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Localization of mRNA for neural cell adhesion molecule (N-CAM) polypeptides in neural and nonneural tissues by *in situ* hybridization

(development/nervous system/alternative splicing)

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ABSTRACT **The differential expression of the mRNA for the neural cell adhesion molecule (N-CAM) has been studied by *in situ* hybridization and compared with protein localization analyzed by immunohistochemical staining. The distribution of mRNA during chicken embryonic development was analyzed in neural and nonneural tissues by using an RNA probe that detects all N-CAM mRNAs and a probe specific for the mRNA of the large cytoplasmic domain (ld) of N-CAM. The results provide a detailed description of the mRNA distribution for N-CAM. The distribution of mRNA for total N-CAM generally corresponded to that of protein but differed at a more detailed level of analysis. For example, the mRNA was localized only within the cell bodies of neurons, whereas the protein was also in neuronal processes; this differential localization was most clearly seen in the alternating layers of cell bodies and fibers in the optic tectum and cerebellum. N-CAM ld mRNA, which arises from alternative RNA splicing, was expressed only in neural tissues, confirming previous biochemical and histological studies. Differential expression of the ld mRNA was detected in specific neural cell types: N-CAM mRNA was present in the ependymal cells of the spinal cord and optic tectum, but mRNA for the ld form was absent. In contrast, the ld mRNA was among the N-CAM mRNAs found in the Purkinje cells and internal granule cells in the cerebellum. The differential expression of mRNAs for the N-CAM forms emphasizes the potential importance of alternative mRNA splicing in modulating adhesive events during embryonic development, particularly in the nervous system.**

Molecules that mediate cell adhesion (CAMs) have been shown to play an important morphogenetic role during embryonic development (1). One of these molecules, the neural cell adhesion molecule (N-CAM), is expressed from the earliest stages of development on derivatives of all three embryonic germ layers (2), and its activity is essential for pattern-forming events—particularly in the nervous system (3–5). N-CAM is a large cell-surface glycoprotein that mediates homophilic binding between neurons and other cells on which it is found (6). The protein is a member of the immunoglobulin superfamily and may be the protein most closely related to the primordial molecules that gave rise to this family (7). There are at least 10 polypeptide forms of N-CAM (8–10) that are generated by alternative splicing of mRNA transcribed from a single gene (11). Two major forms of N-CAM in the chicken, the large cytoplasmic domain (ld) form (a 180-kDa protein) and the small cytoplasmic domain (sd) form (a 140-kDa protein), are identical in their extracellular and transmembrane domains but differ in length of their cytoplasmic domains. The ld form of N-CAM has an additional 261 amino acids encoded by a single exon (12). The splicing events that generate this form are of special importance because they occur only in tissues of the nervous system (13, 14). Moreover, the additional segment of the ld form contains phosphorylation sites (15) in addition to those shared with the cytoplasmic domain of the small cytoplasmic domain form and also provides a region for possible interactions with the cytoskeleton (16, 17). This comparison suggests a differential role of various N-CAM forms that is connected with their polar distribution in nerve cells.

N-CAM is ubiquitously distributed in the central nervous system and peripheral nervous system as well as in many nonneural tissues throughout development, and it is present on both cell bodies and processes of neurons (5, 18). Although distribution of the various forms of the protein on the cell surface has been examined in detail, localization of N-CAM mRNAs has not been extensively studied, except by Northern (RNA) blot analysis. A comparison of the relative location of mRNA and protein is of particular interest in neurons because recent observations suggest that mRNAs can be selectively transported into processes before translation (19, 20).

We have employed the technique of *in situ* hybridization (21) with a probe encoding regions common to all forms of N-CAM and a probe specific for sequences encoding the ld form of N-CAM to localize the respective mRNAs in neural and nonneural tissues. The results show that, in general, distribution of mRNA for N-CAM parallels that of the protein and also reveal certain special features: (*i*) different levels of expression of the N-CAM message in a given tissue, such as the gray matter of the spinal cord, (*ii*) differential distribution of the mRNA and the protein in layered structures, such as the optic tectum and the cerebellum, and (*iii*) differences in expression of N-CAM mRNA and ld mRNA in different cell types as seen in the cerebellum. These results provide information regarding the detailed cellular localization of mRNAs for N-CAM and are consistent with previous studies (13, 14, 18, 22) of N-CAM expression during development.

MATERIALS AND METHODS

Tissues. Tissues from White Leghorn chicken embryos were fixed overnight in 4% (wt/vol) paraformaldehyde/240 mM phosphate buffer, pH 7.6, and then cryoprotected successively in sucrose solutions (12, 16, and 18% in water) for 2 hr each. Cryostat sections of 10 μm were collected on gelatin/chrom-alum-coated slides and stored desiccated at 4°C. Sections used for immunofluorescence staining and *in situ* hybridization underwent the same treatment.

Hybridization Probes. Two different RNA probes were used in this study: a 3184-base-pair (bp) probe that recognized all forms of N-CAM mRNA, referred to hereafter as

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Abbreviations: CAM, cell adhesion molecule; N-CAM, neural CAM; E, embryonic day; ld, large cytoplasmic domain of N-CAM.

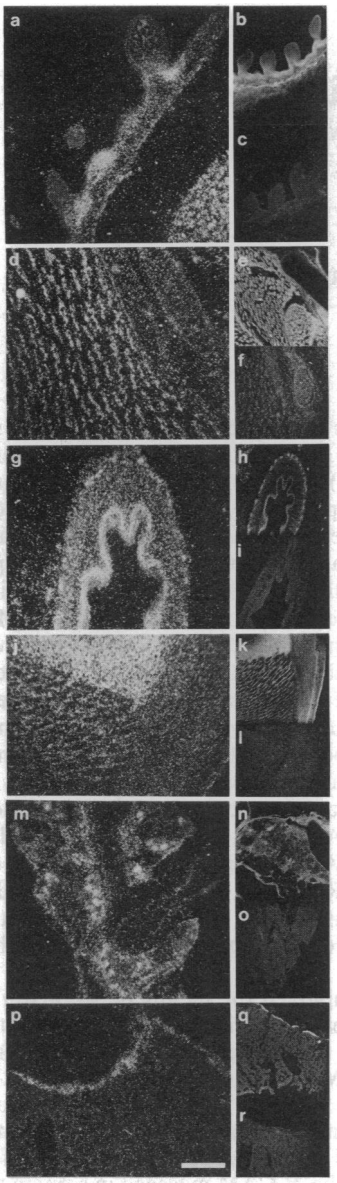

FIG. 1. Expression of N-CAM protein, total N-CAM mRNA, and ld mRNA in nonneural tissues. Parasagittal sections of embryonic day 11 (E11) trunks stained with polyclonal anti-N-CAM (*b, e, h, k, n,* and *q*) and hybridized with total N-CAM antisense probe (*a, d, g, j, m,* and *p*) or with ld antisense probe (*c, f, i, l, o,* and *r*). The tissues shown are skin (*a–c*), skeletal muscle (*d–f*), esophagus (*g–i*), gizzard (*j–l*), kidney (*m–o*), and liver (*p–r*). [Bar = 200 μm (*p*); *a, d, g, j,* and *m*, equivalent length = 200 μm; all other sections, equivalent length = 480 μm.]

N-CAM probe, and a 382-bp probe that exclusively recognized the ld form mRNA.

The N-CAM template contained 2550 bp of coding sequence, 26 bp of 5′-untranslated sequence, and 608 bp of 3′-untranslated sequence. The template was constructed by ligating a 2.8-kilobase (kb) *Eco*RI fragment from pEC281 (23) and a *Hae* III–*Eco*RI fragment from pEC265 (12) into the *Sma* I/*Eco*RI sites of pGEM1 (Promega Biotec). Transcripts were generated in either orientation using SP6 and T7 promoters after linearizing the plasmids with *Nco* I and *Sal* I, respectively. The ld template was constructed by ligating a *Eco*RI–*Eco*RI fragment from pEC252 (13) into a Bluescript KS plasmid (Stratagene). A 382-bp *Eco*RI–*Pst* I fragment from this plasmid was ligated into the *Eco*RI/*Pst* I sites of Bluescript KS plasmid. Transcripts were generated in either orientation with T7 and T3 promoters after linearizing the plasmids with *Xho* I and *Xba* I, respectively.

Transcription reactions were done by using Riboprobe system (Promega Biotec) for pGEM1 and RNA transcription kit (Stratagene) for Bluescript, following reaction conditions suggested by the manufacturers. ^{35}S-labeled CTP (New England Nuclear; 1350 Ci/mmol; 1 Ci = 37 GBq) was used at a final concentration of 12 μM with no unlabeled CTP.

***In Situ* Hybridization.** The described protocol (24) was used without modification. After hybridization and washes, the slides were air-dried, dipped in NTB2 (Kodak nuclear track emulsion), melted at 43°C, and diluted 2:1. The slides hybridized with total N-CAM probe were exposed 3–7 days, and those with the ld probe were exposed for 15 days. The higher background levels seen with the ld probe were probably due to the longer exposure times. The slides were developed for 2.5 min with Kodak D_{19} (1:2), rinsed, fixed with Kodak Ektaflo fixer (1:2) for 10 min, counterstained with cresyl violet, and analyzed under the microscope by using dark-field and bright-field illumination. Sense probes labeled to the same specific activity and hybridized to parallel sections under the same conditions as the antisense probes served as negative controls.

Immunofluorescence. Indirect immunofluorescence labeling was done as described by using rabbit polyclonal antibodies raised against embryonic chicken N-CAM (2).

RESULTS

The expression of total N-CAM mRNA was examined in different tissues and developmental stages of the chicken embryo and compared with the protein distribution previously reported for N-CAM and the ld form of N-CAM (12). We describe first the distribution of mRNA in nonneural tissues, then show mRNA expression in the peripheral nervous system, and finally present results obtained from the central nervous system, particularly the spinal cord, optic tectum, and cerebellum. Special attention was given to areas where differential expression of N-CAM forms was apparent.

Expression of N-CAM mRNA in Nonneural Tissues. In various nonneural tissues, N-CAM mRNA (Fig. 1 *a, d, g, j, m,* and *p*) and N-CAM protein (Fig. 1 *b, e, h, k, n,* and *q*) were detected, but no hybridization was seen in any of the nonneural portions of these tissues with the ld probe (Fig. 1 *c, f, i, l, o, and r*). N-CAM mRNA was detected in the cephalic portion of the feather bud in the dermal condensations of the skin (Fig. 1*a*), as previously observed (25) for the protein distribution (Fig. 1*b*). N-CAM mRNA was also present in skeletal muscle (Fig. 1*d*). In the esophagus and gizzard (Fig. 1 *g* and *j*), there was strong hybridization in the tunica propria subjacent to the epithelium. The mesenteric plexus in the esophagus also expressed total N-CAM mRNA. In the gizzard there was also evident hybridization in the muscularis mucosae, in radial muscle fibers, and in the lateral tendon (Fig. 1*j*). In the developing kidney (Fig. 1*m*), N-CAM mRNA could be detected in the metanephric tubules, but the adjacent mesonephros appeared negative at this stage. In the liver, both N-CAM protein (Fig. 1*q*) and mRNA (Fig. 1*p*) were present in connective tissue (and possibly in nerve fibers) lining the sinusoids.

Distribution of N-CAM in the Peripheral Nervous System. Although the mRNA for the ld form of N-CAM was not found in nonneural tissues, it was present in the nerve plexuses and ganglia within these organs (Fig. 2). In the intestine, both N-CAM and ld probes hybridized to the ganglion of Remak (Fig. 2 *b* and *c*). This ganglion innervates the mesorectum, mesocolon, and mesentery of the chicken (26). The developing enteric plexus within the muscular layer also hybridized with the N-CAM probe (Fig. 2*b*). Similar results were seen in the gizzard, where the ganglia hybridized with both the N-CAM and ld probes (Fig. 2 *f* and *g*). This result confirms previous interpretations of data obtained by immu-

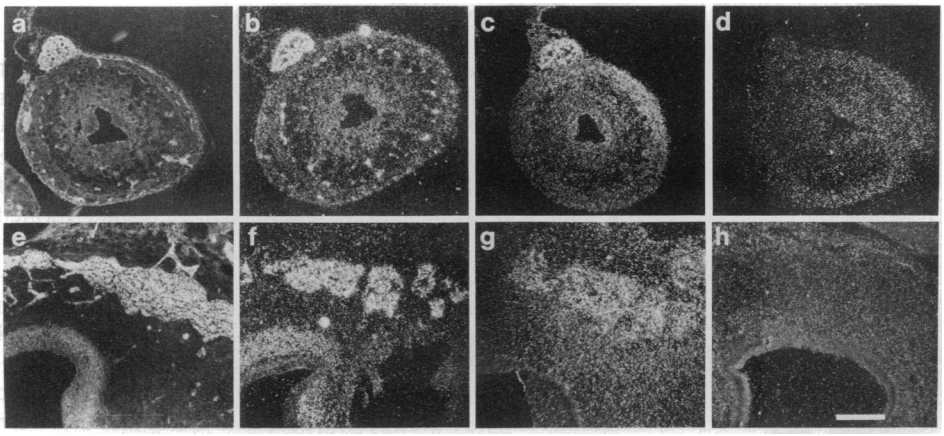

FIG. 2. Total N-CAM mRNA and ld mRNA in peripheral nervous system: gut and gizzard. Transverse sections through the gut (*a–d*) and sagittal sections through the gizzard at E11 (*e–h*) were used for antibody staining with polyclonal anti-N-CAM (*a* and *e*) and hybridized with total N-CAM antisense probes (*b* and *f*) or with ld-specific antisense probe (*c* and *g*). As controls, parallel sections were hybridized using the sense orientation of the total N-CAM probe (*d* and *h*). [Bar = 200 μm (*h*).]

nohistology and RNA blot analysis that the presence of the ld form of N-CAM in the gizzard is due to the innervation of the tissue (13, 22).

Differential Levels of Total N-CAM mRNA and ld mRNA Expression in Central Nervous System: The Spinal Cord. At E11, N-CAM mRNA was present in the gray matter of the spinal cord, including the ependymal layer, and prominently in the lateral and medial ventral motor columns (Fig. 3*b*). The ld form of N-CAM was also prominent in the motor columns but was absent in the ependymal layer (Fig. 3*c*). High levels of protein were seen throughout the motor columns, presumably due to staining of dense fiber bundles (Fig. 3*a*). The white matter appeared positive for protein, as previously reported (18) (Fig. 3*a*), but no hybridization was detected there with either N-CAM probe. The dorsal root ganglia and part of the sympathetic trunk strongly expressed both N-CAM mRNA and the ld mRNA. Consistent with levels of protein detected by immunolocalization, N-CAM hybridization levels were lower in the developing muscle than in the spinal cord or ganglia and were at background in developing cartilage.

Contrasting Levels of mRNA and Protein Expression in a Layered Central Nervous System Structure: The Optic Tectum. The distribution of the N-CAM mRNAs in the optic tectum of the chicken was examined at the period of development when stratification is complete and the only subsequent changes are from further growth of the layers and completion of the cytoarchitecture (27). N-CAM mRNA (Fig. 4*b*) including the ld mRNA were widely distributed in the strata and their corresponding lamina in the optic tectum at E11. This overall pattern of distribution did not appear changed for E15 (Fig. 4 *c* and *d*) and E17 (Fig. 4 *e* and *f*). The ld mRNA was absent from the ependymal layer at all stages, as shown at high magnification in Fig. 4 *c* and *d*. Fiber-rich strata such as stratum fibrosum periventriculare, stratum album centrale, stratum opticum, and lamina h of stratum griseum et fibrosum superficiale (27) (Fig. 4) were strongly positive for protein but were negative by *in situ* hybridization (Fig. 4*b*). In contrast, cell body-rich layers showed strong hybridization signals with both probes and lower levels of immunofluorescent staining for protein. These findings are in accord with localization of mRNA in the soma and with high levels of protein on the fibers. This pattern is especially evident in the cell body-rich strata, the stratum griseum centrale and stratum griseum periventriculare (Fig. 4 *a* and *b*), where there was strong hybridization in neuronal cell bodies but only weak staining for protein by immunofluorescence.

Differential Expression in Different Cell Types in the Cerebellum. In the cerebellar cortex (Fig. 5) at E17, the individual Purkinje cells and the internal granular layer hybridized with the N-CAM (Fig. 5*a*) and ld probes (Fig. 5*b*). Some hybridization was seen with the N-CAM probe (Fig. 5 d_2, *e*, and *g*) and with the ld probe (Fig. 5*f*) in the more external

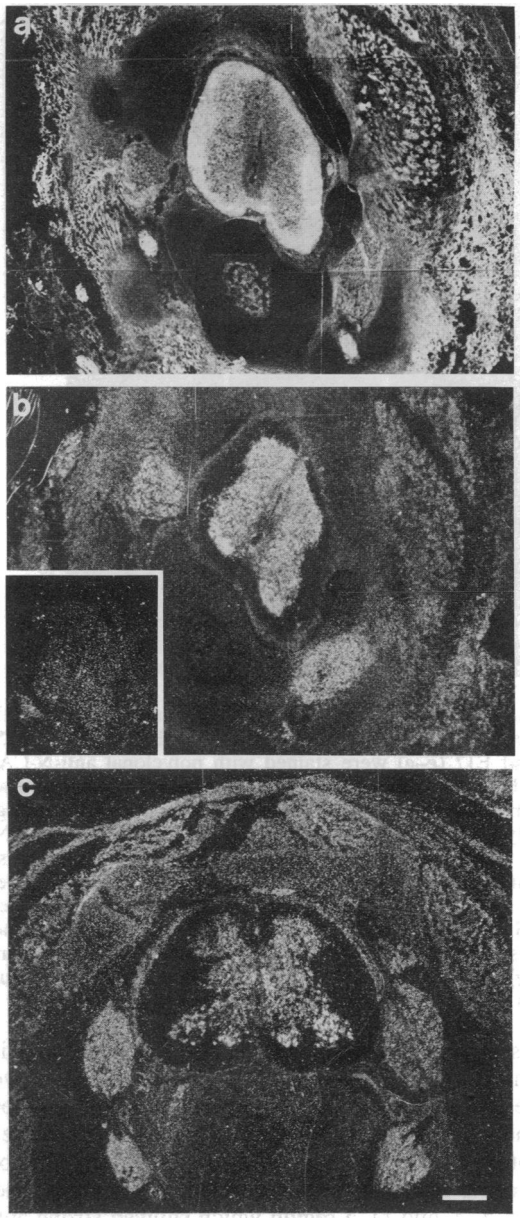

FIG. 3. Distribution of N-CAM in the spinal cord. Transverse sections of E11 were stained with anti-N-CAM (*a*), hybridized with the total N-CAM antisense probe (*b*), or with the ld antisense probe (*c*). (*Inset* in *b*) A parallel section was hybridized with N-CAM sense probe as control. (Bar = 200 μm.)

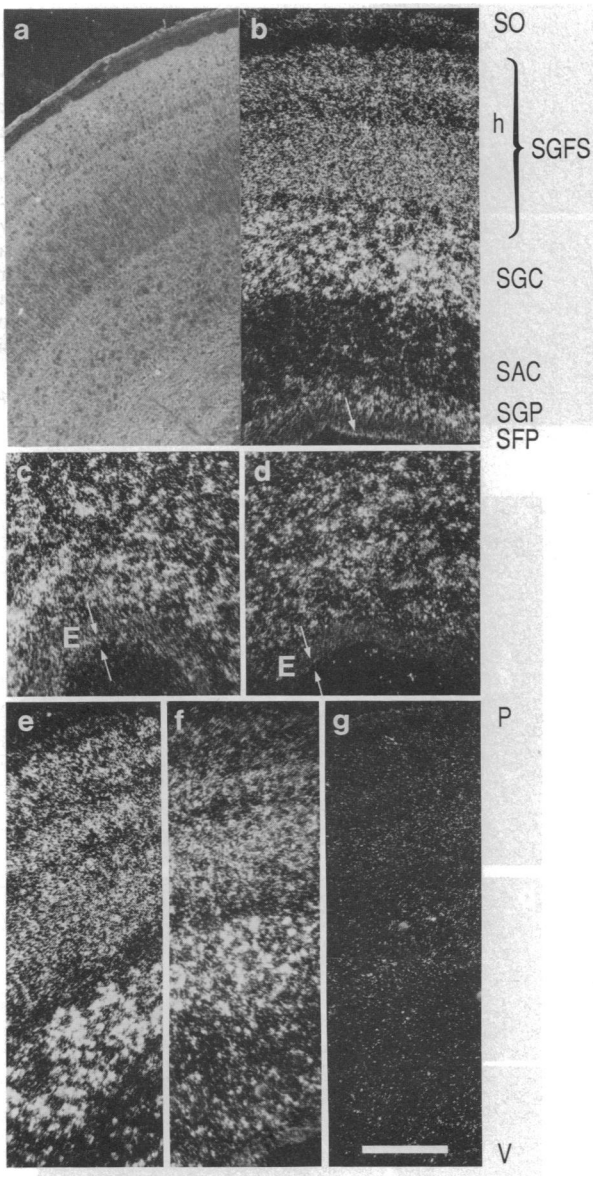

FIG. 4. Localization of total N-CAM and ld-specific mRNAs in the optic tectum. Parasagittal sections from E11 (*a* and *b*), E15 (*c* and *d*), and E17 (*e–g*) were stained with polyclonal anti-N-CAM (*a*), hybridized with the N-CAM antisense probe (*b*, *c*, and *e*) or with the ld antisense probe (*d* and *f*). Arrows in *c* and *d* highlight the ependymal layer. A parallel section hybridized with a total N-CAM sense probe is shown in *g*. SO, stratum opticum; SGFS, stratum griseum et fibrosum superficiale; SGC, stratum griseum centrale; SAC, stratum album centrale; SGP, stratum griseum periventriculare; SFP, stratum fibrosum periventriculare; E, ependyma shown by arrows in *b*, *c*, and *d*; P, pial surface; and V, ventricle. [Bar = 200 μm (*g*); *a* and *b*, equivalent length = 180 μm; *c* and *d*, equivalent length = 240 μm; *e* and *f*, equivalent length = 200 μm.]

layers at E15. This pattern agrees with N-CAM localization by immunohistochemistry in the internal granular layer and suggests that the high levels of immunofluorescence found in the molecular layer result from N-CAM staining of the fibers of both Purkinje cells and granule cells. Hybridization was low in the fiber-rich region surrounding the Purkinje cells (Fig. 5 *d*, *e*, and *f*), a region which showed strong antibody staining (Fig. 5 *d* and *h*). This is consistent with the expression of N-CAM mRNA primarily in cell bodies. Like the optic tectum, the cerebellum represents an example of a layered structure in which the protein and the mRNA distributions do not completely correspond.

DISCUSSION

In this study, we have examined the distribution of mRNA for N-CAM and mRNA specific for the ld polypeptide of this molecule by *in situ* hybridization. The specificity of hybridization was verified by the paired use of sense and antisense probes, as well as by correlation with protein levels analyzed by immunohistochemistry. In general, the results verified earlier descriptions of the distribution of N-CAM (18) and the ld polypeptide (13). More significantly, they revealed important new details regarding the distribution of the ld form in subsets of cells and a differential distribution of mRNA and N-CAM protein in neurons.

In layered structures, such as the cerebellum and optic tectum, N-CAM mRNA was localized in cell bodies that were surrounded by fibers containing large amounts of N-CAM protein. In contrast, *in situ* hybridization analysis for myelin basic protein (19) and microtubule-associated protein 2 mRNAs (20) has shown that the mRNAs encoding these proteins actually are transported to their final destination in processes before translation. Our observations that the N-CAM mRNA was localized in cell bodies, whereas protein was also found at high levels in fibers, suggest that the compartmentalization of the N-CAM protein occurs post-translationally at the level of protein transport, confirming previous studies of the axonal transport of N-CAM (28). The observations of polarity modulation of N-CAM (5, 18) and of interaction of N-CAM with cytoskeletal molecules (16, 17) are also consistent with the idea that the protein is synthesized in the cell body and then transported.

The mRNA for the ld form of N-CAM was present only in nervous tissues, confirming previous immunohistological and RNA blot hybridization studies (13, 29). The ld mRNA was found in the neural organs of the gizzard and intestine, tissues in which both the neural and nonneural components express N-CAM mRNA. These findings are in accord with the previous conclusion (13) that splicing of mRNA leading to the ld polypeptide of N-CAM is restricted to the nervous system.

Cell types within the nervous system could also be distinguished in their capacity to express the ld mRNA. In the spinal cord and optic tectum the ependymal cells expressed N-CAM mRNA but failed to express the ld mRNA. In the cerebellum, both Purkinje cells and internal granule cells expressed the ld mRNA. It was previously difficult to demonstrate N-CAM in these cell types by immunohistochemical techniques because of the contrast between strong surface staining and low levels of intracellular staining (5, 18, 22). The question remains as to which N-CAM mRNAs other than those specifying the ld polypeptide are expressed in these cell types or in any cell type that expresses the ld polypeptide. Such a study will require the use of probes specific for other N-CAM forms.

With the exception of the ependymal layer discussed above, layers in the optic tectum that expressed N-CAM mRNA also expressed ld mRNA. It remains possible, however, that the ld mRNA is restricted to a subset of cell types inasmuch as each layer of the optic tectum of the chicken is composed of at least two or three different kinds of neuronal cells distinguishable by morphological criteria (24). Further analysis of mRNA expression in various neuronal cell types will require combining tissue culture techniques to identify these cells by morphological and biochemical criteria with *in situ* hybridization. Additional applications of *in situ* hybridization should prove fruitful in further studies of the various spatial and temporal patterns of tissue-specific splicing events known to occur during N-CAM expression (6–12).

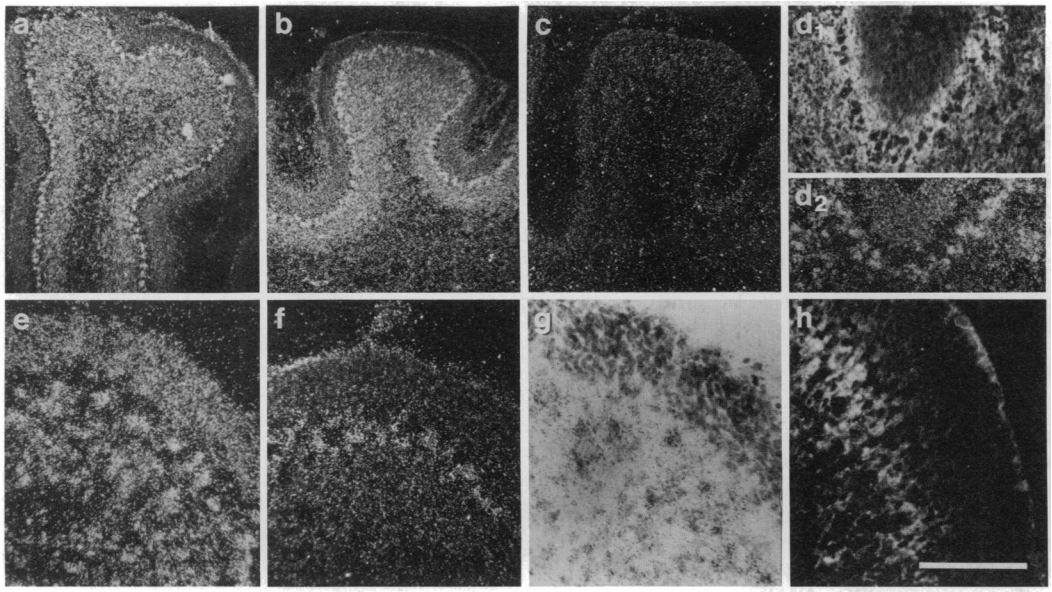

FIG. 5. Localization of total N-CAM mRNA and ld mRNA in the cerebellum. Parasagittal sections from E17 (*a–c*) and E15 (d_1, d_2, *e–h*) were hybridized with the total N-CAM antisense probe (*a*, d_2, *e*, and *g*), the ld antisense probe (*b* and *f*), the ld sense probe as control (*c*), or stained with polyclonal anti-N-CAM (d_1 and *h*). [Bar = 63 μm (*h*); *a–c*, equivalent length, 400 μm; d_1 and d_2, equivalent length, 150 μm; *e–g*, equivalent length, 100 μm.]

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