

# Murine Clusterin: Molecular Cloning and mRNA Localization of a Gene Associated with Epithelial Differentiation Processes during Embryogenesis

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**Abstract.** Clusterin is a broadly distributed glycoprotein constitutively expressed by various tissues and cell types, that has been shown to be involved in cell-cell adhesion and expressed during cellular differentiation *in vitro*. To assess the suggested participation of clusterin in these processes *in vivo*, we have cloned the cDNA encoding murine clusterin and studied the cellular distribution of clusterin mRNA during murine embryogenesis. Sequence analysis of the cDNA encoding murine clusterin revealed 92 and 75% sequence identity with the rat and human cDNAs, respectively, and conservation of the predicted structural features which include  $\alpha$ -helical regions and heparin-binding domains. From 12.5 d of development onwards, the clusterin gene is widely expressed in developing epithelia, and selectively localized within the differen-

tiating cell layers of tissues such as the developing skin, tooth, and duodenum where proliferating and differentiating compartments are readily distinguished. In addition, transient and localized clusterin gene expression was detected in certain morphogenetically active epithelia. In the lung, abundant gene transcripts were detected in cuboidal epithelial cells of the terminal lung buds during branching morphogenesis, and in the kidney, clusterin gene expression in the epithelial cells of comma and S-shaped bodies coincided with the process of polarization. Our results demonstrate the *in vivo* expression of the clusterin gene by differentiating epithelial cells during murine embryogenesis, and provide novel evidence suggesting that clusterin may be involved in the differentiation and morphogenesis of certain epithelia.

**C**LUSTERIN is a heterodimeric glycoprotein found in serum and seminal plasma which is constitutively synthesized and secreted by various cell types (Murphy et al., 1988; Jenne and Tschopp, 1989; Burkey et al., 1991; Hartmann et al., 1991; Diemer et al., 1992). *In vivo*, the distribution of clusterin within tissues is broad; clusterin mRNA being relatively abundant in the testes, brain, liver, and ovary, and detectable in several other tissues including the kidney, thymus, spleen, and heart (for review see Jenne and Tschopp, 1992).

Originally, clusterin was identified as a major constituent of ram rete testis fluid and shown to induce the aggregation of Sertoli cells *in vitro* and favor homotypic aggregation within mixed cell suspensions (Fritz et al., 1983; Blaschuk et al., 1983). More recently, clusterin has been characterized and sequenced in several other species (Kirszbaum et al., 1989; Jenne and Tschopp, 1989; Collard and Griswold, 1987; Palmer et al., 1990; Michel et al., 1989; for review see Jenne and Tschopp, 1992). Sequence analysis revealed considerable homology between different species and predicts potential binding sites such as a heparin-binding domain and several amphiphilic regions thought to mediate the

interactions of clusterin with hydrophobic substances or hydrophobic domains in other proteins (Jenne and Tschopp, 1992). Some of clusterin's known properties seem to be explained by its avidity for exposed hydrophobic domains on macromolecules. First, clusterin, which is a potent inhibitor of complement mediated lysis *in vitro* (Jenne and Tschopp, 1989; Choi et al., 1989; Murphy et al., 1989b), and colocalizes with membrane attack complex (MAC) deposits *in vivo* (Murphy et al., 1988, 1989a; French et al., 1992a,b), most probably binds to newly exposed hydrophobic domains in nascent C5b-7, C5b-8, and C5b-9 complexes thereby inhibiting membrane insertion (Tschopp et al., 1993a). Second, clusterin associates with certain high density lipoprotein (HDL) particles (de Silva et al., 1990; James et al., 1991; Jenne et al., 1991) and is dissociated from these particles by non-ionic detergents, suggesting that it interacts directly with lipids (Jenne et al., 1991).

There is growing evidence that clusterin may be involved in tissue remodeling. Clusterin gene expression is highly increased during the involution of certain tissues in response to hormonal modulations or injury (Buttayan et al., 1989; Bandyk et al., 1990; Grima et al., 1990; Sensibar et al.,

1991; Connor et al., 1991; May et al., 1990), in particular under circumstances where cell death occurs by apoptosis. Recent reports indicate, however, that clusterin is not expressed by apoptotic cells in vivo (Garden et al., 1991; French et al., 1992c), and it has been suggested that clusterin may be part of the repair and remodeling response within regressing tissues (Jenne and Tschopp, 1992).

Recently, clusterin gene expression has been correlated to the *in vitro* differentiation of aortic smooth muscle cells (Diemer et al., 1992) and the oncogenic transformation of neuroretinal cells by various retroviral oncogenes (Michel et al., 1989). We have also reported that clusterin gene expression may be related to the terminal differentiation of thymic epithelial cells in vivo (French et al., 1992c). Taken together, the wide tissue distribution, high degree of interspecies homology, propensity to induce cell aggregation, and recent evidence of its expression during cellular differentiation processes, suggest that clusterin may play a more general role in cellular physiology than those described to date.

To determine whether clusterin is implicated in the cellular processes leading to the histogenesis and ultimate shaping of tissues during development, we have cloned the cDNA encoding murine clusterin and used it to study by *in situ* hybridization the pattern of clusterin gene expression during murine embryogenesis. We report here that clusterin mRNA is detected from 12.5 d onwards essentially in the epithelial components of developing organs undergoing active morphogenesis or terminal differentiation.

## Materials and Methods

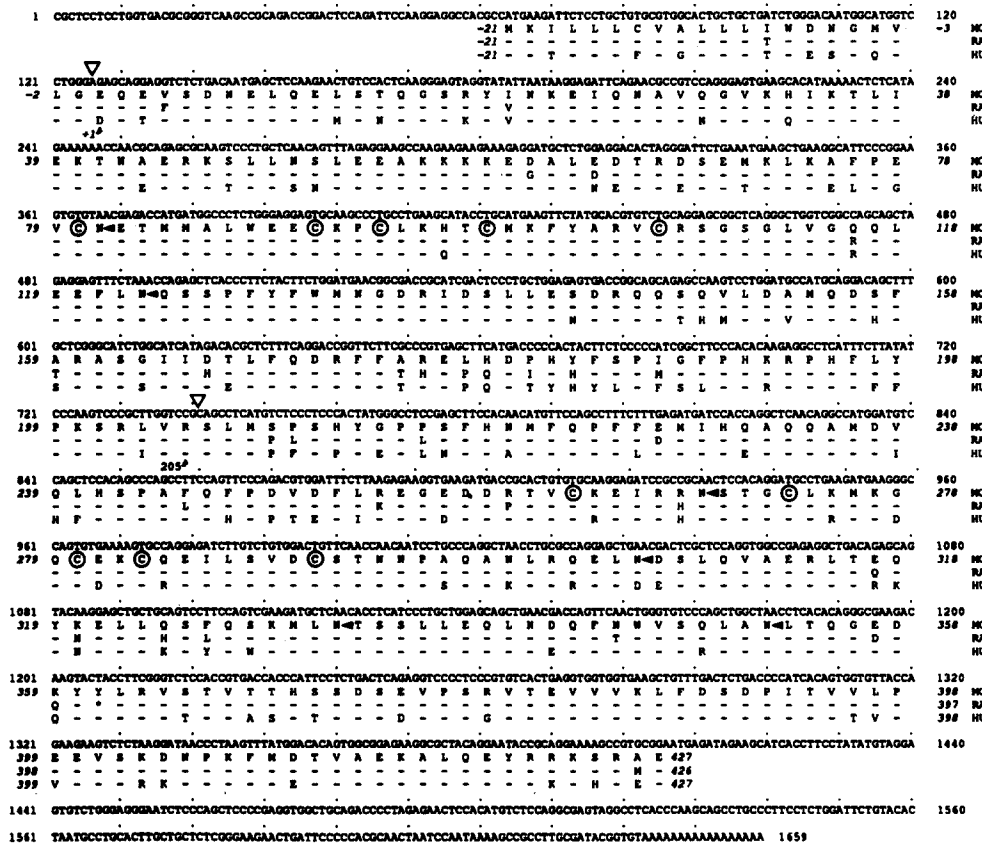
### Materials

Adult NMRI female mice (Kleintierfarm, Madörin, Basel) were housed with males for one night and monitored for the appearance of a vaginal plug the following morning. Noon on the day of the vaginal plug was considered as day 0.5 of gestation. Segments of uteri, or at later stages whole embryos were dissected, embedded in Tissue-Tek (Miles Ames Div., Elkhart, IN), frozen down in precooled methylbutane and stored at  $-70^{\circ}\text{C}$ . For each time point, three to four embryos were analyzed, and 20–40 sections were prepared per embryo.

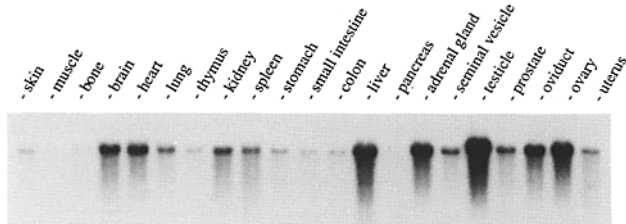
### Isolation of Murine Clusterin cDNA

Poly(A)<sup>+</sup> RNA purified from total murine testicular RNA with oligo(dT)-cellulose (type 77F, Pharmacia LKB Biotechnology, Uppsala, Sweden), was used to synthesize murine testicular cDNA by reverse transcription (cDNA synthesis kit; Boehringer, Mannheim, Germany). This cDNA was used to obtain a 638-bp murine clusterin cDNA fragment by oligonucleotide-primed amplification using the PCR. The oligonucleotides were chosen within the most conserved regions of rat and human clusterin cDNAs (Collard and Griswold, 1987; Kirsbaum et al., 1989; Jenne and Tschopp, 1989): 5'-ATG ATG GCC CTC TGG GAA GAG TG-3' corresponds to residues 511–533 of the human clusterin cDNA (Jenne and Tschopp, 1989), and 5'-GGA TTG TTG GTC GAA CAG TCC AC-3' is complementary to residues 1126–1148 of the human clusterin cDNA with the exception of residue 1146. The PCR amplified 638-bp fragment of murine clusterin was subsequently cloned into the *Sma*I site of pBluescript KS(+) vector and both strands sequenced by the dideoxy methodology with T7 and T3 sequencing primers and a commercial kit (Sequenase 2.0; United States Biochemical Corp., Cleveland, Ohio).

To obtain the full length murine clusterin cDNA, a Balb/c female murine heart cDNA library (Stratagene, La Jolla, CA) constructed into the lambda Zap II vector was screened with the partial murine cDNA probe described



**Figure 1.** Nucleotide and derived amino acid sequences of murine clusterin. The deduced amino acid sequence (MO) is shown in single-letter code beneath the cDNA sequence, together with the protein sequence of rat (RA) and human (HU) clusterin. Amino acid residues  $-21$ – $-1$  represent the signal peptide; residues 1–205 encode the  $\alpha$  subunit, and residues 206–427 the  $\beta$  subunit. The respective boundaries are marked by open arrowheads. The six predicted asparagine-linked glycosylation sites are indicated by filled triangles. Each subunit contains five closely clustered cysteines (encircled). These sequence data are available from GenBank under accession number L08235.



**Figure 2.** Northern blot analysis of clusterin mRNA in adult mouse tissues. 5  $\mu$ g of total RNA was prepared from the indicated tissues and analyzed using a clusterin cRNA probe. The membrane was exposed for 12 h at  $-80^{\circ}\text{C}$ , between intensifying screens. Equal loading and the integrity of RNAs were verified by staining membranes after transfer with methylene blue.

above and the full length human cDNA probe (Jenne and Tschopp, 1989). The method of Church and Gilbert (1981) was used under stringent washing conditions to scan for double positive clones. Conversion of lambda Zap II vector to pBluescript SK(-) was achieved by excision of lambda DNA using the Exassist/Solr system (Stratagene). A single clone containing an insert of  $\sim 1.7$  kb was identified by EcoRI restriction enzyme digest of plasmid DNA prepared by the alkaline lysis method (Maniatis et al., 1989). DNA was prepared from this clone using the Qiagen Plasmid Midi Prep procedure (Kontron Instruments, Zürich, Switzerland). Sequencing was performed as described above. All kits were used according to the manufacturer's instructions.

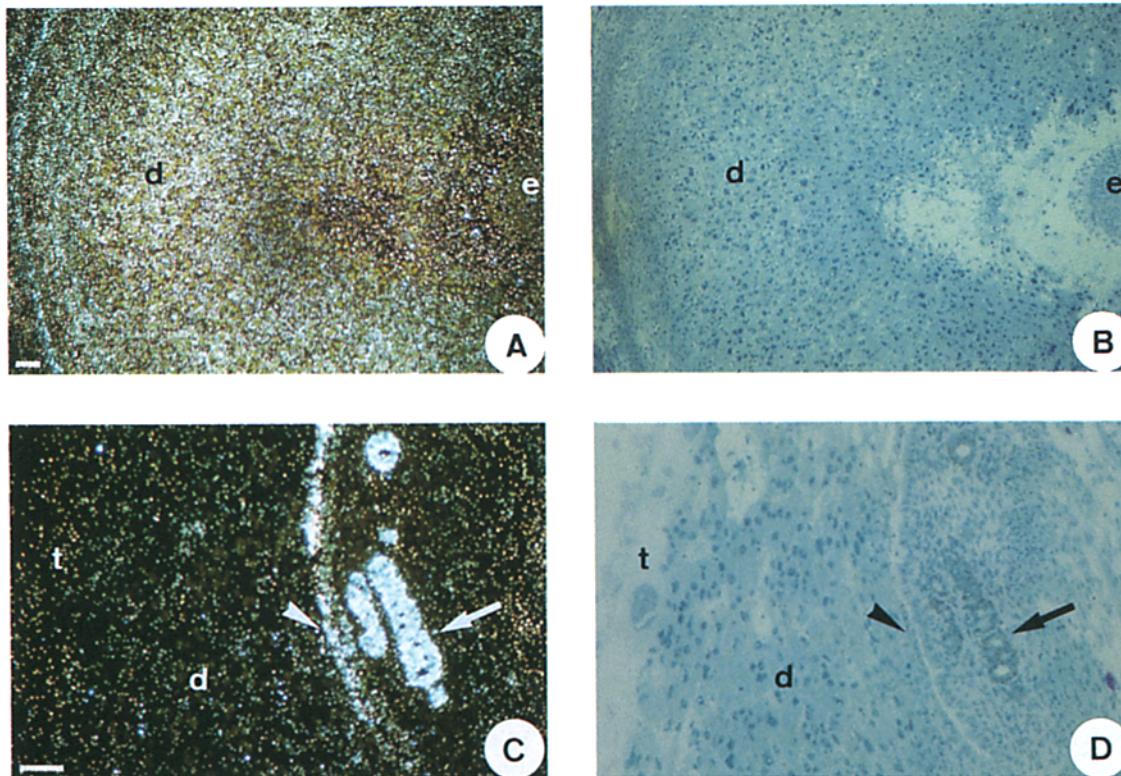
### Plasmid Construction, In Vitro Transcription, and Northern Blot Analysis

The murine clusterin sense and antisense probes were prepared from pBluescript KS m-CLI containing the 638-bp murine clusterin cDNA fragment described above. Radiolabeled cRNAs were synthesized by in vitro transcription in the presence of 12.5  $\mu\text{M}$  of  $\alpha$   $^{32}\text{P}$ -labeled UTP (400 Ci/mmol; Amersham International, Amersham, UK) or 30  $\mu\text{M}$   $^3\text{H}$ -labeled UTP and 30  $\mu\text{M}$   $^3\text{H}$ -labeled CTP (40 and 20 Ci/mmol, respectively; Amersham International).  $^3\text{H}$ -labeled probes were reduced to an average size of 50–100 nucleotides by mild alkaline hydrolysis as previously described (Sappino et al., 1991).

Total RNA was extracted as described elsewhere (Sappino et al., 1987). RNAs were denatured with glyoxal, electrophoresed in 1.2% agarose gels, and transferred overnight onto Hybond nylon membranes (Hybond-N; Amersham International). Prehybridizations, hybridizations, and posthybridization washes were carried out as described (Sappino et al., 1991).

### In Situ Hybridizations

Minor modifications were brought to the procedure previously described (Sappino et al., 1991). 5- $\mu\text{m}$  cryostat tissue sections were mounted on poly-L-lysine (Sigma Immunochemicals, St. Louis, MO) coated microscope slides, fixed in 4% glutaraldehyde in PBS for 1–5 min, rinsed in PBS, and stored in 70% ethanol at  $4^{\circ}\text{C}$  until analyzed.  $1\text{--}3 \times 10^6$  cpm of  $^{32}\text{P}$ -labeled RNAs or  $0.4\text{--}1.0 \times 10^6$  cpm of  $^3\text{H}$ -labeled RNAs were applied to each section in 20–70  $\mu\text{l}$  of hybridization mixture. After graded ethanol dehydration, sections hybridized to  $^{32}\text{P}$ -labeled cRNAs were directly exposed to x-ray films (SB5; Eastman Kodak Co., Rochester, NY) between intensifying screens and the films developed after 3–5 d exposure at room temperature, while sections hybridized to  $^3\text{H}$ -labeled RNAs were immersed in NTB-2 emulsion (Eastman Kodak Co.), diluted 1:1 in deionized water. After 3–15-



**Figure 3.** Localization of clusterin mRNA in uterine decidual cells by in situ hybridization of a  $^3\text{H}$ -labeled anti-sense cRNA probe to frozen sections of a gravid uterus containing a 7.5 (A and B) or 9.5-d-old (C and D) embryo. Strong clusterin gene expression is detectable in uterine decidual cells (d) at 7.5-d after conception only. Uterine glands (arrow) and certain epithelial cells lining uterine crypts (arrowhead) also contain abundant clusterin transcripts (C and D), whereas the embryo (A and B; e) and embryonic trophoblast cells (E and F; t) are unlabeled. A and C are dark-field micrographs; B and D are the corresponding light-field micrographs. Bar, 15  $\mu\text{m}$ .

wk exposure, they were developed in Kodak D-19 developer, fixed in 30% Na thiosulfate, and counterstained in 1% methylene blue.

Controls of specificity included the systematic use of the sense mRNA probe in each experiment, and always revealed the absence of significant labeling. Tissue sections of mouse testes were also included in several experiments as a positive control, the Sertoli cells containing large amounts of clusterin mRNA. Photographs of hybridizations using  $^{32}\text{P}$ -labeled RNAs were taken with a conventional camera (Canon USA Inc., Lake Success, NY), using PANF black and white film (Ilford, Knutsford, Cheshire, England). Microphotographs were taken with a photomicroscope (Zeiss, Oberkochen, Germany), equipped with an immersion dark-field condenser, using Kodak Ektachrome T64 color film.

## Results

### Isolation of the cDNA Encoding Murine Clusterin

A partial murine clusterin cDNA of 638-bp obtained by PCR was used with the full length human clusterin cDNA to screen a murine heart cDNA library. One positive clone was identified by EcoRI restriction enzyme digestion and sequenced.

The nucleotide sequence of the 1,659-bp insert contains an open reading frame of 1,344 nt from the first ATG at position 64 to TGA at position 1407, and encodes a protein of 448-amino acid residues of which the first 21 amino acids represent a typical signal peptide (Von Heijne, 1986) (Fig. 1). The context of the ATG at position 64 is in accordance with those required for translation initiation (Kozak, 1984). Both this ATG and the cleavage site situated between Arg-205 and Ser-206 which produces the mature  $\alpha$  and  $\beta$  clusterin subunits are located at the same position in human and rat clusterin (Collard and Griswold, 1987; Jenne and Tschopp, 1989). Two potential asparagine-linked glycosylation sites in the  $\alpha$  subunit, four in the  $\beta$  subunit, as well as five closely clustered cysteines in each subunit are predicted from this sequence (Fig. 1).

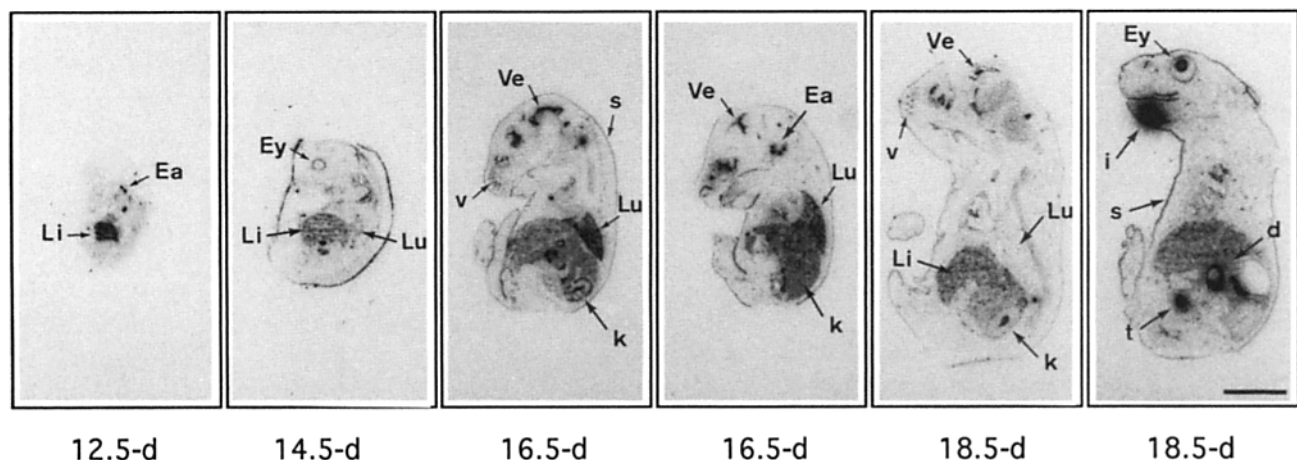
Comparison of the deduced amino acid sequence for murine clusterin with those of the human (Jenne and Tschopp, 1989) and rat (Collard and Griswold, 1987) homologs revealed 75 and 92% sequence identity, respectively. The putative heparin-binding domains and amphipathic  $\alpha$ -helical regions predicted from the human and rat sequences are conserved in the mouse sequence.

### The Clusterin Gene Is Widely Expressed in the Adult Mouse

Northern blot analysis of clusterin gene expression in adult mouse tissues revealed a single RNA species of  $\sim 2$  kb in a broad array of tissues, the highest levels being detected in the brain, heart, testes, ovary, liver, and adrenal gland (Fig. 2). Moderate levels of clusterin mRNA were detected in the lung, kidney, spleen, seminal vesicle, prostate, and uterus, and low levels in the skin, bone, thymus, and digestive tract. Upon longer exposures, all tissues studied showed detectable levels of clusterin mRNA.

### Uterine Decidual Cells Transiently Express Clusterin mRNA After Implantation

During blastocyst implantation, differentiation of stromal cells into decidual cells, a process referred to as decidualization, takes place in the uterine stroma (O'Grady and Bell, 1977; Welsh and Enders, 1985). Analysis of implanting embryos at different stages of development by in situ hybridization revealed that in uteri containing 7.5-d-old embryos, when this process is well engaged, high levels of clusterin mRNA were detectable within cells recognizable by their morphology and distribution as anti-mesometrial decidual cells (Fig. 3, A and B). However, both at the beginning of decidualization in uteri containing 5.5- and 6.5-d-old embryos as well as when decidualization is complete, in uteri



**Figure 4.** Macroscopic localization of clusterin mRNA by in situ hybridization of  $^{32}\text{P}$ -labeled cRNA probes to cryostat tissue sections of 12.5–18.5-d-old mouse embryos. Autoradiograms revealed wide expression of clusterin mRNA in embryos from 12.5 d onwards. Clusterin mRNA is detectable in the liver (Li) and developing inner ear (Ea) from 12.5 d onwards, and in the ventricular cavities of the brain (Ve), the developing eye (Ey), and the kidney (k) from 14.5 d onwards. By 16.5 d of gestation, clusterin mRNA is also macroscopically detectable in the developing skin (s) and vibrissa (v). Note the transient clusterin gene expression in the lung (Lu) of 14.5- and 16.5-d-old embryos, while no clusterin mRNA is macroscopically detectable in the lungs of 18.5-d-old embryos. Finally, the 18.5-d-old embryo shown on the far right illustrates the high level of clusterin gene expression detected in the developing eye (Ey), incisor (i), duodenum (d), and testes (t). Photographs were taken after 10 d of exposure at room temperature. Bar, 0.44 cm.

containing embryos aged of 8.5-d and older, little or no clusterin message was detectable within decidual cells (Fig. 3, C and D). Hybridization with the sense cRNA probe revealed no significant labeling (not shown).

Throughout implantation and postimplantation phases, uterine glands as well as certain epithelial cells lining uterine crypts devoid of blastocytes were intensely labeled with the clusterin cRNA probe (Fig. 3, C and D). The reduced size of these epithelial cells suggests that they may be implicated in the complex reorganization of the luminal epithelial surface associated with the closure of the uterine lumen (Pollard and Finn, 1974; O'Grady and Bell, 1977).

Finally, no clusterin mRNA was detected in embryonic cells between 5.5 and 11.5 d of gestation (Fig. 3).

#### **Embryos Contain Clusterin mRNA from 12.5 d Onwards**

In situ hybridization of <sup>32</sup>P-labeled probes to whole embryos revealed that the clusterin gene is specifically expressed within certain macroscopically identifiable organs from 12.5 d of development onwards. Clusterin gene expression is already widespread in the developing testes, liver, lung, eye, and inner ear of 14.5-d-old embryos (Fig. 4 and Table I). By 16.5 d of development clusterin mRNA was detectable in the developing kidney, vibrissa, and skin. All these organs continued to express the clusterin gene in 18.5-d-old embryos except for the lung in which the intensity of labeling strikingly decreases between 16.5 and 18.5 d (Fig. 4).

#### **Clusterin mRNA Is Predominantly Localized within Epithelia during Organogenesis**

Histological examination of 14.5-, 16.5-, and 18.5-d-old embryos hybridized to <sup>3</sup>H-labeled cRNA probes revealed that clusterin mRNA was present in cells derived from all three germ layers and most abundant in the epithelial cells of developing organs. Particularly strong labeling was observed for example in the epithelial components of the testes and choroid plexus from 14.5 d onwards, as well as in the cylindrical epithelium of the developing gall bladder of 16.5-, and 18.5-d-old embryos (Fig. 5).

Clusterin mRNA was also abundant in epithelia of the developing auditory, olfactory, and visual apparatus. From 12.5 d of gestation onwards, clusterin message was detectable in the developing ear (Fig. 4), and localized within the sensory epithelial cells comprising the epithelial thickenings that are known to appear in the utricle, saccule, and cochlea at this early stage of organogenesis (Swanson et al., 1990). In contrast, the adjacent simple epithelium of the inner ear remained unlabeled at all stages of development studied (Fig. 6).

In 14.5-d and older embryos, when morphogenesis of the nasal cavities is nearly complete, considerable amounts of clusterin mRNA were detected in the pseudostratified olfactory epithelium (Fig. 6). Within the developing olfactory epithelium which consists of dividing cells situated at its base, surrounded by differentiating receptor and supporting cells (Smart, 1971; Cuschieri and Bannister, 1975a,b), it is essentially within the differentiating cell populations situated in the most superficial portion of the epithelium that abundant clusterin transcripts were localized.

**Table I. Localization of Clusterin mRNA in 14.5–18.5-d-old Mouse Embryos**

	14.5 d	16.5 d	18.5 d
<b>Epithelia</b>			
Suprabasal keratinocytes	+	++	++
Whisker follicles	+	++	++
Choroid plexus	+++	+++	+++
Olfactory epithelium	+	++	++
Otic epithelium	+++	+++	+++
Retinal pigment epithelium	++	++	++
Proliferative lens epithelium	+	+++	+++
Salivary glands	–	–	–
Tooth (ameloblasts)	NS	+++	+++
Bronchial epithelium	++	++	–
Esophagus	NS	–	–
Stomach - body and fundus	NS	–	–
- pyloric antrum	NS	++	++
Duodenum	NS	+++	+++
Intestine	+	+	+
Kidney tubular epithelium	++	++	++
Ureter epithelium	NS	+	+
Testes	+++	+++	+++
Bladder epithelium	NS	–	–
Biliary epithelium	+++	+++	+++
Pancreatic acini and ductules	NS	++	++
Hepatocytes	–	+	++
Ventricular neuro-epithelium	+	+	+
Thymus	NS	+	+
Spleen	NS	+	+
Adrenal gland	NS	+	+
Hematopoietic tissue (megakaryocytes)	++	++	++
Neuronal tissue - dorsal root ganglia	NS	+	+
- ventral spinal cord	+	+	++
<b>Cartilage</b>	–	–	–
Bone (osteoclasts or osteoblasts)	–	+	+
Endothelia	–	–	–
Mesenchyme	–	–	–
Muscle	–	–	–

Expression of clusterin mRNA in a variety of tissues was assessed semi-quantitatively by in situ hybridization of the <sup>3</sup>H-labeled murine clusterin cRNA probe to frozen cryostat sections of 14.5-, 16.5-, and 18.5-d-old embryos. (–) no clusterin mRNA detectable; (+) weak labeling; (++) moderate labeling; (+++) strong labeling; and NS, not studied.

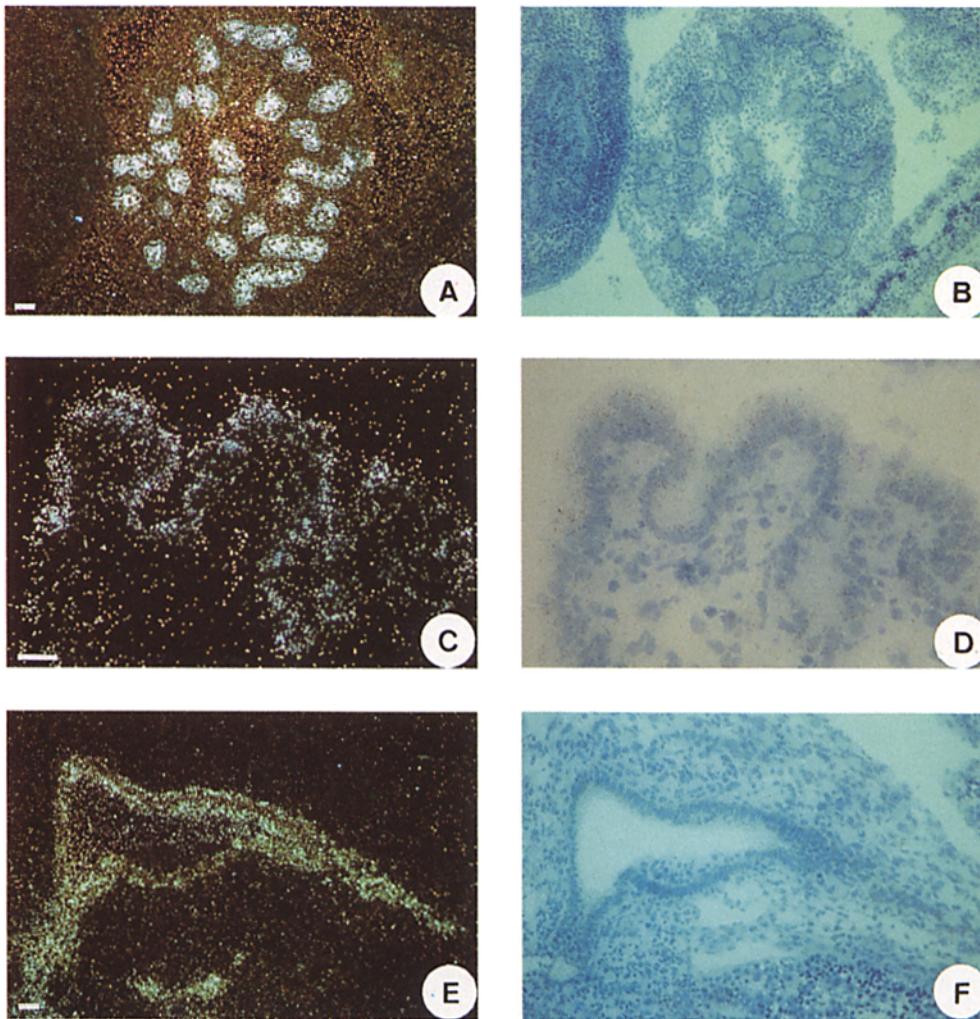
Between 14.5 and 18.5 d of gestation, clusterin mRNA is also detectable in the retinal pigment epithelium, the epithelium of the lens, the cubic epithelium of the ciliary body, and in the epithelium of the lacrymal glands (not shown).

Clusterin gene expression is not, however, exclusively confined to developing epithelia, as not all epithelia express the clusterin gene, and conversely some non-epithelial cell types do express the gene (Table I).

#### **Differentiating Compartments of Developing Epithelia Selectively Express the Clusterin Gene**

Within the skin, duodenum, and developing tooth it is possible to distinguish the proliferative or stem cell compartment from the differentiated compartment. In each of these epithelia, in situ hybridization revealed selective expression of clusterin mRNA in cells of differentiated cell compartments.

In the skin, the low levels of clusterin mRNA detectable in the 2–3-layer-thick, poorly differentiated epidermis of 14.5-d-old embryos (Fig. 7, A and B), contrasted with the



**Figure 5.** Localization of clusterin mRNA in developing epithelia of mouse embryos. In situ hybridizations to frozen sections of 16.5-d-old embryos revealed the presence of clusterin gene transcripts in the epithelial components of the testes (*A* and *B*), the choroid plexus of the fourth ventricle (*C* and *D*), and the epithelium of the gall bladder (*E* and *F*). *A*, *C*, and *E* are dark-field micrographs; *B*, *D*, and *F* are the corresponding light-field micrographs. Bars, 15  $\mu$ m.

high levels detectable in the several-layer-thick well differentiated epidermis of 18.5-d-old embryos (Fig. 7, *C* and *D*). In addition, in 18.5-d-old embryos, clusterin mRNA was clearly localized in the differentiating suprabasal cell layers of the epidermis (Fig. 7, *C-E*), suggesting that during epidermal development, clusterin gene expression coincides with both epidermal stratification and epidermal differentiation.

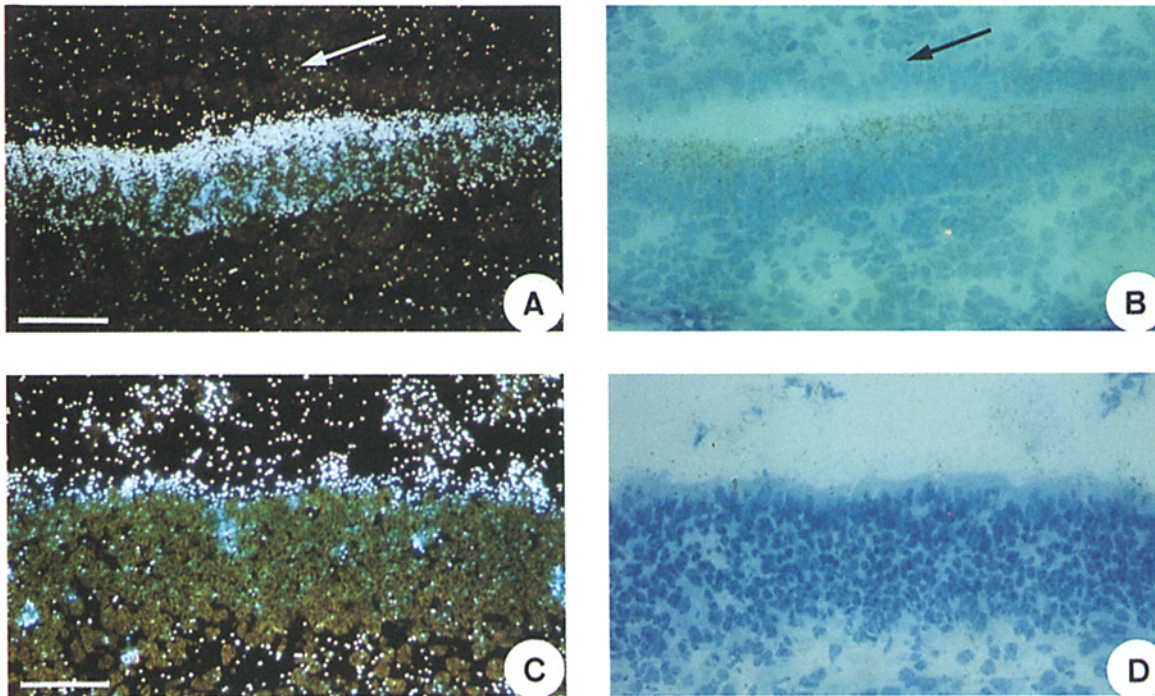
In the duodenum, during the week preceding birth, rapid growth and differentiation leads to the simultaneous appearance of villi covered by a columnar epithelium and marked clusterin gene expression (Fig. 7, *F* and *G*). As in the skin, labeling is weak in the dividing epithelial cells situated at the base of the villi in 16.5- and 18.5-d-old embryos, and contrasted sharply with the abundant labeling of more differentiated cells that line the rest of the villus (Fig. 7 *H*).

Lastly, in the developing tooth, differentiated enamel secreting ameloblasts of the enamel organ also showed marked clusterin gene expression, which contrasted sharply with that of morphologically distinct undifferentiated, proliferating preameloblasts (Fig. 7, *I* and *J*).

#### ***Transient and Localized Expression of Clusterin mRNA in Bronchial Buds during Lung Branching Morphogenesis***

Macroscopic analysis of embryos between 14.5 and 18.5 d of development by in situ hybridization using  $^{32}$ P-labeled clusterin cRNAs, revealed abundant clusterin message in the lungs of 14.5- and 16.5-d-old embryos contrasting with the selective absence of message in the lungs of 18.5-d-old embryos (Fig. 4).

At 14.5 and 16.5 d of development clusterin mRNA was localized exclusively in the cuboidal epithelial cells of morphogenetically active bronchial buds (Fig. 8). By 18.5 d of gestation, when the process of branching morphogenesis is virtually complete and remaining bronchial buds rare, the overall level of pulmonary clusterin gene expression was drastically diminished (Fig. 4). Interestingly, during pulmonary organogenesis, within bronchioles the morphological transition from proximal, columnar to distal, cuboidal epithelium, and just before birth, to completely differentiated alveolar epithelial cells corresponds chronologically to dis-



**Figure 6.** Localization of clusterin mRNA in epithelia of the developing auditory, and olfactory apparatus by in situ hybridization. In 18.5-d-old embryos, sensory epithelial cells of the inner ear contain abundant clusterin transcripts, whereas the adjacent simple epithelium of the inner ear is not labeled (*arrow*) (*A* and *B*). In the developing nose of 16.5-d-old embryos, selective labeling was observed in the apical differentiating cell population of the olfactory epithelium (*C* and *D*). *A* and *C* are dark-field micrographs; *B* and *D* are the corresponding light-field micrographs. Bars, 15  $\mu$ m.

tinct events in pulmonary epithelial cell differentiation (Joyce-Brady and Brody, 1990). Thus the clusterin gene is selectively and transiently expressed by differentiating bronchial epithelial cells and furthermore, in a manner that coincides precisely with the process of branching morphogenesis that is known to involve epithelial proliferation, differentiation, cell-cell and cell-matrix interactions (Wessels, 1970; Ten Have-Opbroek, 1981; Roman et al., 1991; Schuger et al., 1990; Hirai et al., 1989).

#### ***Transient and Locally Restricted Clusterin Gene Expression by Developing Epithelial Cells during Kidney Organogenesis***

Throughout kidney embryogenesis, the epithelial cells of the ureter and collecting ducts form by branching morphogenesis, whereas glomerular and tubular epithelial cells develop by direct conversion of mesenchyme into epithelium, a process that is induced by the branching ureter tips (Sorokin and Ekblom, 1992). In situ hybridization revealed that the clusterin gene was abundantly expressed in both the epithelial cell lineage of the branching ureter (Fig. 9, *A* and *B*) and the lineage that develops from the mesenchyme (Fig. 9, *C* and *D*).

During the development of nephric units, clusterin mRNA is first detectable in the newly polarized cells of comma and S-shaped bodies of 16.5- and 18.5-d-old kidneys (Fig. 9, *C* and *D*). Clusterin mRNA was not detectable in the subcapsular area of the cortex which is known to contain uninduced mesenchyme or induced mesenchymal cells that do not yet display morphological signs of polarization (not shown). In

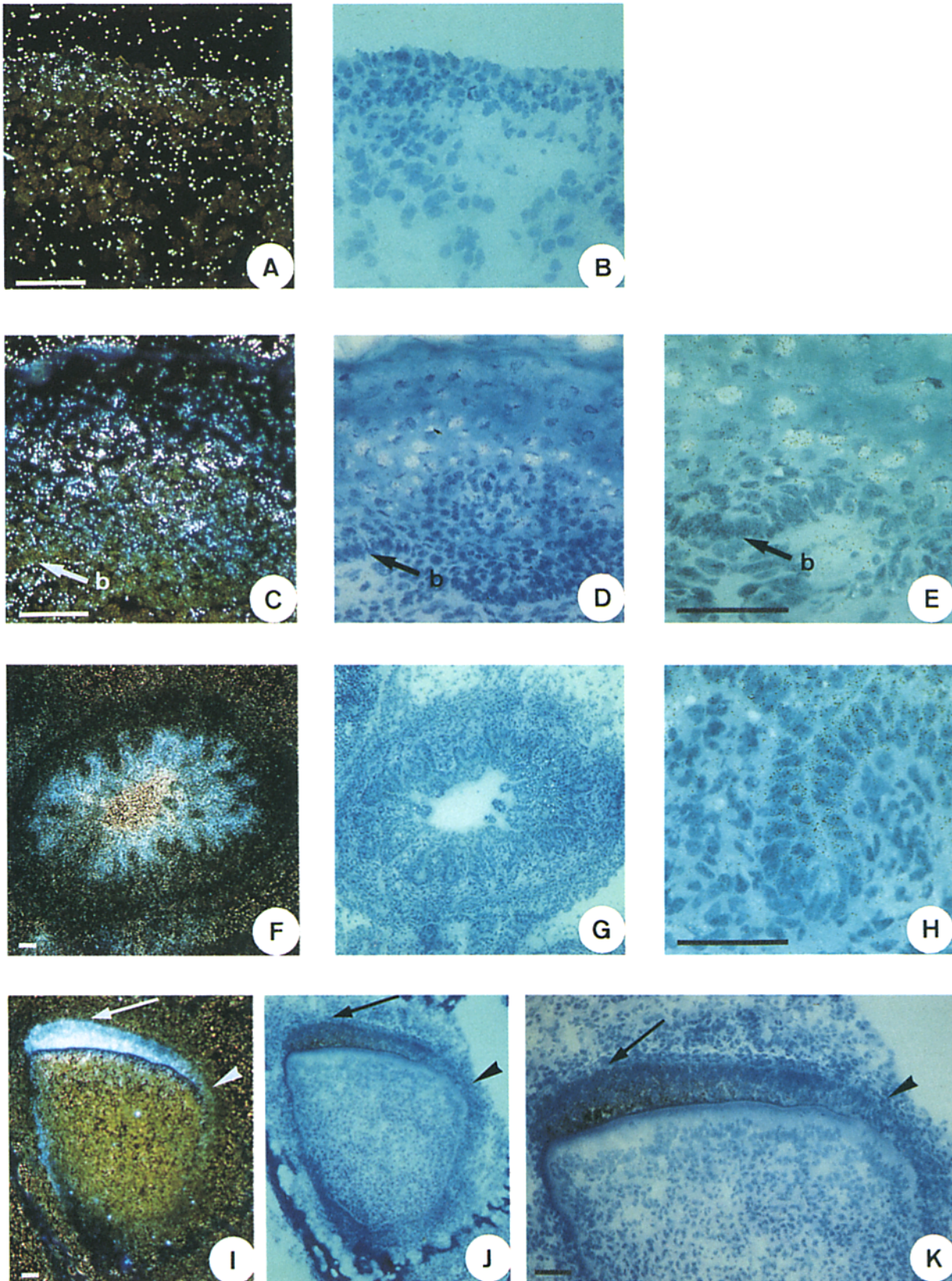
addition, the pattern of clusterin gene expression was markedly different from that of genes like *N-myc* which are expressed at earlier stages of nephric unit development and localized to the most cortical areas of the developing kidney (Mugrauer et al., 1988). Clusterin mRNA is also abundantly expressed in developing kidney tubules including the collecting tubes situated within the medulla (Fig. 9, *E* and *F*). No clusterin message was however detectable in morphologically recognizable glomerules of 18.5-d-old embryos (Fig. 9, *G* and *H*).

Thus, during morphogenesis of the kidney, clusterin gene expression is induced in areas of the nephric unit where epithelial cell polarization occurs, as well as in the collecting tubes and certain developing tubules.

#### ***Discussion***

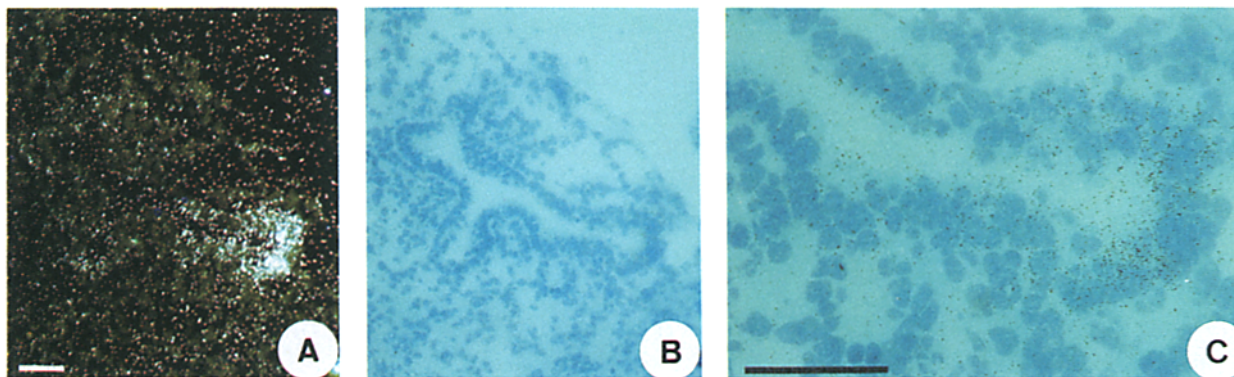
Our results demonstrate that the clusterin gene is widely expressed during murine embryogenesis, nearly exclusively in developing epithelia, and often in association with distinct stages of their differentiation. They suggest that clusterin is not only a constitutively expressed glycoprotein, but also the product of a gene that may be implicated in epithelial differentiation.

Sequence analysis of the murine clusterin cDNA and comparison with available clusterin cDNA sequences from other mammalian species revealed a high degree of sequence homology (Fig. 1), and conservation of the protein's predicted structural features between different species. Moreover, the



**Figure 7.** Distinct localization by in situ hybridization of clusterin mRNA in differentiated compartments of developing epithelia. Clusterin gene expression in the skin correlates with epidermal stratification and differentiation: the two to three layer thick epidermis of 14.5-d-old embryos (*A* and *B*) is very weakly labeled in comparison to the multilayered epidermis of 18.5-d-old embryos (*C* and *D*). Furthermore, as illustrated in *C*, *D*, and *E*, clusterin mRNA is clearly localized to the suprabasal layers of the skin in 18.5-d-old embryos (*b*, basal cell layer). In the duodenum of 16.5-d-old embryos, high levels of clusterin mRNA detectable in differentiated epithelial cells lining the villi





**Figure 8.** Distribution of clusterin mRNA during lung development. Abundant clusterin mRNA is localized in cuboidal epithelial cells of bronchial buds (A–C) whereas the more proximal, less differentiated bronchial epithelium is unlabeled. A, dark-field micrograph; B, corresponding light-field micrograph; and C, higher magnification of B. Bars, 15  $\mu$ m.

pattern of gene expression observed in adult murine tissues was very similar to that reported for other species (Jenne and Tschopp, 1992), clusterin mRNA being detectable in most tissues analyzed, and particularly abundant in the testes. Taken together, these results suggest that the biological function(s) of clusterin is (are) well conserved in mammals.

During murine embryogenesis clusterin gene expression has been localized by *in situ* hybridization to various differentiating cell types, most of which are epithelial. For instance, shortly after implantation, clusterin gene transcription is strongly and transiently induced in antimesometrial decidual cells of the uterus at a time where these stromally derived cells are undergoing profound morphological and biochemical modifications that lead to the fully differentiated uterine decidual cells (O'Grady and Bell, 1977; Welsh and Enders, 1985). During development of the olfactory epithelium, clusterin mRNA is specifically localized to differentiating receptor and supporting cells. In addition, in developing epithelia where a clear distinction between the proliferative and differentiating compartments can be made, clusterin mRNA was also specifically localized to the latter compartment, as was shown in the skin, duodenum, and tooth. Finally, during morphogenesis of the lung and kidney, expression of the clusterin gene was also associated with differentiating bronchial and tubular epithelial cells respectively. Thus, during murine embryogenesis, a clear *in vivo* correlation exists between clusterin gene expression and cellular differentiation.

The exact role of clusterin in cellular differentiation remains to be clarified. Clusterin, which has been shown to favor homotypic cell aggregation (Fritz et al., 1983; Blaschuk et al., 1983) may simply confer a new adhesive phenotype to cells in a certain state of differentiation, thereby participating in the spatial distribution of cells within tissues and/or affecting cell movement. Alternatively, clusterin synthesized by differentiating cells may be involved in locally

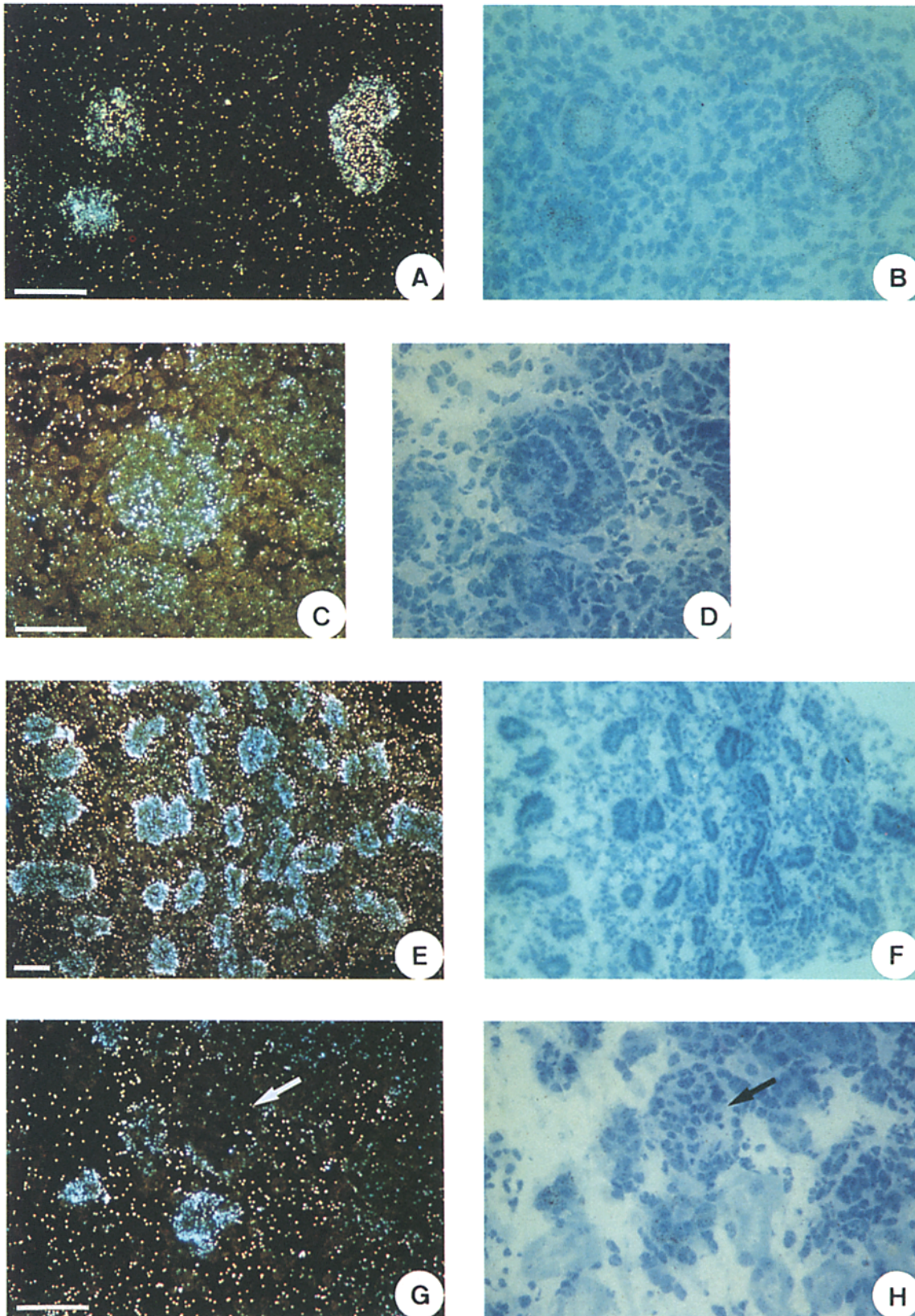
binding and concentrating certain growth factors, or else directly implicated in autocrine or paracrine growth control.

The pattern of clusterin gene expression during murine embryogenesis was very similar to that of the gene encoding TGF- $\beta$ 2 (Fergus et al., 1991), a widely expressed multifunctional cytokine known to be a potent inhibitor of epithelial cell growth and modulator of differentiation (Roberts and Sporn, 1990; Massagué, 1990; Sporn and Roberts, 1992). Indeed, widespread epithelial expression of TGF- $\beta$ 2 RNA was correlated with epithelial differentiation per se, being present for example in suprabasal keratinocytes of the developing skin and differentiating cells of the olfactory epithelium, but also in morphogenetically active tissue such as the cuboidal epithelial cells of bronchial buds during lung branching morphogenesis (Fergus et al., 1991; Pelton et al., 1991; Schmid et al., 1991). Further similarities exist between clusterin and TGF- $\beta$ 2. Like clusterin, TGF- $\beta$ 2 is constitutively expressed by a wide variety of differentiated cell types (Miller et al., 1989; Massagué, 1990), and stored in high concentrations within  $\alpha$ -granules of platelets (Tschopp et al., 1993b; Cheifetz et al., 1987). The striking similarities between clusterin and TGF- $\beta$ 2, together with the predominant localization of clusterin mRNA within differentiating cells and morphogenetically active tissues, raises the possibility that clusterin may somehow be involved in the control of cellular differentiation. We can reasonably speculate that clusterin RNA is translated and secreted as has been demonstrated for most cell types studied so far (Urban et al., 1987; Hartmann et al., 1991; Burkey et al., 1991; Jenne and Tschopp, 1992). If this is the case, secreted clusterin may modulate cellular differentiation by (a) binding to TGF- $\beta$ 2 and thereby locally increasing its availability to cells, or alternatively by (b) directly acting upon cellular differentiation.

Alternatively, clusterin may be a marker of differentiation, contributing to the cellular adhesive processes that are

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contrast with the absence of mRNA in proliferating cells situated at the villi's base (F, G, and H). Differentiated ameloblasts (arrow) of the developing tooth also contain abundant clusterin mRNA in comparison to undifferentiated preameloblasts (arrowhead) (I, J, and K). A, C, F, and I are dark-field micrographs; B, D, G, and J are the corresponding light-field micrographs. E, H, and K are higher magnifications of D, G, and J, respectively. Bar, 15  $\mu$ m.



**Figure 9.** Localization of clusterin mRNA in the developing kidney. In 14.5-d-old embryos, epithelial cells of the branching ureter are strongly labeled by the clusterin cRNA probe (*A* and *B*). *C* and *D* illustrate the localization of clusterin mRNA in an S-shaped body of a 16.5-d-old embryo. Developing collecting tubes (*E* and *F*) and kidney tubules (*G* and *H*) of 18.5-d-old embryos contain large amounts of clusterin mRNA, but no clusterin message was detectable within glomeruli (*G* and *H*, arrow). *A*, *C*, *E*, and *G* are dark-field micrographs; *B*, *D*, *F*, and *H* are the corresponding light-field micrographs. Bars, 15  $\mu$ m.

thought to participate in the morphogenetic rearrangement of cells in embryonic tissues as well as in the disposition of cells within complex multilayered tissues (Gumbiner, 1992; Edelman and Crossin, 1991). Localization of clusterin gene transcripts in the developing kidney is very similar, for example, to that of two adhesion molecules; the cell-cell adhesion molecule uvomorulin (Vestweber et al., 1985) and the cell-substratum adhesion molecule laminin (Ekblom et al., 1990), which are both expressed in mesenchymally derived epithelial cells of comma and S-shaped bodies. In addition, clusterin is known to promote homophilic cell adhesion in vitro (Fritz et al., 1983), and has been localized at the surface of cells in vivo (Sylvester et al., 1984; Hartmann et al., 1991), leading Hartmann et al. to suggest that the protein may be a constituent of the cell surface coat. These adhesive properties may be mediated by cell surface proteoglycans (Jackson et al., 1991; Ruoslahti and Yamaguchi, 1991), to which clusterin could bind via one of its predicted heparin-binding domains, thus creating a molecular link between proteoglycan molecules of adjacent cells. Although no experimental evidence exists at present, similar interactions between cell-associated clusterin and extracellular-matrix proteoglycans may also participate in cell-substratum interactions which are prominent during morphogenesis. Further investigations will however be needed to define the proposed role of clusterin in these cell adhesion processes.

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