Localization during Development of Alternatively Spliced Forms of Cytotactin mRNA by In Situ Hybridization

Anne L. Prieto, Frederick S. Jones, Bruce A. Cunningham, Kathryn L. Crossin, and Gerald M. Edelman

Laboratory of Developmental and Molecular Biology, The Rockefeller University, New York 10021

Abstract. Cytotactin, an extracellular glycoprotein found in neural and nonneural tissues, influences a variety of cellular phenomena, particularly cell adhesion and cell migration. Northern and Western blot analysis and in situ hybridization were used to determine localization of alternatively spliced forms of cytotactin in neural and nonneural tissues using a probe (CT) that detected all forms of cytotactin mRNA, and one (VbVc) that detected two of the differentially spliced repeats homologous to the type III repeats of fibronectin.

In the brain, the levels of mRNA and protein increased from E8 through E15 and then gradually decreased until they were barely detectable by P3. Among the three cytotactin mRNAs (7.2, 6.6, and 6.4 kb) detected in the brain, the VbVc probe hybridized only to the 7.2-kb message. In isolated cerebella, the 220-kD polypeptide and 7.2-kb mRNA were the only cytotactin species present at hatching, indicating that the 220-kD polypeptide is encoded by the 7.2-kb message that contains the VbVc alternatively spliced insert. In situ hybridization showed cytotactin mRNA in glia and glial precursors in the ventricular zone throughout the central nervous system. In all regions of the nervous system, cytotactin mRNAs were more transient and more localized than the polypeptides. For example, in the radial glia, cytotactin mRNA was observed in the soma whereas the protein was present externally along the glial fibers. In the telencephalon, cytotactin mRNAs were found in a narrow band at the edge of a larger region in which the protein was widespread. Hybridization with the VbVc probe generally overlapped that of the CT probe in the spinal cord and cerebellum, consistent with the results of Northern blot analysis. In contrast, in the outermost tectal layers, differential hybridization was observed with the two probes.

In nonneural tissues, hybridization with the CT probe, but not the VbVc probe, was detected in chondroblasts, tendinous tissues, and certain mesenchymal cells in the lung. In contrast, hybridization with both probes was observed in smooth muscle and lung epithelium. Both epithelium and mesenchyme expressed cytotactin mRNA in varying combinations: in the choroid plexus, only epithelial cells expressed cytotactin mRNA; in kidney, only mesenchymal cells; and in the lung, both of these cell types contained cytotactin mRNA.

These spatiotemporal changes during development suggest that the synthesis of the various alternatively spliced cytotactin mRNAs is responsive to tissue-specific local signals and prompt a search for functional differences in the various molecular forms of the protein.

CYTOTACTIN, an extracellular glycoprotein found in neural (5, 26, 36) and nonneural tissues (8, 9, 13, 26), has been implicated in a variety of morphogenetic phenomena. In various reports, this protein has been called glioma mesenchymal extracellular matrix protein, myotendinous antigen, hexabrachion protein, J1 220/200, and tenascin (5, 8–10, 18, 27, 36). Immunohistochemical analysis has revealed that its expression during embryonic development is restricted both spatially and temporally (13, 14, 41, 50), in marked contrast to other matrix proteins such as fibronectin and laminin (48). The dynamic, restricted, and extracellular localization of cytotactin polypeptides underlines the importance of comparing the polypeptide and mRNA distributions. Identification of the particular cells synthesizing various forms of this molecule may help to clarify its role in a variety of cellular interactions.

In the embryonic chicken, cytotactin first appears during gastrulation (13), and is present in neural crest pathways (7, 13, 41, 50). Later in development, it is present in the extracellular matrix of the central and peripheral nervous systems where it is expressed by glial cells (17, 26). It is also present in nonneural tissues, such as smooth muscle (29), tendon (8, 9), cartilage (53), and in the lung and kidney (2, 13, 26) where it may influence mesenchymal–epithelial interactions.

Cytotactin is composed of major polypeptides of 220, 200, and 190 kD when isolated from chick brain. In electron micrographs of the rotary shadowed molecule, it appears as

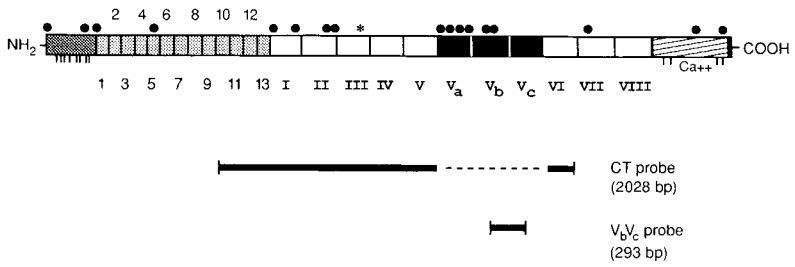

Figure 1. Alignment of RNA probes (CT and VbVc) on the structure of the cytotactin polypeptide. The CT probe included regions encoding four (*10–13*) of the 13 epidermal growth factor-like repeats (*1–13, stippled boxes*) and six of the fibronectin type III repeats (*I–VI, open rectangles*), but not the three type III repeats (VaVbVc, *solid rectangles*) found in large mRNAs of cytotactin. (*Dark stippled box*) Amino-terminal region; (*vertical lines*) cysteine residues; (*diagonally striped box*), carboxyl-terminal segment with homology to fibrinogen; (*solid circles*) potential N-linked glycosylation sites; (*) RGD sequence.

a six-armed structure termed a hexabrachion (18). The amino acid sequence of cytotactin has been deduced by sequencing cDNA clones encoding the complete protein (27, 34, 35, 44, 49) and a model has been formulated mapping the protein domains onto the hexabrachion structure (35, 49). The amino-terminal segment contains 8 cysteine residues that link cytotactin into multimers at the hub of the hexabrachion followed by a segment composed of 13 complete epidermal growth factor–like repeats. The carboxyl-terminal segment resembles the distal globular domain of the β and γ chains of fibrinogen. The central portion of the molecule is composed of a variable number (8–15) of consecutive segments resembling fibronectin type III repeats.

Alternative RNA splicing generates the variation in the number of type III repeats. Two cDNAs have been identified from brain libraries: one that contains eight type III (I-VIII) repeats and the other that contains an additional three type III repeats between repeats V and VI (designated VaVbVc) (Fig. 1). Based upon the polypeptide size of a single type III repeat (35), these two cDNAs are presumed to encode the 190- and 220-kD polypeptides. Another cDNA has been isolated that includes only the Vc repeat and that presumably encodes the 200-kD polypeptide (49). The sizes of the polypeptides and mRNAs found in chicken gizzard and in human tissues suggests that additional splice variants exist (27, 35). The presence of different type III repeats may have implications for cytotactin function as has already been demonstrated for fibronectin (30, 31, 43, 54).

In this study, we have used the techniques of Northern and Western blot analysis and in situ hybridization to show that the levels of cytotactin mRNA and protein changed in characteristic patterns in various tissues throughout development and that particular alternatively spliced forms of both the mRNA and protein occur selectively within certain tissues, such as the cerebellum. Not only were there temporal differences in the expression of cytotactin mRNA and polypeptides among tissues, but also differential expression of alternatively spliced forms of cytotactin within different regions of the same organ was observed, for example, in the optic tectum and gizzard. As might be expected for an extracellular matrix protein, the mRNA was generally more localized than the protein, allowing a more precise identification of sites of cytotactin synthesis. Moreover, in nonneural tissues, cytotactin was synthesized by epithelial as well as mesenchymal cells, an observation that was not apparent in previous immunohistochemical studies.

Materials and Methods

Tissues

Tissues from White Leghorn chickens were fixed overnight in 4% paraformaldehyde (wt/vol)/240 mM phosphate buffer, pH 7.6 and then cryoprotected as previously described (45). Cryostat sections of 10 μm were collected on chrome-alum coated slides and stored desiccated at 4°C.

Probes for In Situ Hybridization

Two different RNA probes were used in this study, as shown in Fig. 1: a 2,028-nt probe, CT, was transcribed from an Eco RI insert derived from pEC802 (34) subcloned in Bluescript KS (Stratagene, La Jolla, CA). The 293-nt probe, VbVc, was transcribed from a 293-bp Pst I–Hind III insert derived from λgt11 cytotactin cDNA (35) subcloned into Bluescript KS. The plasmids were linearized with Xho I and Xba I and mRNA was obtained in the sense and antisense orientation by transcribing from the T7 and T3 promoters, respectively. Probes in the sense orientation served as controls for nonspecific hybridization. Transcription reactions were carried out using an RNA transcription kit (Stratagene) following conditions suggested by the manufacturers. ^{35}S-labeled CTP (New England Nuclear, Boston, MA; 1,350 Ci/mmol, 1 Ci = 37 GBq) was used at a final concentration of 12 μM without unlabeled CTP.

In Situ Hybridization

The protocol for in situ hybridization and subsequent washes was as previously described in detail (42) and used in this laboratory (45). After hybridization and washes, the slides were air dried and then dipped in NTB2 (Kodak Nuclear Track Emulsion), melted at 43°C and diluted 2:1 with distilled water. The slides were then exposed 7–15 d and developed for 2.5 min with D_{19} 1:2, rinsed in water, and fixed with Kodak Ektaflo Fixer (1:2) for 10 min. They were counterstained with cresyl violet and analyzed using dark-field and bright-field microscopy. Specificity of the hybridization in the different tissues was tested using ^{35}S-labeled probes in the sense orientation for both the CT and VbVc inserts on parallel sections under the same hybridization and washing conditions as the probe in the antisense orientation.

Immunohistochemistry

Indirect immunofluorescence labeling was done as described (12) using rabbit polyclonal antibodies raised against purified embryonic chicken cytotactin (29).

RNA and Protein Blot Analysis

Whole brains and cerebella from chicken embryos at various stages of development were dissected on ice and collected. RNA was purified as described in 23. For RNA blot analysis, 5 μg of whole brain RNA or 20 μg of cerebellar RNA was electrophoresed on a 0.8% agarose formaldehyde gel as described (34), and transferred to nitrocellulose. Filters were hybridized in a buffer containing 50% formamide at 42°C with either ^{32}P-labeled CT or VbVc cDNA probe (^{32}P dCTP; New England Nuclear, Boston, MA;

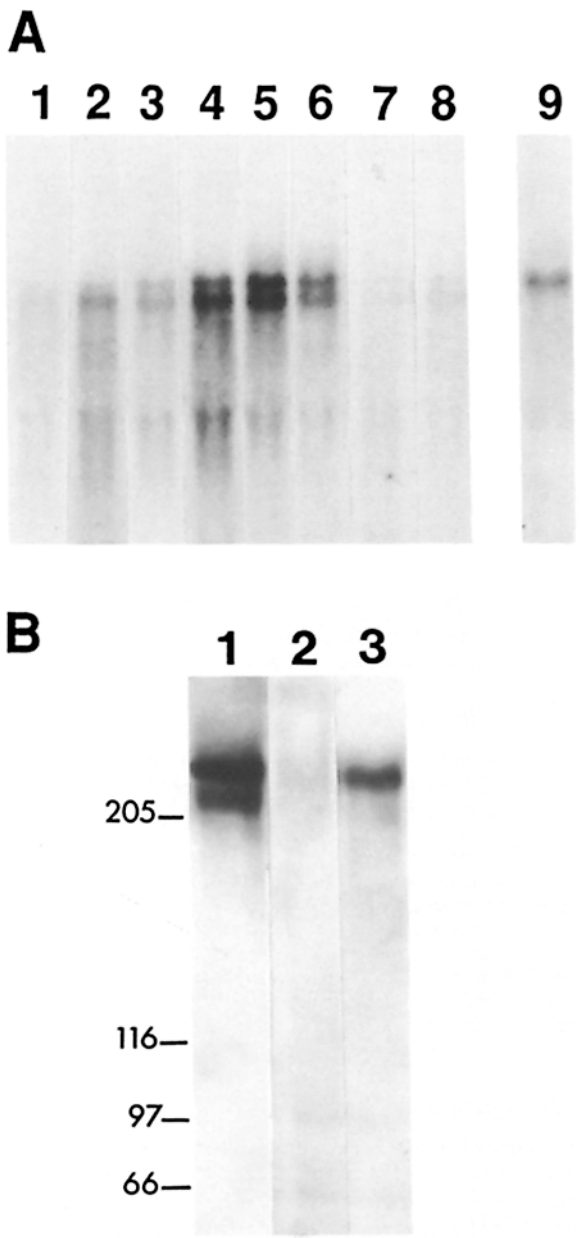

Figure 2. Cytotactin mRNAs and polypeptides during brain development. (*A*) RNA blot analysis of cytotactin mRNAs during embryonic development of the brain. 5 μg of total RNA was electrophoresed on a formaldehyde gel transferred to nitrocellulose and hybridized with the CT probe (lanes *1–8*) or to the VbVc probe (lane *9*). Lane *1*, RNA from total embryo at E5; lanes *2–9* are RNA from whole brain of E8, E11, E13, E15, E17, hatchling, P3, and E15, respectively. (*B*) Immunoblot analysis of cytotactin polypeptide species. 40 μg of protein were electrophoresed by SDS-PAGE (on a 6% gel), transferred onto nitrocellulose and immunoblotted with cytotactin polyclonal antibodies (29). Lane *1*, purified cytotactin from E14 chick brains; lane *2*, extracts from whole brain minus cerebellum; lane *3*, extracts from cerebellum. The numbers on the left represent the molecular mass × 10^{-3} of standard proteins.

3,000 Ci/ mmol; 1 mCi = 37 MBq). Filters were washed at 65°C for 20 min, twice in 0.2% SDS, 0.2× SSC, and autoradiographed.

For protein extracts, tissues from appropriate ages were pulverized in a minidounce homogenizer in 0.2% NP-40 in PBS, and spun for 10 min in an Eppendorf centrifuge (Brinkmann Instruments Co., Westbury, NY) at 4°C. Protein quantitation was performed on the supernatant of this fraction using Bradford's method (6) (Bio-Rad Laboratories, Richmond, CA). Samples were boiled in 2× Laemmli sample buffer (38) and subjected to 6% SDS-PAGE. Protein was electrotransferred to nitrocellulose and subjected to immunoblot analysis with polyclonal antisera prepared against purified cytotactin (29).

Results

The distribution of cytotactin mRNA during chick embryogenesis was studied using two different probes: one probe (CT) contained 2,028 nucleotides corresponding to a region (Fig. 1) that is present in all forms of cytotactin analyzed thus far (35, 49). It encodes a segment that spans the last four epidermal growth factor–like repeats and six fibronectin type III repeats (I–VI). The other probe (VbVc), contained 293 nucleotides corresponding to a region that is spliced into mRNAs for the larger cytotactin polypeptides (35); it includes portions of the Vb and Vc type III repeats (see Fig. 1) and should detect any cytotactin mRNA containing either of these two repeats.

Analysis of Cytotactin mRNA and Polypeptide Species during the Development of the Brain and Cerebellum

RNA blotting using the CT probe established the presence of at least two cytotactin mRNA species (7.2 and 6.6/6.4 kb) (35) in E5 whole embryos (Fig. 2 *A*, lane *1*) and in E8 brain (Fig. 2 *A*, lane *2*). The levels of mRNA in brain increased from E8 through E15 (Fig. 2 *A*, lanes *2–5*) and then gradually decreased to barely detectable levels between E17 and P3 (Fig. 2 *A*, lanes *6–8*). The larger message (7.2 kb) increased with respect to the other messages (6.6/6.4 kb) until E15. The VbVc probe hybridized only to the 7.2-kb message (Fig. 2 *A*, lane *9*) demonstrating that this species contains all or part of the VaVbVc insert.

Previous immunohistological experiments indicated that the cerebellum continues to express cytotactin late in development (11) when the level of the protein in the whole brain is low (13, 14, 29) and is detected primarily as the 220-kD component (14, 29). To extend these findings, the composition at hatching of cytotactin polypeptides present in cerebellum and in whole brain minus cerebellum were examined. The isolated cerebellum, which comprised approximately one-tenth of the wet tissue weight of the whole chicken brain, expressed high levels of cytotactin that consisted almost exclusively of the 220-kD component (Fig. 2 *B*, lane *3*). The remaining 90% of the brain contained low levels of cytotactin as compared to the cerebellum (Fig. 2 *B*, lane *2*).

More detailed analyses of cytotactin mRNA and protein in isolated cerebella showed that the prevailing mRNA species was the 7.2-kb mRNA from E15 to P3, while the 6.4 kb species decreased steadily (Fig. 3 *A*). The 220-kD polypeptide was the predominant form throughout development (Fig. 3 *B*) and by E19 it was the only species present. Taken together, these results support the conclusion that the 220-kD polypeptide is encoded by the 7.2-kb message and that this mRNA contains the VbVc insert shown in Fig. 1.

Cytotactin mRNA Species Are Differentially Regulated during the Development of the Chicken Cerebellum

Cytotactin mRNA was found in the cell bodies that surround the unlabeled Purkinje neurons (see * in Fig. 4 *D*) from E15

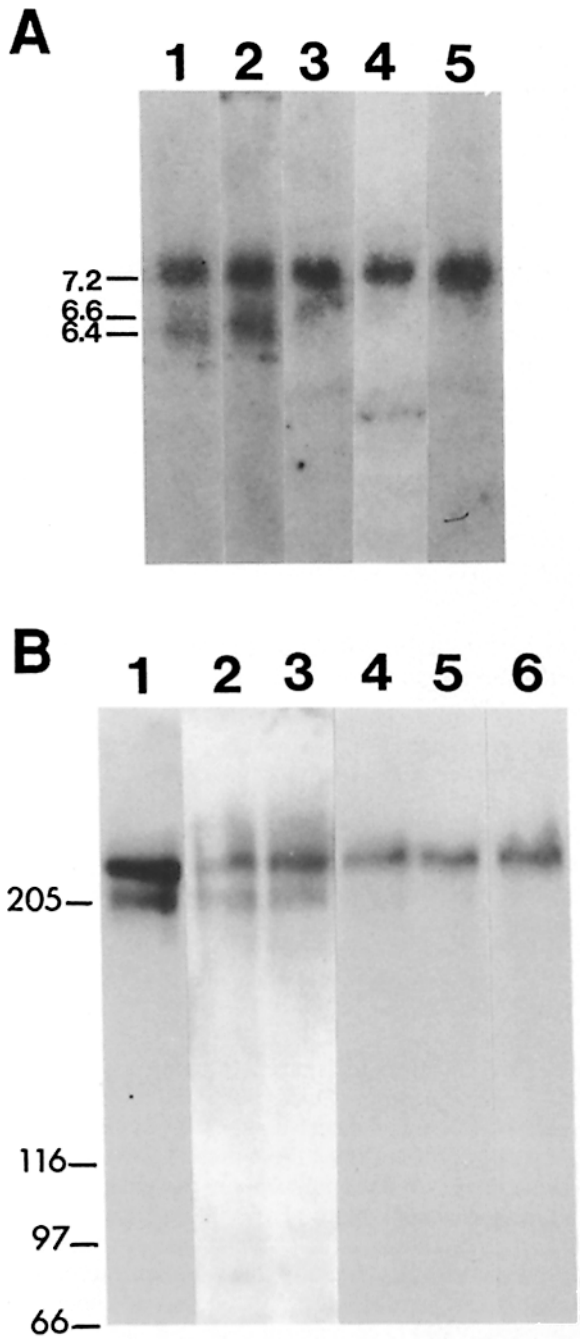

Figure 3. Cytotactin RNA and polypeptide species in the development of the chick cerebellum. (*A*) 20 μg of total RNA extracted from cerebella were electrophoresed on formaldehyde agarose gels, transferred to nitrocellulose, and hybridized with the CT probe. Lanes *1–5*, RNA from E15, E17, E19, hatchling, and P3. The sizes of cytotactin mRNA species previously characterized (35) are indicated in kilobases by the numbers to the left. (*B*) Immunoblot analysis of cytotactin polypeptides in the development of the cerebellum. 40 μg of protein extracted from cerebella were electrophoresed by SDS-PAGE (on a 6% gel), transferred to nitrocellulose and immunoblotted. Lane *1*, purified cytotactin from 14-d chick brains; lanes *2–6* are cerebellar protein extracts from E15, E17, and E19, hatchling, and P3, respectively. The numbers on the left represent the molecular mass × 10^{-3} of standard proteins.

(Fig. 4, *A* and *B*) to hatching (Fig. 4, *C–H*). These cells are the Bergmann glia as suggested by antibody staining of their processes (Fig. 4, *C* and *G*). In the cerebellum, these Bergmann glial fibers are oriented radially through the molecular layer, with their somata lying between the Purkinje cell bodies and their processes projecting into the outer layers (3). In the molecular or external granular layers (egl) where the glial fibers project (Fig. 4 *D*), no cytotactin mRNA was detected with the CT probe when compared with the sense strand (Fig. 4 *H*), indicating that the protein on the fibers must be made by the Bergmann glia themselves. Hybridization was also observed in the glia located in the developing internal granular layer (igl) (Fig. 4, *A, B, D, E,* and *F*); these cells probably secrete the cytotactin seen in this area by antibody staining (Fig. 4, *C* and *G*). At E15 (Fig. 4, *A* and *B*), the pattern of hybridization with the VbVc probe was the same as that observed for the CT probe and remained unchanged until hatching (Fig. 4, *E* and *F*), when the only mRNA species present was the 7.2-kb message (Fig. 3 *A*).

Differential Distribution of Alternatively Spliced Forms of Cytotactin in the Optic Tectum

In the optic tectum, at E9, a stage characterized by active cell migration (40), strong hybridization was observed in the ventricular zones where the neuroepithelial cells are proliferating and the cell bodies of the radial glia are located (Fig. 5 *A*) (15). Hybridization using the VbVc probe (Fig. 5 *B*) was restricted to a narrow region adjacent to the ventricle. The higher background signal (39, 40) in more external layers was due to the large number of cells accumulating in this area as evidenced by the fact that the sense strand control gave a similar signal in these regions (not shown). The antibody staining (Fig. 5 *C*) showed that the protein accumulated not only in the ventricular zone, but also around the radial glial cell bodies, along the radial glial fibers (rgf) and at the pial surface.

At E13, the mRNA hybridization pattern resembled that found at earlier stages but there were a few labeled cells in regions adjacent to the ventricular zone (not shown). Antibody staining at E15 (Fig. 5 *F*), showed extensive distribution of cytotactin polypeptides, with the exception of laminae viii and vi of the stratum griseum et fibrosum superficiale (SGFS), which are predominantly neuronal (39), and the stratum album centrale (SAC) formed mainly by the myelinated efferent fibers of the tectum. Intense antibody staining was seen in the stratum opticum (SO) where the fibers of the optic nerve penetrate the tectum. From E15 through hatching (Fig. 5, *D* and *E*), the CT probe (Fig. 5 *D*) and VbVc probe (Fig. 5 *E*) labeled cells in every layer in the tectum, but selective hybridization on specific cells with the CT probe but not with the VbVc probe was observed between laminae ix and xii of the SGFS (compare Figure 5 *D* with 5 *E*). This differential distribution first appeared between E13 and E15 (not shown) and persisted until hatching, the last stage of development that we analyzed. In general, the levels of protein staining and of hybridization for both probes decreased in the optic tectum from E15 until hatching.

Developmental Distribution of Cytotactin in the Chicken Telencephalon

The distributions of cytotactin mRNA and polypeptide were analyzed in the telencephalon from E8 through hatching. As

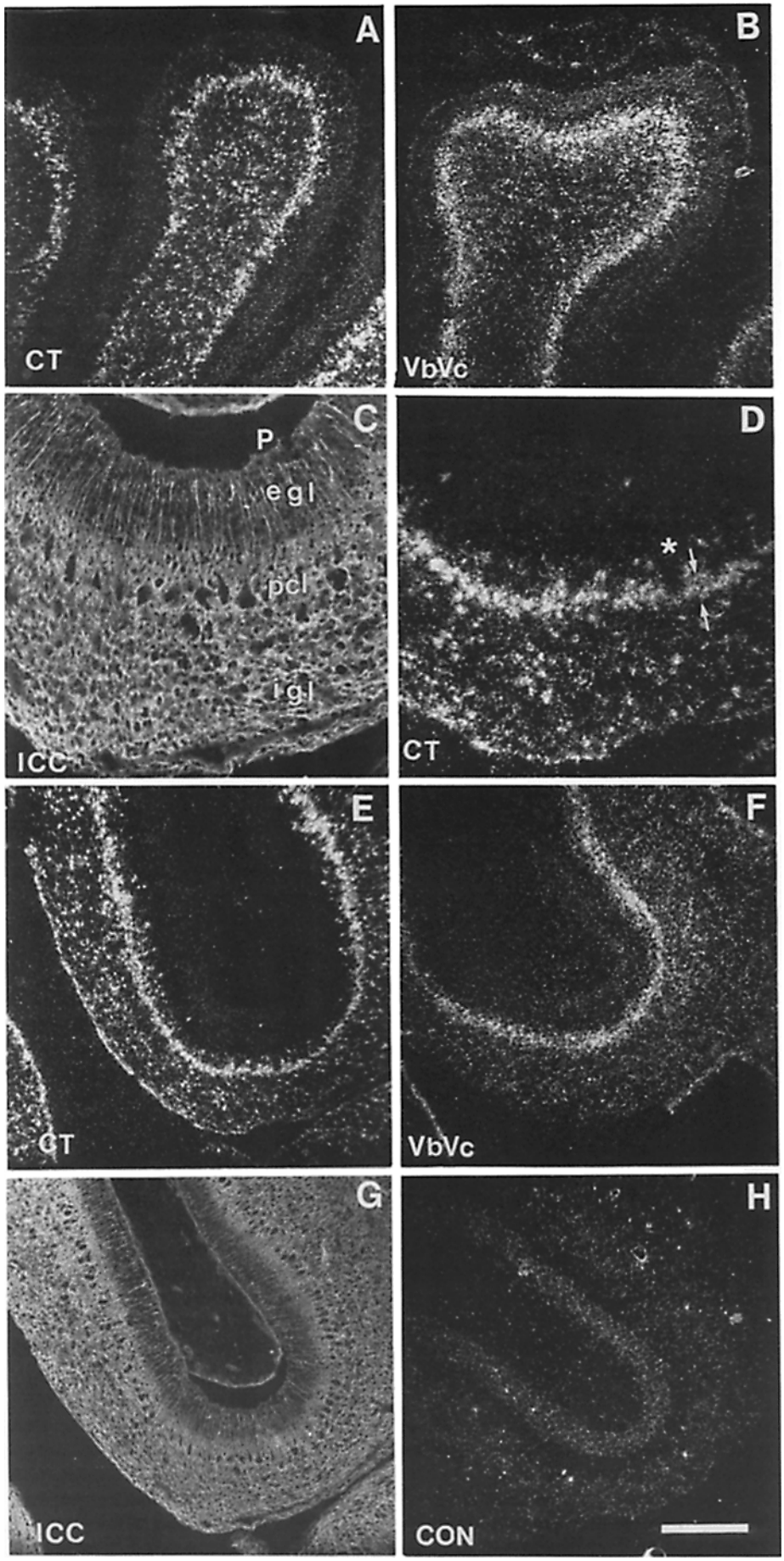

Figure 4. Differential expression of cytotactin mRNA in the cerebellum. Parasagittal sections of cerebella from E15 (*A* and *B*), and hatchling (*C–H*) were hybridized with the antisense CT probe (*A*, *D*, and *E*) and sense CT probe (*H*), VbVc antisense probe (*B* and *F*), or stained with cytotactin polyclonal antibodies (*C* and *G*). Pial surface (*P*); external granule cell layer (*egl*); Purkinje cell layer (*pcl*); internal granule cell layer (*igl*); below the asterisk (*) an unlabeled Purkinje cell. The arrows in *D* enclose Bergmann glia somata in the pcl. *ICC*, immunocytochemistry. *CON*, control. Bars: (*A*, *B*, and *E–H*) 100 μm; (*C* and *D*) 40 μm.

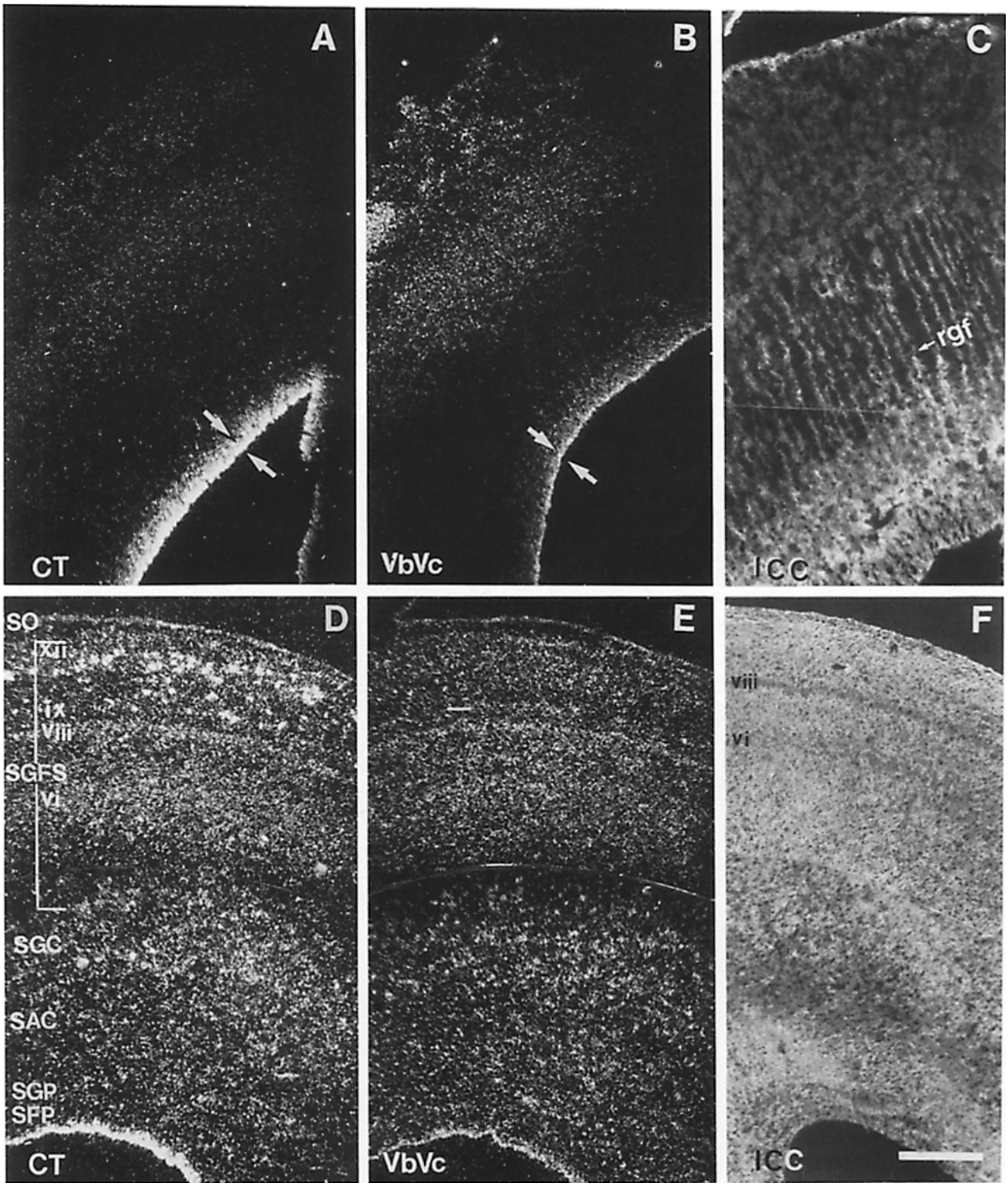

Figure 5. Polypeptide and mRNA localization in the optic tectum. Parasagittal sections of optic tecta of E9 (*A–C*), E15 (*F*), and hatchling (*D* and *E*) were hybridized with: CT antisense probe (*A* and *D*); VbVc probe (*B* and *E*). Immunofluorescent staining using cytotactin polyclonal antibodies is shown in *C* and *F*. Arrows in *A* and *B* enclose the ventricular zone; radial glial fiber (*rgf*). Stratum opticum (*SO*); stratum griseum et fibrosum superficiale (*SGFS*); stratum griseum centrale (*SGC*); stratum album centrale (*SAC*); stratum griseum periventriculare (*SGP*); stratum fibrosum periventriculare (*SFP*). The laminae of the SGFS are designated by Roman numerals. Bars: (*A* and *B*) 125 μm; (*D–F*) 100 μm; (*C*) 40 μm.

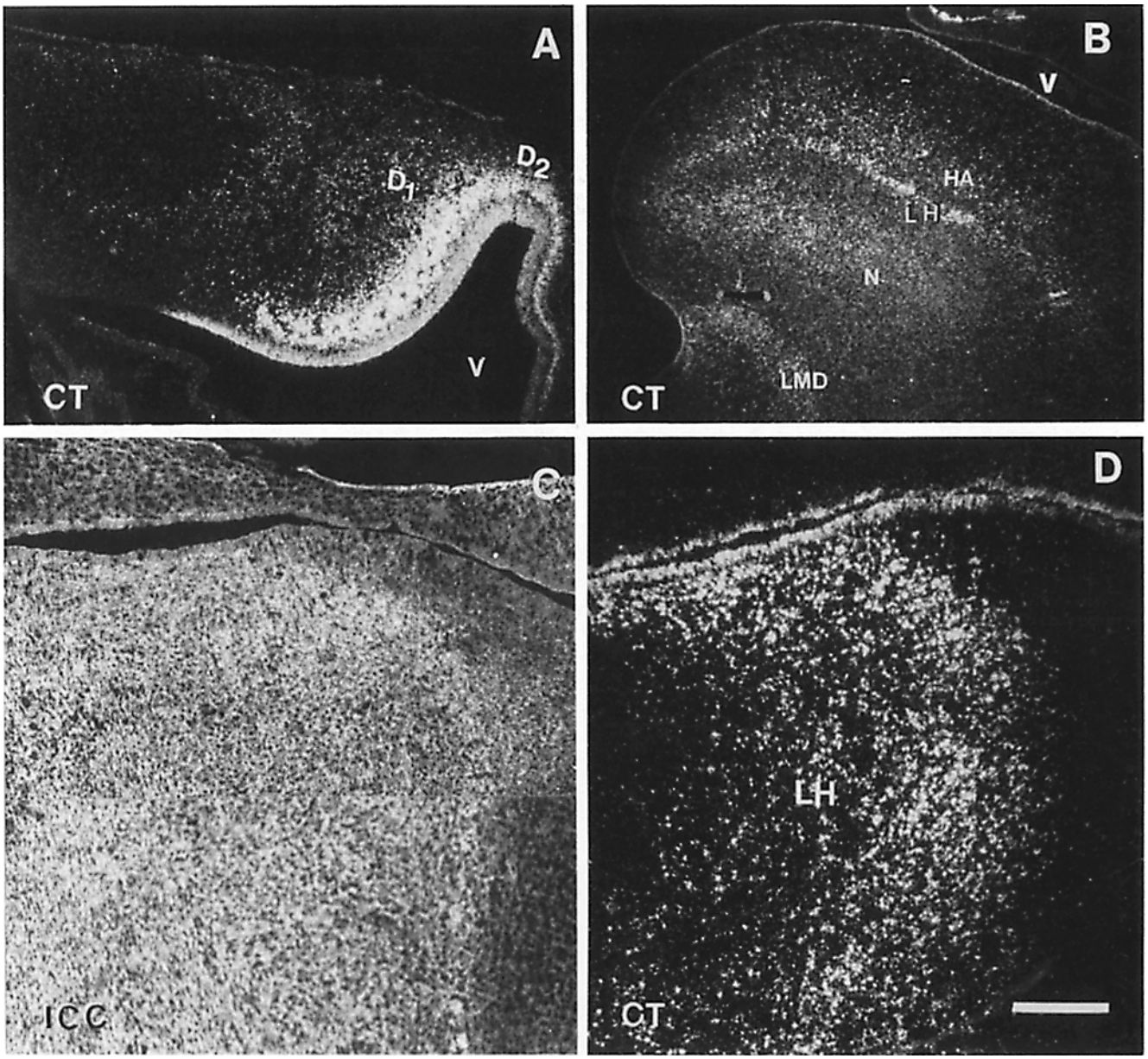

Figure 6. Laminated distribution of cytotactin polypeptides and mRNA in the telencephalon of the chick. Cross sections of the telencephalon of E8 (*A*), E11 (*C* and *D*), and E16 (*B*), were hybridized with the CT antisense probe (*A*, *B*, and *D*); or stained with cytotactin polyclonal antibodies (*C*). The cross sections presented in *A*, *C*, and *D* are from rostral portions of the telencephalon, *B* is from the occipital region. Lamina medullaris dorsalis (*LMD*); lamina hyperstriatica (*LH*); neostriatum (*N*); hyperstriatum accesorium (*HA*). Pallial (upper) zones of the telencephalon are designated D1 and D2 from ventral to dorsal. Bars: (*A*) 100 μm; (*B*) 240 μm; (*C* and *D*) 40 μm.

seen in other regions of the nervous system, strong hybridization with the CT probe was seen at E8 in the ventricular and mantle zone in the basal aspect of the ventricle, an area of extensive cell proliferation and cell migration (areas D_1 and D_2) (Fig. 6 *A*) (37, 47). The hybridization was not continuous throughout the ventricular zone even in regions where cells are proliferating, indicating that the expression of cytotactin mRNA is not strictly correlated with proliferative zones.

Cytotactin message was found in definite laminated patterns across the entire telencephalon. At E11, for example, the lamina hyperstriatica is well defined by in situ hybridization with the CT probe of which only the boundary of the mRNA localization coincided with the protein staining (Fig. 6, *D* and *C*). The protein was more widespread (Fig. 6 *C*), presumably reflecting transient RNA synthesis in these regions. At E16, when the cytoarchitecture of the telencephalon is well defined (24), cytotactin mRNA was found in the following regions (Fig. 6 *B*): a band of scattered labeled cells in the hyperstriatum (HA), a narrow, well-defined band corresponding to the lamina hyperstriatica (LH), a broad area in the neostriatum (N), and strong hybridization in the lamina medullaris dorsalis (LMD).

Localization of Cytotactin mRNAs and Polypeptides in the Spinal Cord and Surrounding Nonneural Tissues during Embryonic Development

At E5 (Fig. 7 *A*), no cytotactin mRNA was detected in the

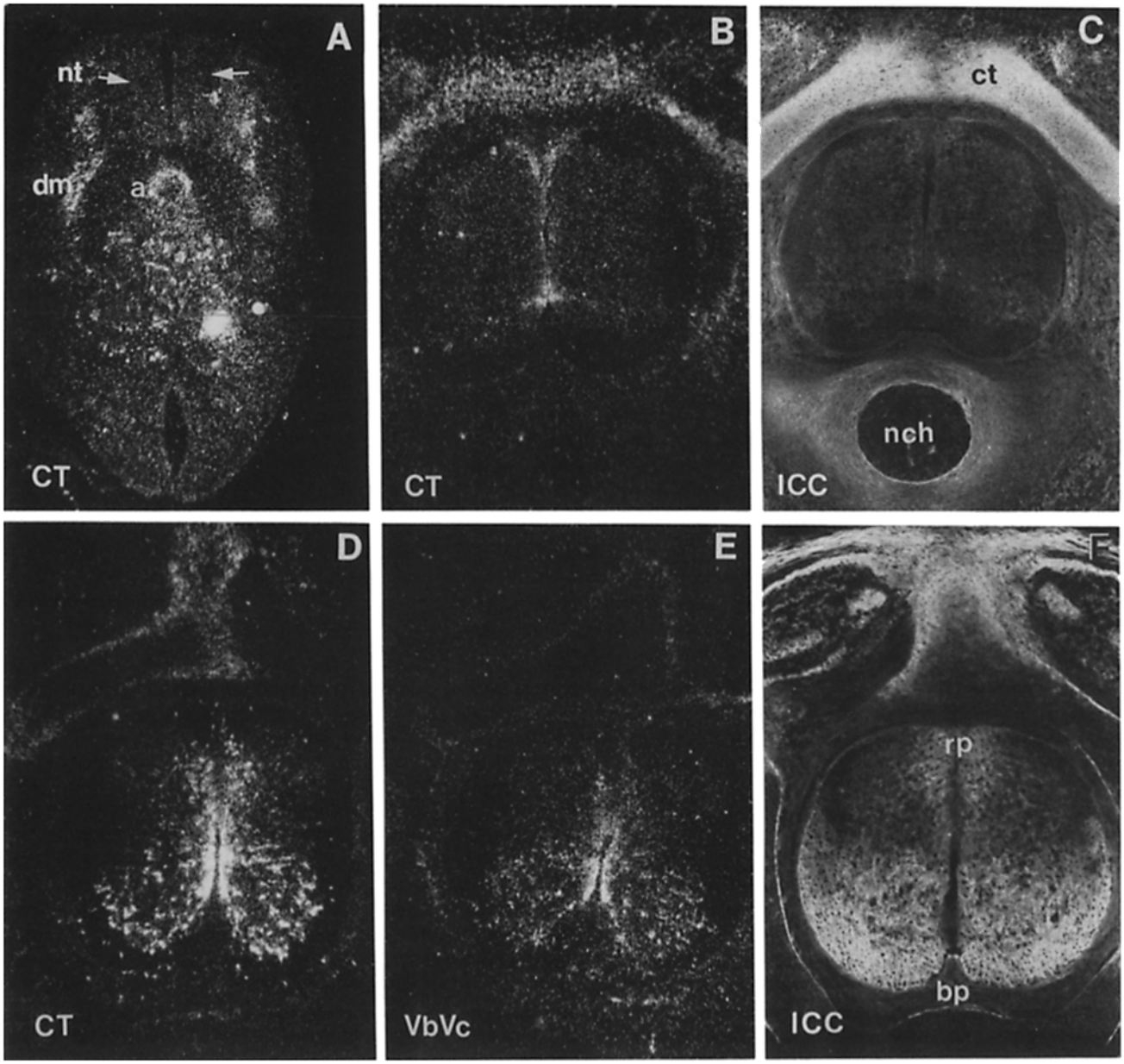

Figure 7. Developmental analysis of the distribution of polypeptides and mRNA of cytotactin in the spinal cord. Cross sections of the spinal cord from E5 (*A*); E8 (*B* and *C*); E11 (*D–F*) were hybridized with the CT antisense probe (*A*, *B*, and *D*), with the VbVc antisense probe (*E*) or stained with cytotactin polyclonal antibodies (*C* and *F*). The neural tube is enclosed within arrows (*nt*); dermamyotome (*dm*); aorta (*a*); notochord (*nch*); cartilage (*c*); roof plate (*rp*); basal plate (*bp*). Bar, 100 μm.

neural tube (nt), although hybridization was apparent in the dermamyotome (dm), aorta (a), and developing gut. Cytotactin mRNA was first detected in the spinal cord at E8 (Fig. 7 *B*), appearing in the ventricular zone along the borders of the roof plate and the basal plate. Strong hybridization was also seen throughout the developing vertebral cartilage at this stage (see below). The protein distribution (Fig. 7 *C*) corresponded to that of the message in the spinal cord (Fig. 7 *B*), however, the levels of cytotactin protein surrounding the notochord were significant but no mRNA was observed (compare Fig. 7 *B* with *C*). The distribution of cytotactin mRNA and protein varied depending on the level of the spinal cord. In lumbar segments (Fig. 7, *D–F*), the CT (Fig. 7 *D*) and VbVc (Fig. 7 *E*) probes detected mRNA on the ventricular zone, and distinct labeled cells were observed along the borders of the roof plate, in ventromedial portions of the spinal cord disposed in a radial orientation, and in the ventral region. The protein (Fig. 7 *F*) filled the extracellular space around the cells that hybridized with the CT and VbVc probes. In thoracic segments, in which development has proceeded further than in more caudal segments, mRNA hybridization was more extensive and areas of cell accumulation in the ventral motor columns were obvious (not shown).

Distribution of Cytotactin mRNAs and Polypeptides in Nonneural Tissues

Differential Expression of Cytotactin Forms during Chondrogenesis. During chondrogenesis, cytotactin appeared homogeneously in the condensing chondroblasts as shown

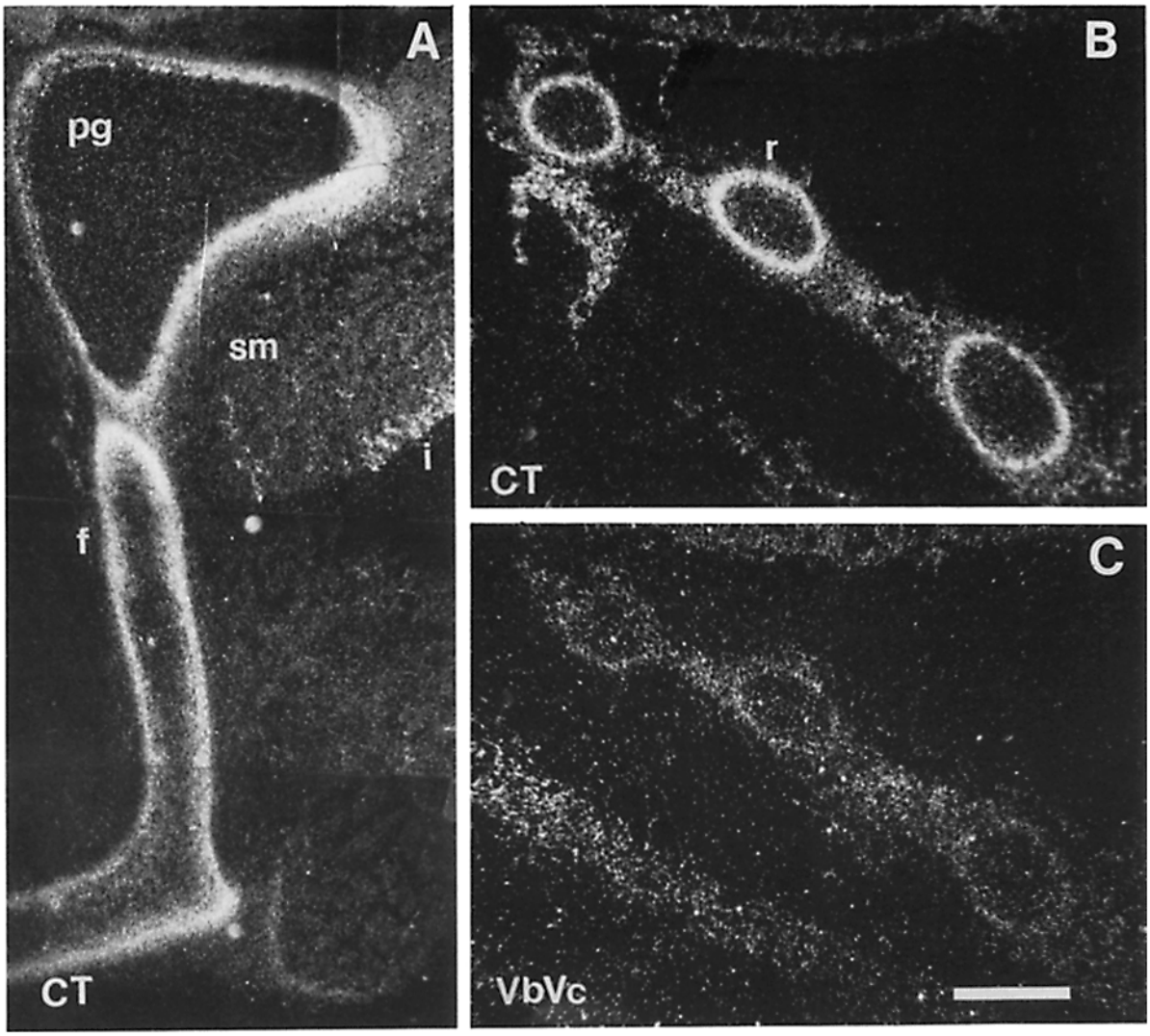

Figure 8. In situ hybridization of cytotactin mRNA in the developing cartilage. Parasagittal section (*A*) and cross sections (*B* and *C*) of E11 were hybridized with the CT antisense probe (*A* and *B*) or the VbVc antisense probe (*C*). *A* shows part of the hindlimb, and *B* and *C* show part of the rib cage. Femur (*f*); innervation (*i*); pelvic girdle (*pg*); rib (*r*); skeletal muscle (*sm*). Bar, 100 μm.

by in situ hybridization (Fig. 7 *B*) and antibody staining (Fig. 7 *C*). As development of the cartilage proceeded, the CT probe labeled the perichondrial region, surrounding the developing cartilage (Fig. 8, *A* and *B*), a pattern that was widespread in cartilage throughout the skeletal system. In the hindlimb of an E11 chick embryo, mRNA detected by the CT probe was limited to the perichondrium of the pelvic girdle (pg) and femur (f) (Fig. 8 *A*). The innervation (i) of the skeletal muscle was also evident by hybridization on the otherwise unlabeled skeletal muscle (sm). In contrast, when the VbVc probe was used, no hybridization above background was detected when compared with the CT and VbVc sense strand probes (not shown) in cartilage as illustrated for the ribs (compare Fig. 8 *B* and *C*).

Localization of Alternatively Spliced Forms of Cytotactin in Feather Buds and Gizzard. Differential hybridization with the CT and VbVc probes was also seen in other nonneural tissues. During feather bud formation (E11), hybridization in the dermis of the bud, probable precursors to smooth muscle cells of the erector muscles, was observed at similar levels with both the VbVc and CT probes (Fig. 9, *B* and *C*) corresponding to the localization of cytotactin by antibody staining (Fig. 9 *A*). Cytotactin mRNA was detected in the muscular layers along the gut. In the gizzard, the CT probe hybridized with mRNA in the smooth muscle (sm) and tendon (t) (Fig. 9 *G*) coincident with the antibody staining pattern (Fig. 9 *E*). The highest levels of protein were detected in the tendon, with less in smooth muscle, and none in epithelia (e) and submucosa. Hybridization using the VbVc probe above the background levels was found only in the smooth muscle but not in the tendon or epithelia (Fig. 9 *F*). These results are consistent with previous observations (35) indicating that the VbVc insert was present in gizzard cytotactin mRNA. This probe also detected cytotactin mRNA in the smooth muscle in other organs besides the gizzard, including large blood vessels such as the aorta (not shown).

Localization of Cytotactin mRNA in Epithelial and Mesenchymal Cells. In situ hybridization was used to determine whether cytotactin was synthesized by epithelial or mesenchymal cells in those tissues in which it is expressed at the epithelial–mesenchymal interface. The localization of the protein and the mRNA was compared in the choroid

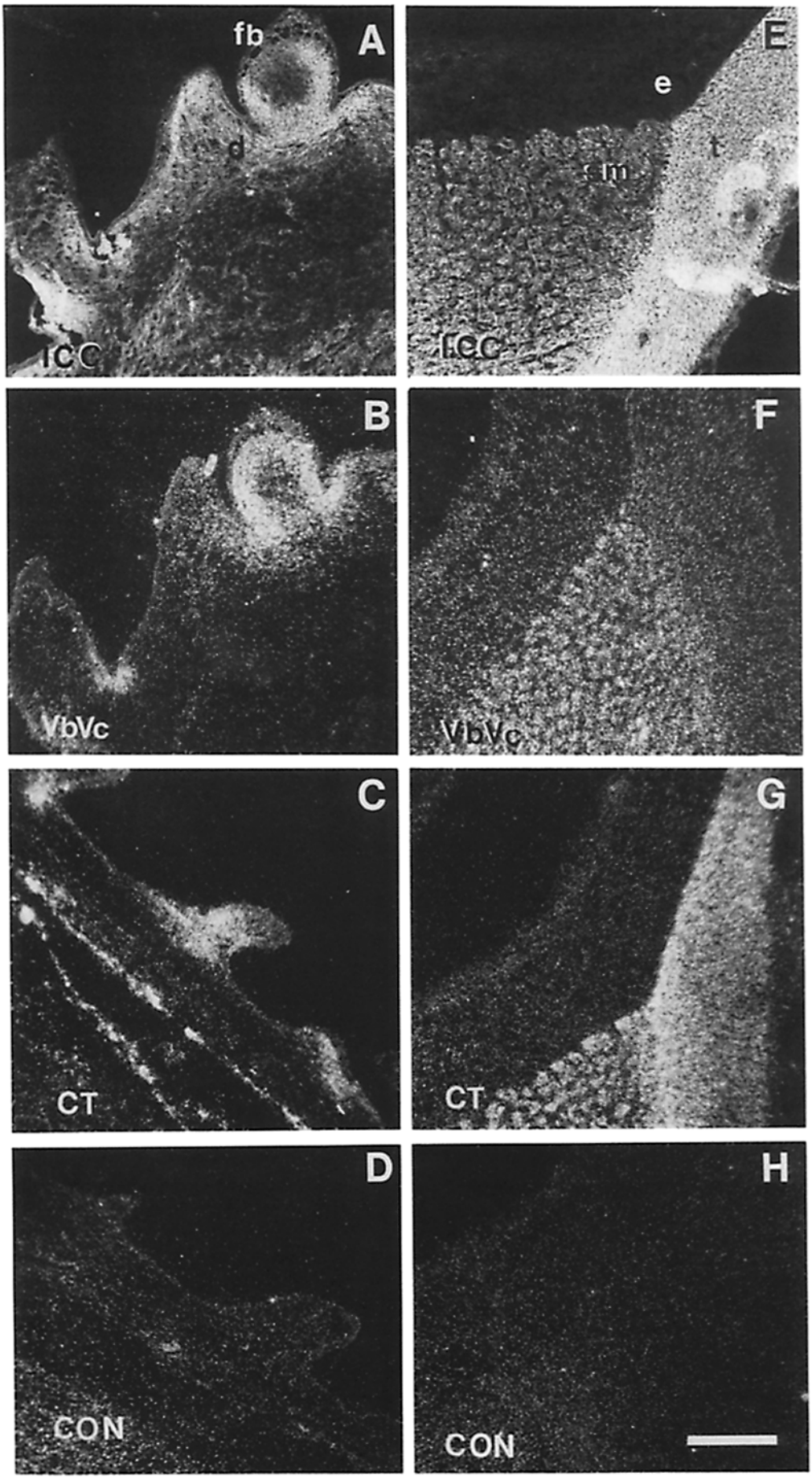

Figure 9. Localization of mRNA and polypeptide forms in the feather bud and in the gizzard at E11. Cross sections of feather buds (*A–D*), and parasagittal sections of gizzard (*E–H*), were stained with cytotactin polyclonal antibodies (*A* and *E*) or hybridized with: the VbVc antisense probe (*B* and *F*), CT antisense probe (*C* and *G*), and the CT sense probe (*D* and *H*). Feather bud (*fb*); dermis (*d*); smooth muscle (*sm*); tendon (*t*); epithelium (*e*). Bar, 100 μm.

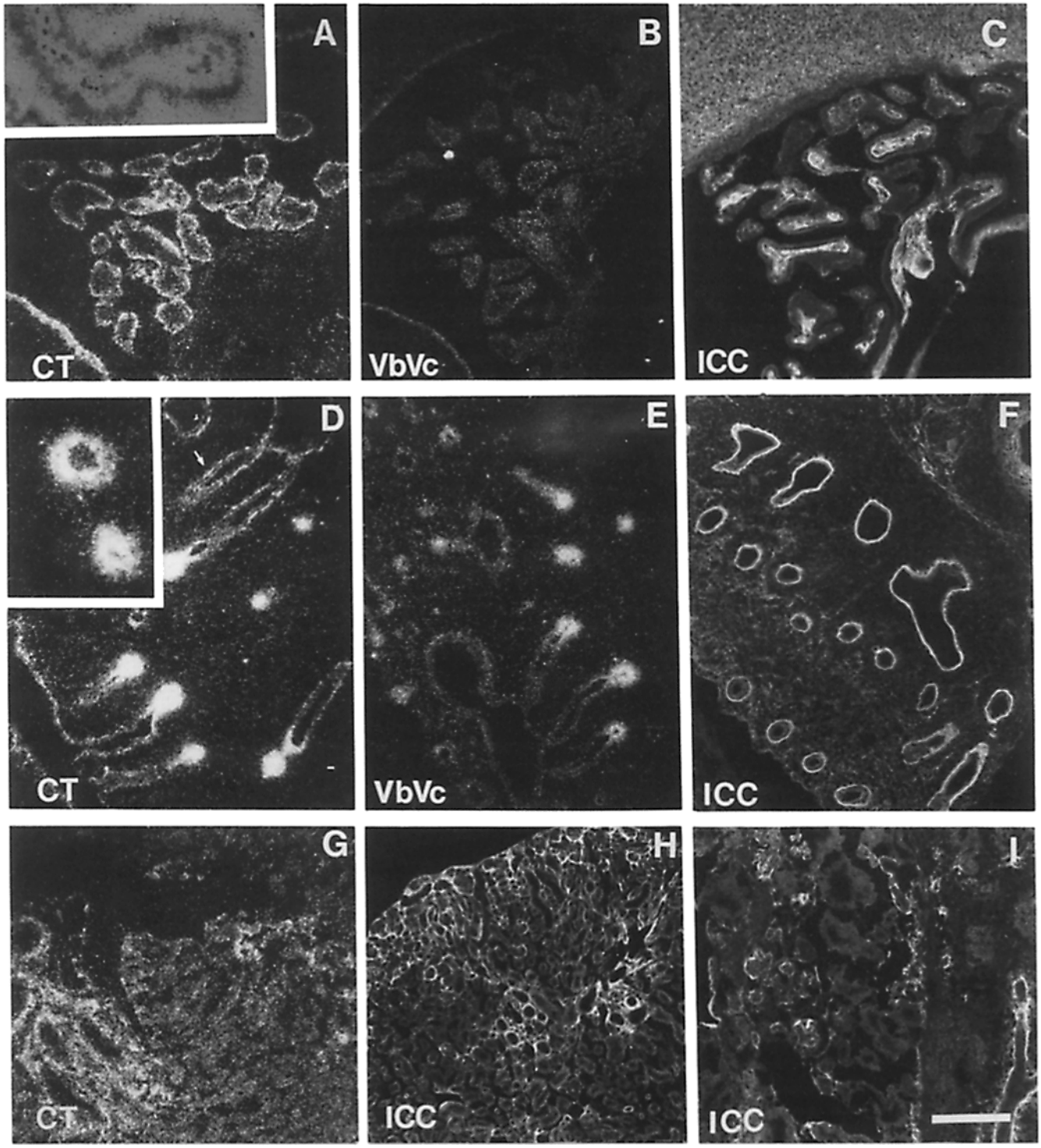

Figure 10. Epithelial and mesenchymal localization of cytotactin mRNAs. Parasagittal sections of E15 choroid plexus (*A–C*), E11 lung (*D–F*), and E11 kidney (*I*) and cross sections of hatchling kidney (*G* and *H*) were hybridized with CT antisense probe (*A*, *D*, and *G*), VbVc antisense probe (*B* and *E*), or stained with cytotactin polyclonal antibodies (*C*, *F*, *H*, and *I*). Arrow in *D* indicates hybridization signal in mesenchymal cells. Bars: (*inset* in *A*) 25 μm; (*inset* in *D*) 40 μm; (*A–I*) 100 μm.

plexus, the kidney, and the lung. In the choroid plexus (Fig. 10, *A–C*), strong antibody staining was observed in the basal lamina between the epithelia and the mesenchyme and in the underlying vasculature (Fig. 10 *C*). In situ hybridization using the CT probe showed that the mRNA was restricted to the epithelia (Fig. 10 *A* and *inset*). Hybridization above background levels was not detected with the VbVc probe (Fig. 10 *B*).

In the lung (Fig. 10, *D–F*), cytotactin protein (Fig. 10 *F*) was found in the basal lamina surrounding the parabronchi, as previously reported (13, 26). Strong hybridization with both the CT and VbVc probes (Fig. 10, *D* and *E*) was ob-

served in the bronchial epithelium with the highest levels in the tips of the budding tubules. In contrast, some cells adjacent to the epithelium also showed specific hybridization with the CT probe but not with the VbVc probe; the counterstain used did not allow us to identify these cell types that were probably fibroblasts or single smooth muscle cells.

In the kidney (Fig. 10, *G–I*) at E11, when the mesenchyme is still in contact with the inducing epithelia (25), small patches of antibody staining (Fig. 10 *I*) and diffuse hybridization with the CT probe were observed (not shown) in the loose mesenchyme surrounding tubules. In the hatchling kidney (Fig. 10, *G* and *H*), cytotactin and its mRNA were detected at low levels, while higher levels were detected in cortical regions, mainly in loose tissue surrounding the glomeruli. As observed for the protein distribution, most of the hybridization with the CT probe was associated with blood vessels, connective tissue, and around the glomeruli. This pattern clearly contrasts with that found in the lung where intense epithelial mRNA localization was observed.

Discussion

In situ hybridization together with mRNA blot analysis has been used to study the dynamic patterns of transcription of alternatively spliced mRNAs of cytotactin during development of the chick. The results of this study show that: (*a*) the forms and amounts of cytotactin mRNAs change throughout development in a specific spatiotemporal pattern; (*b*) in the cerebellum, the 220-kD polypeptide of cytotactin is translated from the 7.2-kb message, which contains the VbVc insert; (*c*) single alternatively spliced forms of cytotactin mRNA appear specifically in certain tissues; (*d*) cytotactin mRNAs are more sharply localized than the polypeptides; (*e*) in certain cell types, cytotactin expression decreases upon differentiation; (*f*) the insert recognized by the VbVc probe is only present in a subset of the cells expressing cytotactin, including neuroepithelial cells, glia, smooth muscle cells, epithelia of the budding bronchiolus; (*g*) both epithelial and mesenchymal cells can express cytotactin.

In chicken brain, the levels of cytotactin mRNAs decreased steadily during embryonic development. In accord with previous observations (29), cytotactin was expressed at the highest levels between E9 and E15 and decreased to low levels by hatching. In the brain, at late stages of development, the major mRNA species was the 7.2-kb mRNA and the major polypeptide was the 220-kD form of cytotactin; these were found primarily in the cerebellum, consistent with the fact that the cerebellum develops later than other brain structures. The results further support the conclusion (35) that the 220-kD form of cytotactin is encoded by the 7.2-kb message and that in some tissues this splice variant of cytotactin can be the only form expressed.

Comparison of in situ hybridization with immunocytochemistry showed that, as might be expected for an extracellular matrix protein, the distribution of cytotactin mRNA was more localized than that of the protein. This difference was not only apparent in specific cell types such as the radial glia, but also in larger areas in the telencephalon, spinal cord, and optic tectum. In general, the distribution of cytotactin protein in the embryonic brain paralleled the dynamic changes observed in the formation of well-defined anatomical structures; this was preceded by the transient expression of cytotactin mRNA. For example, in the developing telencephalon at E8, the regions of protein staining and mRNA hybridization were coincident, but as the telencephalon increased in size, the protein was found both with cells expressing the mRNA but also surrounded cells in adjacent regions. Anatomical gradients of cytotactin expression due to differences in cell maturation or cell rearrangement could result in extensive distribution of protein in areas where the mRNA is not present. The restricted spatiotemporal distribution of cytotactin mRNA, nevertheless, suggests that there is tight developmental control of signals for its expression at these various sites.

Cytotactin mRNA was found in radial glial cells both in the cerebellum and in the optic tectum; the mRNA was restricted to the somata and the protein was distributed along fibers, as had been previously reported (11, 26, 29). In contrast to other molecules in the nervous system for which mRNA is transported within fibers (22, 51), cytotactin is transported posttranslationally, and then distributed along the external surface of the Bergmann glial fibers and radial glia of the optic tectum. After its release into the extracellular space, the protein may attach to the glial cell surface, consistent with previous observations that glia can bind cytotactin (21). Binding may be mediated by integral membrane receptors, of which integrins are good candidates (4, 32), or by other molecules such as the cytotactin-binding proteoglycan (28).

Differential localization of at least two cytotactin splice variants occurred in both neural and nonneural tissues. Both the CT and the VbVc probes detected mRNAs in the ventricular zone throughout the nervous system. In the outer layers of the optic tectum at late stages of development, a subpopulation of cells preferentially hybridized with the CT probe. Messenger RNAs reacting with both the CT and the VbVc probes are also expressed in lung epithelia, dermal condensations of the feather bud, and smooth muscle, but in other regions, such as perichondroblasts, epithelia of the choroid plexus, and tendons, no reactivity with the VbVc probe was observed (Table I). The differentially spliced type III repeats of cytotactin detected by the VbVc probe are located in the outer half of the arms of the hexabrachion (Fig. 1), a region shown to be important in its cell and molecular binding properties. The possibility arises that different combinations of type III repeats may have an effect on the binding of cytotactin (28, 29, 49) as has been shown for fibronectin (30, 31, 43, 54). Differential localization of alternatively spliced type III repeats in fibronectin mRNAs has also been observed during embryonic development in the chick (19, 20).

The identification of cell types synthesizing cytotactin at the epithelial–mesenchymal interface is essential in order to determine the sequence of reciprocal interactions in which cytotactin is involved during organogenesis. Our study indicates that both epithelia and mesenchyme can express cytotactin, a point that had been unclear in previous studies. In the case of the lung, the epithelia of the growing parabronchi expressed cytotactin mRNA and its levels of synthesis were higher at the end of the growing tubule, where differentiation and branching of the epithelia occur (16). Cytotactin mRNA was also detected in lung mesenchymal cells that are apposed to the tubules. In contrast, in the kidney, the mesenchyme appears to be the major source of cytotactin mRNA; no epithelial synthesis of cytotactin was observed in this or-

Table I. Distribution of Cytotactin mRNA and mRNA Containing the VbVc Differential Splice

	CT probe	VbVc probe
Lung		
Epithelia	+	+
Fibroblast, smooth muscle	+	−
Gizzard		
Epithelia	−	−
Smooth muscle	+	+
Tendon	+	−
Ganglia	+	+
Cartilage		
Perichondroblasts	+	−
Skeletal muscle	−	−
Gut		
Epithelia	−	−
Smooth muscle	+	+
Kidney	+	−
Brain		
Cerebellum		
ML	−	−
PCL	+	+
IG	+	+
Choroid plexus		
Epithelia	+	−
Tectum		
(Ventricular)	+	+

gan at any stage. Other authors have reported the induction in mesenchyme of cytotactin/tenascin in response to epithelial–mesenchymal interactions (1, 2, 33, 52). As demonstrated in this study, especially in cases where cytotactin is accumulated adjacent to the epithelia, in situ hybridization is required to determine definitively whether the epithelia or mesenchyme are synthesizing the molecule.

In the nervous system, cytotactin modulates both cell adhesion and cell migration. It was identified in this laboratory as a molecule mediating interactions between neurons and glia in vitro (26). Evidence supporting a role of cytotactin in cell migration in the cerebellum was obtained in perturbation experiments in cerebellar explant cultures (11). During cerebellar development, postmitotic granule cells in the external granular layer migrate along the Bergmann glial fibers crossing the molecular layer into the internal granule layer (46). Antibodies against cytotactin caused cells to pile up along the Bergmann glia and accumulate in the molecular layer suggesting that the cytotactin expression facilitates cell migration. In tissue culture assays, however, cytotactin inhibited the migration of neural crest cells in a dose-dependent manner (50). These apparently contradictory functions, adhesion and repulsion, could coexist given that the cytotactin molecule is composed of different domains (35, 49), an idea supported by recent studies on cytotactin/tenascin binding (49). The differential distribution of the spliced forms observed here may reflect the influence of various repeats on these functions.

In this context, it must be remembered that cytotactin does not act alone but in concert with other extracellular matrix proteins to which it binds, such as fibronectin (21, 29) and the cytotactin-binding proteoglycan (28). Thus the ability of the molecule to mediate cell adhesion and migration may itself be modulated by interaction with these other proteins. The design of appropriate biological assays should allow the dissection of these complex modulatory functions in morphogenesis as well as their relationship to the pattern of synthesis described here.

We thank Kim Drozdoski and Caroline Albanese for excellent technical assistance. We also thank Dr. Arturo Alvarez-Buylla for advice on avian brain development.

This work was supported by United States Public Health Service grants HD-09635, DK-04256, and Senator Jacob Javits Center of Excellence in Neuroscience Award, NS-22789. A. L. Prieto is supported by National Institutes of Health training grant GM-07524. F. S. Jones is a fellow of the Lucille P. Markey Charitable Trust. K. L. Crossin is a Becton Dickinson Young Faculty Fellow.

Received for publication 23 January 1990 and in revised form 4 April 1990.

References

1. Aufderheide, E., and P. Ekblom. 1988. Tenascin during gut development: appearance in the mesenchyme, shift in molecular forms, and dependence on epithelial–mesenchymal interactions. *J. Cell Biol.* 105:2341–2349.
2. Aufderheide, E., E. R. Chiquet-Ehrismann, and P. Ekblom. 1987. Epithelial–mesenchymal interactions in the developing kidney lead to expression of tenascin in mesenchyme. *J. Cell Biol.* 105:599–608.
3. Bignami, A., and D. Dahl. 1974. Astrocyte specific protein in neuroglial differentiation. An immunofluorescence study with antibodies to glial fibrillary acidic protein. *J. Comp. Neurol.* 153:27–37.
4. Bourdon, M. A., and E. Ruoslahti. 1989. Tenascin mediates cell attachment through an RGD-dependent receptor. *J. Cell Biol.* 108:1149–1155.
5. Bourdon, M. A., C. J. Wikstrand, H. Furthmayer, T. J. Matthews, and D. D. Bigner. 1983. Human glioma-mesenchymal extracellular matrix antigen defined by monoclonal antibody. *Cancer Res.* 43:2796–2805.
6. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
7. Bronner-Fraser, M. 1988. Distribution and function of tenascin during cranial neural crest development in the chick. *J. Neurosci. Res.* 21:135–147.
8. Chiquet, M., and D. M. Fambrough. 1984. Chick myotendinous antigen. II. A novel extracellular glycoprotein complex consisting of large disulfide-linked subunits. *J. Cell Biol.* 98:1937–1946.
9. Chiquet, M., and D. M. Fambrough. 1984. Chick myotendinous antigen. I. A monoclonal antibody as a marker for tendon and muscle morphogenesis. *J. Cell Biol.* 98:1926–1936.
10. Chiquet-Ehrismann, R., E. J. Mackie, C. A. Pearson, and T. Sakakura. 1986. Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell.* 47:131–139.
11. Chuong, C.-M., K. L. Crossin, and G. M. Edelman. 1987. Sequential expression and differential function of multiple adhesion molecules during the formation of cerebellar cortical layers. *J. Cell Biol.* 104:331–342.
12. Crossin, K. L., C.-M. Chuong, and G. M. Edelman. 1985. Expression sequences of cell adhesion molecules. *Proc. Natl. Acad. Sci. USA.* 82: 6942–6946.
13. Crossin, K. L., S. Hoffman, M. Grumet, J.-P. Thiery, and G. M. Edelman. 1986. Site-restricted expression of cytotactin during development of the chicken embryo. *J. Cell Biol.* 102:1917–1930.
14. Crossin, K. L., S. Hoffman, S.-S. Tan, and G. M. Edelman. 1989. Cytotactin and its proteoglycan ligand mark structural and functional boundaries in somatosensory cortex of the early postnatal mouse. *Dev. Biol.* 136: 381–392.
15. Dahl, D., and A. Bignami. 1973. Immunochemical and immunofluorescence studies of the glial acidic protein in vertebrates. *Brain Res.* 61: 279–293.
16. Dameron, F. 1961. L'influence de divers mésenchymes sur la différenciation de l'épithélium pulmonaire de l'embryon de poulet en culture *in vitro*. *J. Embryol. Exp. Morphol.* 9:628–633.
17. Daniloff, J. K., K. L. Crossin, M. Pinçon-Raymond, M. Murawsky, F. Rieger, and G. M. Edelman. 1989. Expression of cytotactin in the normal and regenerating neuromuscular system. *J. Cell Biol.* 108:625–635.
18. Erickson, H. P., and J. L. Iglesias. 1984. A six-armed oligomer isolated from cell surface fibronectin preparations. *Nature (Lond.).* 311:267–269.
19. ffrench-Constant, C., and R. O. Hynes. 1988. Patterns of fibronectin gene expression and splicing during cell migration in chicken embryos. *Development (Camb.).* 104:369–382.
20. ffrench-Constant, C., and R. Hynes. 1989. Alternative splicing of fibronectin is temporally and spatially regulated in the chicken embryo. *Development (Camb.).* 106:375–388.
21. Friedlander, D. R., S. Hoffman, and G. M. Edelman. 1988. Functional

mapping of cytotactin: proteolytic fragments active in cell-substrate adhesion. *J. Cell Biol.* 107:2329–2340.
22. Garner, C. C., R. P. Tucker, and A. Matus. 1988. Selective localization of messenger RNA for cytoskeletal protein MAP2 in dendrites. *Nature (Lond.).* 336:674–677.
23. Geliebter, J. 1987. Dideoxynucleotide sequencing of RNA and uncloned cDNA. *BRL Focus.* 9:5–8.
24. Grassi, S., F. Magni, M, Maurizi, and F. Ottaviani. 1984. A Stereotaxic Atlas of the Brain of the Domestic Chick. (*Gallus gallus*). Maggioli editore. Rimini.
25. Grobstein, C. 1956. Transfilter induction of tubules in mouse metanephric mesenchyme. *Exp. Cell Res.* 10:424–440.
26. Grumet, M., S. Hoffman, K. L. Crossin, and G. M. Edelman. 1985. Cytotactin, an extracellular matrix protein of neural and non-neural tissues that mediates glia-neuron interaction. *Proc. Natl. Acad. Sci. USA.* 82:8075–8079.
27. Gulcher, J. R., D. E. Nies, L. S. Marton, and K. Stefansson. 1989. An alternatively spliced region of the human hexabrachion contains a novel repeat of potential N-glycosylation sites. *Proc. Natl. Acad. Sci. USA.* 86:1588–1592.
28. Hoffman, S., and G. M. Edelman. 1987. A proteoglycan with HNK-1 antigenic determinants is a neuron-associated ligand for cytotactin. *Proc. Natl. Acad. Sci. USA.* 84:2523–2527.
29. Hoffman, S., K. L. Crossin, and G. M. Edelman. 1988. Molecular forms, binding functions, and developmental expression patterns of cytotactin and cytotactin-binding proteoglycan, an interactive pair of extracellular matrix molecules. *J. Cell Biol.* 106:519–532.
30. Humphries, M. J., A. Komoriya, S. K. Akiyama, K. Olden, and K. M. Yamada. 1987. Identification of two distinct regions of the type III connecting segment of the human plasma fibronectin that promote cell-type specific adhesion. *J. Biol. Chem.* 262:6886–6892.
31. Humphries, M. J., S. K. Akiyama, A. Komoriya, K. Olden, and K. M. Yamada. 1988. Neurite extension of the chicken peripheral nervous system neurons on fibronectin: relative importance of specific adhesion sites in the central cell-binding domain and alternatively spliced type III connecting segment. *J. Cell Biol.* 106:1289–1297.
32. Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Ceil.* 48:549–554.
33. Inaguma, Y., M. Kusakabe, E. J. Mackie, C. A. Pearson, R. Chiquet-Ehrismann, and T. Sakakura. 1988. Epithelial induction of stromal tenascin in the mouse mammary gland: from embryogenesis to carcinogenesis. *Dev. Biol.* 128:245–255.
34. Jones, F. S., M. P. Burgoon, S. Hoffman, K. L. Crossin, B. A. Cunningham, and G. M. Edelman. 1988. A cDNA clone for cytotactin contains sequences similar to epidermal growth factor-like repeats and segments of fibronectin and fibrinogen. *Proc. Natl. Acad. Sci. USA.* 85:2186–2190.
35. Jones, F. S., S. Hoffman, B. A. Cunningham, and G. M. Edelman. 1989. A detailed structural model of cytotactin: Protein homologies, alternative RNA splicing, and binding regions. *Proc. Natl. Acad. Sci. USA.* 86: 1905–1909.
36. Kruse, J., G. Keilhauer, A. Faissner, R. Timpl, and M. Schachner. 1985. The J1 glycoprotein: a novel nervous system cell adhesion molecule of the L2/HNK-1 family. *Nature (Lond.).* 316:146–148.
37. Kuhlenbeck, H. 1938. The ontogenic development and phylogenetic significance of the cortex telencephali in the chick. *J. Comp. Neurol.* 69:273–301.
38. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
39. LaVail, J. H., and W. M. Cowan. 1971. The development of the chick optic tectum. I. Normal morphology and cytoarchitectonic development. *Brain Res.* 28:391–419.
40. LaVail, J. H., and W. M. Cowan. 1971. The development of the chick optic tectum II. Autoradiographic studies. *Brain Res.* 28:421–441.
41. Mackie, E. J., R. P. Tucker, W. Halfter, R. Chiquet-Ehrismann, and H. H. Epperlein. 1988. The distribution of tenascin coincides with pathways of neural crest cell migration. *Development (Camb.).* 102:237–250.
42. Miller, F. D., C. C. G. Naus, M. Durand, F. Bloom, and R. Milner. 1987. Isotypes of α-tubulin are differentially regulated during neuronal maturation. *J. Cell Biol.* 105:3065–3073.
43. Obara, M., M. S. Kang, and K. M. Yamada. 1988. Site-directed mutagenesis of the cell-binding domain of human fibronectin: separable, synergistic sites mediate adhesive function. *Cell.* 53:649–657.
44. Pearson, C. A., D. Pearson, S. Shibahara, J. Hofsteenge, and R. Chiquet-Ehrismann. 1988. Tenascin: cDNA cloning and induction by TGF-B. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2977–2982.
45. Prieto, A. L., K. L. Crossin, B. A. Cunningham, and G. M. Edelman. 1989. Localization of mRNA for neural cell adhesion molecule (N-CAM) polypeptides in neural and nonneural tissues by *in situ* hybridization. *Proc. Natl. Acad. Sci. USA.* 86:9579–9583.
46. Rakic, P. 1985. Mechanisms of neuronal migration in developing cerebellar cortex. *In* Molecular Bases of Neural Development. G. M. Edelman, W. E. Gall, and W. M. Cowan, editors. John Wiley & Sons, Inc., New York. 139–160.
47. Romanoff, A. L. 1960. The Avian Embryo: Structural and Functional Development. MacMillan Publishing Co., New York. 211–362.
48. Sanes, J. R. 1989. Extracellular matrix molecules that influence neural development. *Annu. Rev. Neurosci.* 12:491–516.
49. Spring, J., K. Beck, and R. Chiquet-Ehrismann. 1989. Two contrary functions of tenascin: dissection of the active sites by recombinant tenascin fragments. *Cell.* 59:325–334.
50. Tan, S.-S., K. L. Crossin, S. Hoffman, and G. M. Edelman. 1987. Asymmetric expression in somites of cytotactin and its proteoglycan ligand is correlated with neural crest cell distribution. *Proc. Natl. Acad. Sci. USA.* 84:7977–7981.
51. Trapp, B. D., T. Moench, M. Pulley, E. Barbosa, G. Tennekoon, and J. Griffin. 1987. Spatial segregation of mRNA encoding myelin-specific proteins. *Proc. Natl. Acad. Sci. USA.* 84:7773–7777.
52. Vainio, S., M. Jalkanen, and I. Thesleff. 1989. Syndecan and tenascin expression is induced by epithelial–mesenchymal interactions in embryonic tooth mesenchyme. *J. Cell Biol.* 108:1945–1954.
53. Vaughan, L., S. Huber, M. Chiquet, and K. H. Winterhalter. 1987. A major, six-armed glycoprotein from embryonic cartilage. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:349–353.
54. Wayner, E. A., A. Garcia-Pardo, M. J. Humphries, J. A. McDonald, and W. G. Carter. 1989. Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. *J. Cell Biol.* 109:1321–1330.