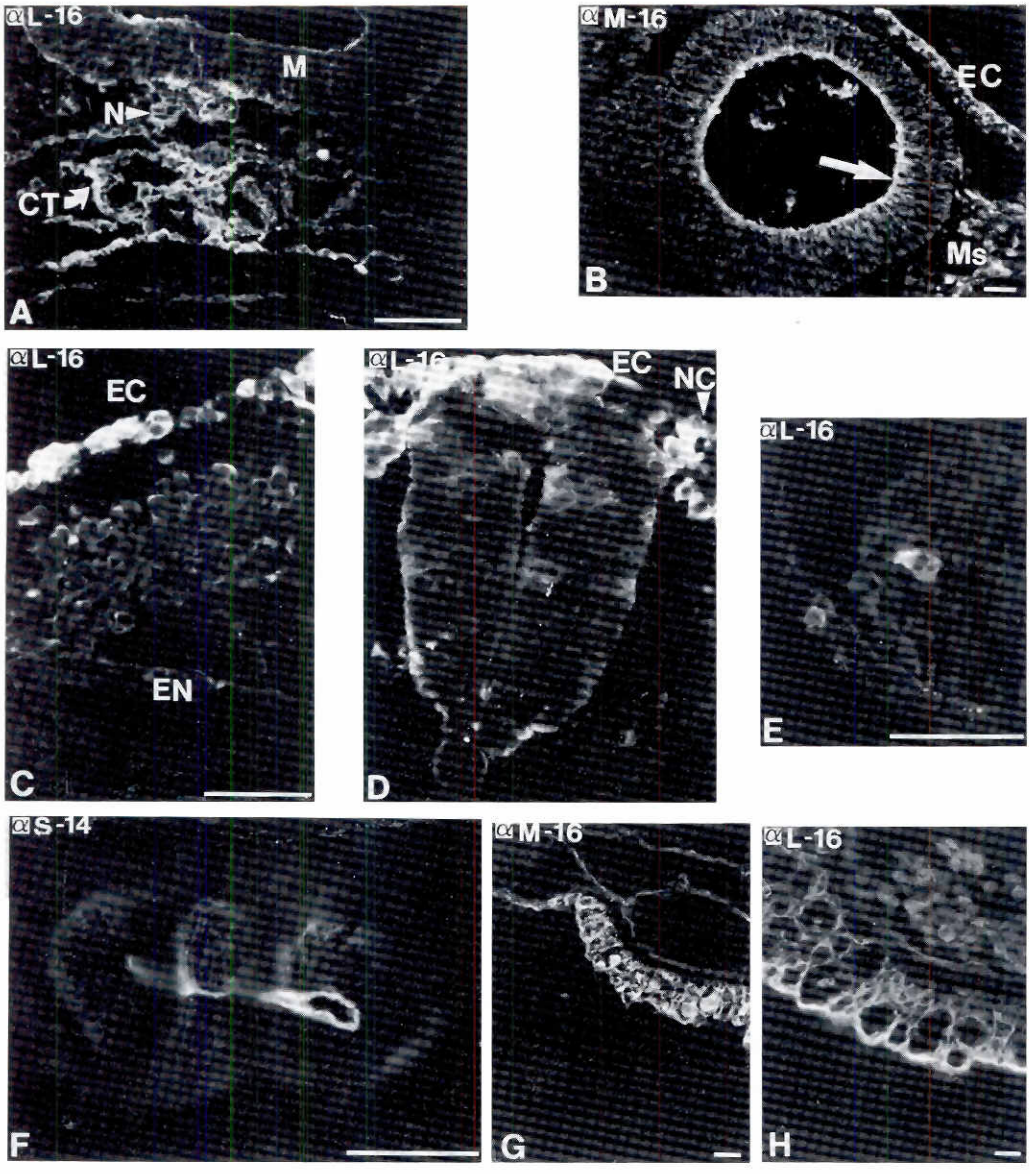

Fig. 3. Sections of embryos at different developmental stages reacted with various anti-lectin antisera. (A,C,D,E and G) *are from stage 10 embryos;* **(B and F)** *are from embryos at stage 11-12;* **(H)** *is from a stage 14 embryo.* **(A,C, D, E and H)** *were stained with anti,L-16,* **(B, and G)** *were stained with anti,M-16, and* **(F)** *was reacted with anti,S-14.* **(A)** *Section at the mesencephalic region of the embryo, material that reacts with anti,L-16, is present in the inner and outer limiting membranes of the mesencephalon around the forming notochord and around the recently fused myocardial tubes. Some staining is present in the endoderm of the foregut.* **(B)** *In a cross section at the anterior level of the embryo the lectin is expressed in the ectoderm, in some cells of the cephalic mesenchyme and in the inner limiting membrane of the prosencephalon (arrow).* **(C and D)** *show sections at the trunk level of the embryo.* **(C)** *is a region of the embryo slightly lateral to that shown in* **D.** *Staining is present at the external limiting membrane of the neural tube; weak but definite staining also occurs at the external surface of the notochord, the cells of the ectoderm immediately dorsal and dorso-lateral to the neural tube, possibly belonging to the neural crest show intense intracellular staining, in* **C**, *some cells in the somite are also stained.* **(E)** *A section at the level of the anterior mesencephalon showing a region of the head mesenchyme. A lectin-rich cell is present in the dorso-lateral cephalic mesenchyme, another lectin-rich cell is present in the ectoderm.* **(F)** *Section posterior to the mesencephalic area of the embryo, reactivity with anti,S-14 is present in the inner limiting membrane of the neural tube.* **(G and H)** *show that in the region of the developing yolk sac intense lectin expression occurs in the yolk sac endoderm. Bars, A,C,D,E,F: 50 μM; B,G,H: 20 μM. (CT) cardiac tubes, (EC) ectoderm, (EN) endoderm, (M) mesencephalon, (Ms) mesenchyme, (N) notochord, (NC), putative neural crest cells, (NT) neural tube. The antiserum used in the section is shown in the upper side; α stands for anti.*

recently been found to bind laminin and type IV collagen (Mecham *et al.*, 1989; Mecham, 1991). This topic has been a subject of several recent reviews (Barondes, 1988; Caron *et al.*, 1990; Harrison, 1991a,b; Zalik, 1991; Hughes, 1992).

Two main lectins of 14 kDa and 16 kDa have been described in the chick. These lectins are differentially expressed during tissue differentiation in late embryonic and fetal stages (Kobiler and Barondes, 1977; Nowak *et al.*, 1977; Kobiler *et al.*, 1978; Beyer, *et*

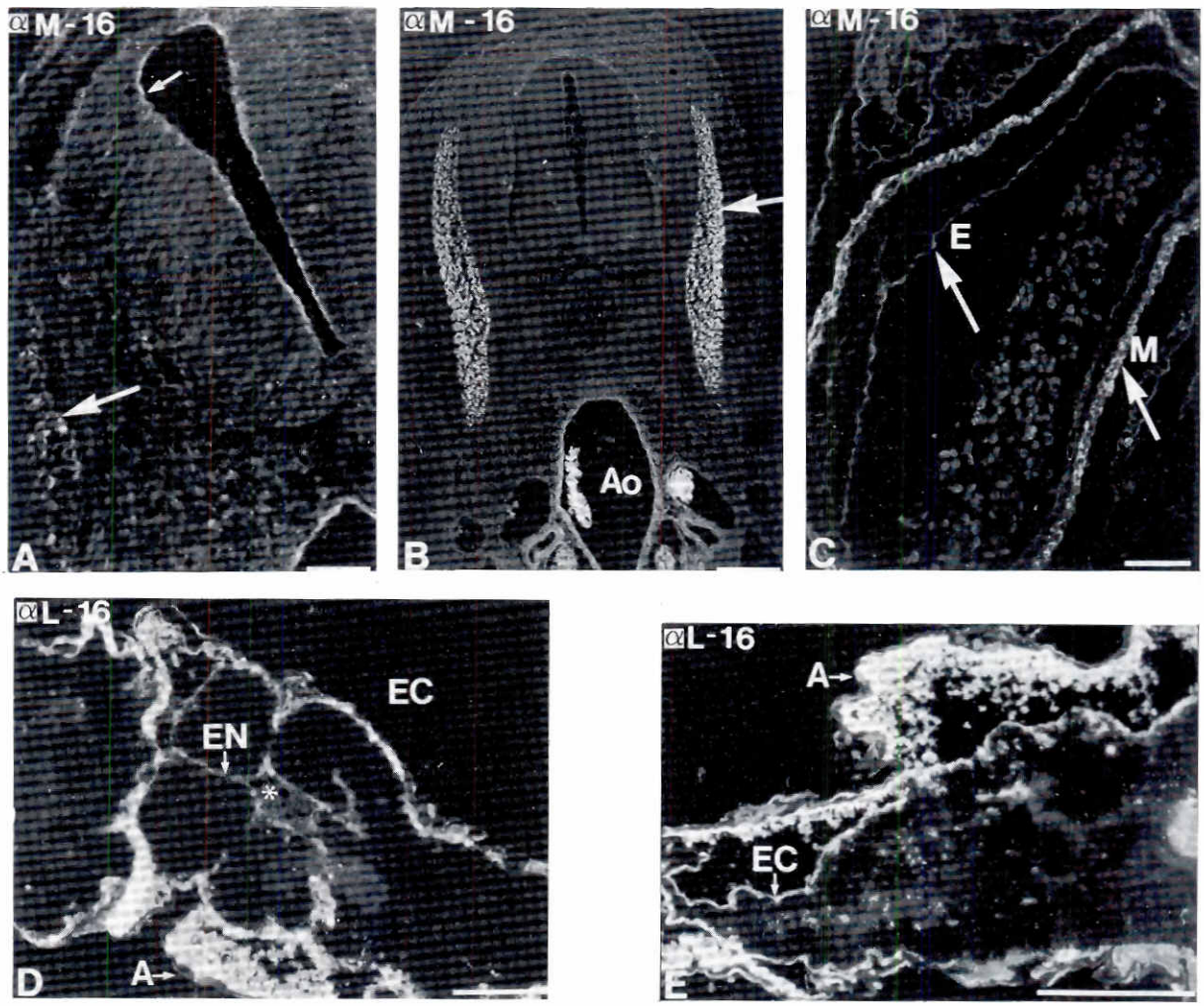

Fig. 4. Lectin expression at later stages of development; A, B and C,were reacted with anti,M-16; D and F, were stained with anti,L-16. (A) *Section of the trunk region of a stage 18-19 embryo. Lectin is expressed in the inner limiting membrane of the neural tube (small arrow), and in some cells of the segregating myotome (large arrow).* **(B)** *A section at the trunk region of a stage 24 embryo showing that lectin expression has intensified in the differentiating myotome (arrow). The fluorescence in the cellular material inside the aorta and its lateral branches is due to hemoglobin autofluorescence of the erythrocytes.* **(C)** *Section at the level of the ventricle in a stage 14 embryo, observe that the cells of the myocardium (arrow), express the lectin intracellularly.* **(D and E)** *are sections from the cervical and lumbar regions of a stage 19 embryo. At this stage anti,L-16 reacts only with material present in the lumen of the pharynx in* **D** *(*), and in the contents of the amniotic cavity in* **(D and E)**, *this material appears to have a granular texture. The endoderm of the branchial arches* **(C)**, *as well as well as the ectoderm* **(D)**, *are also stained. (A) amnion, (Ao) aorta, (E) endocardium, (EC) ectoderm, (EN) endoderm (M) myocardium. Bars, A: 40 μM; B: 160 μ; C, 63 μM; D and E: 50 μM. The antibody used is shown in the upper left, α stands for anti.*

al., 1980; Kitamura, 1980; Oda and Kasai, 1983; Oda *et al.*, 1989; Sakakura *et al.*, 1990; Akimoto *et al.*, 1992), and the distribution of a 15 kDa lectin variant has been documented (Levi and Teichberg, 1989). In some tissues, such as muscle, lectin distribution changes during development from a predominantly intracellular to an extracellular location (Barondes and Haywood-Reid, 1981), while during embryonic chick kidney differentiation, the 16 kDa lectin has been detected as extracellular (Didier *et al.*, 1988). The 14 kDa lectin has been reported to bind a polylactosaminoglycan (Oda and Kasai, 1984). During epidermal differentiation the extracellular 16 kDa and 14 kDa lectins are associated with desmosomes and are expressed differentially during mucous metaplasia (Oda *et al.*, 1989; Akimoto *et al.*, 1992, 1993).

We have previously shown that the 14 and 16 kDa galactose-binding lectins are already present in the chick blastoderm at the time of gastrulation (Zalik *et al.*, 1990, 1992; Didier *et al.*, 1993). These lectins occur in the area pellucida and in the area opaca of the embryo. In the embryonic cell, these proteins are localized in the yolk platelets and in pleiomorphic organelles associated with the yolk granules, as well as at the cell surface (Zalik *et al.*, 1990, 1992; Sanders *et al.*, 1990; Zalik, 1991).

Most of the studies on lectin expression during chick development have been performed either in very early embryos or in embryos at advanced stages of organ development. It was of interest, therefore, to determine the expression of the 14 and 16 kDa lectins at the stages of organization of tissue primordia. We have used a panel of antibodies to these lectins to examine the expression of these proteins in embryos up to 4 days of incubation, using several protocols of tissue fixation and preparation at both the light and electron microscope levels. In the extraembryonic

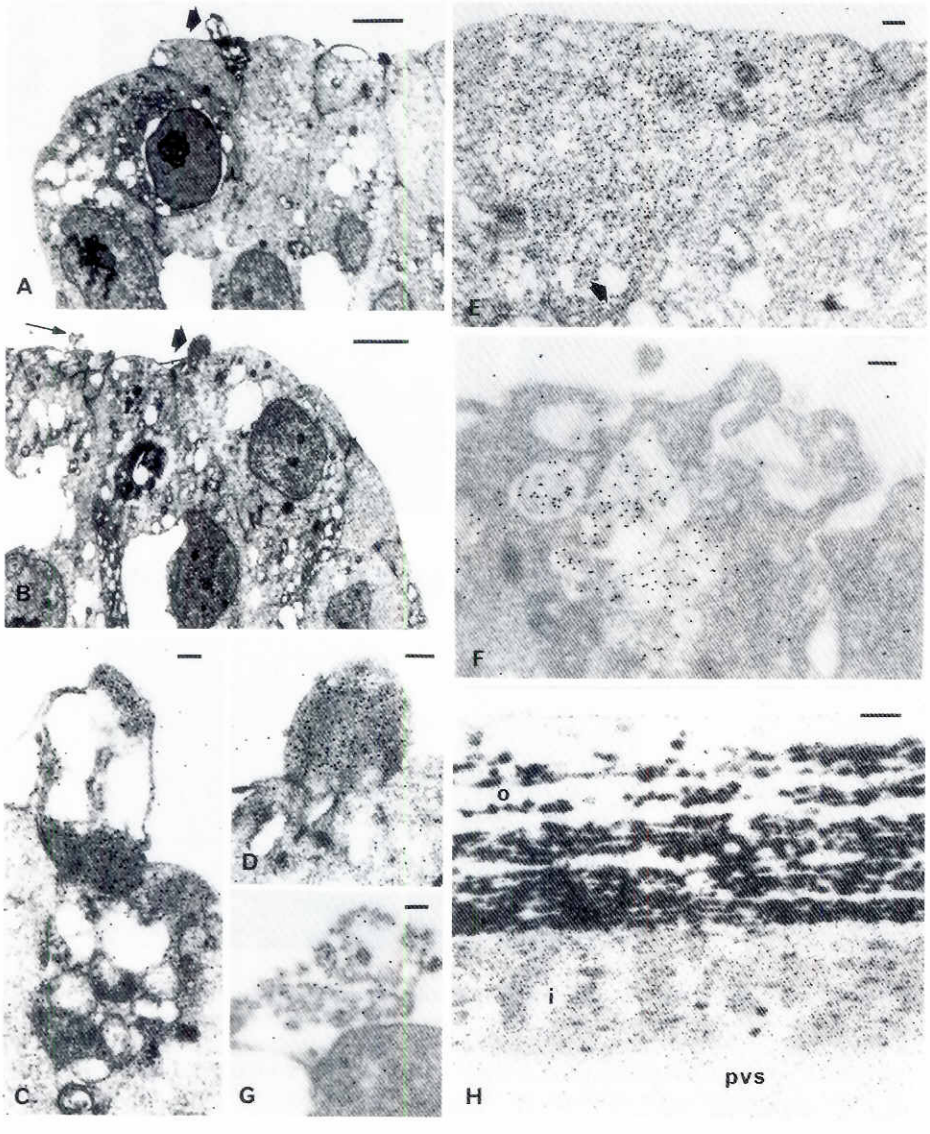

Fig. 5. Ultrastructural immunocytochemistry of sections of stage 9-10 chick embryos reacted with anti,L-16 and protein A gold. **(A)** *Electron micrograph showing the tip of the neural fold. The arrow indicates a cytoplasmic fragment that is being extruded from one of the tip cells. This region is enlarged in* **(C).** *Bar, 100 μm.* **(B)** *is a section similar to that in* **(A)** *showing two cells extruding lectin rich material at their apical surfaces (arrows). The region indicated by the larger arrow is enlarged in* **(D)**. *Bar, 100 μm.* **(C)** *The region indicated by an arrow in* **(A)** *showing lectin rich cytoplasmic material being extruded. Bar, 10 μm.* **(D)** *Detail indicated by the large arrow in* **(B)**. *Bar, 10 μm.* **(E)** *A lectin rich cell from a region similar to that shown in* **(A and B)**. *Note the juxtaposition of this labelled cell with the one on the right which does not show any immunoreactivity. Vacuoles within the lectin-rich cell (arrow) are not reactive. Bar, 10 μm.* **(F)** *Vacuoles containing immunoreactive material in the cytoplasm of a cell lining the lumen of the neural tube. Bar, 10 μm.* **(G)** *Extracellular immunoreactive material similar to that shown in* **(F)**, *in the lumen of the neural tube. Bar, 10 μm.* **(H)** *A section through the vitelline membrane, showing that both the electron dense outer layer (o) and the electron lucent inner layer (i) are strongly immunoreactive. The perivitelline space (pvs) shows no reaction product. Bar, 20 μm.*

area opaca, the lectins are expressed throughout the stages studied and are detected by all the antibodies used in these studies. Within the embryo, however, an antiserum to the liver 16 kDa lectin and two antisera to the 14 kDa lectin recognize an extracellular form of the lectin that is transiently expressed at the stages at which the organization and segregation of the axial structures and the formation of the tubular heart are taking place. An antiserum to the 16 kDa embryonic muscle lectin recognizes mainly a cellular form of the lectin which is expressed during cardiogenesis and myotome formation. Results suggest that the endogenous galactose-binding lectins in the chick embryo may perform diverse roles during organogenesis.

Results

Immunoblot analysis and Immunoprecipitation

The immunoreactivities of the anti-16 kDa lectin antisera used in these experiments is shown in Fig. 1A ,B and C. Both anti,L-16 and anti,M-16 antibodies reacted with a single band in crude (Fig. 1A) and affinity purified lectin preparations of stages 5 to 8 embryos (Fig. 1B). In extracts of embryos at stages 5 to 23, anti, M-16 immunoprecipitates a single band (Fig. 1C). We have shown previously that the anti,S-14 antiserum recognizes the 14 kDa lectin in affinity purified lectin preparations of chick blastoderms (Zalik *et al.*, 1990, 1992; Zalik, 1991), of chick embryonic pectoral muscle and of adult chick liver (Didier *et al.*, 1993).

Immunocytochemistry

The histochemical procedures for optimum lectin detection by the above-mentioned antibody probes are described in detail elsewhere (Didier *et al.*, 1993). In general, anti,M-16 binds primarily to cytoplasmic lectins and reacts weakly with matrix-type lectins. Anti,L-16 has a strong reactivity towards the matrix-type lectin and, with one exception (see below), is a rather weak probe for the cytoplasmic lectin. The anti-14 kDa antibodies in general have similar staining characteristics to those of the anti,L-16 lectin

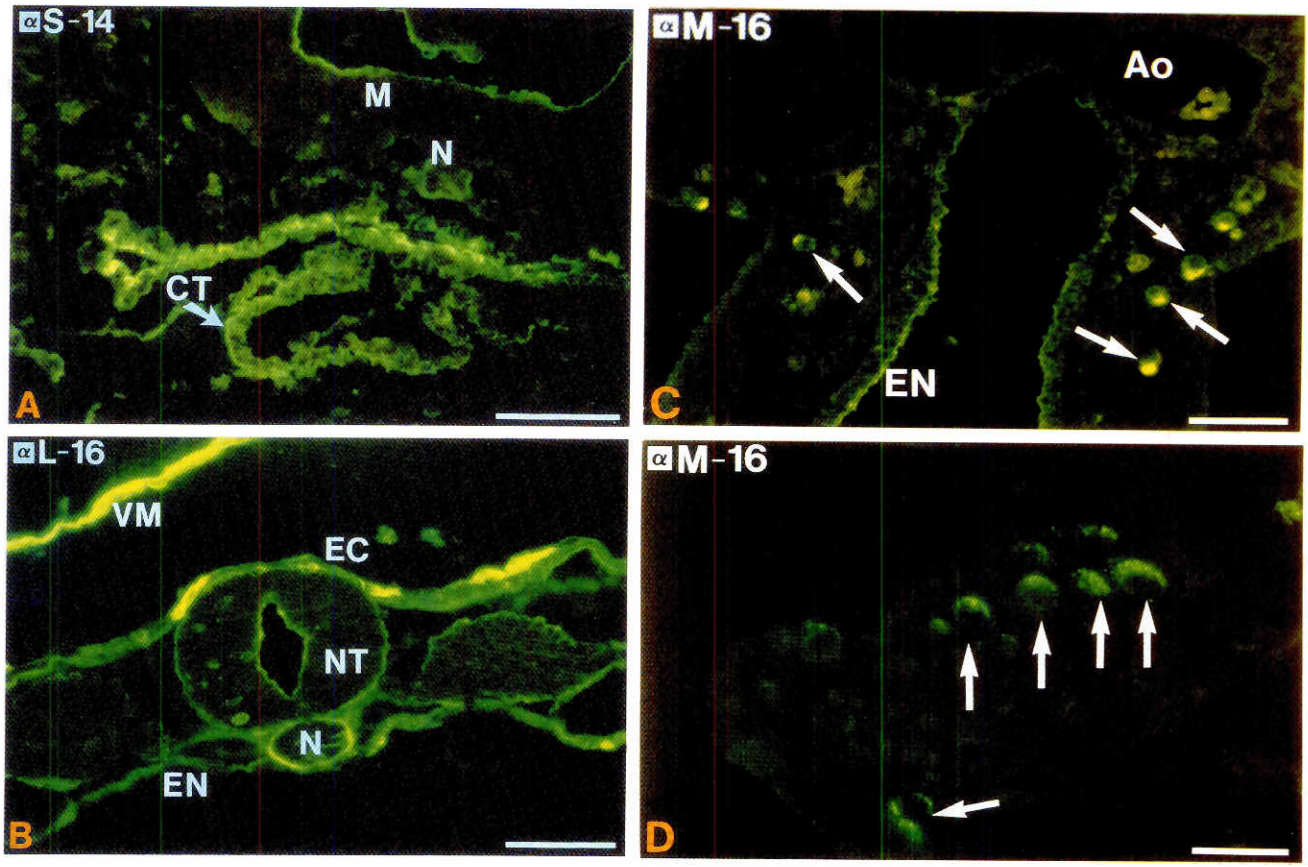

Fig. 6. Sections of embryos at several developmental stages reacted with various anti lectin antisera. (A) *Stage 10 embryo reacted with anti,S-14; this antiserum reacts with the cells of the recently fused myocardial tubes and stains intensely the cells' periphery at their coelomic surface. The apical surface of the endoderm lining the gut is also stained as is the inner limiting membrane of the mesencephalon and the external surface of the notochord.* **(B)** *Section at the thoracic region of a stage 11-12 embryo stained with anti,L-16. Lectin is expressed in the outer and inner limiting membranes of the neural tube, the outer surface of the notochord and the gut endoderm. Some cells of the ectoderm, dorsal and lateral to the neural tube, react with this antiserum; material surrounding the somite is also stained. The vitelline membrane reacts strongly with this antiserum. The yellow color of the ectodermal cells lateral to the neural tube as well as in the vitelline membrane is due to their intense staining by this antibody.* **(C)** *Section at the trunk region of a stage 19 embryo reacted with anti,M-16. Lectin is expressed in the primordial germ cells that are migrating through the mesentery (arrows), and at the surface of the endoderm. The staining in the cells in the dorsal aorta is due to the hemoglobin autofluorescence of the erythrocytes.* **(D)** *Section through the differentiating gonad of a stage 24 embryo. Lectin continues to be expressed in the primordial germ cells (arrows). (Ao) aorta, (CT) cardiac tubes, (EC) ectoderm, (EN) endoderm, (M) mesencephalon, (N) notochord, NT) neural tube, (VM) vitelline membrane. Bars, A and B: 50 μM; C and D 27 μM. The antiserum used in each section is shown on the upper left, α stands for anti.*

antibodies. All the antibodies reacted with the intracellular lectin of the yolk sac endoderm. The immunoreactive sites recognized by anti,L-16 and the anti-14 kDa lectin antisera are best preserved in embryos processed according to procedures 1 and 2, while the epitopes recognized by anti,M-16 are best retained in embryos prepared according to procedure 3 (see Materials and Methods).

An interesting finding in this work is that several of the anti-lectin antibodies had a strong reactivity towards the vitelline membrane. This was most noticeable with the anti,L-16 and was less intense with the anti-14 kDa lectin antisera (Figs. 2C,D, 5H, 6B, Tables 1, 3). Staining with anti,L-16, in most cases, was homogeneous throughout the thickness of the membrane (Figs. 5H, 6B). Anti,M-16 did not stain the vitelline membrane.

Ectoderm and derivatives

From the earliest stages of neurulation, lectin is present in irregular amorphous flocculent material located at the surface of the neural groove (Fig. 2A). From stages 9 to 12, this lectin-rich material is also present as a lectin-rich band that either forms, or is associated with, the inner limiting membrane of the neural tube. This neural tube material stains strongly with the anti-16 kDa and anti-14 kDa lectin antibodies. (Figs. 2C,D,E, 3A,B,F, 4A, 6A,B). The lectin-rich material that lines the inner surface of the neural tube has a discontinuous, patchy distribution along the tube. It is prominent at the level of the brain vesicles, the optic cup and the spinal chord. The distributions of the 14 and 16 kDa lectin in the internal surface of the neural tube exhibit a certain degree of overlapping. However, in regions through the posterior region of the neural tube, sections adjacent to those that stained positively with anti,L-16 did not react with anti,S-14. This suggests that the 14 kDa lectin does not always colocalize with the 16 kDa lectin throughout the lumenal surface of the neural tube. The flocculent

TABLE 1

LIGHT MICROSCOPE IMMUNOREACTIVITY OF THE ANTI,L-16 LECTIN ANTISERUM

Stage	histol. procedure	ectoderm	mesoderm	endoderm	extra-emb. endoderm	neural crest	neural tube	primordial germ cells	pharynx	heart	somite	head mesenchyme	notochord	amnion	v.membrane
5-6	1-2	apical	sporadic cell periphery	apical	intracellular granules & yolk	—	apical	+	A	A	A	—	—	A	+
9-13	1-2	apical	—	apical	intracellular granules & yolk	some cells +++	apical, contents, basal ECM	N.D.	apical & basal	basal myocardium	surface	few cells intracellular	surrounding ECM	A	+
	3	—	—	±	intracellular yolk	—	—	—	—	—	—	—	—	—	—
14-19	1-2	apical	—	—	intracellular yolk	—	—	—	granular contents	—	—	—	—	apical ectoderm, granular contents	A, N.D.
	3	apical	—	—	intracellular yolk	—	—	—	—	—	—	few cells +	—	N.D.	A, N.D.

1. Paraformaldehyde fixation and polyethilene glycol embedding; 2. Paraformaldehyde-glutaraldehyde fixation, frozen sections; 3. Acetic acid-ethanol fixation and paraffin embedding; + staining; — no stainng; ± weak staining; A, absent; N.D. not determined.

amorphous material in the internal surface of the neural tube, resembles in appearance, the cytoplasmic shreds in which the lectin is externalized described by Cooper and Barondes (1990) and Harrison and Wilson (1992) in differentiating myoblasts. In experiments using electron microscope immunocytochemistry, the only immunoreactivity preserved in tissues was that recognized with anti,L-16. In these experiments, the material associated with the inner surface of the neural tube has the appearance of cytoplasmic fragments which sometimes appear to be in the process of being shed from the apical surface of the neural tube cells (Fig. 5A,B,C,D,G). These fragments appeared to be composed of condensed cytoplasm, or of portions of condensed cytoplasm associated with abundant large vacuoles (Fig. 5C,D,F). In some cases, the shed cytoplasm appeared to be disintegrating into a matrix-like material (Fig. 5G). In many embryos lectin is also present in the external surface of the neural tube (Figs. 2B,C, 3A, 6B). The lectin layer present at the external surface of the neural tube was best detected with anti,L-16 and anti,14kDa lectin antisera. All of the anti-lectin antisera reacted with the external surface of the ectoderm (Figs. 2C, 3B, 4D,E, Tables 1 to 3).

Anti,L-16 also reacts strongly with cells in the ectoderm located immediately dorsal or dorsolateral to the invaginating neural tube (Figs. 2E, 3C,D, 6B, Table 1). The location of these cells suggests that they may belong to the neural crest population. Much of the intracellular staining in these cells appeared to be distributed homogeneously in the cytoplasm (Figs. 3C,D, 6B). When examined by ultrastructural immunocytochemistry, lectin immunoreactivity in these cells appears to be present throughout the cytoplasmic matrix but it seems to be excluded from the vacuolar contents (Fig. 5E). As cells penetrate into the embryo proper, lectin staining is reduced and is retained only in some lectin-rich cells in the head mesenchyme (Fig. 3E). These cells are present in stages 9 to 12 and disappear after stage 13 of development (Tables 1 to 3).

Controls with preimmune serum to anti,L-16 did not stain any embryonic or extraembryonic tissues. An additional control was performed using the antiserum to VLDL Apo II a lipoprotein of the very low density lipoproteins. In primitive streak embryos this apolipoprotein copurifies and colocalizes with the 14 and 16 kDa lectins (Sanders *et al.*, 1990; Zalik *et al.*, 1990). The antiserum to VLDL Apo II did not stain any of the tissues within the embryo (Fig. 2F), and staining was restricted to the cells of the extraembryonic endoderm where it was associated with the intracellular yolk (not shown). This indicates that the cellular and matrix-type lectins, present within the differentiating embryo as such, are not associated with this apolipoprotein.

Mesoderm and derivatives

In embryos at stages 8 to 13, lectin is present in the extracellular matrix material that surrounds the notochord, (Figs. 2B, 3A,D, 6B), and the periphery of the somite (Fig. 6B, Tables 1, 2). At stage 8, the cells composing the newly formed somites and the unsegmented dorsal mesoderm express lectin. With subsequent development strong lectin expression is observed in the myotomal component of the differentiating somite (Fig. 4A,B). Such distribution is similar to the one described recently by Levi and Teichberg (1989) using an anti 15 kDa lectin antiserum. Anti,M-16 stained the cytoplasmic lectin of the myotome strongly but did not bind to the extracellular material surrounding the somites, while anti,L-16 did (Fig. 6B). The latter antiserum had an immunoreactivity restricted for the most part to the periphery of the somite cells (Figs. 3C, 6B). At later stages of somite development, anti,L-16 did not stain the myotome.

Lectin was present in the cells and at the coelomic surface of the cardiac primordium (Figs. 3A, 4C, 6A). It should be noted that the anti,L-16 and anti,S-14 preferentially bound to surface material situated in the coelomic side of the myocardium with a weaker immunoreactivity towards the cells forming the myocardium of the heart primordium. The cellular myocardial lectin was stained strongly by anti,M-16 (Fig. 4C, Table 2). Some mesenchymal cells located sporadically between the developing organ primordia at different levels of the embryo were seen to have strong lectin expression. Most of these lectin-positive cells were located in the cephalic mesenchyme; others were seen dorsal to the neural tube in trunk sections (not shown). Lectin immunoreactivity in the cardiac primordium was not preserved under the conditions used for electron microscopical immunocytochemistry.

Endoderm

Previously we reported that anti,L-16, anti,M-16 and anti,S-14 react with the endoblast and hypoblast of stage 4 to 6 embryos

(Zalik *et al.*, 1990, 1992; Didier *et al.*, 1993). During development of the digestive system, the endoderm continues to express lectin (Figs. 2C, 3A, 4D, 6A,B,C, Tables 1 to 3). In embryos at stages 10 to 13, lectin is present in cells of the pharyngeal endoderm (Figs. 3A, 6A) as well as the posterior embryonic endoderm (Fig. 6B). In embryos at 3 to 4 days of incubation (stages 19 to 22), lectin continues to be expressed in the pharyngeal endoderm (Fig. 4D). At these stages of development intense lectin immunoreactivity with anti,L-16 and anti-14 kDa lectin antibodies appeared within flocculent aggregates present in the amniotic cavity of embryos (Fig. 4D,E, Tables 1 and 3). The extraembryonic endoderm of the yolk sac is rich in lectin at all stages of development; these cells stained with all of the antibodies used in this study (Fig. 3G,H).

We have previously shown that the primordial germ cells from early embryos, are rich in lectin (Zalik, 1991; Didier *et al.*, 1993). These cells continue to express lectin as they migrate through the embryo and settle into the developing gonad (Fig. 6C,D, Table 2). Within the migrating primordial germ cells the lectin-rich cytoplasm appears to be segregated to one pole of the cell (Fig. 6C). This distribution is retained, to a certain extent, when cells reach the developing gonad (Fig. 6D). This lectin could only be detected with the anti,M-16 kDa antiserum. The immunoreactivity patterns of developing embryos with these antisera is summarized in Tables 1 to 3.

Discussion

Immunoreactivity of the anti-16 kDa and anti-14 kDa lectin antisera

The polyclonal antisera used in these studies consist of mixtures of antibodies to different epitopes of the lectin molecule(s), and the relative concentration of these antibodies varies among different antisera to the same protein. In crude and purified lectin extracts from chick embryos, immunoblot analyses indicate a very strong immunoreactivity of both anti,L-16 and anti,M-16 antisera with the 16kDa band. These antisera also recognize a 16kDa band in affinity purified lectin preparations of pectoral muscle and liver which consists of a mixture of 16 and the 14 kDa lectins (Didier *et al.*, 1993). These results indicate that the two anti-16 kDa lectin antisera used in these studies appear to be lectin-specific. This is in contrast with the purified anti 15 kDa lectin antibody used by Levi and Teichberg (1989) which also reacted with a 6.5 kDa band.

Results using immunohistochemistry, indicate that the immunoreactivity patterns of the above anti-16 kDa lectin antisera with different embryonic structures show a certain degree of overlap, in that both antisera react with the extraembryonic endoderm, with some lectin-rich cells in the mesenchyme and with the internal limiting membrane of the neural tube. However, while anti,L-16 reacts predominantly with an extracellular matrix type of lectin as well as with some of the cytoplasmic lectin of ectodermal and of neural crest cells, anti,M-16 reacts primarily with cytoplasmic lectin present in early and more advanced embryos (Table 2). The differences in the immunoreactivity pattern between the 16 kDa lectin antisera may depend on the differential display of a particular protein configuration in the matrix and some cytoplasmic-associated lectins. It is possible that subjecting these proteins to different fixatives, temperatures, embedding compounds during the histological processing, leads to differential exposure of particular lectin epitopes recognized by specific antibodies which differ in abundance in the two anti-16 kDa lectin antisera. Paraformaldehyde fixation followed by PEG embedding or cryostat sectioning may preserve a lectin variant which predominates in the extracellular matrix, in a more native form required by its recognition by anti,L-16. In contrast, the antibodies that predominate in anti,M-16 may recognize epitopes buried within the protein, most of which become available only after exposure to extensive denaturation conditions. Alternatively, the lectins, once externalized, may undergo molecular modifications or associate with glycoproteins which may prevent them from being recognized by some antisera. The epitopes recognized by the antibodies of the anti-14 kDa lectin antisera used in these studies, (anti,l-14 and anti,S-14) may be of a similar nature to those reactive with anti,L-16, since the pattern of embryo staining was similar with these three antisera and the immunoreactivity was best preserved in

TABLE 2

LIGHT MICROSCOPE IMMUNOREACTIVITY OF THE ANTI,M-16 LECTIN ANTISERUM

Stage	histol. procedure	ectoderm	mesoderm	endoderm	extra-emb. endoderm	neural crest	neural tube	primordial germ cells	pharynx	heart	somite	head mesenchyme	notochord	amnion	v.membrane	wing bud ectoderm
	2	apical	N.D.	N.D.	intracellular yolk	N.D.	apical	N.D.	apical	N.D.	—	—	+	A	N.D.	A
5-8	3	apical	cell periphery	+	intracellular yolk	—	apical	+	intracellular	intracellular	intracellular	—	—	A	—	A
9-13	1	—	—	—	intracellular yolk	—	—	—	—	—	—	—	—	A	—	A
	3	apical	—	apical	intracellular yolk	—	apical contents	+	granular contents	myo-cardium	intracellular presumptive myotome	few cells intracellular	—	A	—	A
14-19	1	—	—	—	intracellular yolk	—	—	—	—	—	myotome	—	—	—	A	—
	3	apical	—	apical	intracellular yolk	—	apical	+	apical, granular contents	myo-cardium, endocardium	myotome	—	—	N.D.	A	+

1. Paraformaldehyde fixation and polyethylene glycol embedding; 2. Paraformaldehyde-glutaraldehyde fixation, frozen sections; 3. Acetic acid-ethanol fixation and paraffin embedding; + staining; — no stainng; ± weak staining; A, absent; N.D. not determined.

TABLE 3

LIGHT MICROSCOPE IMMUNOREACTIVITY OF THE ANTI 14 kDa LECTIN ANTISERA

Stage	anti-serum	histol. procedure	ectoderm	mesoderm	endoderm	emtra-emb. endoderm	neural crest	neural tube	primordial germ cells	pharynx	heart	somite	head mesenchyme	notochord	amnion	v.membrane
5-6	anti,S-14	1	apical	sporadic cell periphery	apical	intracellular yolk	—	apical	N.D.	A	A	A	—	—	A	+
9-13	anti,S-14	1	apical	—	apical	intracellular yolk	—	apical contents, basal ECM	—	apical	basal myocardium	—	few cells	cellular surrounding ECM	A	+
		3	—	—	—	intracellular yolk	—	—	—	—	—	—	—	—	A	—
	anti,I-14	1	apical	—	apical	intracellular yolk	—	apical contents, basal ECM	N.D.	N.D.	+	N.D.	few cells	cellular surrounding ECM	A	+
14-19	anti,S-14	1	—	—	apical	intracellular yolk	—	—	—	apical	—	—	—	—	apical ectoderm, granular contents	A
		3	—	—	—	intracellular yolk	—	—	—	—	—	—	—	—	—	A

1. Paraformaldehyde fixation and polyethilene glycol embedding; 2. Paraformaldehyde-glutaraldehyde fixation, frozen sections; 3. Acetic acid-ethanol fixation and paraffin embedding; + staining; — no stainng; ± weak staining; A, absent; N.D. not determined.

embryos processed according to method 1 (Tables 1 and 3). Based on the immunoreactivity patterns with the antisera used in these studies, the lectins present in embryos up to 4 days of incubation can be tentatively divided into three types: a) the *matrix* type lectin(s), recognized predominantly by anti,L-16, anti,S-14 and anti,I-14, and weakly by anti,M-16; b) the *cellular* or *cytoplasmic* form of lectin(s) recognized predominantly by anti,M-16 and less effectively by anti,L-16 and the anti-14 kDa lectin antisera and, c) the *storage* form of lectin associated with the intracellular yolk present in the early blastoderm cells and in the cells of yolk sac endoderm; this lectin type is recognized by all of the anti-lectin antisera used in this study.

Possible functions of the lectins in the embryo

Our results show that at the time of the segregation of organ primordia the 14 and 16 kDa lectins are located in the vitelline membrane, the perivitelline space and in the matrix material associated with organ primordia. The association of these lectins with the vitelline membrane suggests that these proteins may play a role in the expansion of the chick blastoderm. A fucoidan/mannan-specific lectin is present in the outer layer of the chick vitelline membrane (Cook *et al.*, 1985), while the galactose-specific lectins, in particular the 16 kDa lectin, are located in the inner layer as well as throughout the width of this membrane. The association of the blastoderm with the inner side of the vitelline membrane is crucial for blastoderm expansion since the latter is inhibited when blastoderms are cultured on the outer surface of this membrane (New, 1959). This inhibition seems to be due to the formation of strong adhesions by the cells of the edge of the blastoderm with the outer surface of the membrane. Evidence indicates that externalized 14kDa lectins inhibit adhesion to the extracellular matrix (Allen *et al.*, 1990; Cooper *et al.*, 1991), and results from our laboratory indicate that externalized blastoderm lectin in which the 14 and 16 kDa lectin predominate, inhibits the adhesion of cells of early embryos (Milos and Zalik 1981, 1982, 1983). It is possible that the galactose-binding lectins in the vitelline membrane as well as those released from cells could be involved in blastoderm spreading by allowing for transitory adhesion and deadhesion. Galactose binding lectins bind with high affinity to oligosaccharides containing polylactosamine (Oda and Kasai, 1984) or poly-N-acetyllactosamine residues (Merkle and Cummings, 1988). Fibronectin is present in the interface of the migrating edge cells of the blastoderm and the vitelline membrane (Lash *et al.*, 1990). Some forms of embryonic fibronectin contain lactosaminyl residues (Cossu and Warren, 1983; Zhou and Laine, 1985), and some galactose-binding lectins bind to the glycosylated forms of this protein (Sato and Hughes, 1992). By binding and saturating lactosaminyl groups in an embryonic fibronectin, the lectins could weaken the adhesion of cells at the leading edge of the blastoderm allowing them to detach and form new adhesions as they migrate over the vitelline membrane. Experiments in our laboratory indicate that anti,L-16 as well as hapten inhibitors of these endogenous lectins inhibit expansion of the blastoderm over the vitelline membrane (Jeeva and Zalik in preparation). The presence of a galactose binding-lectin in the vitelline membrane, i.e. the extracellular matrix of the egg, has been also described for the early amphibian embryo (Roberson and Barondes, 1982, 1983; Outenreath *et al.*, 1988; Yoshizaki, 1989; Hedrick and Nishihara, 1991).

In embryos up to stage 13 we have observed extracellular lectin in the apical and basal surfaces of the early neural tube, the notochord, the apical and basal surfaces of the endoderm and the coelomic surfaces of the heart primordium. The presence of the 14 and 16kDa lectins in the extracellular matrix has been documented (Beyer *et al.*, 1979, 1982; Barondes and Haywood-Reid, 1981; Cooper and Barondes, 1990; Cooper *et al.*, 1991; Kawamura *et al.*, 1991; Akimoto *et al.*, 1992; Harrison and Wilson, 1992). The 14kDa lectin of mammalian myoblasts has been reported to colocalize with laminin and to bind to the lactosaminyl residues of this molecule (Cooper and Barondes, 1990; Cooper *et al.*, 1991). The lectin associated with the basement membrane of the neural tube and notochord, and with the endoderm and the coelomic surface of the myocardial tubes could also bind to putative lactosaminyl residues of fibronectin (Duband and Thiery, 1982a,b; Linask and Lash, 1986; Drake *et al.*, 1990), laminin (Drake *et al.*, 1990), or of cytotactin /tenascin (Crossin *et al.*, 1986); proteins that are asso-

ciated with the basal lamina of these primordia. The lectins, by binding to these residues, could inhibit the formation of strong permanent adhesions between other cells in the embryo and the extracellular matrix (Dean *et al.*, 1990), and allow for the segregation of the rudiments of organ primordia. A galactose-binding lectin associated with extracellular material surrounding the early neural tube has also been described in the *Xenopus* embryo (Outenreath *et al.*, 1988). Likewise, the lectins present in the inner limiting membrane of the neural tube could bind to apical N-cadherin molecules (Hatta *et al.*, 1987; Duband *et al.*, 1988), preventing homotypic adhesion at the apical surfaces of the neuroepithelial cells and preserving the lumen of the tube. These lectins could also modulate proliferation, (Lipsick *et al.*, 1980; Pitts and Yang, 1981; Wells and Malluci, 1991; Yamaoka *et al.*, 1991), and differentiation (Matsutani and Yamagata, 1982; Kawaguchi *et al.*, 1991) in the cells of the neural tube. Currently it is not possible to make any assumptions concerning the origin of the lectin-rich internal limiting membrane of the neural tube, but the release of lectin by disintegrating cytoplasmic fragments extruded from certain cells, as shown by our ultrastructural observations, remains a strong possibility. Alternatively, the lectin of this internal limiting membrane could also be released after the death of some of the lectin-rich neuroectodermal cells. The differential lectin distribution could also be associated with the acquisition of polarity by the neuroepithelium of the neural tube as well as by other epithelia.

The cellular type of lectin occurs in a cohort of cells which are possibly neural crest. Some of the cellular lectins present in these cells could be released by the cells, bind to lactosaminoglycans of the extracellular matrix, and aid in cell dislodgment and cell separation. It is also possible that these lectin-rich cells represent a subpopulation of neural crest cells with distinct commitments. The uniform distribution of lectin in the cytoplasm (Figs. 3D,E, 5E) is similar to that described by Cooper and Barondes (1990) and Harrison and Wilson (1992) in differentiating myoblasts, and by Lindstedt *et al.* (1993) in a kidney cell line.

The primordial germ cells express a high concentration of cytoplasmic lectin (Didier *et al.*, 1991; Zalik, 1991), as well lactose/lactosamine-containing glycoconjugates (Didier *et al.*, 1990). One of the possible roles of the lectin in these cells could be as a factor involved in the processing of storage materials present in the yolk which could be used as a source of energy necessary for migration. This galactose-binding protein could also be associated with vitellogenesis during oocyte growth since, in oocytes at post-hatching stages, this protein is abundant in the cytoplasm and is also associated with the nucleus (Didier *et al.*, 1990, 1991). Alternatively, these lectins could be released by the primordial germ cells in the gonad and promote proliferation.

The presence of cytoplasmic galactose-binding lectins in the early myotome has been also reported by other investigators (Levi and Teichberg, 1989; Poirier *et al.*, 1992). In our experiments we found strong immunoreactivity in the myotome in embryos stained with anti, M-16. Since the anti,M-16 antiserum and the antiserum used by Levi and Teichberg (1989) were prepared against the main lectin of the embryonic chick pectoral muscle, it is probable that both antisera detect the same lectin isotype. Since both antisera react with the myotome as well as the myocardium, this lectin could be a factor important in skeletal and cardiac myogenesis. The cytoplasmic lectin in the myotome could be synthesized in the early myoblast and released as these cells migrate away from the myotome and undergo fusion to form myotubes (Cooper *et al.*, 1991).

The presence of the 14 and 16 kDa lectins in the extracellular matrix as well as in the cytoplasm and yolk granules of cells of several embryonic and extraembryonic tissues suggests that these proteins play several roles in embryonic development. The fact that antibodies reacting with seemingly the same protein in immunoblot analysis recognize different structural lectins in the embryo, suggests that diverse versions of the same proteins may play different functional roles in embryonic development and tissue differentiation.

Materials and Methods

Preparation of embryos

Embryos from non-incubated stages up to stage 24 (Hamburger and Hamilton, 1951), obtained from the University of Alberta farm or from a local hatchery close to Aubiere, France were used. Embryos were fixed either in: 1) freshly prepared 3.7% formaldehyde in Panett and Compton's saline and embedded in polyethylene glycol, 2) a mixture of 3.5% formaldehyde and 0.05% glutaraldehyde in phosphate buffered saline and processed for cryostat sections (Didier *et al.*, 1990) and, 3) a mixture of 1% acetic acid in 95% ethanol and embedded in paraplast. The fixation and processing procedures as well as their influence on the detectability of the matrix and cellular forms of lectin by the different anti-lectin antisera is reported in detail elsewhere (Didier *et al.*, 1993).

Antibodies

Several rabbit anti lectin antisera were used: a) anti-liver 16kDa lectin antiserum (anti,L-16) was prepared in Edmonton and was raised by injecting the 16kDa band cut from gels in which affinity purified adult chicken liver lectin preparations were separated; b) anti-muscle 16 kDa lectin antiserum (anti,M-16) was produced in Aubiere by injecting affinity purified lectin fractions from 15-day old embryonic chick pectoral muscle; c) anti-skin 14 kDa lectin antiserum (anti,S-14) prepared against the 16-18 day chick embryonic skin lectin was a gift from Drs. Y. Oda and K.I. Kasai, Teikyo University, Japan; d) the anti-intestine 14 kDa (anti,1-14) lectin antiserum prepared against the lectin from adult chick intestine, was a gift from Dr. S.H. Barondes, University of California, San Francisco. An antiserum to a chick apolipoprotein (Apo) of the very low density lipoproteins Apo VLDL II, was prepared using the protein from early embryos as an antigen as described previously (Zalik *et al.*, 1990).

Immunoblot analysis

Crude and affinity purified lectin extracts from stages 4-6 embryos (Hamburger and Hamilton, 1951), as well as extracts and purified lectin preparations from adult chick liver were prepared as described previously (Zalik *et al.*, 1990). Muscle lectins were affinity purified from crude extracts of 15 day old embryonic pectoral muscle (Didier *et al.*, 1988, 1993). Extracts and purified lectin preparations were separated by SDS-PAGE by the procedure of Laemmli (1970), using gradient gels with the Miniprotean II system (Bio Rad), as described previously (Zalik *et al.*, 1990), or the Mighty Small II electrophoresis unit (Hoefer Scientific) using 15% gels. Transfer to nitrocellulose or Immobilon P (Millipore), was performed according to Towbin *et al.*, (1979) and immunoreactivity in immunoblot strips was assessed using peroxidase labelled goat anti-rabbit immunoglobulin (Bio Rad) and detected with 4-chloro-1-naphthol (Hawkes *et al.*, 1982). The antisera to the 16kDa adult liver and embryonic pectoral muscle lectins as well as that for the 14 kDa skin lectin were used at a dilution of 1:1000. The antiserum to the 14 kDa intestinal lectin was used at a 1:100 dilution; all antisera were diluted in TBS-BSA (50 mM Tris-HCl in 100 mM NaCl pH 7.2, containing 1% bovine serum albumin (BSA, Sigma). Blots were exposed overnight (18-19 hours) to the first antiserum and 2 h to the second antiserum.

Immunoprecipitation

Lectin extracts of chick embryos at stages 5 to 23 prepared according to Nowak *et al.* (1977) were submitted to immunoprecipitation on Protein A-

Sepharose (PAS, Pharmacia). Extracts ranging in volume from 2.5 to 10 ml were incubated with 5 µl of anti,M-16 antiserum for 2 h at room temperature with mild shaking. The antigen-antibody complex was then incubated for 2 h with 20 mg PAS, previously swollen in 60 µl of 0.12 M Tris-HCl pH 7.2 containing 0.1% BSA and 1% Triton X-100. After centrifugation, beads were carefully rinsed in 0.12 M Tris-HCl pH 7.2 with 1% Triton X-100. PAS-bound immune complexes were eluted in Laemmli's sample buffer (1970), heated at 90°C for 10 min and processed for SDS-PAGE and immunoblotting.

Immunohistochemistry

Immunohistochemical staining procedures were performed at room temperature. Primary antisera were used at the following dilutions: anti,L-16 antiserum 1:50; anti,M-16 antiserum 1:100; anti,S-14 antiserum 1:100; anti,I-14 antiserum 1:100. All dilutions were performed with phosphate-buffered saline (PBS, 150 mM NaCl, in 5mM Na/K phosphate buffer, pH 7.5), containing 1% BSA. Embryos processed according to procedures 1 and 2, were stained using immunofluorescence as follows: sections of polyethylene-glycol embedded tissues (procedure 1) were treated with acetone for 3 min, washed in three changes of PBS, 5 min each, reacted with the primary antisera for 1 h at room temperature, washed in 3 changes of PBS, 5 min each, and exposed to fluorescein-labelled goat anti-rabbit immunoglobulin (Sigma, 1:80 dilution) for 1 h. After washing with PBS as before, tissues were mounted in Mowiol (Calbiochem) with DABCO (Sigma), prepared as reported previously (Zalik *et al.*, 1990). In some experiments, nuclei were stained with DAPI (Sigma), dissolved in PBS (0.25 mg/ml) for 1 min followed by washing sections in PBS prior to mounting. Sections were observed using the Zeiss Photomicroscope II using the appropriate filters. Photographs were taken using Kodak Tri-X Pan or Ektachrome 200 films. For immunofluorescent staining of embryos processed using procedures 2 and 3, cryostat sections or deparaffinized sections, were rinsed in PBS and incubated in 0.05 M NH_4Cl. Subsequently, sections were exposed to the primary antisera for 1h at room temperature, washed in PBS as above and exposed to fluorescein labelled goat-anti rabbit IgG (Biosys, Compiegne, France) (1:400 dilution) for 1h, washed in PBS and mounted in 90% glycerol in 10% PBS containing 2% n-propylgallate. In some sections nuclei were stained with Bisbenzimide H 33258 (Fluka, 0.05 mg/ml). Observations were made with a Leitz Orthoplan microscope equipped with the PLOEMOPAK II fluorescence system. Photographs were taken with Kodak TMAX (400) or Ektachrome 400.

Electron microscope immunocytochemistry

Embryos were removed from the yolk and fixed while attached to the vitelline membrane in a solution of 2% glutaraldehyde and 0.2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 30 min at room temperature and left overnight in PBS at 4°C. The following day embryos were placed in freshly prepared 0.5% sodium borohydride in PBS for 30 min and subsequently washed for 15 min, both at 4°C. Embryos were then transferred to 50% ethanol at 4°C and further dehydration and embedding were performed at -20°C. Samples were transferred through 70% ethanol for 20 min, 95% ethanol for 25 min, 90% ethanol LR gold resin (1:1) (Polysciences) 2 h, 90% ethanol LR gold 7:3 1h, LR gold resin overnight, LR gold resin+initiator 24 h. Embryos were then polymerized in capsules under a UV lamp (360 µm) for 24 h. Thin sections were cut and mounted on uncoated nickel grids. For immunogold cytochemistry, grids were floated on a drop of filtered 1% BSA in PBS for 10 min and transferred without washing to a drop of primary antibody diluted 1:50 with PBS for 2 h at room temperature. Control grids were floated on pre-immune or non-immune serum. After washing with jets of filtered PBS, each grid was floated on three successive drops of PBS 10 min each. Grids were then floated for 1 h at room temperature on drops of protein-A gold (E-Y Laboratories), diluted 1:10 with filtered PBS. Washing was performed by directing jets of filtered PBS at the grids, followed by floating the grids on drops of PBS. Final washing was carried out with jets of filtered distilled water and blotting with filter paper. Grids were stained with uranyl acetate and lead citrate and examined with a Phillips 300 electron microscope.

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