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Cell adhesion molecules in early chicken embryogenesis

(neural cell adhesion molecule/embryonic induction/mesenchyme–epithelium interconversion/cell surface modulation)

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ABSTRACT **N-CAM, the neural cell adhesion molecule, has been found at a number of regions in the early (1–5 days) chicken embryo by fluorescent antibody techniques. These regions appear to be those concerned with induction of the primary developmental axis (neural plate, neural tube, notochord, somites) or those in which later inductive events occur (neural crest cells, optic, otic, and pharyngeal placodes, cardiac mesoderm, mesonephric primordium, limb buds). The staining patterns in the latter group of regions are highly dynamic and transient and are limited to the epithelial components of the placodes and to the precursors of mesonephric tubules. In neural crest cells, N-CAM appears early, disappears during migration of the cells on fibronectin, and reappears at sites where ganglia are formed. In other regions of the nervous system, particularly those related directly to the neural tube, the N-CAM molecule is stained at all stages. The results raise the possibility that adhesion mediated by N-CAM plays a primary role in early embryogenesis as well as in later histogenesis.**

In the avian embryo, the primordia of the main structures of the body are formed during the first 3 or 4 days of development. Rapid proliferation, active migration, and adhesive interactions of cells contribute to the shaping of these embryonic structures. Transitory condensation of primary mesenchyme into well-defined epithelium and reorganizations of epithelial sheets are most frequently observed during these early stages of development. A better understanding of the precisely coordinated mechanisms that direct individual cells into tissues requires an analysis of the different adhesive processes mediated by cell surface molecules, by specialized junctions, and by basement membranes. Fibronectin (FN) associated with basement membranes has been correlated with the existence of stable epithelium; this molecule disappears locally at sites of cell detachment preparatory to migration (1–3). Intercellular junctions occur transiently and are redistributed during morphogenetic events (4).

A number of cell adhesion molecules have been identified in studies of histogenesis (see ref. 5 for a brief review; see also refs. 6 and 7) but so far they have not been studied extensively in connection with events in early embryogenesis. In the present paper, we describe the appearance of a neural cell adhesion molecule (N-CAM) as an early embryonic marker, its transient appearance in a number of regions classically associated with embryonic induction, and its final localization in structures destined to become neural tissues.

MATERIALS AND METHODS

Embryos. White Leghorn chicken embryos were used. Development of embryos older than 4 days of incubation was determined by reference to the staging series of Hamburger and Hamilton (8). In younger embryos, age was determined by the number of somite pairs.

Preparation of Specimens. Embryos were fixed in 3.7% formaldehyde in phosphate-buffered saline (P_i/NaCl) for 1 hr at 20°C, dehydrated with ethanol in a graded series (70% to 100%), and embedded in polyethylene glycol 1500 (Serva, Heidelberg, Germany) at 48°C. Sections (5 μm thick) were attached to glass slides previously coated with rubber cement (9).

Labeling of Sections. Affinity-purified antibodies to N-CAM were prepared as described (6, 10) and staining for FN was carried out as described (1). Sections were incubated with rabbit IgG (10 μg/ml in 0.5% bovine serum albumin in P_i/NaCl) for 1 hr at room temperature. After washes in P_i/NaCl, sections were incubated with fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG (Institut Pasteur, Paris) diluted 1:150 in P_i/NaCl-containing 0.5% albumin. Sections were then washed in P_i/NaCl, mounted in glycerol/P_i/NaCl, 9:1 (vol/vol), and examined under epifluorescence with a Leitz Orthoplan microscope equipped with a varioorthomat camera. Control sections were incubated with preimmune serum in place of the first antibody. The pattern of N-CAM distribution was found to be identical in frozen sections, indicating that the polyethylene glycol embedding procedure did not alter N-CAM antigenicity.

RESULTS

Neurulation. At the end of gastrulation, the central part of the epiblast ahead of Hensen's node rapidly formed the neural plate under control of the closely associated notochord. N-CAM was found to appear for the first time in the neural plate/notochord territory (not shown). Neurulation was studied throughout the posterior half of a 15-somite-stage embryo (Fig. 1). N-CAM was localized in the neural plate overlying the epiblast and presumptive notochord; this staining provides a landmark in the epiblast for neural tissue and epidermis. The boundary between the presumptive notochord and the neural plate is not obvious because basement membranes are not yet established (2). N-CAM was found only in the neural tube (Fig. 1*B*); the notochord, which was partly separated from the neural tube, remained unstained at this stage. Sections made at the penultimate somite (Fig. 1*C*) revealed that N-CAM was homogeneously distributed around all the neutral epithelial cells and in the well-defined notochord. The ectoderm, endoderm, and blood vessel walls remained unstained but the dorsal border of the neural tube corresponding to the crest cells was still rich in N-CAM. N-CAM was also found in the condensed somites. Clearly, N-CAM is found in precursor cells of the central nervous system prior to their differentiation into neurons.

Peripheral Nervous System. Neural crest cells appearing at the dorsal border of the neural tube stained for N-CAM (Fig. 1*C*) prior to migration. In the trunk, crest cells at the origin of

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Abbreviations: N-CAM, neural cell adhesion molecule; FN, fibronectin; P_i/NaCl, phosphate-buffered saline.

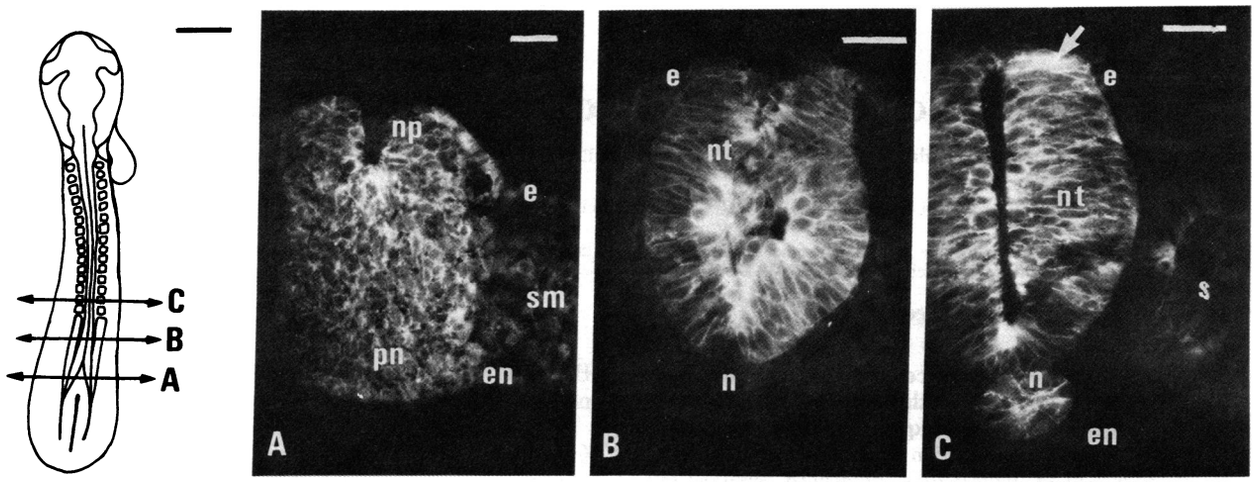

FIG. 1. Neural tube formation: Levels of the various transverse sections are shown on the drawing of the 15-somite-stage embryo (bar = 0.5 mm). (*A*) Neural plate. At the vicinity of Hensen's node, N-CAM is found in the axial structures (np, neural plate; pn, presumptive notochord) and in the connecting ectoderm, whereas the lateral structures (e, ectoderm; sm, somitic mesenchyme; en, endoderm) remained unstained. (*B*) Middle region of the unsegmented trunk. N-CAM is exclusively located in the closing neural tube (nt), mostly at its apical aspect; the notochord (n) and ectoderm (e) are unstained. (*C*) Somite 14. After complete closure of the neural tube (nt), N-CAM was found in the neural epithelial cells including crest cells (arrow) and notochordal (n) cells. The ectoderm (e) and the endoderm (en) remained unstained whereas N-CAM appeared in the apical region of the somite (s). (Bar = 25 μm.)

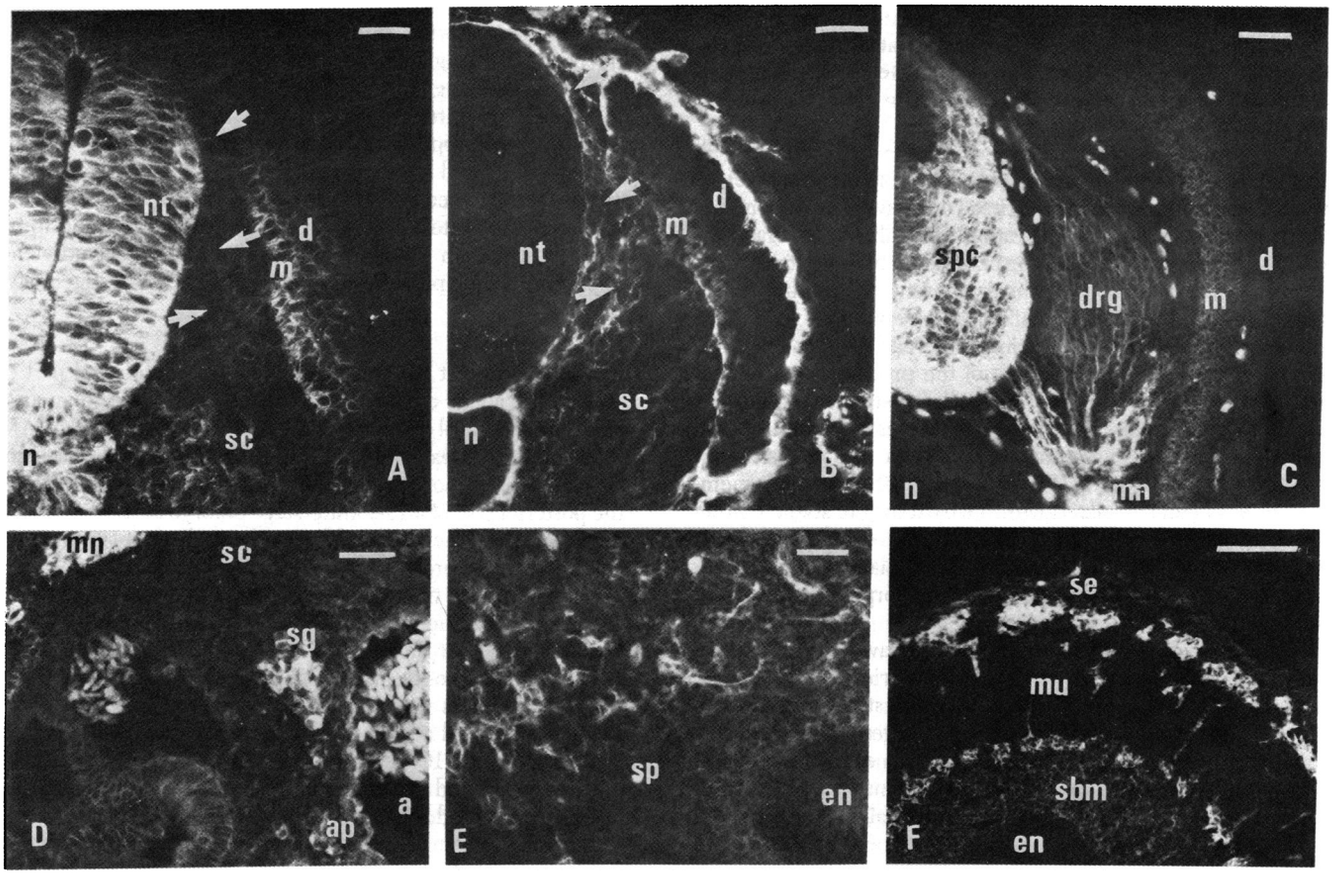

FIG. 2. Ontogeny of the peripheral nervous system: 15th somite level. (*A* to *C*) Dorsal root ganglion. (*A*) At 35-somite stage. Crest cells (arrows) accumulating between the neural tube (nt), the dermomyotome (d, m), and the sclerotome (sc) remain unstained. N-CAM is found in the neural tube, the notochord (n), and the myotome (m). (*B*) Same stage. Labeling of FN showed it to be present as a basement membrane around the neural tube (nt), notochord (n), and dermomyotome (d, m). Crest cells (arrows) are enmeshed in a FN-rich extracellular matrix. (*C*) Stage 25 (4 1/2 days). Crest cells aggregated by stage 21 and formed a ganglion rudiment (drg). All the cells are labeled with anti-N-CAM antibodies, including the first axons that connected to the developing spinal cord (spc); all nerves in the spinal cord and the motor root outgrowth (mn) are also heavily stained. Some of the fibers have already reached the myotome (m) which also contained N-CAM. Note the presence of many blood vessels with autofluorescent erythrocytes. (*D*) Sympathetic ganglion: Stage 21 (3 1/2 days), 15th-somite level. Crest cells accumulate near the aorta (a) to form metameric ganglia (sg) and diffuse aortic plexuses (ap) within the sclerotome (sc) concomitantly with the appearance of N-CAM at their surface. Note the presence of stained motor nerve (mn). (*E* and *F*) Enteric ganglia. (*E*) Stage 25 (5 days), level of gizzard. After cessation of migration, crest cells collected into

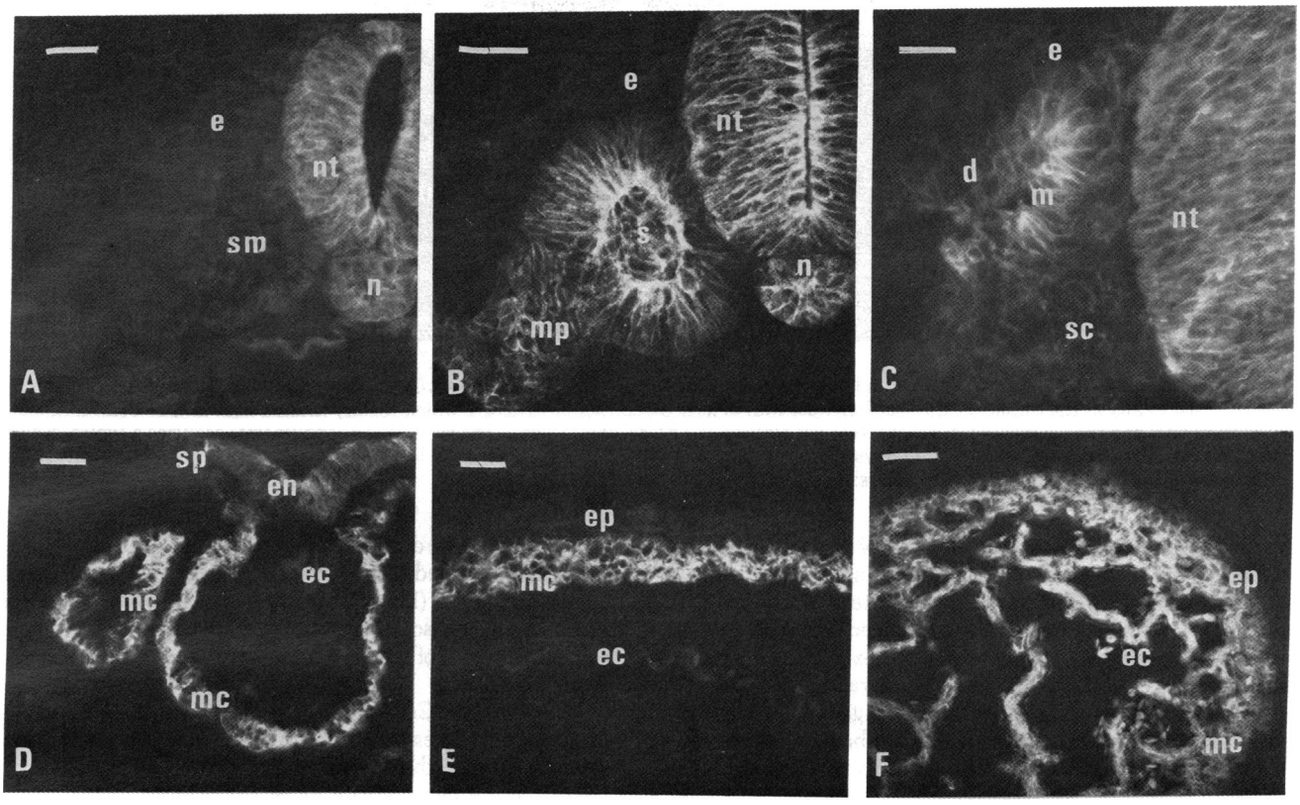

FIG. 3. Somitic mesoderm and cardiac mesoderm. (*A–C*) Transverse sections at the 15th-somite level. (*A*) At 14-somite stage. The somitic mesenchyme (sm) is devoid of N-CAM prior to metamerization as opposed to the neural tube (nt) and the notochord (n). (*B*) At 18-somite stage. After compaction, most somitic cells (s) are stained by N-CAM antibodies, particularly near the cavity; cells in the mesonephric primordium (mp) remained poorly stained. (*C*) At 28-somite stage. The somitic epithelium is disrupted; N-CAM is localized in the expanding myotome (*m*) whereas the dermis (*d*), which is still epithelial, progressively loses CAM along with the dissociated sclerotomal cells (sc). The neural tube (nt) contained N-CAM. At all stages, the ectoderm (e) was unstained. (*D–F*) Transverse sections at the rhombencephalic level. (*D*) At 14-somite stage. The myocardium (mc) is the only segment of the splanchnic mesoderm (sp) which is stained. The endocardium (ec) and the foregut endoderm (en) do not show N-CAM. (*E*) At 35-somite stage. The myocardium (mc) is strongly stained whereas the epicardium (ep) and the endocardium (ec) are totally devoid of N-CAM. (*F*) Stage 23 (4 days). N-CAM is found in the myocardium (mc) of the ventricle, forming multiple outpocketings. The thin layer of endocardium (ec) and the epicardium (ep) remain unstained. (Bars: *A, B, C, D,* and *E,* 25 μm; *F,* 50 μm.)

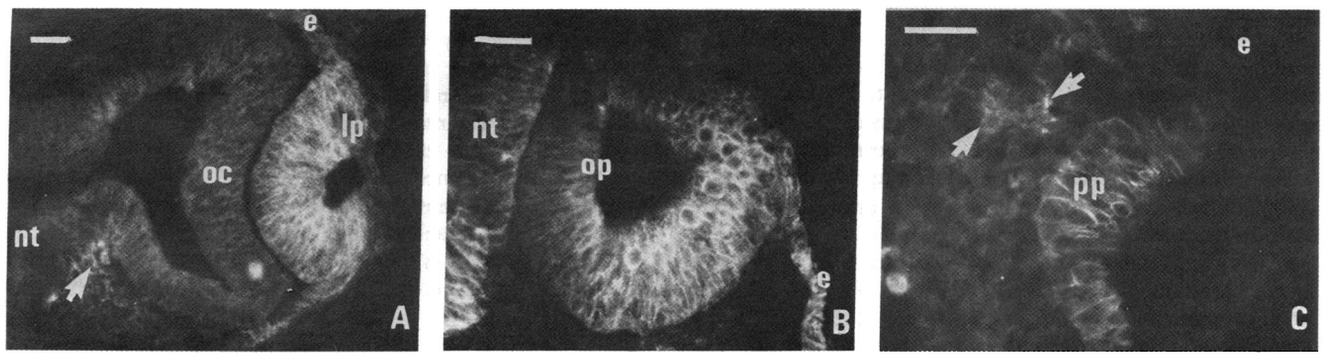

FIG. 4. Placodes. (*A*) At 28-somite stage, prosencephalic level. The lens placode (lp) and its attached surface ectoderm (e) are stained. The optic cup (oc) and lateral aspect of the prosencephalic neural tube (nt) remain unstained. At this stage, the medial region only is stained (not shown). Ciliary ganglion cell precursors deriving from the crest cells are already labeled (arrow). (*B*) At 28-somite stage, median rhombencephalon. The otic placode (op) in the process of invagination is labeled with its connecting ectoderm (e). The dorsally associated rhombencephalic neural tube (nt) is also stained. (*C*) At 25-somite stage, posterior rhombencephalon. The pharyngeal placode (pp) at the origin of the neurons of the nodose sensory ganglion is labeled prior to invagination. Crest cells (arrows) interpersed in the mesenchyme are also labeled; after fusion with the placode, they differentiate into glial cells. (Bars, 25 μm.)

small clusters connected to each other by processes. Cell bodies and processes are stained. The surrounding splanchnopleural cells (sp) corresponding to future smooth muscle layers as well as the endoderm (en) do not stain. (*F*) Stage 37 (11 days), level of rectum. The two ganglion plexuses and interconnecting axons are strongly stained. The presence of N-CAM is also apparent in the submucosa (sbm). The endoderm (en), smooth muscle layer (mu), and serosa (se) are unstained. (Bars: *A, B, D,* and *E,* 25 μm; *C* and *F,* 100 μm).

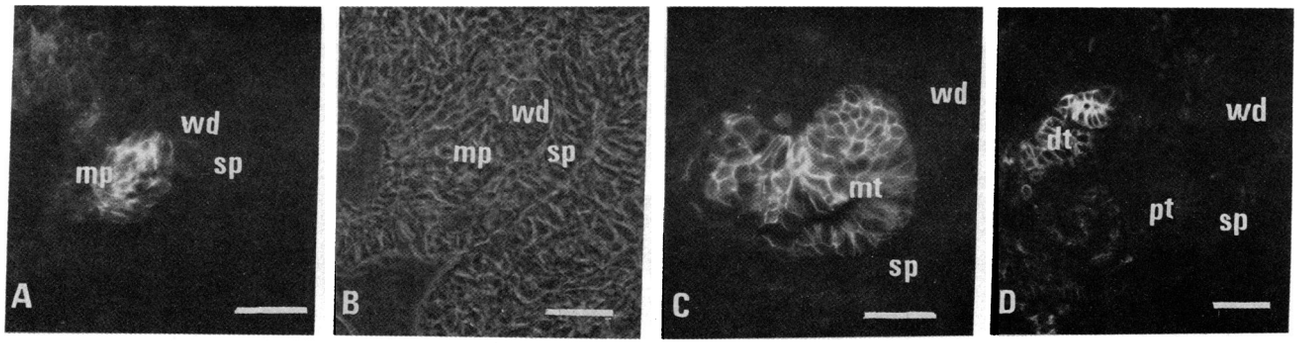

FIG. 5. Mesonephros, 20th-somite level. (*A* and *B*) Immunofluorescence (*A*) and phase-contrast (*B*) of 28-somite stage. Mesonephric primordium (mp) condensed locally at the contact of the Wolffian duct (wd) to form an epithelial structure which readily labeled with anti-N-CAM antibodies. The Wolffian duct and the splanchnopleure (sp) were not stained. (*C*) At 35-somite stage. The mesonephric tubule (mt) acquires a typical S shape in which all cells are stained. (*D*) Stage 23 (4 days). The proximal mesonephric tubules (pt) have lost N-CAM, whereas it appeared at the surface of cells aggregating into distal tubules (dt). (Bars: *A*, *B*, and *C*, 25 μm; *D*, 50 μm.)

the sensory ganglia utilize a ventral pathway between the dissociating somite and neural tube (3). Migrating crest cells were devoid of N-CAM (Fig. 2*A*). A reciprocal pattern of staining was found for N-CAM and FN which was localized in the basement membranes of epithelial tissues and in the extracellular matrix which serves as a substrate for crest cell migration (Fig. 2*B*). At the site of arrest, crest cells collecting into a ganglion rudiment were again all labeled with anti-N-CAM antibodies (not shown) and FN staining had disappeared within the rudiment. At 5 days, the ganglion and its nerve outgrowth could be easily distinguished by N-CAM staining from the surrounding sclerotomal cells (Fig. 2*C*). At this time, FN was absent from neural tissues including the neural crest cells. The autonomic nervous system originates from crest cells migrating in transient narrow spaces between adjacent somites (3). Crest cells accumulated along the lateral and ventral aspect of the aorta where they formed metameric sympathetic ganglia and a diffuse network concomitantly with the appearance of N-CAM on their surfaces (Fig. 2*D*). The enteric nervous system is derived mainly from cervical crest cells that have migrated within the gut. N-CAM appeared in differentiating crest cells and in their processes behind the front of migration in the developing gizzard by 4 days (Fig. 2*E*). The network interspersed in the dense mesenchyme eventually will become organized as two plexuses located on either side of the muscular layer of the gut. The submucous plexus of Meissner and the myenteric plexus of Auerbach and interconnecting fibers stained (Fig. 2*F*) where they traversed the smooth muscle layer in which no staining was detected.

Striated Muscles. Trunk skeletal muscles originate from the somitic mesoderm. Just prior to condensation, the somitic mesenchyme was unstained (Fig. 3*A*) whereas the neural tube and the notochord contained N-CAM (see Fig. 1). N-CAM was present in the radially oriented somitic cells (Fig. 3*B*). After disruption of the somites, staining was maintained only in the developing myotome (Fig. 3*C*).

Cardiac Muscle. At the end of gastrulation, the region of rhombencephalic mesenchyme that gives rise to the heart was stained for N-CAM (not shown). N-CAM was found to be present in the region of the splanchnopleure giving rise to the cardiac primordium and was absent in the newly formed endocardium. Later on, when the epicardium was formed, N-CAM remained only in the growing myocardium (Fig. 3 *D*, *E*, and *F*).

Placodes. Many of these local thickenings of the cephalic ectoderm participate in forming sensory specializations as well as the lens. The lens placode became labeled at the 4-somite stage and remained stained during the process of its invagination (Fig. 4*A*). The otic placode appeared at the 10-somite stage and became labeled by the 16-somite stage (Fig. 4*B*). The epibranchial placode (Fig. 4*C*) became stained by the 20-somite stage. We have also observed (data not shown) that the apical ectodermal ridge of the limb buds was stained at the 40-somite stage.

Mesonephros. Cells from the level of the intermediate piece of the first somites form the Wolffian duct, which elongates caudally along with the newly formed somites. Mesenchymal cells from the intermediate piece are induced to aggregate at the front of extension of the Wolffian duct. The mesonephric cells became labeled for N-CAM (Fig. 5*A*) as soon as they formed a tight aggregate. Seven hours later, a well-developed S-shaped mesonephric tubule was labeled but the surrounding tissues including the Wolffian duct were devoid of CAM. One day later, the mesonephric tubules that were first stained became unstained. The same transient staining was observed in the case of the metanephros (not shown).

DISCUSSION

The most striking findings in the present study are the extensive distribution of N-CAM in a number of embryonic regions and its presence at very early stages of development. These findings were unexpected, inasmuch as the motives for isolating the molecule (11, 12) related to its possible role in neural histogenesis at later embryonic stages. In addition to its widespread occurrence in the early embryo, N-CAM showed a dynamic pattern of appearance and disappearance. In structures that are destined to form most of the central nervous system, however, the molecule is stably represented even in the early embryo. Although N-CAM is ubiquitous in the neural epithelium, it is not found on glial cells and therefore must be lost during glial differentiation.

It was not surprising that N-CAM, which contributes to the interaction of neurons with striated muscle cells (13), is found as an early marker on somites and in the cardiac mesoderm. Consistent with these ideas, there is evidence to suggest that neural interaction with striated muscle is essential for proper maintenance of both tissues (14). Moreover, the absence of N-CAM in smooth muscle rudiments of the gut is in accord with the relative independence of the development of this tissue and the enteric plexuses (15).

Neural crest cells show N-CAM when they first appear but do not stain for the molecule during migration; N-CAM appears again at their surface just before they form peripheral ganglia. The disappearance of N-CAM staining is coordinated with the appearance of FN along their path of migration (3). The factors

regulating the conjugate appearance and disappearance of these cell–cell adhesion and cell–substrate adhesion molecules are not known.

Perhaps the most surprising feature of the present study was the transient appearance of N-CAM in placodes and in precursors of the mesonephric tubule. These are regions of intense inductive interactions. This observation suggests the possibility that N-CAM holds cells together in the tissue to be induced for a critical period necessary for maintenance and proximity. In any case, the present observations suggest that N-CAM may be essential for cell-to-cell adhesion during early embryogenesis when structures have to be rapidly established but also need to be remodeled. One of the most challenging tasks in understanding this process is to relate the role of CAMs to the roles played by substrate adhesion molecules such as FN as well as by intercellular junctions and their associated molecules.

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1. Duband, J.-L. & Thiery, J.-P. (1982) *Dev. Biol.* **92**, in press.
2. Duband, J.-L. & Thiery, J.-P. (1982) *Dev. Biol.* **92**, in press.
3. Thiery, J.-P., Duband, J.-L. & Delouvée, A. (1982) *Dev. Biol.* **92**, in press.
4. Revel, J.-P. & Brown, S. S. (1976) *Cold Spring Harbor Symp. Quant. Biol.* **40**, 443–455.
5. Edelman, G. M. & Rutishauser, U. (1981) *Supramol. Struct. Cell. Biochem.* **16**, 259–268.
6. Hoffman, S., Sorkin, B. C., White, P. C., Brackenbury, R., Mailhammer, R., Rutishauser, U., Cunningham, B. A. & Edelman, G. M. (1982) *J. Biol. Chem.* **257**, 7720–7729.
7. Chuong, M.-C., McClain, D. A., Streit, P. & Edelman, G. M. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4234–4238.
8. Hamburger, V. & Hamilton, H. L. (1951) *J. Morphol.* **88**, 49–92.
9. Drews, V. (1975) *Prog. Histochem. Cytochem.* **7**, 1–52.
10. Rutishauser, U., Hoffman, S. & Edelman, G. M. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 685–689.
11. Brackenbury, R., Thiery, J.-P., Rutishauser, U. & Edelman, G. M. (1977) *J. Biol. Chem.* **252**, 6835–6840.
12. Thiery, J.-P., Brackenbury, R., Rutishauser, U. & Edelman, G. M. (1977) *J. Biol. Chem.* **252**, 6841–6845.
13. Grumet, M., Rutishauser, U. & Edelman, G. M. (1982) *Nature (London)* **295**, 693–695.
14. Dennis, M. N. (1981) *Annu. Rev. Neurosci.* **4**, 43–68.
15. Gershon, M. D. (1981) *Annu. Rev. Neurosci.* **4**, 227–272.