

Expression Sequences and Distribution of Two Primary Cell Adhesion Molecules during Embryonic Development of *Xenopus laevis*

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Abstract. Studies of chicken embryos have demonstrated that cell adhesion molecules are important in embryonic induction and are expressed in defined sequences during embryogenesis and histogenesis. To extend these observations and to provide comparable evidence for heterochronic changes in such sequences during evolution, the local distributions of the neural cell adhesion molecule (N-CAM) and of the liver cell adhesion molecule (L-CAM) were examined in *Xenopus laevis* embryos by immunohistochemical and biochemical techniques. Because of the technical difficulties presented by the existence of multiple polypeptide forms of CAMs and by autofluorescence of yolk-containing cells, special care was taken in choosing and characterizing antibodies, fluorophores, and embedding procedures. Both N-CAM and L-CAM were found at low levels in pregastrulation embryos. During gastrulation, N-CAM levels increased in the presumptive neural epithelium and decreased in the endoderm, but L-CAM continued to be expressed in all cells including endodermal cells. During neurulation, the level of N-CAM expression in the neural ectoderm increased considerably, while remaining constant in non-neural ectoderm and diminishing in the somites; in the notochord, N-CAM was expressed transiently. Prevalence modulation was also seen at all sites of secondary induction: both CAMs increased in the sensory layer of the ectoderm during condensation of the placodes. During organogenesis, the expression of L-CAM gradually diminished in the nervous system

while N-CAM expression remained high. In all other organs examined, the amount of one or the other CAM decreased, so that by stage 50 these two molecules were expressed in non-overlapping territories. Embryonic and adult tissues were compared to search for concordance of CAM expression at later stages. With few exceptions, the tissue distributions of N-CAM and L-CAM were similar in the frog and in the chicken from early times of development. In contrast to previous observations in the chicken and in the mouse, N-CAM expression was found to be high in the adult liver of *Xenopus*, whereas L-CAM expression was low. In the adult brain, N-CAM was expressed as three components of apparent molecular mass 180, 140, and 120 kD, respectively; in earlier stages of development only the 140-kD component could be detected. In the liver, a single N-CAM band appears at 160 kD, raising the possibility that this band represents an unusual N-CAM polypeptide. L-CAM appeared at all stages as a 124-kD molecule. Although the relative amounts of the two primary CAMs of chicken and frog differ as a function of time of development, the final tissue distributions of these molecules mapped back onto fate maps of the early gastrula show remarkable similarities. This is consistent with the regulator hypothesis which proposes that CAMs play important regulatory roles in establishing the basic body plan at sites of induction and also in histogenesis.

A number of cell adhesion molecules (CAMs)¹ have now been identified, isolated, and studied in detail (Edelman, 1986a). Two of these, the neural CAM, (N-CAM) and the liver CAM (L-CAM) have been found to appear at very early stages of development in the chicken and have therefore been called primary CAMs. N-CAM is a cell surface glycoprotein that mediates calcium-independent ag-

gregation of cells by a homophilic mechanism (Hoffman et al., 1982; Hoffman and Edelman, 1983). Sequence studies indicate that it is a member of the Ig superfamily and that it has five Ig-like extracellular domains in all of its polypeptide variants (Cunningham et al., 1987). The polypeptides differ only in their cell-associated domains as a result of alternative splicing of RNA (Murray et al., 1986a; Owens et al., 1987; Hemperly et al., 1986a, b; Cunningham et al., 1987) transcribed from a single N-CAM gene (D'Eustachio et al., 1985). L-CAM is a calcium-dependent adhesion molecule first isolated from chicken embryo liver (Gallin et al.,

1. *Abbreviations used in this paper:* CAM, cell adhesion molecule; L-CAM, liver CAM; N-CAM, neural CAM.

1983) that has been shown to be closely involved in intercellular binding of hepatocytes and various epithelia (Bertolotti et al., 1980; Gallin et al., 1983). It appears to be related to a class of other calcium-dependent adhesion molecules that have been isolated from other species (Peyrieras et al., 1983; Shirayoshi et al., 1986; reviewed in Edelman, 1986a). Complete sequence analysis (Gallin et al., 1987) indicates that it is not related to N-CAM.

CAMs show defined sequences of expression during embryogenesis and histogenesis and appear to play important roles in embryonic induction (Edelman 1986a, b; Crossin et al., 1985; Chuong and Edelman, 1985a, b; Richardson et al., 1987; Kintner and Melton, 1987). In all of these studies, a strong correlation was observed between CAM expression sequences and major morphogenetic events, particularly those related to epithelial-mesenchymal transformations and embryonic induction. Several regularities of primary CAM expression in epithelia and mesenchyme have been observed at sites of induction: N-CAM disappears transiently from a mesenchyme to be reexpressed later, whereas one or the other of N-CAM or L-CAM disappears from epithelia that originally express both CAMs (Crossin et al., 1985). More recently, it has been possible to perturb inductive events with antibodies to a CAM: during feather induction (Gallin et al., 1986), antibodies to L-CAM, which is present in the skin ectoderm, dramatically altered the morphology of the underlying mesodermal condensates, which expressed N-CAM and not L-CAM. This result suggests that the exchange of inductive signals leading to pattern formation is dependent on the integrity of CAM-linked cell collectives.

Evolutionary conservation of the CAM expression rules in different vertebrate species, while altering the developmental timing of CAM expression, might preserve the generalized vertebrate body plan by ensuring the proper contiguity of interacting tissues involved in embryonic induction and at the same time allow morphologic variance (Edelman, 1984, 1986b). We undertook the current study to determine whether CAM expression sequences are conserved at sites of embryonic induction in *Xenopus laevis*, a species that is quite different from the chicken in terms of its early morphogenesis and timing of developmental processes. The comparison of CAM expression in chicken and *Xenopus* development revealed that the rules for CAM expression were generally maintained in these two species but major differences occurred in the relative timing of CAM modulations. Such "molecular heterochrony" is consistent with the heterochronic changes (Gould, 1977) that are observed in comparing morphologic development in these species.

Materials and Methods

Animals

Oocyte positive *Xenopus laevis* females and sexually mature males were purchased from Xenopus I (Ann Arbor, MI). Embryos were obtained through artificial fertilization. Eggs were stripped from females injected 12 h earlier with human chorionic gonadotropin (1,000 IU; Sigma Chemical Co., St. Louis, MO) and fertilized with the minced testis of males injected 12 h earlier with 400 IU of human chorionic gonadotropin. The embryos were maintained in 10% filter-sterilized Holtfreter's solution at room temperature. Stages of development were determined according to Nieuwkoop and Faber (1967).

Antibodies

N-CAM was prepared from *Xenopus* tadpoles by affinity purification with an immobilized cross-reactive monoclonal antibody directed against chicken N-CAM. Monoclonal (7C8 and 10H4) and polyclonal (684) antibodies against *Xenopus* N-CAM were raised as described previously (Fraser et al., 1984). Polyclonal antibodies against chicken L-CAM (Gallin et al., 1983) were found to be cross-reactive with a similar molecule in *Xenopus* as shown by immunoblot analysis. The functional reactivity of this antibody in *Xenopus* was established by its ability (Gallin et al., 1983) to inhibit the aggregation of tadpole skin cells in the presence of calcium. Skin cells were prepared by immersing stage 32 or 38 tadpoles in HEPES-buffered, Ca^{2+} / Mg^{2+} -free medium supplemented with 0.05% trypsin (Worthington Biochemical Corp., Freehold, NJ) and 1 mM EDTA for 25 min at 37°C. The dissociated cells were collected and resuspended in DME containing 1 mg/ml Fab' fragments of polyclonal rabbit anti-L-CAM antibody or of rabbit nonimmune IgGs. The capacity of the antibody to prevent the reaggregation of the cells was scored 30 and 60 min later by direct microscopic observation or by counting the number of suspended particles with a Coulter counter (Coulter Electronics Inc., Hialeah, FL) as described (Hoffman and Edelman, 1983).

Immunohistochemistry

Paraffin sections were prepared for staining according to the method of Gurdon et al. (1976). Briefly, whole embryos or dissected organs were frozen in isopentane cooled in liquid nitrogen and immediately immersed in methanol at -70°C . After 40–70 h, the embryos were serially transferred to methanol equilibrated at -20° , 4° , and 20°C incubating them for at least 3 h at each step. The samples were then immersed twice in xylene for 15 min and then transferred to a solution of 50% Paraplast (Monoject Scientific, St. Louis, MO) in xylene at 45°C for 15 min, infiltrated twice with Paraplast in a vacuum oven at 45°C for 10 min, and embedded in Paraplast. Sections 10- μm thick were cut using a microtome (model 3010; Reichert Scientific Instruments, Buffalo, NY). For immunofluorescent staining, the deparaffinized sections were incubated sequentially with the primary antibody (10 $\mu\text{g}/\text{ml}$ in PBS, 5% normal goat serum) and an appropriate phycoerythrin-conjugated secondary antibody (Biomed Corp., Foster City, CA) and observed in a Zeiss microscope using a barrier filter at 550 nm. This entire procedure considerably reduced the intensity of the yolk autofluorescence commonly observed in *Xenopus* frozen sections, and was found to be essential to determine low levels of the primary CAMs reliably.

Biochemical Techniques

To detect the presence of N-CAM in early stages of development (stage 12 to stage 25), the jelly coats were removed manually from ~200 embryos leaving intact the vitelline membrane. The embryos were then homogenized on ice in 10 ml of 0.1 M NaCl/20 mM Tris-HCl (pH 7.6)/1 mM phenylmethylsulfonyl fluoride (PMSF)/2.5% (vol/vol) Trasylol (Moby Chemical Corp., Pittsburgh, PA)/1% Triton X-100. The homogenate was then centrifuged at 10,000 g in a microcentrifuge and the supernatant was collected avoiding the lipid pellicle. N-CAM was then immunoprecipitated from the supernatant by incubating overnight with 50 μl of Sepharose CL-4B beads directly coupled to the monoclonal antibody 7C8 directed against *Xenopus* N-CAM. The beads were collected, washed twice, and boiled 3 min in 80 μl of SDS sample buffer. Embryos between stage 28 and stage 42 were homogenized in 20 mM Tris-HCl (pH 7.6)/1 mM PMSF/2.5% (vol/vol) Trasylol/10% (wt/vol) sucrose. The pellet obtained after centrifugation at 10,000 g was gently extracted with 2 vol of PBS containing 0.5% NP-40 and centrifuged again. The resulting extract was mixed with an appropriate volume of 3 \times SDS sample buffer and boiled 3 min. For later stage embryos, tissues were dissected, homogenized in 20 mM Tris-HCl (pH 7.6)/1 mM PMSF/2.5% (vol/vol) Trasylol, and centrifuged at 10,000 g. The pellet obtained was extracted with 5 vol of PBS containing 0.5% NP-40, the protein concentration of the extract was measured, and SDS sample buffer was added to yield a final protein concentration of 2 $\mu\text{g}/\mu\text{l}$. To reveal the presence of L-CAM, tissues were directly homogenized in 5 vol of 1 \times boiling SDS sample buffer, boiled 3 min, centrifuged at 10,000 g, and resolved on SDS-PAGE. Immunoprecipitations and neuraminidase treatment of N-CAM were performed as previously described (Cunningham et al., 1983). Samples were resolved by SDS-PAGE (Laemmli, 1970) followed by electrophoretic transfer of the polypeptides to nitrocellulose paper (Towbin et al., 1979). The presence of the antigens was revealed by incubation with 50 μg of antibodies followed by [^{125}I]protein A and autoradiography. When mono-

clonal antibodies were used on immunoblots, a rabbit anti-mouse polyclonal antibody was added before the protein A.

Results

Characterization of Antibodies

In comparative studies of CAMs, particularly N-CAM, which has multiple polypeptide forms (Murray et al., 1986b), it is essential to determine as accurately as possible the specificity of the reagents used. The distribution of N-CAM in the development of *Xenopus* was studied with a polyclonal rabbit antibody (684) and with two mouse monoclonal antibodies (7C8 and 10H4), all of which were prepared against *Xenopus* N-CAM (Fraser et al., 1984). All these antibodies have been shown to be able to block the aggregation of *Xenopus* brain membrane vesicles in *in vitro* assays, and to perturb the retinotectal projection pattern during regeneration of the optic nerve *in vivo* (Fraser et al., 1984). The specificity of these antibodies was tested by immunoblot analysis on NP-40 extracts of adult *Xenopus* brain. Polyclonal antibodies and monoclonal antibody 7C8 recognized the typical three components of the adult form of N-CAM at 180, 140, and, at lower levels, 120 kD, respectively (Fig. 1, lanes 1 and 2), presumably corresponding to the ld, sd, and ssd chains found in chicken N-CAM. Monoclonal antibody 10H4 recognized predominantly a component at 180 kD and only very faintly a band at 140 kD (Fig. 1, lane 3). To confirm that all these antibodies were indeed recognizing N-CAM, we coupled them to Sepharose 4B beads and used these beads to immunoprecipitate adult *Xenopus* brain NP-40 extracts. The immunoprecipitated material was then tested for cross-reactivity with a bona fide polyclonal antibody directed against chicken N-CAM. This antibody recognized only the 180- and 140-kD components of N-CAM when tested on immunoblots of NP-40 extracts of *Xenopus* brain (Fig. 1, lane 4). The same components were recognized in the immunoprecipitates obtained with polyclonal antibodies and monoclonal antibody 7C8 (Fig. 1, lanes 5 and 6), whereas only the 180-kD component was present in the immunoprecipitate of antibody 10H4 (Fig. 1, lane 7). To confirm further that the material recognized by 7C8 and 10H4 was related to the molecule recognized by the polyclonal antiserum, we immunoblotted the material immunoprecipitated by 7C8 and 10H4 with the polyclonal rabbit antibody. Again we observed the three components of N-CAM immunoprecipitated by 7C8 and a predominant 180-kD component immunoprecipitated by 10H4 (Fig. 1, lanes 8 and 9). When these results are compared with the known polypeptide structure of N-CAM (Cunningham et al., 1987), they suggest that 7C8 recognizes a determinant in the extracellular portion of the molecule, whereas 10H4 recognizes an epitope located either within or in the proximity of a cytoplasmic domain of N-CAM. Such monoclonal antibodies of differing specificity provide powerful tools for distinguishing the expression of the different chains of N-CAM: because of tissue specific differences in sequential expression of N-CAM chains as a result of alternative RNA splicing (Murray et al., 1986b), the use of only a single antibody could easily result in erroneous conclusions about times and sites of appearance.

To detect L-CAM, we used a rabbit polyclonal antibody prepared against the affinity-purified chicken F11 fragment of

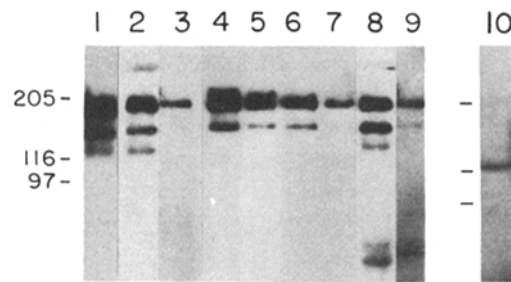


Figure 1. Characterization of the antibodies used in these studies. Lanes 1–4, immunoblotting pattern obtained with antibodies 684 (lane 1), 7C8 (lane 2), and 10H4 (lane 3) directed against *Xenopus* N-CAM and with antibody 541 (lane 4) against chicken N-CAM on NP-40 extracts of adult *Xenopus* brain membranes; 60 μ g of total protein per lane. Lanes 5–7, recognition with antibody 541 against chicken N-CAM of material immunoprecipitated from NP-40 extracts of adult *Xenopus* brain membrane using antibodies 684 (lane 5), 7C8 (lane 6), and 10H4 (lane 7). Lanes 8–9, recognition with antibody 684 of material immunoprecipitated with antibodies 7C8 (lane 8) and 10H4 (lane 9) from NP-40 extracts of adult *Xenopus* brain membranes. Lane 10, immunoblotting pattern obtained with antibody 701 against chicken L-CAM on adult *Xenopus* skin extract. Molecular mass markers are at 205, 116, and 97 kD, respectively.

the molecule (Gallin et al., 1983) and known to perturb adhesion between chicken hepatocytes *in vitro* and to disrupt inductive interactions during feather pattern formation (Gallin et al., 1986). Fab fragments of this antibody readily prevented the calcium-dependent aggregation of skin cells isolated from stage 32 or 38 *Xenopus* tadpoles. When tested in immunoblots of *Xenopus* skin extracts, this antibody recognized a single component at 124 kD (Fig. 1, lane 10). The pattern of distribution of L-CAM was also confirmed using a second polyclonal antibody that we recently prepared against affinity-purified L-CAM from adult dog liver and that cross-reacts with frog L-CAM. In all cases tested, the staining pattern obtained with both antibodies was identical.

Cleavage, Gastrulation, and Primary Induction

To determine the localization of CAM expression in the *Xenopus* embryo, an indirect immunofluorescence method was used. A major difficulty in using such histological techniques for amphibian embryos has been the strong autofluorescence of the yolk platelets that renders problematic the recognition of low level signals using conventional staining protocols. We have here combined the use of a gentle paraffin embedding technique (Gurdon et al., 1976), which preserves well the antigenic properties of tissues, with the use of phycoerythrin-coupled second antibodies. Phycoerythrins, when excited with a 490-nm wavelength, emit light at 576 nm; the large Stoke's shift makes it easy to distinguish the emitted light (bright yellow) from the tissue autofluorescence (green) that can then be eliminated using appropriate filters.

Both N-CAM and L-CAM were detected at stage 2 of development. The staining for both molecules was diffuse in the cytoplasm of the blastomeres; it was not associated with yolk platelets but with fibrous structures, possibly of cytoskeletal origin. Several control antibodies gave no positive signal. A uniform, low-level staining of the embryo could

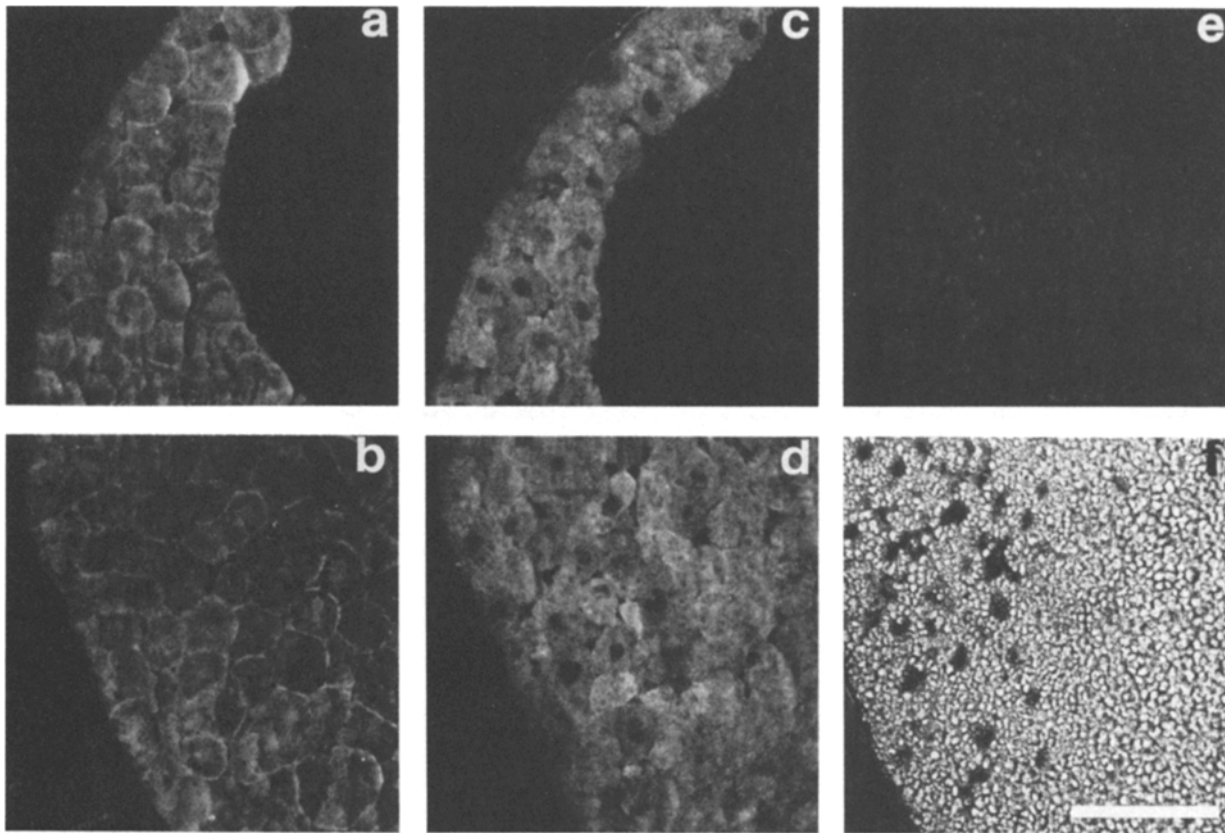


Figure 2. Stage 9 blastula stained with rabbit polyclonal antibodies. Both N-CAM (*a, b*) and L-CAM (*c, d*) are expressed in all cells of the embryo, in the animal (*a* and *c*) and in the vegetal regions (*b, d, e, f*). N-CAM staining appears to be associated with the plasma membrane, while L-CAM appears to be more diffuse in the cytoplasm. (*e*) Control staining with normal rabbit serum. (*f*) Phase contrast. Bar, 100 μ m.

be observed until stage 8–9 of development (mid to late blastula) (Fig. 2). At this stage, the two molecules appeared to be uniformly distributed between the animal (Fig. 2 *a, c*, and *e*) and vegetal (Fig. 2, *b, d*, and *f*) hemispheres of the embryo. N-CAM staining was primarily associated with the plasma membrane (Fig. 2, *a* and *b*); L-CAM staining (Fig. 2, *c* and *d*) was more diffuse throughout the cell bodies of the blastomeres.

During gastrulation (Fig. 3), we observed an increase in N-CAM staining levels in the presumptive neural epithelium. N-CAM persisted at low levels in all non-neural ectoderm and in mesodermal cells, and decreased substantially in endodermal cells (Fig. 3, *a* and *c*). The increase of N-CAM levels in the presumptive neural plate could be observed beginning at stage 11–11.5, shortly after primary induction, and appeared to be more intense in the inner layer of the ectoderm. N-CAM staining was predominantly associated with the plasma membrane. Absorption of the antibodies with purified N-CAM completely removed the staining at this stage (not shown) and staining with nonimmune Ig revealed only a low level of autofluorescence, confined to the yolk plug and a few cells of the external ectodermal layer (Fig. 3 *d*). The presence of N-CAM in the late gastrula (stage 12) was also confirmed biochemically. As discussed below, N-CAM at this stage appeared as a single molecular species of 140 kD (see Fig. 9, lane 2). The staining of the gastrula with anti-L-CAM antibodies was more uniform (Fig. 3, *a'*

and *c'*). Positive staining could be detected in all cells of the embryo, although it appeared to be brighter in the ectoderm.

Neurulation

During neurulation and formation of the neural tube (stage 14–20) (Fig. 4), the level of N-CAM expression continued to increase in the sensorial layer of the neural ectoderm. Cells of the neural plate, neural fold, and neural tube expressed N-CAM at higher levels than the surrounding non-neural ectoderm (Fig. 4, *a–c*), particularly after neural tube closure (Fig. 4 *c*). The staining was arranged radially around the neural folds and was apparently associated with the cell membrane. The notochord was weakly positive for N-CAM throughout neurulation and, at stage 20, the level of N-CAM expression in the notochord was clearly higher than in the surrounding mesoderm. It decreased later, and virtually disappeared by stage 24 when vacuolation of notochordal cells begins. The somitic mesoderm remained weakly N-CAM positive throughout neurulation including the period of somite rotation (stage 17). At stage 20, N-CAM staining of the somites was diminished and by stage 24 it had almost disappeared. The sensorial layer of the non-neural ectoderm continued to express low levels of N-CAM, while in the external layer of the ectoderm and in the endoderm, N-CAM staining was barely detectable by stage 24.

L-CAM staining remained generalized during neurulation

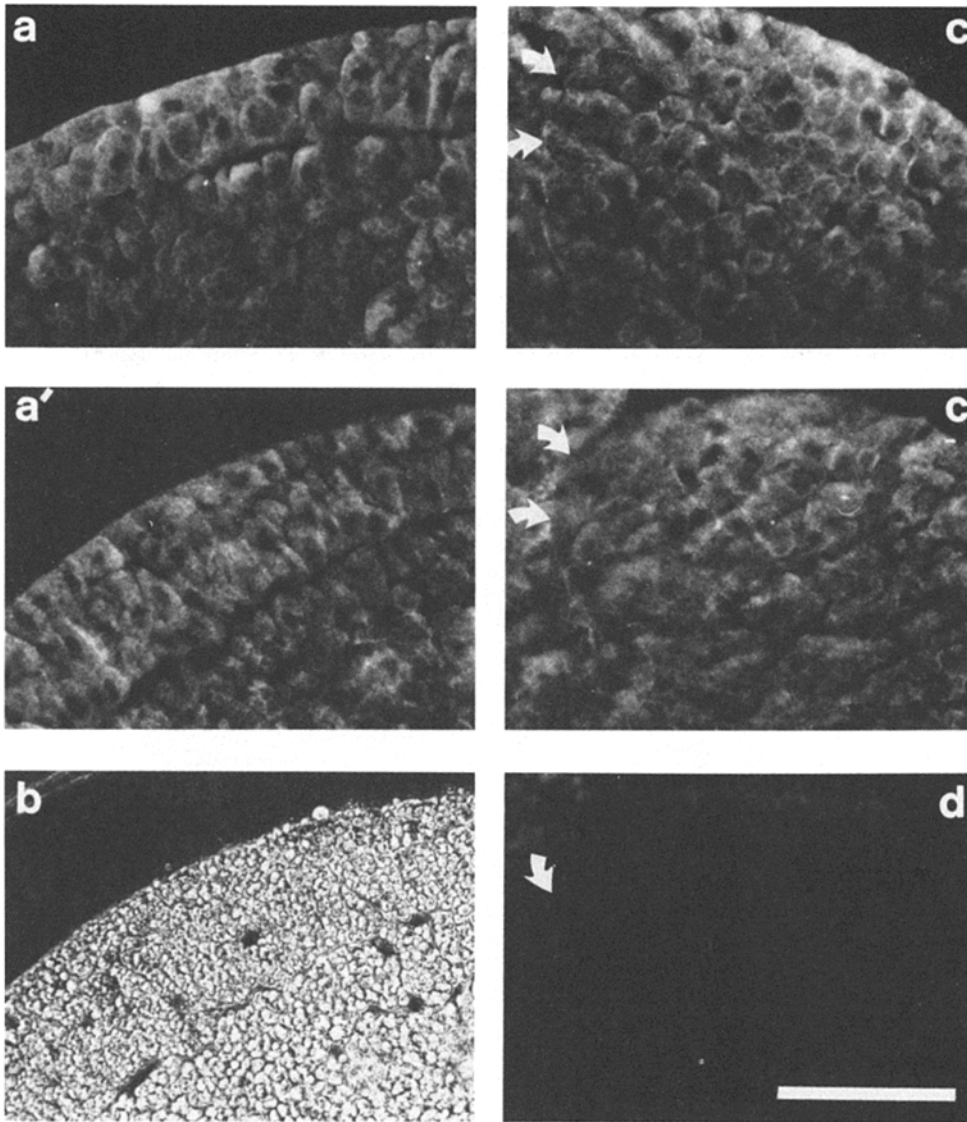


Figure 3. Stage 11.5 gastrula stained with polyclonal antibodies against N-CAM (*a*, *c*) or L-CAM (*a'*, *c'*) in the prospective neural ectoderm (*a*, *a'*, *b*) and the dorsal lip of the blastopore (*c*, *c'*, *d*). Sagittal sections in a plane passing through the induced presumptive neural plate. The expression of N-CAM increased in the presumptive neural ectoderm and the molecule is still present in the underlying mesoderm and at low levels in the endoderm. L-CAM continued to be expressed in all cells. (*b*) Phase contrast of neural ectoderm. (*d*) Staining pattern with normal rabbit serum on the dorsal lip showing a low level of autofluorescence in the yolk plug and the margin of the dorsal lip. Arrows point to the limit of the dorsal lip. Bar, 100 μ m.

(Fig. 4, *a'*–*c'*); all cells expressed L-CAM including neural plate and neural tube cells. At this time, there was no apparent modulation of L-CAM levels except on migrating cells of neural crest origin that were negative for both CAMS.

Placode Formation and Secondary Induction

In previous studies, prevalence modulation of CAMs was observed at various sites of induction including the placodes. In *Xenopus*, placodes develop as thickenings of the sensorial layer of the ectoderm. As already mentioned, we found that the sensorial layer of the ectoderm remained positive for both N-CAM and L-CAM throughout neurulation. At all sites of secondary induction, we observed an increase in the intensity of staining for both CAMs as soon as the ectoderm began to thicken (Fig. 5, *a* and *a'*). This increased staining was observed in the otic, lens, olfactory, dorsolateral, and epibranchial placodes. During further development, N-CAM staining gradually diminished in non-neural ectoderm of the ear but persisted in the otic ganglia and, at lower levels, in the sensory epithelium of the otic vesicle (Fig. 5, *b* and *c*). L-CAM levels increased and remained high in all of the

epithelia and began to decrease in the ganglia (Fig. 5, *b'* and *c'*). By stage 42, L-CAM staining was limited to the otic vesicle and had disappeared from the ganglia, while N-CAM persisted almost exclusively in the otic ganglia and in the corresponding sensory epithelium of the ear. At the early stages examined here, these findings corresponded to those seen in the chicken (Richardson et al., 1987). A similar series of events occurred also in all other ectodermally derived neural structures; after an initial increase in both CAMs, N-CAM persisted only in the ganglia and disappeared from the epithelia while L-CAM persisted in the epithelia. In the lens, N-CAM persisted in the lens epithelium at least until stage 39 and disappeared from the central lens fibers (Fig. 6 *a*) where L-CAM continued to be expressed (Fig. 6 *a'*). These patterns are consistent with those observed in the chicken (Crossin et al., 1985; Edelman et al., 1983; Thiery et al., 1984).

Organogenesis

During later stages of development, we observed a progressive disappearance of N-CAM or L-CAM from different tis-

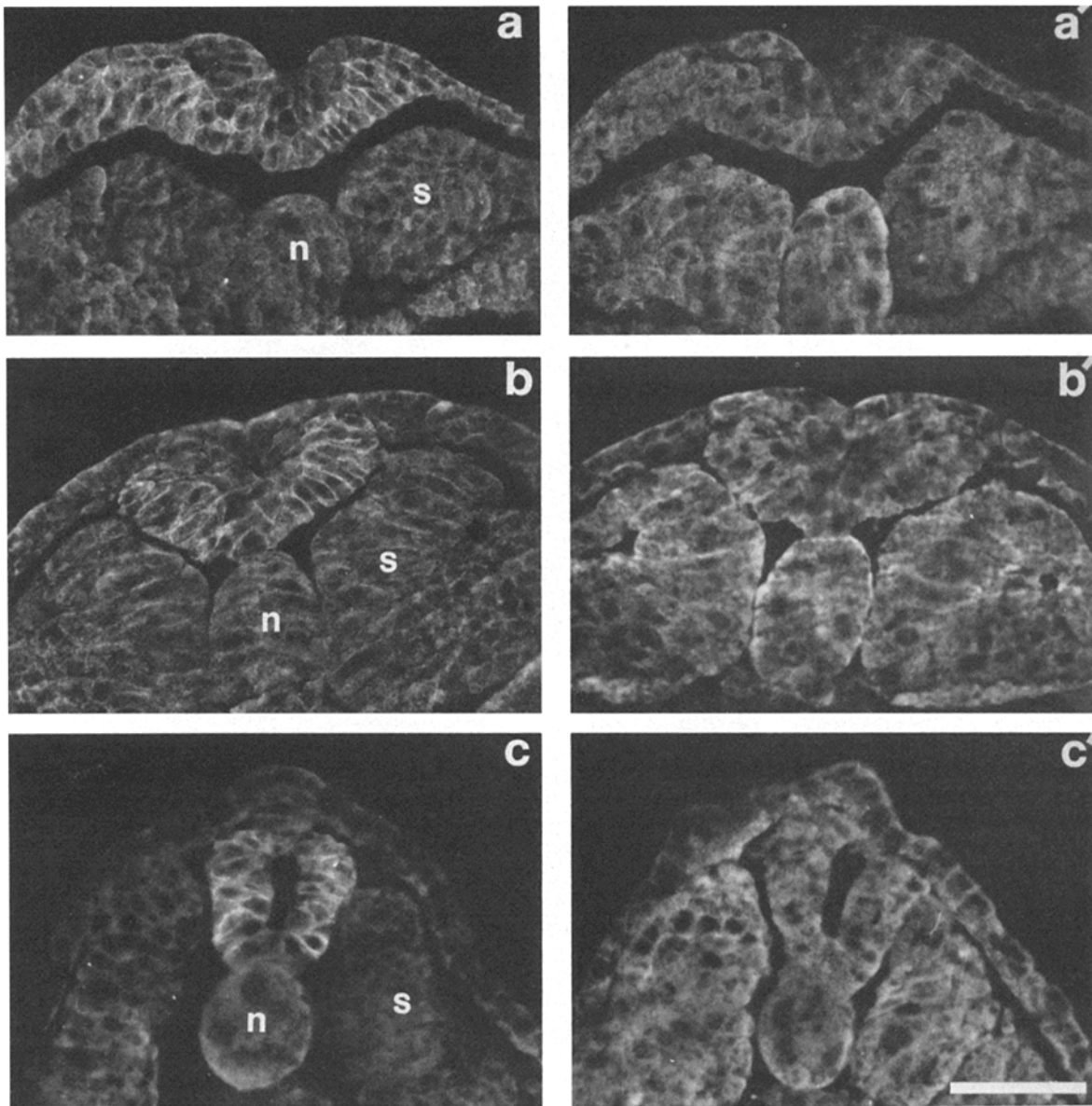


Figure 4. Primary induction. Embryos at stage 16 (*a, a'*), stage 18 (*b, b'*), or stage 20 (*c, c'*) were stained with polyclonal antibodies directed against N-CAM (*a-c*) and L-CAM (*a'-c'*). N-CAM staining gradually increases in the neural ectoderm and persists at lower levels in the notochord and somites. L-CAM continues to be expressed by most cells of the embryo. *n*, notochord; *s*, somite. Bar, 100 μ m.

issues that led eventually to the expression of the two molecules in completely segregated territories. The central and peripheral nervous system remained clearly positive for both adhesion molecules until stage 37. At stage 39, however, N-CAM levels were stabilized and L-CAM levels began to decrease in the nervous system as exemplified by the retina (Fig. 6, *a* and *a'*); by stage 50, only N-CAM was detected both in the central and in the peripheral nervous system. In muscle, somites, and notochord, L-CAM was present until stage 39 (Fig. 6, *b* and *b'*) and then gradually disappeared. The nephric system and the associated tubules were N-CAM and L-CAM positive up to stage 33; by stage 39 N-CAM disappeared and the pronephric tubules were only L-CAM positive (Fig. 6, *c* and *c'*). At stage 39, most of the epithelia lining the surfaces of the body such as the skin (Fig. 6, *c* and *c'*)

and the esophagus (Fig. 6, *b* and *b'*) already expressed only L-CAM.

N-CAM and L-CAM Distribution in the Adult

To determine the relationship between embryonic expression of adhesion molecules and their persistence in later organs, we examined the distribution of N-CAM and L-CAM in several adult tissues. With the exception of the liver (see below), the pattern of distribution of the two molecules in adult *Xenopus* was similar to that found in the chicken (Crossin et al., 1985; Thiery et al., 1984). N-CAM was predominantly found in the central nervous system (Fig. 7, *a* and *b*) and in non-myelinated fibers of the peripheral nervous system (Fig. 7 *c*). No L-CAM staining was found in the central or peripheral

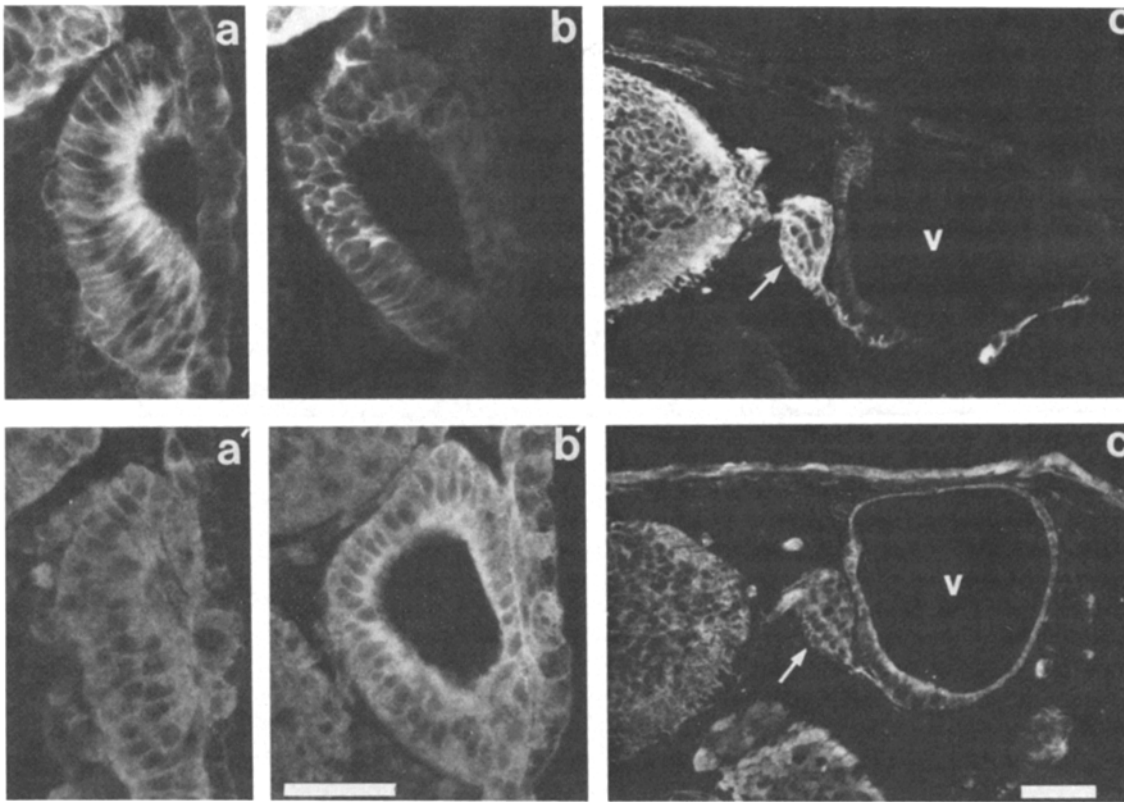


Figure 5. Formation of the otic vesicle. Staining pattern of polyclonal antibodies directed against N-CAM (a–c) and L-CAM (a'–c'). (a, a') Stage 25; (b, b') stage 33; (c, c') stage 39. N-CAM expression strongly increases during condensation of the placodes; during later development N-CAM persists in the ganglia but gradually disappears from the non-neural epithelia. L-CAM expression also increases in the placodes, but unlike N-CAM, L-CAM persists in the epithelia and decreases from the ganglia (arrows in c and c'). L-CAM eventually will disappear from all neural structures. Bars, 50 μ m.

nervous system (Fig. 7, a'–c'). As in the chicken, L-CAM was found in epithelial tissues lining the surfaces of the body of adult *Xenopus*. The stratum germinativum of the skin was stained, while the cells of the dermis were unstained for L-CAM (Fig. 7 d'). The kidney showed staining for L-CAM in all tubular structures and on the parietal cells of the limiting epithelium (Fig. 7 e'); no staining was observed in the cells of the blood vessels or in the glomeruli (Fig. 7 e', arrow). In the intestine, the epithelial cells lining the villi and the crypts were stained for L-CAM (Fig. 7 f'), whereas the tissue in the cores of the villi and the wall of the intestine was weakly positive for N-CAM (Fig. 7 f). All glandular epithelia observed, for example, the secretory glands of the skin (Fig. 7 d'), were brightly positive for L-CAM. The epithelia lining the trachea, lungs, and oviduct were stained for L-CAM in distributions similar to those of the chicken (data not shown). No N-CAM staining was found in L-CAM-positive epithelia. As a rule, no adult tissue was found to express N-CAM and L-CAM simultaneously; the two molecules appeared to be expressed in distinct and non-overlapping regions of the body.

A major exception to the pattern of expression of CAMs encountered in other adult vertebrates was found in the liver. The parenchymal cells of the liver were brightly stained on the whole cell surface by polyclonal antibodies directed against *Xenopus* N-CAM (Fig. 8 a). Polyclonal antibody

against L-CAM gave only a weak intracellular staining (Fig. 8 b) that was clearly above background levels. A similar result for N-CAM was obtained with monoclonal antibody 7C8 (Fig. 8 c) and with several cross-reactive polyclonal anti-N-CAM antibodies from different species (Fig. 8 e). No staining was observed with monoclonal antibody 10H4 (Fig. 8 d) or with other nonrelevant polyclonal antibodies (Fig. 8 f). The presence of N-CAM in the adult *Xenopus* liver was confirmed biochemically with polyclonal anti-N-CAM antibodies, with monoclonal antibody 7C8, as well as with cross-reactive polyclonal antibodies. 10H4 did not immunoprecipitate or immunoblot the form of N-CAM found within the liver. As seen in Fig. 9 (lanes 6–8), liver N-CAM is constituted by a single band of 160 kD similar to the unusual polypeptide found in the chicken heart (Murray et al., 1986b). Small amounts of the 180-kD form of the molecule were occasionally detected in the adult liver of *Xenopus* and are probably attributable to the presence of contaminating neural tissue as confirmed for certain chicken organs (Murray et al., 1986b).

Biochemical Forms of N-CAM Polypeptides during Development

Because of the importance of the polypeptide forms of N-CAM in analyses of expression sequences, we examined the various molecular forms of N-CAM during *Xenopus* develop-

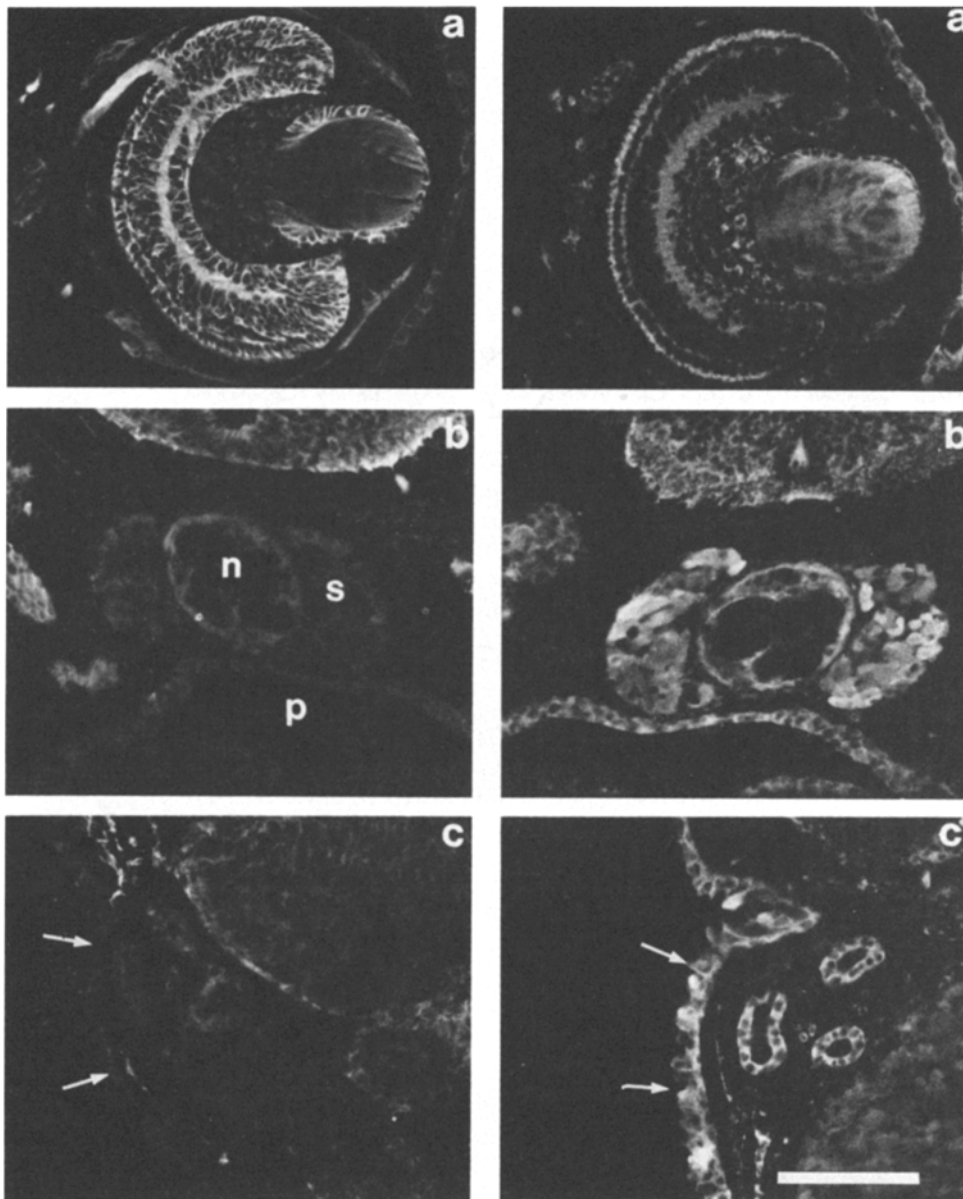


Figure 6. Embryonic organogenesis. Staining pattern of polyclonal antibodies directed against N-CAM (a-c) and L-CAM (a'-c') in stage 39 embryo. (a, a') Eye; (b, b') notochord, somites, and pharynx; (c, c') pronephros and skin (arrows). n, notochord; s, somite; p, pharynx. Bar, 100 μ m.

ment. To reveal the presence of N-CAM before stage 26, it was necessary to concentrate the N-CAM present in several embryos by immunoprecipitation using the monoclonal antibody 7C8 (for details of the procedure see Materials and Methods). N-CAM first appeared as a single molecular species of 140 kD (Fig. 9, lane 2). This was the form present throughout gastrulation and was the predominant species observed during early stages of neurulation up to stage 20. During later stages of development, the 180-kD component of N-CAM became gradually more prominent. At stage 28, the 180- and 140-kD components of N-CAM showed a similar intensity while the 120-kD component was barely visible in the immunoblots (Fig. 9, lane 3). This expression of the different components of N-CAM strongly resembles that of the sd and ld chains during the development of the chicken embryo (Murray et al., 1986b).

During the development of the chick embryo, the N-CAM molecule has a transition from a highly polysialylated, poly-

disperse form (the so-called embryonic, or E form of N-CAM) to adult forms (the A forms) that has only 30% of the sialic acid content of the E form (Hoffman et al., 1982). This transition is found in all species examined so far (Hoffman et al., 1984) and is associated with a change in the kinetics of N-CAM homophilic binding (Hoffman and Edelman, 1983). The appearance of the E form in *Xenopus* occurs during metamorphosis. After stage 50, the N-CAM polypeptides become gradually more sialylated and by stage 58, N-CAM appeared as a polydisperse region (Fig. 9, lane 4). By adult stages (Fig. 9, lane 5), E to A conversion had occurred and N-CAM appeared as three discrete molecular components at 180, 140, and 120 kD.

Discussion

In this study, we examined the distribution of two primary cell adhesion molecules, N-CAM and L-CAM, during the

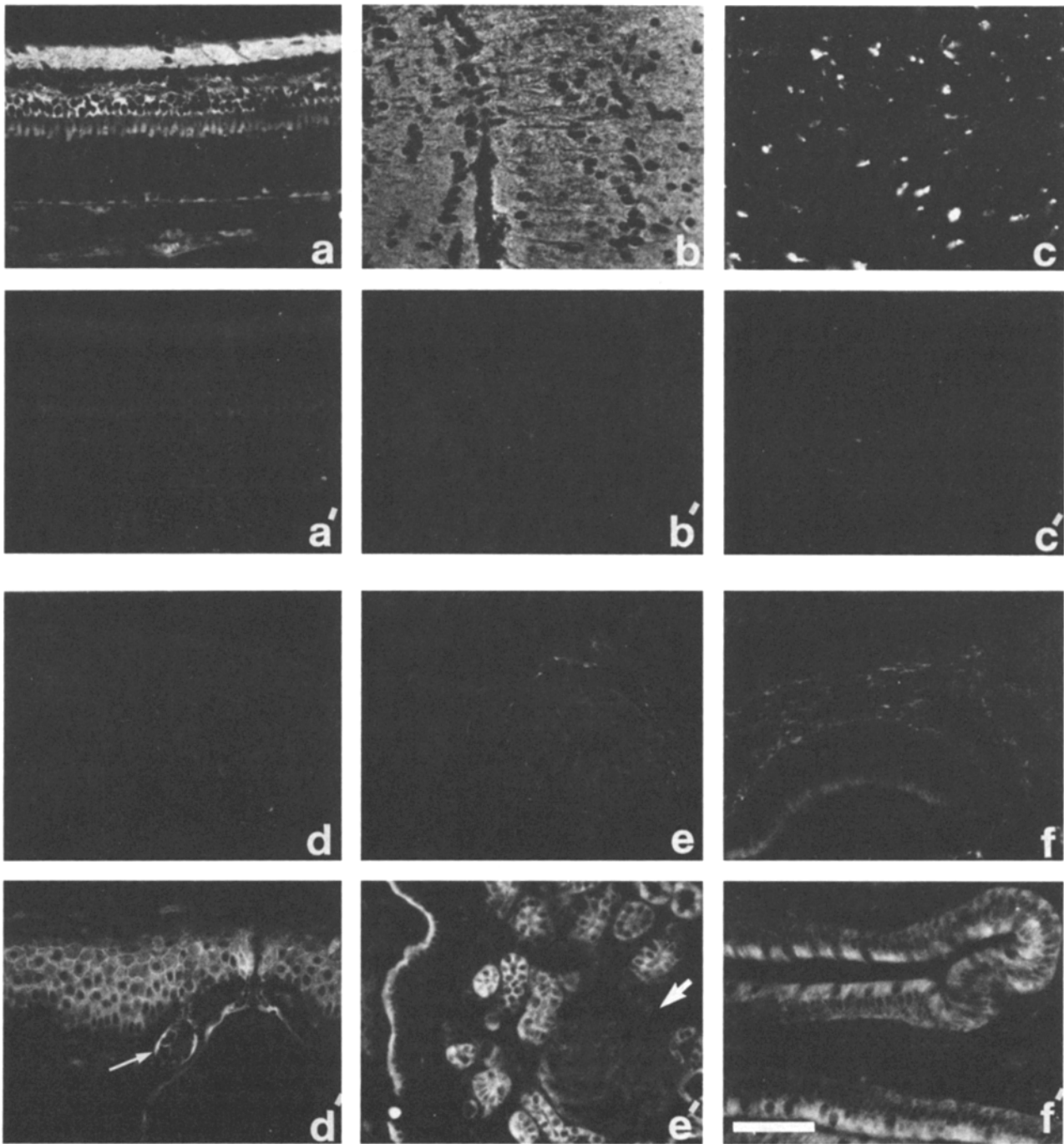


Figure 7. Adult *Xenopus* organs. Staining pattern of polyclonal antibodies directed against N-CAM (a-f) and L-CAM (a'-f'). Retina (a, a'). Brain (b, b'). Peripheral nerve (c, c'). Skin (d, d'). Kidney (e, e'). Intestine (f, f'). N-CAM expression persisted in the central nervous system and in non-myelinated fibers of the peripheral nervous system and it disappeared from most epithelia. No detectable levels of L-CAM expression could be found in the nervous system, the molecule was expressed in the epithelia lining all surfaces of the body such as the stratum germinativum of the skin (d'), glandular epithelium (arrow in d'), the tubuli of the kidney (e'), and the lining of the intestine (f'). L-CAM was not expressed in the glomeruli of the kidney (arrow in e'). Bar, 100 μ m.

development of *Xenopus laevis* embryos. Our major findings may be summarized as follows. (a) Both molecules are present at low levels from the earliest stages of embryonic development. Their presence before and during the mid-blastula transition, the moment at which the first RNA synthesis can be observed (Shiokawa et al., 1981; Newport and Kirschner

1982a, b), suggests their maternal origin (Kintner and Melton, 1987). (b) N-CAM and L-CAM are present in all the cells of the early embryo up to stage 10 of development. During gastrulation, N-CAM levels increase in the induced presumptive neural ectoderm, remain constant in the mesoderm and non-neural ectoderm, and decrease in the endoderm;

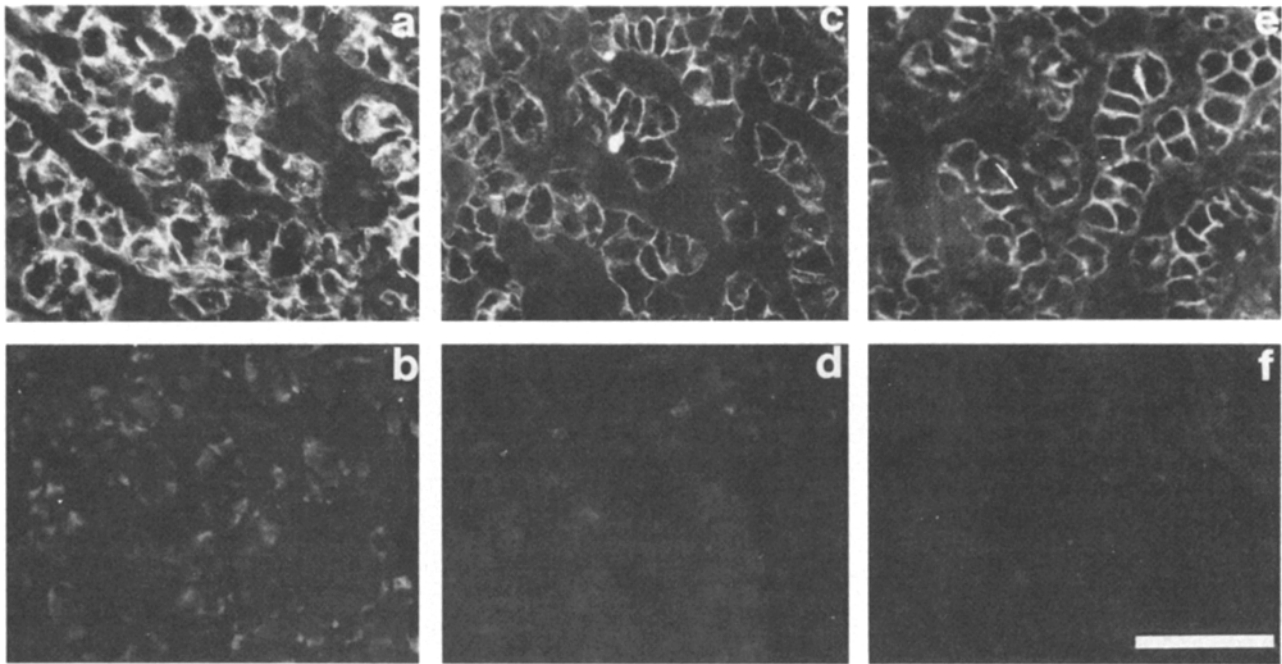


Figure 8. Adult *Xenopus* liver. (a) Polyclonal antibody anti-*Xenopus* N-CAM. (b) Polyclonal antibody anti-L-CAM. (c) Monoclonal antibody 7C8. (d) Monoclonal antibody 10H4. (e) Cross-reactive polyclonal antibody against chicken N-CAM. (f) Normal rabbit serum. The presence of N-CAM in the liver was detected by all the antibodies directed against all the components of N-CAM. Monoclonal antibody 10H4 (d), which recognizes predominantly the 180-kD component of N-CAM, did not give any significant staining in the liver. L-CAM staining appeared as diffuse and intracellular in the hepatocytes. Bar, 100 μ m.

L-CAM remains ubiquitous. (c) N-CAM expression increases at sites of primary and secondary induction. During neurulation, N-CAM levels increase strongly in the sensorial layer of the neural ectoderm during formation of the neural tube. N-CAM persists at lower levels in the nonneural ectoderm, in the somites, and in the notochord. After stage 24, it disappears from somites and notochord. N-CAM expression increases strongly during the thickening of placode epithelium and N-CAM can be detected in the lens epithelium and in the otic vesicle until at least stage 37 of development. L-CAM expression is high in all epithelia and the level of L-CAM expression increases in the placodes during secondary induction. L-CAM levels remain high in the nervous system up to stage 37 and then gradually decrease. Transient L-CAM expression is also observed in the notochord and in the somites. (d) Migrating cells do not express either of the two CAMs. (e) During later development, the regions where the two molecules are expressed become gradually more distinct and, in the adult, no overlap of expression was observed. N-CAM was present in the central and peripheral nervous system and, unusually, in the adult liver. L-CAM was expressed in all epithelial tissues lining the surfaces of the body of the adult *Xenopus*. (f) N-CAM in early stages appeared as a 140-kD component but in adult brain also showed 180- and 120-kD components, consistent with known apparent sizes of ld, sd, and ssd chains. The component in liver was exceptional, having an apparent size of 160 kD and resembled that seen in chicken heart (Murray et al., 1986b). L-CAM at all stages was present at a 124-kD molecule.

Our data are in sharp contrast to recent reports in which the distribution of N-CAM during early *Xenopus* develop-

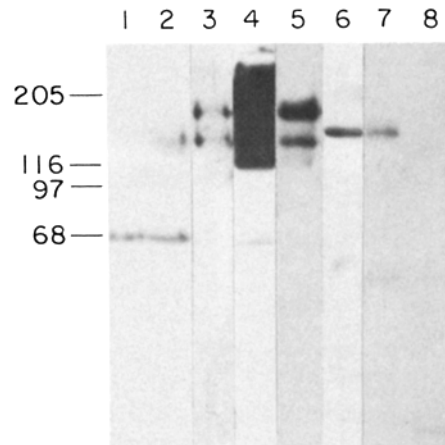


Figure 9. Biochemical characterization of N-CAM at different stages of development and in the adult liver. Lanes 1 and 2, monoclonal antibody 7C8 was coupled to Sepharose beads and used to immunoprecipitate N-CAM from extracts of stage 12 gastrula. The immunoprecipitated material was then resolved by SDS-PAGE, transferred to nitrocellulose paper, and visualized using polyclonal anti-N-CAM antibody followed by radiolabeled protein A and autoradiography. Lane 1, 7C8 beads before incubation with gastrula extract; lane 2, material immunoprecipitated with 7C8 beads from gastrula extracts. Lane 3-5, N-CAM at stage 28 (lane 3), stage 56 (lane 4), and adult brain as revealed by polyclonal rabbit antibodies. Lane 6, immunoblot of adult liver extract (100 μ g) with polyclonal antibody anti-*Xenopus* N-CAM. Lanes 7 and 8, material immunoprecipitated from adult liver extracts using, respectively, immobilized monoclonal antibodies 7C8 and 10H4, and revealed in immunoblots with polyclonal antibody against *Xenopus* N-CAM. Molecular mass markers are at 205, 116, 97, and 68 kD, respectively.

Table I. Comparison of the Expression Sequences of N-CAM and L-CAM in *Xenopus* and in the Chicken

| Stage* | Common features | Differences | |
|--|---|-------------------------------------|---|
| | | <i>Xenopus</i> | Chicken† |
| Cleavage (NF2) | | | |
| N-CAM | | All cells | Unknown |
| L-CAM | | All cells | Unknown |
| Blastula (NF9, HH4) | | | |
| N-CAM | All cells | | |
| L-CAM | All cells | | |
| Gastrula (NF12, HH6) | | | |
| N-CAM | Ectoderm Mesoderm | | |
| L-CAM | Ectoderm Endoderm | Mesoderm | Lost during formation of mesoblast |
| Neural tube (NF23-33, HH7-15) | | | |
| N-CAM | Neural tube Placodes Heart Not in migrating cells | Low levels in notochord and somites | High levels in notochord and somites |
| L-CAM | Placodes Epidermis Pronephros Gut primordium Not in migrating cells | Neural tube Notochord Somites | Disappears after HH stage 7 Not seen Not seen |
| Premetamorphic/hatching (NF39-44, HH45-46) | | | |
| N-CAM | Nervous system Heart | Low levels in muscle Liver | Decreasing in muscle Not present in liver |
| L-CAM | Most epithelia | | |
| Adult | | | |
| N-CAM | Nervous system Low level in muscles‡ | Liver | None in adult liver |
| L-CAM | Most epithelia | Low levels in liver | Liver |

* Different stages of development of *Xenopus* and chicken are roughly compared on the basis of major embryological movements and of the approximate times of organ formation. Corresponding developmental stages of *Xenopus* after Nierwkoop and Faber (1967) and of chicken after Hamburger and Hamilton (1951) are given in parenthesis.

† Distribution of N-CAM and L-CAM in chicken taken from Crossin et al., 1985; Thiery et al., 1984; Edelman et al., 1983.

‡ Low levels of N-CAM can be detected biochemically in adult *Xenopus* muscle (Levi, G., unpublished observation).

ment has been studied with an antibody (Jacobson and Rutishauser, 1986; Balak et al., 1987) prepared against a mixture of *Rana* and *Xenopus* N-CAM that recognizes almost exclusively the 180-kD component of the molecule. In these studies, N-CAM was not found before stage 14 of development, or in placodes, non-neural epithelia, or adult liver. Our present biochemical analysis of the early forms of *Xenopus* N-CAM and of the unusual liver N-CAM, together with studies done in early chicken development (Murray et al., 1986b), indicate that, at all these sites, the expression of the 180-kD component of N-CAM is extremely low. It is therefore not surprising that using an antibody that recognizes predominantly the 180-kD polypeptide, no N-CAM was detected in these tissues. Consistent with this conclusion, our monoclonal antibody 10H4, that in immunoblots recognizes N-CAM in a pattern similar to the antibody used by Jacobson

and Rutishauser (1986), does not recognize liver N-CAM and does not stain placodes. Furthermore, the combination of histological techniques used here might have permitted the detection of signals at a level below the threshold of the methods used by other investigators. Similarly, Kintner and Melton (1987), using in situ hybridization, could not detect N-CAM mRNA before late gastrula stages but using the more sensitive technique of S1 analysis, could detect the presence of N-CAM mRNA as early as in the fertilized egg. The current report supports their observation and suggests that N-CAM protein is translated early in development from maternal mRNA.

The detection of N-CAM and L-CAM in early stages of development of *Xenopus* is consistent with the idea that adhesion mediated by either of these primary CAMs has an important role during the earliest phases of embryonic develop-

ment. A role of uvomorulin and E-cadherin, two molecules closely related or related to L-CAM, has been demonstrated for the compaction of the early mouse embryo (Peyrieras et al., 1983; Shirayoshi et al., 1983). Furthermore, it appears that N-CAM and L-CAM are not merely "markers" of induction, or neural markers, as their expression precedes neural induction and is not restricted to the nervous system. It has been shown, however, that in the chicken embryo the 180-kD form of N-CAM does indeed appear only in the nervous system at some time after primary induction has occurred (Murray et al., 1986b). The differential expression of N-CAM in neural and non-neural tissue is associated with a developmentally regulated event that enables the differential splicing of the N-CAM gene into a mRNA specifying the Id chain, which appears to be expressed exclusively in neural tissue (Murray et al., 1986b). This represents a clear case of CAM modulation at the cell surface but this event is not in itself a marker of induction inasmuch as the 180-kD form of N-CAM appears much after closure of the neural tube. Of course, at induction, prevalence modulation of existing CAMs does occur (Thiery et al., 1982; Crossin et al., 1985; Chuong and Edelman, 1985a, b) consistent with the increase in transcription seen by Kintner and Melton (1987).

In our previous studies on chicken development (Edelman et al., 1983; Thiery et al., 1984; Crossin et al., 1985), it was observed that CAMs have definite sequences of expression that correlate strongly with major morphogenetic events particularly at sites of induction. Two modes of expression (Crossin et al., 1985) were generally observed: (a) in mesenchyme, N-CAM diminishes at the surface and then reappears and (b) one or the other of the two primary CAMs studied here disappears from epithelia that initially express both CAMs. To assess the general validity of these observations and of the regulator hypothesis (Edelman, 1984), it was essential to determine whether the rules governing the sequence of CAM expression in the chicken were also found to hold in *Xenopus*, a species whose early embryonic development involves a completely different set of morphogenetic movements and presumably concurrent differences in epigenetic sequences. In Table I, we have compared the sites of expression of N-CAM and L-CAM in the chicken and in *Xenopus* at comparable stages of development. Although the rates of modulation of the CAMs differ in the two species, the overall expression sequences of CAMs appear to be strikingly similar. The major differences in the frog are a delayed disappearance of L-CAM from the nervous system, somites, and notochord, an early decrease of the level of N-CAM in muscle, and the presence of N-CAM and not L-CAM in the adult liver. Very early stages of development and adult stages are very similar in terms of CAM distribution and the rules of CAM expression found in the chicken also appear to hold for *Xenopus*: (a) All cells start positive for both primary CAMs. (b) Loose mesenchymal cells become transiently negative for both CAMs and then can reexpress N-CAM. (c) All epithelia lose one of the two CAMs so that in later stages of development only one of the two is expressed.

Analysis of the data in Table I shows that although the general patterns of expression are similar in both species during the period between primary induction and early organogenesis, the relative prevalence of the two molecules in various structures at any time is quite diverse. Such heterochrony in sequences of expression should be reflected in di-

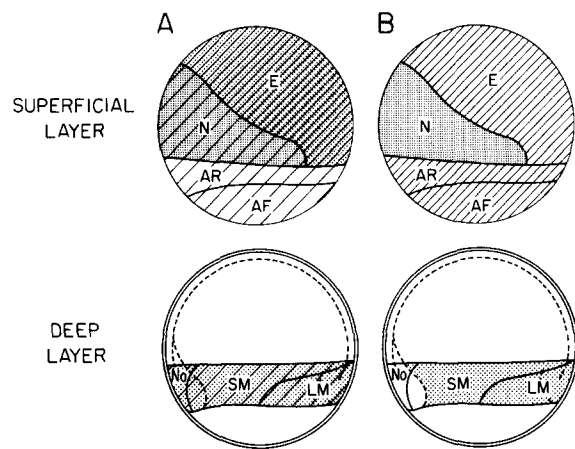


Figure 10. Fate maps of stage 10+ *Xenopus* gastrula (after Keller, 1975, 1976) comparing the areas of the superficial and deep layers of the embryo that will give rise to tissues expressing either N-CAM (dotted) or L-CAM (slashed) at stage 20 of development (A) or in the adult (B). As can be seen, after an initial overlapping of the territories of expression of the two CAMs, these became segregated in two contiguous and simply connected regions in the adult. The presence of N-CAM in adult muscle and heart was determined biochemically (Levi, G., unpublished observation). N, prospective neural area. E, prospective epidermal area. AR, prospective archenteron roof. AF, prospective archenteron floor. SM, prospective somite mesoderm. LM, prospective lateral mesoderm.

verse adhesive behaviors of developing collectives of cells which in turn could affect the path of morphological development of the embryo. As shown in the fundamental studies of Keller (1975, 1976), morphogenetic movements involving convergent extension (Keller et al., 1985) occur during gastrulation in *Xenopus*. The movements that occur during gastrulation in the chicken are quite different (Vakaet, 1985). If one considers that during morphogenesis of frog and chicken, two embryos with totally different starting geometry are transformed into mature phenotypes with a generally similar overall body plan, it is not surprising to observe differences in the temporal schedule of modulation of primary CAMs which otherwise follows similar rules of expression.

We have previously summarized CAM expression in chicken development by constructing composite CAM fate maps. For comparative purposes, we have mapped the areas of presumptive expression of N-CAM and L-CAM at both stage 22 of development and in the adult, onto a fate map of the early *Xenopus* gastrula redrawn from Keller (1975, 1976) (Fig. 10). As suggested by the descriptive study of Nieuwkoop and Florshutz (1950) and definitively shown by the vital dye marking experiments of Keller (1975, 1976), the superficial layer of cells of the early *Xenopus* gastrula is constituted only by prospective neural and epidermal ectoderm and prospective endoderm, while the prospective mesoderm is found only in the deeper cell layers of the marginal zone. A fate map of *Xenopus*, therefore, has to show separately the fate of cells belonging to the surface and to the deeper layers of the embryo. As can be seen (Fig. 10 A) in early stages of frog development, there is a considerable overlap of the territories of expression of the two primary CAMs. Later in development, however, these territories gradually segregate, and in the adult (Fig. 10 B), they occupy two contiguous and simply connected areas. Thus, the late frog map appears to be

topologically similar to the fate map previously obtained for the chicken (Crossin et al., 1985) in which after an initial overlap the territories of N-CAM and L-CAM expression become also contiguous and simply connected. These comparisons fulfill a prediction of the regulator hypothesis (Edelman, 1984) in which CAM expression is related to morphogenesis and evolution. This hypothesis postulates that control of the expression of CAMs by regulatory genes plays a central role in morphogenesis by providing appropriate boundaries between cell collectives, creating borders that contribute to restrain and guide other primary processes of development such as mesenchymal cell movement.

The observation that the expression of CAMs related to an early fate map is topologically similar in two widely different species supports the evolutionary preservation of a common regulatory mechanism that acts to ensure the expression of the appropriate CAM in different cell collectives and maintain the body plan. At the same time, however, alterations in the relative rate of expression of CAMs would be expected as morphological changes are selected for in different species. This suggests that regulatory genes for CAMs respond differently in the two species. Inasmuch as CAM modulations have been found in all morphogenetic events so far examined, we would also expect strong rearrangements to occur during metamorphosis, a process that involves drastic changes in most of the body structures of *Xenopus*. Recent studies in metamorphic salamanders (P. Alberch and K. L. Crossin, unpublished observations) suggest that this is the case. All of these observations are consistent with the hypothesis (Edelman, 1986b) that CAMs may be involved in heterochronic changes in development that are postulated to be major factors in morphologic evolution.

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