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Expression sequences of cell adhesion molecules

(embryogenesis/embryonic induction/histogenesis/cell interactions)

KATHRYN L. CROSSIN, CHENG-MING CHUONG, AND GERALD M. EDELMAN

The Rockefeller University, 1230 York Avenue, New York, NY 10021

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ABSTRACT **A reexamination of the expression of cell adhesion molecules (CAMs) during the development of the chicken embryo was carried out using more sensitive immunocytochemical techniques than had been used previously. While the previously determined sequence of CAM expression was confirmed, neural CAM (N-CAM) was also detected on endodermal structures such as the lung epithelium, gut epithelium, and pancreas and on budding structures such as the pancreatic duct and gall bladder. It was also found on ectodermal derivatives of the skin. In most of these sites, N-CAM expression was transient, but in the chicken embryo lung, the epithelium remained positive for N-CAM and liver CAM (L-CAM) into adult life. Thus, at one time or another, both of these primary CAMs can be expressed on derivatives of all three germ layers. At sites of embryonic induction, epithelial cells expressing both L-CAM and N-CAM, or L-CAM only, were apposed to mesenchymal cells expressing N-CAM. Examples included epiblast (NL) and notochord (N); endodermal epithelium (NL) and lung mesenchyme (N); Wolffian duct (NL) and mesonephric mesenchyme (N); apical ectodermal ridge (NL) and limb mesenchyme (N); and feather placode (L) and dermal condensation (N). The cumulative observations indicate that cell surface modulation of the primary CAMs at induction sites can be classified into two modes. In mode I, expression of N-CAM (or both CAMs) in mesenchyme decreases to low amounts at the cell surface, and then N-CAM is reexpressed. In mode II, one or the other CAM disappears from epithelia expressing both CAMs. As a result of the primary processes of development, collectives of cells linked by N-CAM and undergoing modulation mode I are brought into the proximity of collectives of cells linked by L-CAM plus N-CAM or by L-CAM undergoing modulation mode II. Such adjoining cell collectives or CAM couples were found at all sites of embryonic induction examined.**

Cell adhesion molecules (CAMs) are large cell surface glycoproteins responsible for cell–cell binding during development and for stabilization of certain tissues in adult life (1, 2). Primary CAMs are defined as those that appear in early embryogenesis on derivatives of more than one germ layer; secondary CAMs appear during later histogenesis to link cells of different types following cytodifferentiation. The best-characterized primary CAMs are N-CAM (neural CAM) (3, 4) and L-CAM (liver CAM) (5), named after the tissues from which they were first isolated. It has been proposed (1) that a major means of altering the function of CAMs arises from modulation of their binding properties, either by chemical changes such as E–A conversion (4, 6, 7) or by alteration of their prevalence or distribution at the cell surface. Physicochemical studies suggest that changes in prevalence of N-CAM at the cell surface lead to large, nonlinear changes in binding rates (8).

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In previous studies, it was found that the two known primary CAMs are dynamically expressed in definite sequences during embryonic development (9–11). Now we have carried out a more detailed analysis of the primary CAM expression sequence, using more sensitive immunocytochemical techniques to stain tissue sections at closer time intervals in embryonic development. The experiments confirm previous sequences but also show that N-CAM appears transiently in endodermal derivatives and skin where it had not been previously detected; thus, both primary CAMs can appear on derivatives of all three germ layers. The findings indicate that, at sites of embryonic induction, there are two distinct modes of prevalence modulation of CAMs at the cell surface: (*i*) in mesenchyme, N-CAM diminishes at the surface and then reappears and (*ii*) in epithelia, both N-CAM and L-CAM appear together and one or the other subsequently disappears. Together with analyses of the expression sequence of a known secondary CAM (neuron-glia CAM, Ng-CAM) that acts during neural histogenesis (12, 13), the results suggest that local signals are responsible for specific modulation of CAM expression and for consequent morphological change.

MATERIALS AND METHODS

White Leghorn chicken embryos were staged according to the number of somites or according to Hamburger and Hamilton (14) for later stages. Embryos were fixed in 2.5% paraformaldehyde/0.02% glutaraldehyde in 100 mM phosphate buffer (pH 7.2) (P_i) at room temperature for 10–60 min depending on the age of the embryo. After quenching with 0.1 M glycine in phosphate-buffered saline (P_i/NaCl), the embryos were infiltrated with 30% sucrose in P_i/NaCl at 4°C. Very early embryos were first embedded in 1% low-melting-point agarose (Bethesda Research Laboratories) in 18% sucrose/P_i/NaCl to facilitate handling and orientation. The embryos were mounted in OCT compound (Lab-Tek, Naperville, IL) or Lipshaw's M-1 medium on dry ice; 10-μm cryostat sections (International Equipment model CTF) were attached to gelatin- or poly(L-lysine)-coated slides. Indirect immunofluorescent labeling was carried out as described (9–12) with monoclonal antibodies to N-CAM peptide regions or high-affinity rabbit anti-chicken L-CAM and N-CAM; in some cases, the anti-N-CAM polyclonal antibodies were immunoaffinity purified by using chicken brain N-CAM linked to Sepharose CL-2B (3). Slides were mounted in 90% glycerol/P_i/NaCl/0.1% *p*-phenylenediamine to prevent bleaching. Sections were photographed on Tri-X film with a Nikon UFX camera on a Zeiss Universal microscope equipped with IIIRS epifluorescence optics.

RESULTS

CAM Expression in the Early Embryo. Cells of the early blastoderm express both N-CAM and L-CAM (9–11). At the

Abbreviations: CAM, cell adhesion molecule; L-CAM, liver CAM; N-CAM, neural CAM.

primitive streak stage of gastrulation, epiblast cells (derived from the blastoderm) lost both CAMs as they ingressed through the primitive streak (9) to form the middle layer (Fig. 1 *A* and *B*). As the ingressing cells condensed into the mesoblast, they reexpressed N-CAM. Thus, epiblast cells lost both CAMs while undergoing epithelial–mesenchymal transformation and moving into the middle layer. Some of the ingressing cells become the chordamesoderm, which stained for N-CAM and subsequently takes part in neural induction. At neural induction, epiblast cells lost one or the other CAM in a localized area of the epithelial sheet. Epiblast cells yielding the neural plate lost L-CAM, while those yielding somatic ectoderm more gradually lost N-CAM (Fig. 1 *C* and *D*).

N-CAM and L-CAM Appear on Derivatives of All Three Germ Layers. While N-CAM and L-CAM have previously been found in some ectodermal and mesodermal derivatives (9–11), we had not observed N-CAM to be prevalent after neurulation in the ectodermal portions of the skin (other than placodes) or in endodermal derivatives. Here we compare the expression sequences for both CAMs found in examples of derivatives of all three germ layers. Other examples, experimentally determined by similar methods, are listed in Table 1.

Eye (ectoderm). Epithelial cells of the lens placode stained for both N-CAM and L-CAM (10), and this staining pattern was retained in the presumptive corneal epithelium (Fig. 1*E*). L-CAM was not expressed in the developing lens (Fig. 1*G*) after the time of invagination. Later, as lens fibers were formed, staining for N-CAM in the lens (Fig. 1*E*) was not seen, but N-CAM remained in the proliferating lens epithelium (Fig. 1*F*). More intense N-CAM staining occurred in a region of the optic vesicle (the inductor) corresponding to the central, most mature retinal cells at stage 18 prior to retinal layering (Fig. 1*E*), although no morphological differences were observed at the staining border.

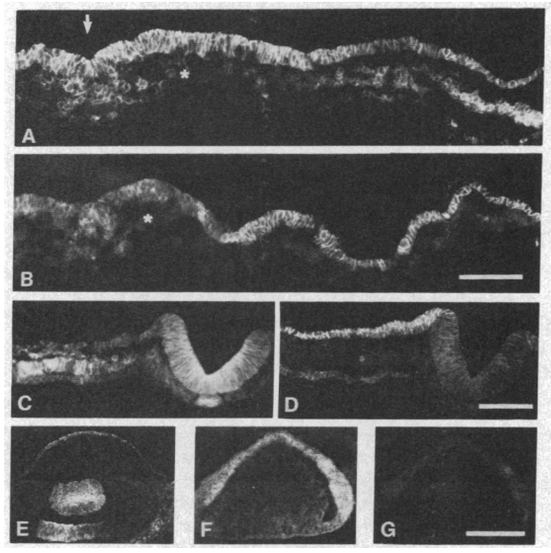

FIG. 1. Immunocytochemical staining of early ectodermal epithelial structures. (*A* and *B*) Head-fold-stage embryo (stage 6) was sectioned transversely through the primitive streak (arrow) and was stained with affinity-purified anti-N-CAM (*A*) or anti-L-CAM (*B*). The epiblast stains for both CAMs, but ingressing cells (*) show no staining. (*C* and *D*) Transverse sections through a five-somite embryo posterior to the last formed somite were stained with affinity-purified anti-N-CAM (*C*) or anti-L-CAM (*D*). Note the enhancement of N-CAM staining in the neural groove with corresponding loss (*D*) of L-CAM staining. (*E*, *F*, and *G*) Transverse section of the eye of a stage 18 embryo shows N-CAM staining (*E*) in the developing lens, retina, and corneal epithelium. Later (stage 30) (*F*), N-CAM staining is restricted to the lens epithelium as L-CAM staining disappears (*G*). (Bar = 100 μm.)

Table 1. Modulation modes of CAM expression during chicken embryogenesis

Mode I: Mesenchymal conversions*	Mode II: Epithelia†
Ectodermal	**Ectodermal**
N → 0 → N	*NL → N*
Neural crest	Neural tube
–Peripheral nerve	Placode-derived ganglia
–Ganglia	*NL → L*
Mesodermal	Somatic ectoderm
N → 0 → N	Stratum germinativum
Somite	Apical ectodermal ridge
–Skeletal muscle (end plate only)	Branchial ectoderm
–Dermal papilla (feather)	*NL → N → ‡*
Nephrotome	Lens
–Germinal epithelium of gonad	Marginal and axial plate of feather
–Gonadal stroma	*NL → L → ‡*
Splanchnopleur	Stratum corneum
–Spleen stroma	Feather barbule, rachis
–Lamina propria of gut	**Mesodermal**
–Some mesenteries	*N → NL → L*
N → 0 → N → ‡	Wolffian duct
Somite	Mesonephric tubules
–Chondrocytes	Müllerian duct
Lateral plates	**Endodermal**
–Smooth muscle	*NL → L*
	Epithelium of
	Trachea
	Gastrointestinal tract
	Hepatic duct
	Gall bladder
	Thyroid
	Pharyngeal derivatives
	NL
	Parabronchi (lung epithelia)

*Mode I shows cyclic changes in N-CAM or disappearance. Some of these transitions occur with movement; 0 represents low levels of CAM. The original tissues are listed at the left margin. Tissues containing high levels of N-CAM are preceded by a dash; in some cases (‡), the CAM at this stage can be replaced by a differentiation product.

†Mode II shows replacement of one CAM by another or disappearance. ‡, Differentiation products (e.g., keratin, crystallin) with disappearance of the CAM.

Respiratory and gastrointestinal tract (endoderm). The avian lung is a classical inductive system in which mesenchyme induces the outgrowth of the tubular structures of the lung (15). Both primary CAMs were detected in the epithelium of the laryngotracheal groove (stage 18) from which the lung buds were induced by the strongly N-CAM-positive surrounding mesenchyme (Fig. 2 *A* and *B*). In the avian lung, both CAMs persisted into adult stages in endodermal derivatives in the parabronchial walls (Fig. 2 *C* and *D*).

In the closing anterior intestinal portal of a stage 15 chicken embryo, the anlage of the pancreas expressed both CAMs (Fig. 2 *E* and *F*) prior to its morphological differentiation. As the pancreas developed, it continued to express L-CAM, while the levels of N-CAM gradually decreased. At stage 18 (3 days), when the pancreatic duct budded from the gastrointestinal tract, strong staining for both CAMs was seen in the bud; at the same time, the gastrointestinal epithelium lost N-CAM while being surrounded by condensing mesenchyme that was N-CAM positive (Fig. 2 *G* and *H*). The enhanced N-CAM staining in a restricted area of an epithelial sheet was

similar to that described above for the early retina. By stage 42, N-CAM disappeared from the pancreas and duct, leaving L-CAM on the pancreatic acinar cells. At this stage, L-CAM strongly stained the gut epithelia (Fig. 2*I*), and intense N-CAM staining was seen in the lamina propria (Fig. 2*J*). This same pattern of CAM expression was seen throughout the gastrointestinal tract; N-CAM was lost from the L-CAM-positive epithelium, while the N-CAM-positive mesenchyme, precursor to the smooth muscle and connective tissue of the gastrointestinal tract, began to condense around the epithelium (Fig. 2 *F*, *H*, and *J*). Sequences of CAM expression similar to those seen in the pancreas were observed for other endodermal buds, such as the thyroid rudiment, the hepatic ducts, and the gall bladder (Table 1).

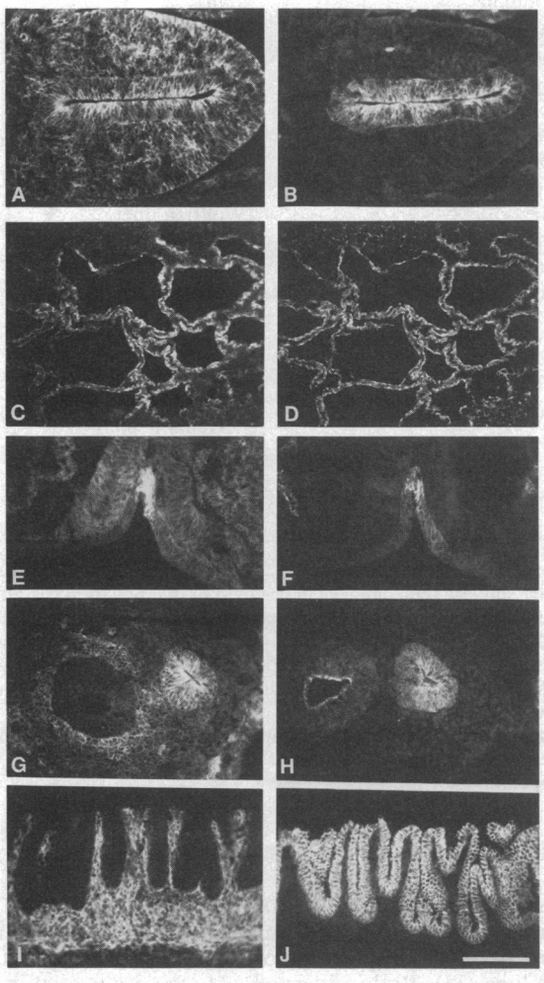

FIG. 2. Colocalization of N-CAM and L-CAM in developing and adult endodermal structures. (*A* and *B*) At stage 18, the epithelium of the laryngotracheal groove (center in *A* and *B*) stains for both N-CAM (*A*) and L-CAM (*B*) and is surrounded by intensely N-CAM-positive mesenchyme (*A*). (*C* and *D*) A section of the adult chicken lung through the walls of the air capillaries was double-stained with monoclonal antibodies (see refs. 3 and 22) to N-CAM polypeptide-region determinants (*C*) and anti-L-CAM (*D*). (*E* and *F*) The early pancreatic rudiment (stage 15) stains for both N-CAM (*E*) and L-CAM (*F*) prior to its morphological differentiation and is adjacent to N-CAM-positive mesenchyme (*E*). (*G* and *H*) N-CAM (*G*) and L-CAM (*H*) are present on the pancreatic duct (right portion of photograph) as it buds from the duodenum (lumen on left) in which N-CAM staining is significantly diminished (*G*) and L-CAM staining (*H*) is highly polarized to the apical surface of the epithelium. The epithelium is surrounded by N-CAM-positive mesenchyme (*H*). (*I* and *J*) In the stage 42 intestine, N-CAM is present on the submucosal layer (*I*), while L-CAM stains only the epithelial layer (*J*). (Bar = 100 μm.)

Mesonephros (mesoderm). At the earliest stages, the intermediate mesoderm stained only with N-CAM (refs. 9 and 10; Fig. 1 *C* and *D*). As the Wolffian duct formed, it expressed both N-CAM and L-CAM. Later, as the duct elongated, it stained weakly for N-CAM and strongly for L-CAM (Fig. 3 *A* and *B*); this resembles the pattern seen for ectodermal and endodermal epithelia discussed above. Mesonephric tubules stained for N-CAM (Fig. 3*B*) as they were organized from the mesonephric mesenchyme under inductive influences of the Wolffian duct (16). The epithelium at the distal ends of the tubule stained most strongly. As noted previously (9–11), as the tubules near the Wolffian duct formed and fused with the duct, they lost N-CAM and stained for L-CAM (in Fig. 3, compare *A* with *B*). In later stages (E12), the well-formed mesonephric tubule epithelia were positive for L-CAM, which was concentrated on the lateral sides of the cells (Fig. 3*D*). A layer of spindle-shaped cells surrounding the tubules was N-CAM positive (Fig. 3*C*). In Bowman's capsule, the parietal layer cells were L-CAM positive; the visceral layer cells (podocytes) and the endothelia within the glomeruli did not stain for either CAM (Fig. 3 *C* and *D*).

Apposition of Cells Showing the Different Modes at Sites of Induction. The foregoing examples suggest that at a number of sites of embryonic induction, mesenchymally derived cells expressing N-CAM are apposed to epithelial cells expressing both N-CAM and L-CAM. Examination of limb development and feather development provided striking additional examples.

Limb. During formation of the limb primordia, the ectoderm overlying the limb bud was both N-CAM and L-CAM positive, and formed a striking border with the ectoderm of the body wall, which contained only L-CAM. In contrast to

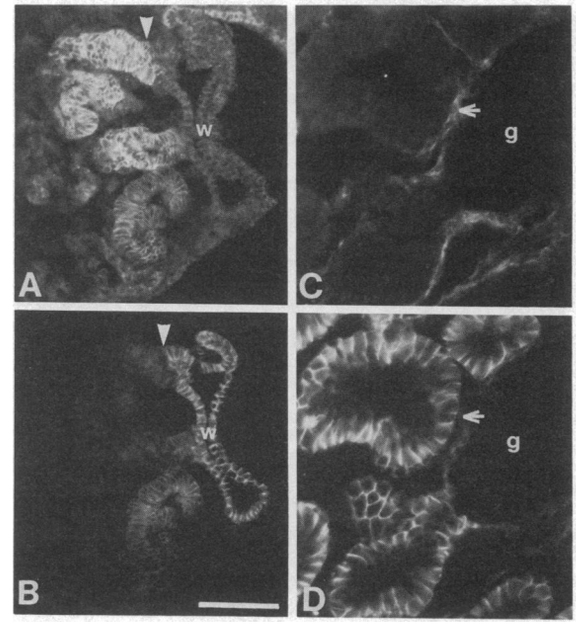

FIG. 3. Primary CAM expression in mesodermal structures exemplified by the mesonephric kidney. (*A* and *B*) Distal regions of the mesonephric tubules of stage 24 chicken embryo show intense staining for N-CAM (*A*), while the tubules nearest the Wolffian duct (marked with w) stain for L-CAM (*B*). (The arrowhead demarcates borders between N-CAM and L-CAM staining.) The Wolffian duct stains brightly for L-CAM and faintly for N-CAM. Mature mesonephric tubules shown in *C* and *D* (stage 38) stain only for L-CAM (*D*), while surrounding spindle-shaped cells (arrow) stain only for N-CAM (*C*). The glomeruli (marked by g) do not stain for either CAM. Both figures are double-stained sections with the same antibodies as described in Fig. 2 *C* and *D*. (Bar = 50 μm.)

the limb bud ectoderm, which stained for N-CAM transiently, the apical ectodermal ridge (Fig. 4 *A* and *B*) stained for both N-CAM and L-CAM throughout its existence. Initially, the apposed underlying mesenchyme stained homogenously for N-CAM (Fig. 4*A*). Later, beginning proximally in all regions that were destined to become chondrocytes, the mesenchyme showed a selective loss of N-CAM staining (Fig. 4 *C* and *D*). At even later stages (stage 34, E8), N-CAM staining was enhanced in muscle, tendons, and perichondrium (Fig. 4*D*).

Feather. We have recently discovered a series of appositions of collectives of N-CAM-positive cells with collectives of L-CAM-positive cells in the feather (17, 18). The main features of the feather sequence are summarized in Fig. 5. (*i*) N-CAM-positive mesodermal mesenchyme (the inductor) forms periodic clusters near L-CAM-positive ectoderm. Cells in the subectodermal region of the developing condensation become N-CAM positive, after which a placode appears in the overlying ectoderm (Fig. 5 *A* and *B*). Later, the L-CAM-positive placode cells transiently express N-CAM. (*ii*) In the formation of the dermal papilla, N-CAM-positive mesodermal cells adjoin L-CAM-positive ectodermal cells (Fig. 5 *C* and *D*). At this stage in the highly proliferative collar epithelium, these ectodermal cells express both L-CAM and N-CAM. The expression of N-CAM in this epithelium is restricted to only a portion of the epithelial sheet as seen with other epithelia (retina, kidney, endoderm, and feather placode, described above). (*iii*) As L-CAM-positive barb ridges form, basilar cells in the ridge valleys become N-CAM positive, leading to alternating N-CAM-positive marginal plates between L-CAM-positive barb ridges (Fig. 5 *E* and *F*). A similar expression of N-CAM-positive cells within each barb ridge occurs during formation of axial plates. The marginal and axial plates subsequently lose L-CAM, leaving alternations of collectives of N-CAM-positive cells between collectives of L-CAM-positive cells. The L-CAM-positive cells of the barbule plate become keratinized as the N-CAM-positive cells of the marginal and axial plates die, leaving spaces between barbs and barbules and leading to the characteristic feather morphology.

DISCUSSION

The major findings of this study are as follows. (*i*) At one or another time, both primary CAMs are expressed on derivatives of all three germ layers. N-CAM and L-CAM are simultaneously expressed on certain epithelial cells from somatic ectoderm, mesoderm, and endoderm in a fashion that resembles their coexpression in the early blastoderm (9–11). Moreover, cells can express N-CAM only, L-CAM only, both CAMs, or very low amounts of either CAM. Such states appear to reflect increases or decreases of the CAMs, which can occur at different rates during development. (*ii*) As summarized in Table 1, the detailed sequences of CAM expression observed in various tissues can be grouped into

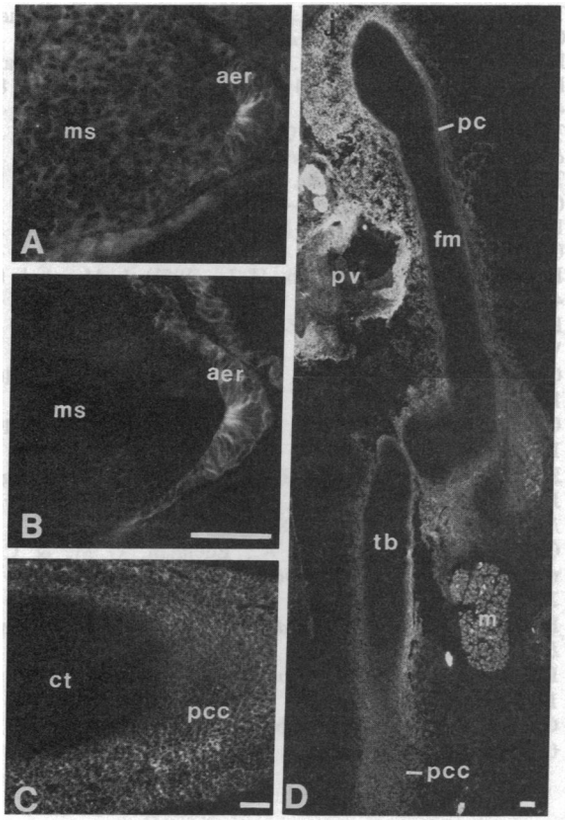

FIG. 4. CAM expression in limb bud epithelium and mesenchyme and in later limb development. (*A* and *B*) The apical ectodermal ridge (aer) of the stage 22 limb bud stains strongly for both N-CAM (*A*) and L-CAM (*B*), while the underlying limb mesenchyme (ms) stains for N-CAM (*A*). (*C*) Precartilage mesenchymal condensations (pcc) of the stage 33 limb are N-CAM positive but lose N-CAM as they differentiate into cartilage (ct). (*D*) The hind limb of a stage 35 embryo shows bright N-CAM staining in the perichondrium (pc), precartilage condensation (pcc), muscle (m), and the region of the presumptive joint between the femur (fm) and pelvis (pv). (Bar = 50 μm.)

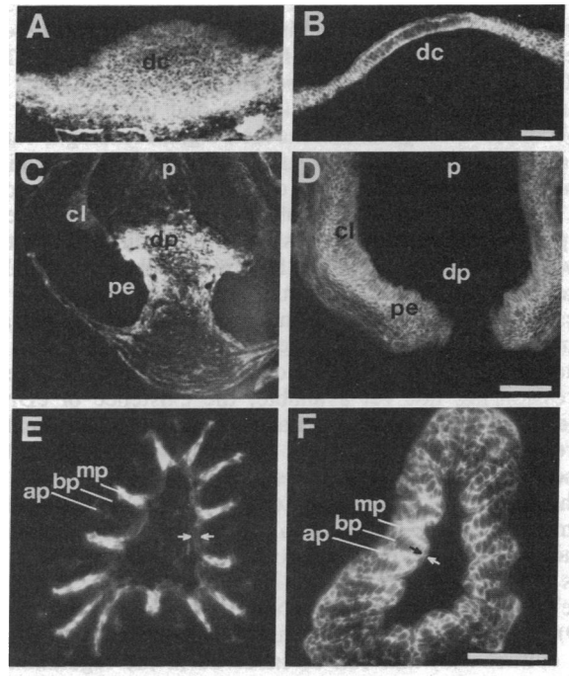

FIG. 5. CAM-linked cell collectives in feather development. (*A* and *B*) In a transverse section of dorsal skin from a stage 33 embryo, cells of the dermal condensation (dc) stain strongly only for N-CAM (*A*); the overlying placode epithelium stains for both N-CAM (*A*) and L-CAM (*B*). (*C* and *D*) In a longitudinal section, feather follicles from the wing skin of a newly hatched chicken show intense N-CAM staining (*C*) in the dermal papilla (dp) and L-CAM staining (*D*) in the papillar ectoderm (pe). The collar epithelium (cl) stains for both N-CAM and L-CAM. p, pulp. (*E* and *F*) Cross sections of feather filaments from the skin of the back of a stage 44 embryo. The marginal (mp) and axial plates (ap) show staining for both N-CAM (*E*) and L-CAM (*F*), which alternates with L-CAM staining in the barbule plate (bp). The marginal, axial, and barbule plates comprise the barb ridge as discussed in the text. The marginal and axial plates later lose L-CAM, leaving N-CAM-positive cells alternating with L-CAM-positive cells of the barbule plate. Arrows point to the basilar layer. (Bar = 50 μm.)

two general modes, mode I in mesenchyme and mode II in epithelia. In mode I, cells expressing N-CAM (or in the blastoderm, both CAMs) go through a stage of decreased CAM expression (particularly during cell movements) and then reexpress N-CAM in a cyclic fashion. By contrast, in mode II, expression of both CAMs in an epithelium is followed by loss of either N-CAM or L-CAM, depending on the locale. Local expression of N-CAM in epithelia can be enhanced to give regions of increased expression bordering on those of low expression. This distinct and localized modulation appears at regions of rapid growth or expansion (see Figs. 1*E*, 2*E*, 2*G*, and 3*A*). (*iii*) In all areas of induction, an epithelial collective of cells linked by L-CAM plus N-CAM (or by L-CAM only) is adjoined by a collective of cells linked by N-CAM alone. Such CAM couples arise either from movement of mesenchymal cells to adjoin epithelia or from differential gene expression and cell division in cells of the same lineage, as seen in the feather. Thus, from the time of primary induction, epithelia expressing both N-CAM and L-CAM are induced by N-CAM-positive mesodermal tissues. This pattern is seen at sites of secondary induction in the lung and gut derivatives, in the skin, and in the limb bud. In the kidney, however, the direction appears to be reversed: the L-CAM- and N-CAM-positive Wolffian duct is the inductor (16) for the N-CAM-positive mesonephric mesenchyme.

These observations reveal that the primary CAMs are ubiquitous and that the uniform patterns of a small number of CAM expression sequences are repeated in many locales. The appearance of mode II modulations in many tissue types, the existence of only one or a few genes for the CAMs (19, 20), the overlapping of tissue-specific borders in fate maps by primary CAM distributions (10, 21), and the existence of CAM couples all suggest that the local signals leading to early expression of CAM gene products differ from those leading to the subsequent expression of tissue-specific gene products. Indeed, the association of CAM expression with definite patterns of primary processes during induction and the known intercellular adhesive function of these molecules suggests that their expression is separately regulated by genes that might be termed morphoregulatory to contrast them with historegulatory genes that control expression of tissue-specific products. While data at the level of direct gene expression are not yet available, the existence of cDNA clones for the CAMs (19, 20) makes it possible to confirm the expression sequences at this level and also to test the proposal (22) of separate stages of gene control.

This proposal would be negated if it were found that CAMs are merely markers that play no causal roles in the regulation of the expression of primary processes of development. Two lines of evidence suggest that this is not the case. (*i*) CAMs have been demonstrated to function as adhesion molecules (4–9); numerous correlative studies (2) suggest that CAMs link epithelia (5) and can be involved in the regulation of morphogenetic movements (22, 23). Moreover, in the feather, the expression of primary CAMs provides boundaries separating morphogenetically significant collectives of cells. Such boundaries are strictly correlated with the expression of different primary processes in each of the cell collectives comprising a CAM couple—e.g., differentiation (keratin expression) for L-linked cells and death for N-linked cells. (*ii*) Recent observations indicate that perturbation of early feather induction *in vitro* by antibodies to either primary CAM affects feather formation (unpublished data). As exhibited in their expression sequences, and as indicated by the results of such perturbation experiments, primary CAMs appear to be important candidates for direct involvement in the complex causal chains of induction.

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