Developmental Expression Pattern of the *cdo* Gene

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ABSTRACT **CDO is a cell-surface protein of the immunoglobulin/fibronectin type III repeat family that positively regulates myogenic differentiation in vitro. To gain a better understanding of the role of *cdo* during vertebrate development, we carried out an extensive in situ hybridization study to characterize its expression pattern from postimplantation to late stages of mouse embryogenesis and in rat brain from E13 to adult. Our results show a broad pattern of *cdo* expression that is spatially and temporally restricted during embryogenesis. In the central nervous system (CNS), *cdo* expression is detected as early as E7.5 and maintained in the dorsal ventricular zones of the brain and spinal cord, becoming increasingly restricted in the adult. High levels of *cdo* are detected in developing sensory organs, such as the eye and ear. Outside the CNS, *cdo* is expressed mainly in neural crest and mesodermal derivatives, including skeletal muscle precursors. Overall, the highest levels of *cdo* expression are seen from E9.0 to E15.5. The temporal onset and restricted expression of *cdo* suggest that *cdo* plays a role in the determination and/or differentiation of a number of cell types during embryogenesis.** © 2000 Wiley-Liss, Inc.

Key words: CDO; immunoglobulin superfamily; in situ hybridization; mouse development; Robo

INTRODUCTION

Vertebrate embryogenesis requires the coordinated proliferation, migration, determination, and differentiation of a number of cell and tissue types. The ability of cells to interact with and respond to their environment is critical for these events. How a particular cell will respond to extracellular cues depends on the repertoire of cell-surface molecules it expresses and the integration of the various signals it receives. A wide variety of cell-surface molecules function during embryogenesis, including growth factor receptors, integrins, and cell adhesion molecules (CAMs). Among the CAMs, the neural cell adhesion molecule (N-CAM) family is of particular interest, especially in the developing nervous system. The N-CAM family is defined by the presence of immunoglobulin (Ig)-like domains and fibronectin type III (FNIII)–like repeats in their extracellular regions. Members of the family include DCC, neogenin, L1, and axonin (Brummendorf and Rathjen, 1995).

We have cloned a cell-surface molecule, CDO (CAM-related, down-regulated by oncogenes; pronounced "kiddo"), whose expression is regulated in fibroblast cell lines by oncogenes, serum growth factors, and cell-substratum adhesion (Kang et al., 1997). CDO contains five Ig-like domains and three FNIII-like repeats in its extracellular region, a transmembrane domain, and a unique 270-amino-acid intracellular region. Along with the ROBO/sax-3 axon guidance molecules and RIG (for which a function is yet to be identified) (Kidd et al., 1998; Zallen et al., 1998; Yuan et al., 1999), CDO is a founding member of the "five (Ig) plus three (FNIII)" subfamily of CAMs. We have mapped the human *cdo* gene to chromosome 11q23-24, a region in close proximity to N-CAM, which suggests that an evolutionary linkage may exist between these two molecules (Kang et al., 1997).

Studies of CDO in myoblast cell lines strongly suggest a role for this protein in skeletal myogenesis (Kang et al., 1998). CDO expression is transiently up-regulated during differentiation of C2C12 myoblasts. Overexpression of CDO in these cells enhanced differentiation, whereas expression of dominant negative forms of CDO inhibited differentiation. Furthermore, the Ras oncoprotein blocked differentiation of C2C12 cells in a manner dependent on down-regulation of CDO, a situation precisely mirrored by MyoD. These and additional studies (Kang et al., 1998) suggested a model in which CDO and MyoD participate in a positive feedback loop.

Although the pattern of *cdo* expression in vivo has yet to be characterized thoroughly, preliminary studies have indicated that *cdo* is present in myogenic lineages and is regulated developmentally. In situ hybridization on mouse embryos indicated that *cdo* is expressed in the developing central nervous system (CNS) and the early myogenic compartment (Kang et al., 1998, and unpublished data). However, *cdo* mRNA is detectable in most adult rat tissues only by use of highly sensitive

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reverse transcriptase polymerase chain reaction techniques, indicating that *cdo* is expressed at extremely low levels postdevelopmentally (Kang et al., 1997). These findings, combined with CDO's ability to regulate cell differentiation, indicate the value in identification of sites of *cdo* expression during development.

In the present study, we report a detailed in situ hybridization analysis of *cdo* expression during mouse embryogenesis and in the developing rat nervous system. Our results show that *cdo* is expressed from early gastrulation through late stages of embryogenesis in several tissues. Furthermore, the spatial and temporal expression pattern suggests that *cdo* plays an important role in the determination and/or differentiation of a number of cell types.

RESULTS

cdo mRNA Expression Begins During Gastrulation and Continues Throughout Embryogenesis

To address the role of *cdo* during postimplantation to late fetal development, whole-mount and thin-section in situ hybridizations were carried out on mouse embryos. *cdo* mRNA was detected throughout development with a dynamic pattern of expression. Using a ^{35}S-labeled antisense probe, we first detect *cdo* expression in the uterine decidua at E6.5, whereas there is no detectable expression in the embryo proper and extraembryonic membranes (data not shown). Expression in the embryo is first observed during gastrulation, around E7.0–7.5, when diffuse signal is detected using either digoxygenin or ^{35}S-labeled antisense probes in the embryonic mesoderm and ectoderm but not in the embryonic endoderm (Fig. 1A and data not shown). By E8.5, high levels of *cdo* expression are restricted to the cephalic region and the dorsal aspects of the neural folds (including neural crest) and somites (Fig. 1B and data not shown). Expression is maintained through the beginning of neural tube closure and during stages of organogenesis and is present in many tissues by E15.5 (Figs. 1–5 and data not shown). Expression persists into late stages of embryogenesis, becoming even more restricted. By late fetal development, expression declines to undetectable levels in most tissues (Figs. 1–5 and data not shown).

cdo Expression in the Developing Musculoskeletal System

The majority of skeletal muscle and axial skeleton originates from the somites (Hauschka, 1994; Christ and Ordahl, 1995; Kaufman, 1995; Kaufman and Bard, 1999). At the earliest stages of somite formation, high levels of *cdo* expression are restricted to the dorsal and lateral aspects of the somite, which give rise to the dermamyotome (Fig. 3A,B). Upon somite maturation and the initiation of myoblast migration (e.g., into the limb buds), *cdo* expression is maintained at high levels in the dermamyotome, myotome, and limb buds (Figs. 1C,D; 2A–C; 3C,D). The level of *cdo* expression in migrating myoblasts could not be discerned because of high levels of *cdo* expression in the limb mesenchyme (see later discussion). *cdo* expression decreases in maturing myoblasts during their differentiation into myotubes around E13.5 (Fig. 3E,F) (Ontell and Kozeka, 1984), though low-level expression appears to be maintained in most muscles as late as E17.5 (Fig. 2E and data not shown). Additionally, *cdo* expression is maintained in another derivative of the dermamyotome, the dermis, as late as E17.5 (Figs. 2D,E; 3F; and data not shown).

No *cdo* expression is seen in the sclerotome, an early stage of vertebra and rib development (Fig. 3A–D). However, by E13.5 *cdo* mRNA is detected in the loose mesenchyme surrounding the ribs (Fig. 2C and data not shown) but is not detectable in the more condensed mesenchyme that is beginning to form cartilage. Progenitors of the appendicular skeleton arise from lateral plate mesodermal condensations, which form a cartilaginous skeleton that eventually gives rise to the limb bones (Kaufman, 1995; Kaufman and Bard, 1999). In contrast to the absence of *cdo* expression in the early rib and vertebrae mesenchymal primordia, *cdo* expression is detected in the proximal anterior region of the limb bud mesenchyme as early as E9.5 (Figs. 1C,D and 3C,D). As the limb mesenchyme condenses to form cartilage at later stages of development (E12.5–15.5), *cdo* expression is maintained in the loose mesenchyme surrounding the condensations with a pattern similar to that seen in rib development (Fig. 3E,F). *cdo* transcripts also are detected in regions harboring proliferating cells of the epiphyseal plate (Fig. 3H).

The bones and muscle of the head derive from neural crest cells and craniofacial mesenchyme (Noden, 1983, 1991). *cdo* expression is detected first in the loose mesenchyme of the head folds at E7.5 and later in the mesenchyme of the head and branchial arches (Figs. 1 and 2B). During later stages of development (after E11.5), expression becomes restricted to the loose mesenchyme and developing musculature (Figs. 2C,D and 3G). No *cdo* expression is observed in the condensed cartilage of the head and neck or in regions harboring mesenchyme undergoing intramembranous ossification (data not shown). Strong *cdo* expression is detected in the regions of joint formation. In the axial skeleton, *cdo* is expressed in the intervertebral discs and the areas where the ribs articulate with the vertebrae (Figs. 2C and 3G). This pattern of expression also is observed in the appendicular skeleton and is illustrated most profoundly in the wrist and ankle joints (Fig. 3H).

cdo Expression in the Developing Nervous System

To obtain a detailed understanding of the dynamic expression of *cdo* in the nervous system, an extensive analysis of *cdo* expression was conducted in the rat

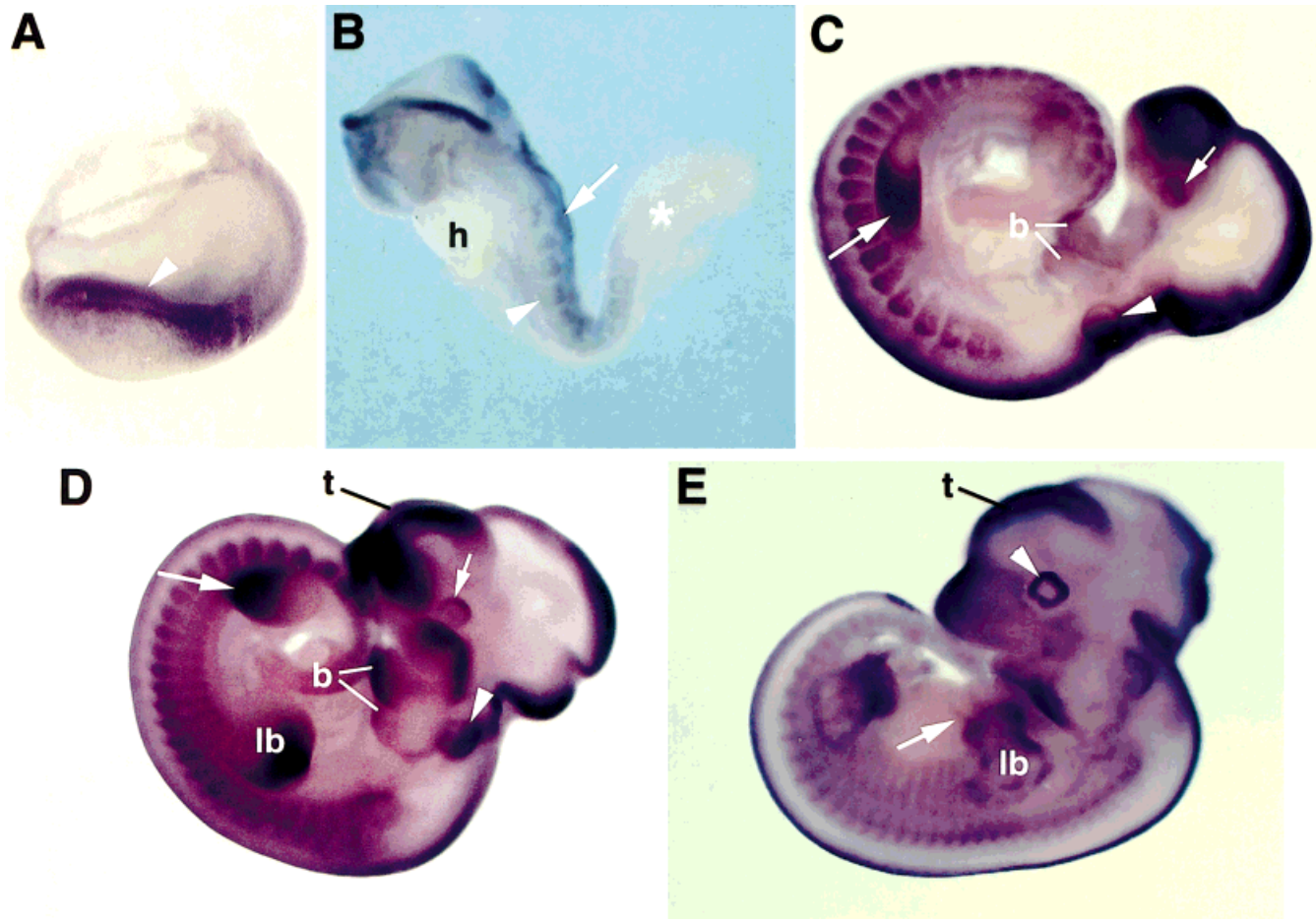

Fig. 1. *cdo* expression in mouse embryos by whole-mount in situ hybridization. **A:** E7.5 embryo. Signal can be detected in the neural folds (arrowhead). **B:** E8.5 embryo. Signal is detected in the dorsal neural tube (arrow), head folds, and somites (arrowhead). No expression is detected in presomitic mesoderm (asterisk) or heart tube. **C,D:** E9.5 and E10.5 embryos, respectively. Expression is maintained in the dorsal neural tube, somites, and head mesenchyme in the region of the nasal processes. Expression also can be detected in the anterior of the limb buds (large arrows), telencephalon, branchial arches, eye (small arrows), and otic vesicle (arrowheads). **E:** E11.5 embryo. Expression is still detected in the structures described in panels C and D, with some differences. In the limb buds, expression of *cdo* is associated with developing structures, such as the digits in the hand plate (arrow). In the eye, high-level expression also is detected in the developing retina (arrowhead). b, branchial arches; h, heart; lb, limb bud; t, telencephalon.

brain and spinal cord from embryonic day 13 (E13) to adult (Fig. 4) and in the rat eye from E13 to E16 (Fig. 4). Comparison of *cdo* expression in several tissues of rat and mouse has revealed that *cdo* expression is similar in these two rodents (P.J.M. and R.S.K., unpublished studies; summarized in Fig. 5). Throughout embryonic and early postnatal development, *cdo* expression in the brain is restricted primarily to dorsal regions, in the telencephalon (Fig. 4A–K) and diencephalon (Fig. 4A–D) as well as in the midbrain (Fig. 4L,M). Little if any *cdo* expression is detected in the ganglionic eminence and other ventral structures.

In the dorsal telencephalon, *cdo* is expressed in the neuroepithelium harboring cortical progenitor cells from E13 to E19 (Fig. 4A–G) (Altman and Bayer, 1995; Bayer and Altman, 1991). Expression of *cdo* at these stages is specific to the ventricular zone, and no expression is detected in the subventricular zone (Fig. 4A–G and data not shown). High levels of *cdo* expression persist in regions of the dorsal telencephalon lining the ventricle throughout late embryonic and early postnatal stages (E20 through P6; Fig. 4H–K), after the disappearance of the cortical neuroepithelium. Cells that express *cdo* at these developmental stages appear to be in the subventricular zone (Fig. 4H–J) (Bayer and Altman, 1991; Altman and Bayer, 1995) and the rostral migratory stream of olfactory interneuron precursors (Fig. 4H, I) (Altman, 1969; Luskin, 1993; Lois and Alvarez-Buylla, 1994). From late postnatal stages (≥ P6) to adult, *cdo* expression in the telencephalon becomes restricted to the hippocampus and to a narrow

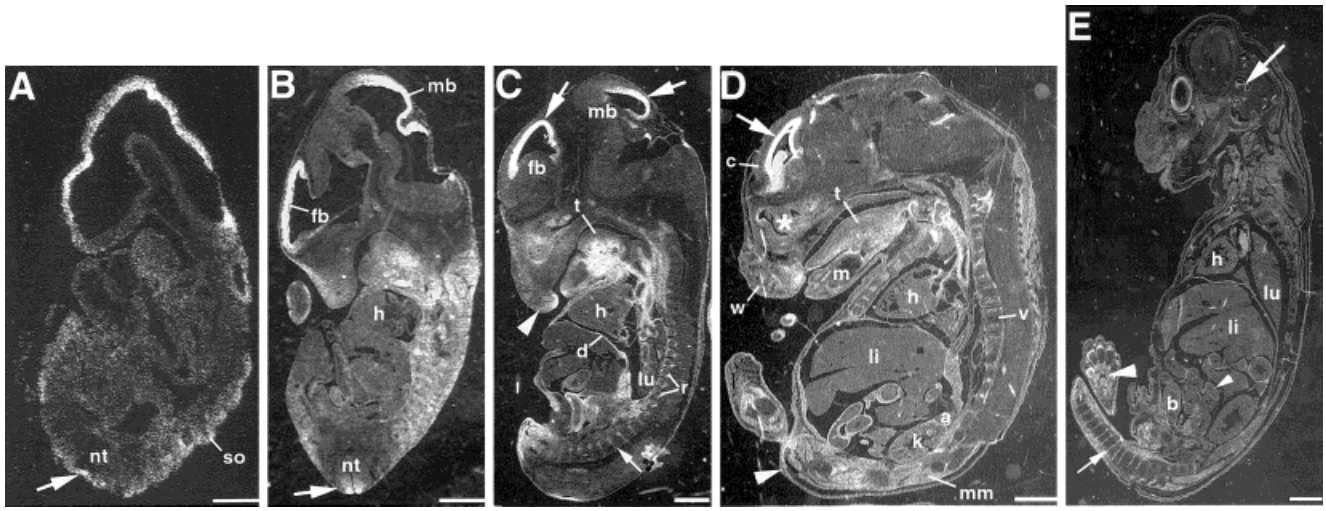

Fig. 2. *cdo* expression in mouse embryos by thin-section in situ hybridization. **A:** Parasagittal section of an E9.5 embryo. High-level *cdo* expression is detected in the dorsal neural tube (arrow), dermamyotome of somites, and dorsal aspects of the developing brain. **B:** Parasagittal section of an E11.5 embryo. *cdo* expression is detected in the dorsal neural tube (arrow), dermamyotome, dorsal forebrain, and midbrain, tongue, and mandibular component of the first branchial arch and the mesenchyme of the nasal process. **C:** Parasagittal section of an E13.5 embryo. *cdo* expression is most prominent in the ventricular zones of the dorsal forebrain and midbrain (arrows) and in the mesenchyme surrounding bronchi, ribs, intestines, and bladder. It is also most prominent in the mesenchyme of facial structures, including the upper lip (arrowhead), tongue, and mandible; the intervertebral disks (thin arrow); the mesenchyme surrounding the ribs, intestines, and bladder; and the diaphragm. **D:** Parasagittal section of an E15.5 embryo. Expression is detected in many structures, including but not limited to the ventricular zone of the cortex (arrow), olfactory epithelium (asterisk), muscle masses, kidney capsule, adrenal cortex, intervertebral disks, smooth-muscle layers of the digestive tract, dermis and mesenchyme of the whisker pad, tongue, trachea, lungs, and kidney (arrowhead). **E:** Parasagittal section of an E17.5 embryo showing *cdo* expression in the intervertebral disks (small arrow), smooth-muscle layers of the digestive tract (small arrowhead), inner ear epithelium (large arrow), dermis and mesenchyme of the whisker pad, and hindpaw (large arrowhead). a, adrenal gland; b, bladder; c, cortex; d, diaphragm; fb, forebrain; h, heart; k, kidney; li, liver; lu, lung; m, mandible; mb, midbrain; mm, muscle mass; nt, neural tube; r, ribs; so, somite; t, tongue; tr, trachea; v, vertebra; w, whisker pad. Scale bars: A = 200 μm; B–E = 1 mm.

band of ependymal cells that line the ventricle (Fig. 4K,O,P).

In the diencephalon, *cdo* expression appears to become increasingly restricted to dorsal regions during development (Altman and Bayer, 1979; Altman and Bayer, 1995). At E13, *cdo* is expressed throughout the thalamic neuroepithelium of the dorsal diencephalon, in a dorsoventral gradient (Fig. 4A). By E14, the region of *cdo* expression is restricted further to the dorsal thalamic neuroepithelium, encompassing about one-fourth of the diencephalon (Fig. 4B). By late embryonic stages *cdo* is expressed in a small region of epithalamic neuroepithelium at the dorsal tip of the diencephalon (E19; Fig. 4D). In coronal sections of E19 brain that include the pineal gland primordium, *cdo* expression is detected in a small region of neuroepithelium adjacent to the posterior commissure (Fig. 4D).

cdo expression also is present in the neuroepithelium of the developing midbrain (Fig. 4L,M). As with forebrain structures, expression in the midbrain also is restricted to neuroepithelium on the dorsal side of the structure, to the superior and inferior tectal neuroepithelium. Expression of *cdo* is not detected in the pontine neurepithelium, which lines the ventral side of the ventricle. *cdo* is not expressed in the developing cerebellum throughout embryonic and early postnatal stages (Fig. 4L–N). However, high levels of *cdo* expression are detected in the adult cerebellum, in regions harboring cerebellar granule cells (Fig. 4Q) (Palay and Chan-Palay, 1974; Altman and Bayer, 1979).

The restricted expression of *cdo* to dorsal regions also occurs in the spinal cord (Altman and Bayer, 1984). In transverse sections of E13 spinal cord at the forelimb level, *cdo* is expressed in a dorsoventral gradient in the ventricular zone (Fig. 4R). In the spinal cord of E14 embryos, the expression of *cdo* is restricted to the very dorsal end of the developing spinal cord, in the roof plate and some surrounding cells (Fig. 4S). After the cessation of proliferation by roof plate cells (Altman and Bayer, 1984), *cdo* expression in the E16 spinal cord is restricted to a narrow band of cells that extends along the anterior–posterior axis (Fig. 4T).

cdo Expression Elsewhere During Development

Although the developing musculoskeletal and nervous systems are the most prominent sites, significant *cdo* expression is seen in a number of developing systems and structures. For example, high-level *cdo* expression is detected in such sensory organs as the eye, inner ear, olfactory apparatus, and whisker pad. In the eye, *cdo* expression is first detected in the neural retina (E9.5) and lens (E10.5), but by E17.5 it becomes re-

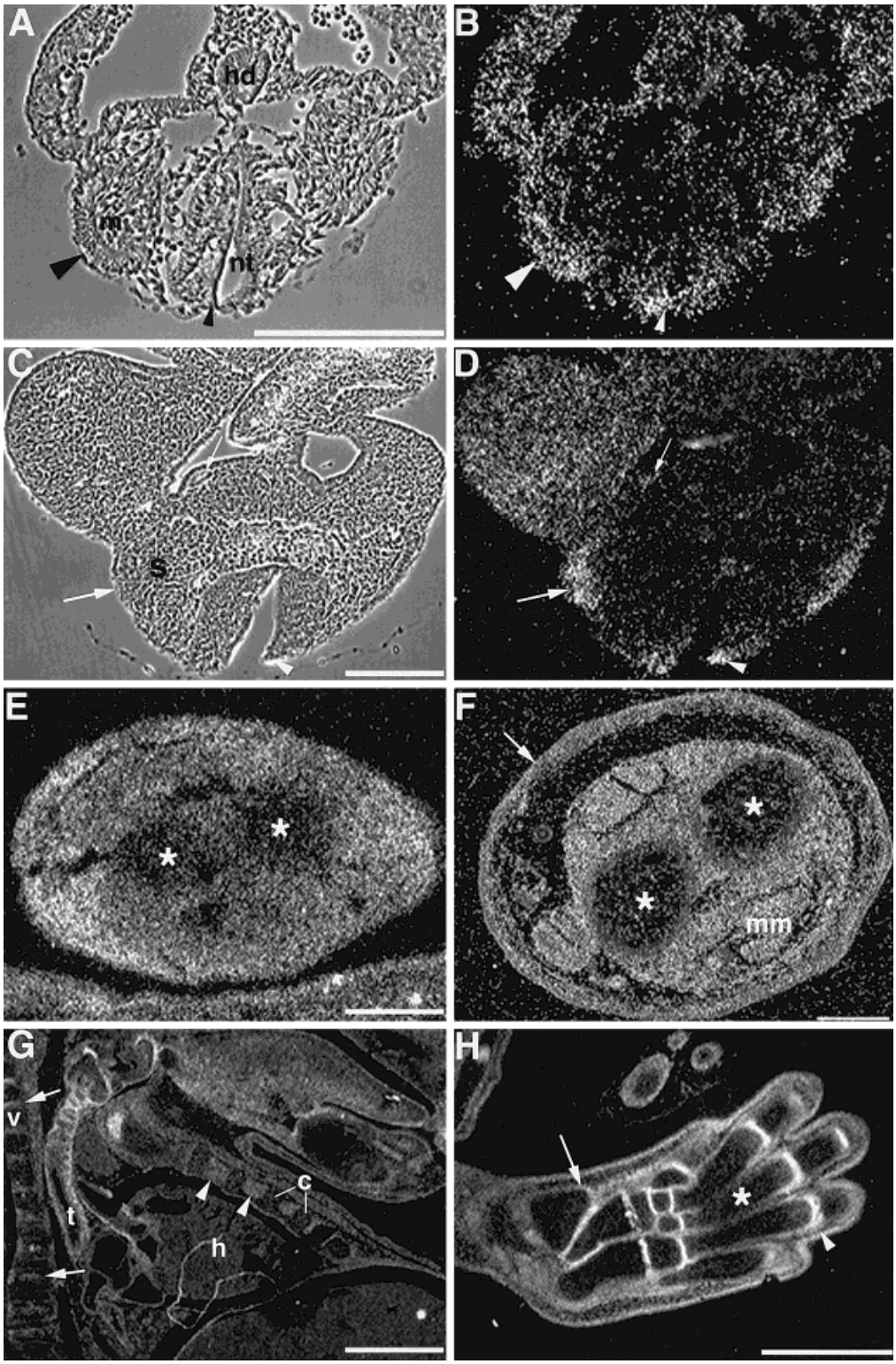

Fig. 3. *cdo* expression in the musculoskeletal system. **A, B:** Phase-contrast (A) and darkfield (B) images of a transverse section through an E9.5 mouse embryo showing *cdo* expression in the dorsal neural tube (small arrowheads) and dorsolateral aspects of epithelialized somites (large arrowheads). Note the lower level of expression in the intermediate and body wall mesoderm and in the mesenchyme surrounding the hindgut diverticulum as well as in the cells of the myocele. **C,D:** Phase-contrast (C) and darkfield (D) images of a transverse section through an E10.5 embryo. *cdo* expression is detected in the dorsal lips of the neural tube (arrowheads), dermamyotome (large arrows), and limb bud mesenchyme. Also note the expression in the mesonephric duct (small arrows). No expression is detected in the sclerotome. **E:** Frontal section through forelimb of an E12.5 embryo. *cdo* is expressed in the mesenchyme and premuscle masses but is not expressed in the condensed mesenchyme of the future limb bones (asterisks). **F:** Frontal section through forelimb of an E15.5 embryo. *cdo* is expressed in the muscle masses and dermis (arrow) of the limb but not in the epidermis or bones (asterisks). **G:** Parasagittal section through an E15.5 embryo. *cdo* expression is detected in the intervertebral disks (arrows), in the mesenchyme of the trachea, and between the costal cartilage (arrowheads); however, no expression is detected in the vertebral bodies, the cartilaginous tracheal rings, or the costal cartilage. **H:** Parasagittal section through an E15.5 embryo showing high levels of *cdo* expression in the mesenchyme surrounding the cartilage condensations of the foot (arrow) and in the regions of joint formation (arrowhead). However, there is no *cdo* expression in the cartilage (asterisk). c, costal cartilage; h, heart; hd, hindgut diverticulum, m, myocele; mm, muscle mass; nt, neural tube; s, sclerotome; t, trachea; v, vertebra. Scale bars: A,C,E,F = 200 μm; G,H = 1 mm.

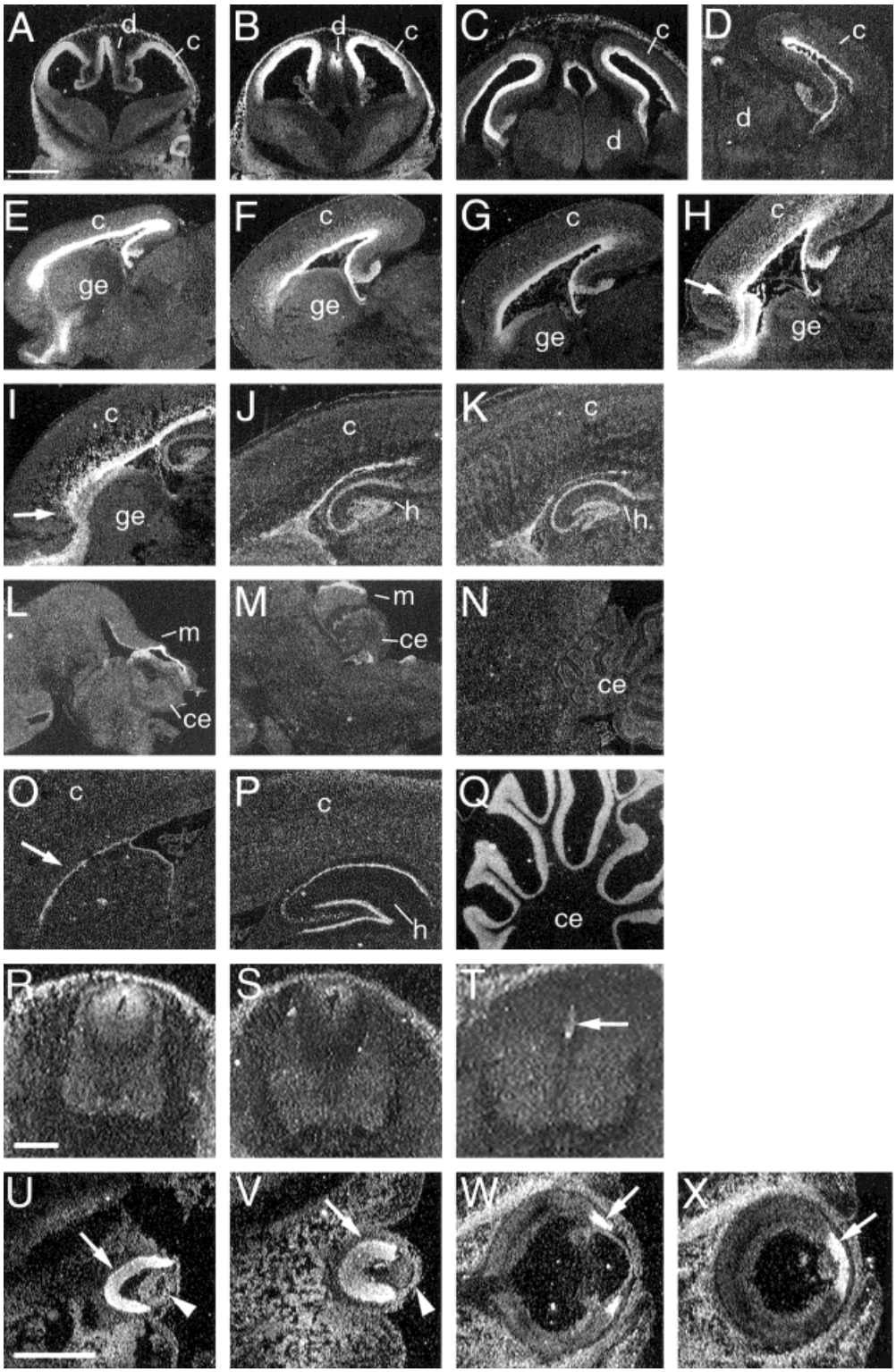

Fig. 4. *cdo* expression in the developing rat brain. **A–Q:** Coronal (A–D) and parasagittal (E–Q) sections through rat brains at E13 (A), E14 (B), E16 (C), E17 (E,L), E18 (F), E19 (D,G), E20 (H,M), P0 (I,N), P3 (J), and P6 (K) and adult (O–Q). Prominent *cdo* expression is detected in the dorsal ventricular (A–G) and subventricular (H,I) zones of the developing cortex, diencephalon, and midbrain. In late embryonic and early postnatal animals, *cdo* expression is detected in the rostral migratory stream (arrow in H,I). In the adult, *cdo* expression is detected in presumptive ependymal cells (arrow in O), the hippocampus (P), and the granule cell layer of the cerebellum (Q). No expression is detected in ventral structures or in the differentiated layers of the cortex. **R–T:** Transverse sections through rat embryos at E13 (R), E14 (S), and E16 (T) showing *cdo* expression in the dorsal ventricular zone of the developing spinal cord. Expression becomes restricted to a narrow band of cells by E16 (arrow in T). c, cortex; ce, cerebellum; d, diencephalon; h, hippocampus; ge, ganglionic eminence. **U–X** Coronal sections through the developing rat eye at E13 (U), E14 (V), E16 (W), and E16 (X). High levels of *cdo* expression are detected in the neural retina (arrows in U and V). Expression becomes restricted to a band of cells around the developing lens (arrows in W and X). Lower *cdo* expression can be detected in the forming lens vesicle (arrowhead in U) and cornea (arrowhead in V) but is lost as these structures mature Scale bars: (A,U) = 1 mm; (R) = 0.5 mm.

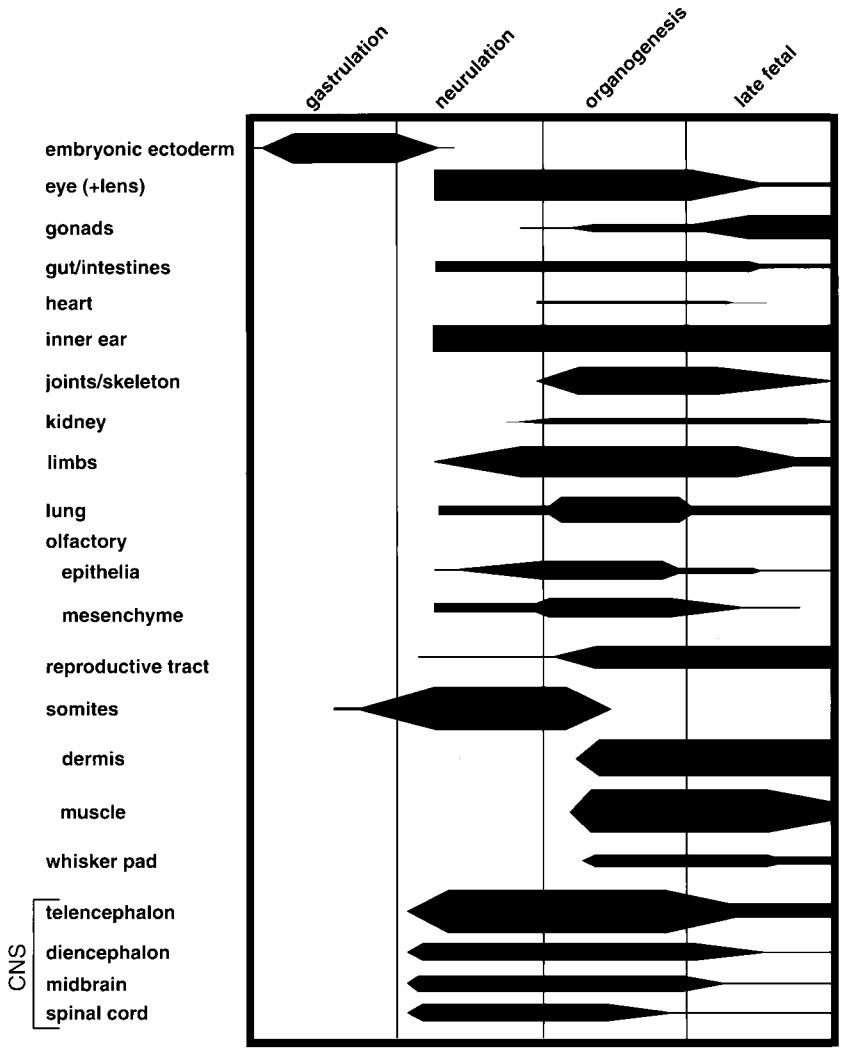

Fig. 5. Summary of *cdo* expression during mouse embryogenesis. Tissues expressing detectable levels of *cdo* transcripts are indicated in the left-hand column. The thickness of the lines indicates relative signal intensity as taken from multiple sections of embryos ranging from E6.5 to E17.5. Gastrulation is defined as E6.5 to E8.0, neurulation as E8.5 to E10.5, organogenesis as E11.5 to E13.5, and late fetal development as E14.5 to E17.5. See text for description of specific sites of expression within the developing structures listed in the left-hand column.

stricted to the ciliary bodies (Fig. 4U–X). Similarly, *cdo* expression in the olfactory mesenchyme and epithelia is high at E11.5, but low by E17.5 and only in the epithelia (Figs. 1B–E and 2A–E). In contrast, the mesenchyme of the whisker pad and the epithelia of the inner ear maintain high-level *cdo* expression throughout their development.

Other sites of *cdo* expression include several internal organs and the urogenital system. From E9.5, high levels of *cdo* expression are detected in the lung mesenchyme surrounding large airways (Fig. 2C and data not shown). However, as development proceeds, high-level expression becomes restricted to the mesenchyme of the trachea, with some low expression persisting in the lung mesenchyme (Figs. 2D,E and 3G). A similar pattern of expression is seen in the developing digestive system. At E9.0, *cdo* expression is detected in the mesenchyme of the primitive gut (Fig. 3A,B). By E11.5, broad expression is detected only in the tongue and uppermost part of the digestive tract, whereas more caudally (esophagus, stomach, and intestines), expression is restricted to the smooth-muscle layers (Figs. 2B–E and 3G and data not shown). Although no *cdo* expression is seen in the endocardium or musculature of the developing heart, *cdo* transcripts are detected transiently (E10.5–E15.5) in the pericardial mesothelium and developing outflow tract (data not shown). In the urogenital system, *cdo* expression is detected at low levels in the mesonephros and gonadal primordium at E10.5 (Fig. 3C,D and data not shown). By E17.5, expression increases and is detected in the mesenchyme and mesothelium of many reproductive tract structures, such as the oviducts and mesorchium, as well as in the stromal cells of the gonads (data not shown). *cdo* expression also is detected in the renal mesenchyme, capsule, and a subset of forming tubules (suggesting very transient expression in all tubules as they develop) and in the smooth-muscle layers of the bladder (Fig. 2D,E and data not shown). A summary of the spatiotemporal pattern of *cdo* expression is presented in Fig. 5.

DISCUSSION

We report here an extensive in situ hybridization analysis of *cdo* expression from postimplantation to late fetal stages of murine development and in the rat CNS from E13 to adult. *cdo* is expressed in a broad spectrum of tissues during development in a temporally and spatially restricted pattern. Expression begins at early stages of gastrulation and is maintained throughout embryogenesis. However, within specific tissues the initiation and maintenance of expression are tightly regulated. In some cases, expression begins even before specific cell types have been determined, whereas in other instances, expression is detected in determined cell types that have not terminally differentiated. In either case, however, *cdo* expression becomes more restricted as development progresses (Fig. 5).

In the musculoskeletal system, *cdo* is expressed in the dorsolateral region of epithelialized somites, a time when cell types have not been determined (Hauschka, 1994). However, this region gives rise to the dermamyotome, and *cdo* expression is maintained in the muscle and dermis, derivatives of the dermamyotome (Hauschka, 1994; Kaufman and Bard, 1999). Expression is reduced at later stages of development, which is consistent with our in vitro data that *cdo* is expressed in undifferentiated myoblasts but is down-regulated in muscle cells (Kang et al., 1998). No *cdo* expression is detected in the sclerotome or vertebral bodies at any time, but expression is detected in the intervertebral disks; the mesenchyme surrounding developing ribs, vertebral arches, and limb bones; and at the sites of bone articulations. Thus, *cdo* expression is detected in specific regions of the developing skeletal system, including regions of joint formation, and may serve as a molecular marker that can differentiate between skeletal elements derived either from sclerotome or from other cell types.

Within the CNS, *cdo* expression is most prevalent in dorsal structures, specifically in regions harboring proliferating cells, such as the ventricular zones of the cortex and thalamus (Altman, 1969; Altman and Bayer, 1979; Bayer and Altman, 1991; Altman and Bayer, 1995). *cdo* is not expressed in developing ventral structures, such as striatum or hypothalamus, and therefore may molecularly distinguish the progenitor cells in these two regions. During the course of development, *cdo* expression becomes increasingly restricted, and in the adult expression is detected only in cerebellar granule cells and some ependymal cells. The expression of *cdo* in ventricular cells of the CNS but not in postmitotic, differentiating neurons is interesting in light of the role of other Ig/FNIII family members in axon guidance. *cdo* mRNA clearly is not expressed in CNS neurons at times of axon outgrowth, strongly suggesting that CDO is not an axon guidance receptor, despite its structural resemblance to Robo-like receptors. Its presence in ventricular cells is more consistent with the role of CDO in the regulation of proliferation, differentiation, or the earliest stages of neuronal migration. Given the known parallels between myogenesis and neurogenesis (Yun and Wold, 1996), it is tempting to speculate that CDO may function to mediate differentiation of neural precursors.

During development of the internal organs, *cdo* expression can be detected in many tissues but only within restricted regions of each. For example, *cdo* expression is restricted to the smooth-muscle layers of the gut, the mesenchyme surrounding larger airways of the lung, and specific areas of the developing kidney and reproductive tract. Taken together, this broad yet restricted expression pattern suggests that *cdo* plays a role during the development of particular structures and/or cell types within these tissues.

Other prominent sites of *cdo* expression are the sensory organs and integumentary system. High levels of *cdo* expression are detected in the early development of the optic, otic, and olfactory systems. Expression becomes more restricted as each of these systems develops. In the integumentary system, *cdo* expression is restricted to the mesodermal derivatives. Therefore, the sensory organs and integumentary system are also potential sites of *cdo* function during development.

While the exact identity of all cell types that express *cdo* could not be determined in this study, the very complex and dynamic expression pattern during vertebrate development suggests that *cdo* functions in a wide variety of cellular contexts. Although the common feature of cells that express *cdo* remains elusive, one decision most cells must make during development is when to stop proliferation and begin differentiation. Perhaps CDO plays a role in coordinating these events. This possibility is consistent with extensive data implicating CDO as a positive regulator of myogenic differentiation in vitro (Kang et al., 1998). It will be of interest, therefore, to identify what role *cdo* plays in other cell types. The identification of sites of *cdo* expression during embryogenesis that is reported here will be a valuable resource in determining the function(s) of *cdo* at the cellular and organismal level.

EXPERIMENTAL PROCEDURES

In Situ Probe Preparation

Digestion of a mouse *cdo* cDNA clone in pBluescript(KS−) (Stratagene, La Jolla, CA) with *Xho*I generated a linearized, 2.4-kb DNA template encompassing part of the fifth Ig domain, all three FNIII repeats, the transmembrane and intracellular domains, and approximately 300-bp of 3′ UTR. The probe used for rat in situ hybridization, a 1.2-kb fragment of the 3′ UTR, was isolated from a cDNA library, generated from individual E14 rat neurepithelial cells using the modified methods of Dulac and Axel (1995), and cloned into PCRII (Invitrogen, La Jolla, CA). Radiolabeled antisense riboprobe was transcribed from 300 ng template

DNA with T7 RNA polymerase in a reaction containing 5 mM each of CTP, ATP, and GTP; 10 mM dithiothreitol (DTT); 1× Transcription Buffer (Boehringer Mannheim, Indianapolis, IN); RNasin (Promega, Madison, WI); and ^{35}S-UTP (>1,000Ci/mmol; NEN, Boston, MA). Digoxygenin-labeled probe was generated from 1 μg template DNA with T7 RNA polymerase according to the manufacturer's DIG RNA labeling kit protocol (Boehringer Mannheim). Both probes were incubated with hydrolysis solution A (50 mM DTT, 40 mM $NaHCO_3$, 60 mM Na_2CO_3) for 1 hr at 37°C, followed by treatment with neutralization solution B (50 mM DTT, 5% acetic acid, 100 mM NaAcetate). Probes were precipitated before use.

Whole-Mount In Situ Hybridization

Whole-mount in situ hybridization was performed as previously described (Hemmati-Brivanlou et al., 1990; Henrique et al., 1995). Briefly, embryos were dissected in phosphate-buffered saline (PBS) and fixed overnight in 4% paraformaldehyde. Embryos were dehydrated through a methanol series and stored at −20°C. Embryos were rehydrated into PTW (1× PBS, 0.1% Tween-20) and bleached with 6% hydrogen peroxide in PTW. After washing, embryos were treated with 10 μg/mL proteinase K in PTW according to stage (5 min for E7.5 up to 20 min for E10.5 or older). Embryos were rinsed, postfixed, and equilibrated into hybridization mix (50% formamide, 1.3× SSC, 5 mM EDTA, 50 μg/ml yeast RNA, 0.2% Tween-20, 0.5% CHAPS [3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate], 100 μg/mL heparin) at 65°C. Embryos were incubated with digoxygenin-labeled probe in hybridization mix overnight at 65°C. After washing, embryos were blocked and incubated overnight at 4°C with a 1/2,000 dilution of alkaline phosphatase–conjugated anti-digoxygenin antibody (Boehringer Mannheim) in blocking buffer [2% Boehringer Blocking Reagent (Boehringer Mannheim), 20% heat-inactivated sheep serum in MABT (100 mM maleic acid at pH7.5, 150 mM NaCl, 0.1% Tween-20)]. Following washes in MABT and NTMT [500 mM NaCl, 100 mM Tris HCl at pH 9.5, 50 mM $MgCl_2$, 1% Tween-20], color was developed using BM Purple AP Substrate, Precipitating (Boehringer Mannheim).

Thin-Section In Situ Hybridization

Hybridizations were performed as previously described (Sassoon and Rosenthal, 1993). Briefly, embryos were dissected and fixed in 4% paraformaldehyde overnight at 4°C. Following dehydration through an ethanol series, embryos were embedded in paraffin, sectioned at 5–10 μm, and placed on 3-aminopropyltriethoxysilane-treated slides. Sections were rehydrated, incubated with 20 μg/mL proteinase K, and treated with acetic anhydride in 0.1 M triethanolamine. Dehydrated sections were hybridized for 16 hr at 50°C with 30,000 cpm/μL ^{35}S-labeled RNA probe in hybridization buffer [50% formamide, 300 mM NaCl, 20 mM Tris HCl (pH 7.4), 5 mM EDTA, 10 mM $NaP0_4$ (pH 8.0), 100 mM DTT, 10% dextran sulfate, 1× Denhardt, 50 μg/mL total yeast RNA] under siliconized coverslips. Coverslips were floated off in 5× SSC, 10 mM DTT at 50°C, followed by two stringent 65°C washes in 50% formamide, 2× SSC, 100 mM DTT. Slides were rinsed in washing buffer [400 mM NaCl, 10 mM Tris HCl (pH 7.5), 5 mM EDTA] and incubated with 20 μg/mL RNase A in washing buffer, followed by 37°C washes in 2× SSC and 0.1× SSC. Slides were dehydrated, dipped in Kodak NTB-2 radiosensitive emulsion, and exposed for 7–10 days at 4°C under desiccant.

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