DEVELOPMENTAL BIOLOGY **192,** 211–227 (1997)
ARTICLE NO. DB978760

metadata, citation and similar papers at core.ac.uk
brought to you by
provided by Elsevier - Publisher C

Mindin/F-spondin Family: Novel ECM Proteins

Shin-ichi Higashijima,*,†,[1] Akinao Nose,* Goro Eguchi,*,[2] Yoshiki Hotta,*,‡ and Hitoshi Okamoto*,§,[1]

**National Institute for Basic Biology, Myodaiji-cho, Okazaki Aichi 444, Japan; †PRESTO, Japan Science and Technology Corporation; ‡Graduate School of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan; and §Department of Physiology, Keio University, School of Medicine, Shinanomachi, Shinjuku-ku, Tokyo 160, Japan*

F-spondin is a secreted protein expressed at high levels by the floor plate cells. The C-terminal half of the protein contains six thrombospondin type 1 repeats, while the N-terminal half exhibited virtually no similarity to any other protein until recently, when a *Drosophila* gene termed *M-spondin* was cloned; its product was found to share two conserved domains with the N-terminal half of F-spondin. We report the molecular cloning of four zebrafish genes encoding secreted proteins with these conserved domains. Two are zebrafish homologs of *F-spondin*, while the other two, termed *mindin1* and *mindin2*, encode mutually related novel proteins, which are more related to the *Drosophila* M-spondin than to F-spondin. During embryonic development, all four genes are expressed in the floor plate cells. In addition to the floor plate, *mindin1* is expressed in the hypochord cells, while *mindin2* is expressed in the sclerotome cells. When ectopically expressed, Mindin proteins selectively accumulate in the basal lamina, suggesting that Mindins are extracellular matrix (ECM) proteins with high affinity to the basal lamina. We also report the spatial distribution of one of the F-spondin proteins, F-spondin2. F-spondin2 is localized to the thread-like structure in the central canal of the spinal cord, which is likely to correspond to Reissner's fiber known to be present in the vertebrate phylum. In summary, our study has defined a novel gene family of ECM molecules in the vertebrate, all of which may potentially be involved in development of the midline structure.
© 1997 Academic Press

INTRODUCTION

The floor plate is a small group of nonneuronal cells with secretory function located at the ventral midline of the developing nervous system. This group is one of the first cell types to differentiate within the embryonic nervous system. During embryonic development, the floor plate secretes a number of morphogenetic molecules. For example, the floor plate along with the notochord secretes Sonic hedgehog (Shh), which acts as a polarizing signal that controls cell identity and pattern formation in the neural tube (for review see Tanabe and Jessell, 1996). The floor plate also secretes Netrin1 which provides long-range guidance cues that promote the growth of axons to the ventral midline of the spinal cord (reviewed in Kennedy and Tessier-Lavigne, 1995).

F-spondin was originally identified as a gene that was expressed at high levels in the developing floor plate (Klar *et al.*, 1992). It encodes a secreted protein with six thrombospondin type 1 repeats [TSR(I)] in the C-terminal half. The TSR(I) motif was originally found in the vertebrate extracellular matrix (ECM) proteins, thrombospondin I and II (for review see Bornstein and Sage, 1994; Bornstein, 1995), and this motif has subsequently been found in several other ECM molecules. Several lines of evidence implicate this domain in cell adhesion. Proteolytic fragments of thrombospondin which contain the TSR(I) promote tumor cell adhesion (Prater *et al.*, 1991). *In vitro* analyses showed that recombinant F-spondin protein promoted neural cell adhesion and neurite outgrowth (Klar *et al.*, 1992). However, the

[1] To whom correspondence should be addressed at Shin-ichi Higashijima, National Institute for Basic Biology, Myodaiji-cho, Okazaki Aichi 444, Japan, Fax: +81-564-55-7571. E-mail: shinichi@nibb.ac.jp; or Hitoshi Okamoto, Laboratory for Developmental Gene Regulation, Brain Science Institute, RIKEN, Hirosawa, Wako, Saitama 351-01 Japan, Fax: +81-48-462-4914. E-mail: QZB00077@niftyserve.or.jp.

[2] Present address: Kumamoto University, Kurokami, Kumamoto 860, Japan.

0012-1606/97 $25.00
Copyright © 1997 by Academic Press
All rights of reproduction in any form reserved.

physiological function of F-spondin *in vivo* still remains largely unknown. The N-terminal half of the protein has no clear similarity to any known proteins except for a low degree of similarity at a short region near the N-terminus to Reelin, a candidate gene product for *reeler* mutant mice (D'Arcangelo *et al.*, 1995). In addition, the localization of F-spondin protein within the nervous system remains unclear, making it difficult to assess its physiological function *in vivo*.

Recently, a *Drosophila* gene the product of which exhibits extensive sequence similarity to F-spondin was cloned using an enhancer trap method (Umemiya *et al.*, 1997). The gene, called *M-spondin*, is expressed mainly in muscle cells and encodes a secreted protein sharing a high degree of similarity in two domains with the N-terminal half of F-spondin. Although the physiological function of these newly identified motifs (FS1 and FS2) remains unclear, this remarkable evolutionary conservation suggests the functional importance of these motifs.

Based on the strong evolutionary conservation of these motifs, we searched for genes with FS1 and FS2 domains in the zebrafish, *Danio rerio*. The zebrafish has many advantages as an experimental model of vertebrate development, including a simple nervous system and transparent embryos (Eisen, 1991). In this paper, we report the cloning and characterization of four genes. Two are zebrafish homologs of *F-spondin*, and two other genes, termed *mindin1* and *mindin2*, encode novel secreted proteins, which are closely related to each other, and are more closely related to the *Drosophila* M-spondin than to F-spondin. During embryonic development, all four genes are expressed in the floor plate cells and variously in other tissues. We show the data which suggest that the Mindin proteins are ECM molecules having potential to bind to the basal lamina. We also show the spatial distribution of F-spondin protein for the first time. One of the F-spondin proteins, F-spondin2, is localized to the thread-like structure in the central canal, which is likely to correspond to Reissner's fiber known to be present in the vertebrate phylum. Previous immunohistochemical analyses suggested that materials of Reissner's fiber are mainly secreted from the floor plate cells in the early stages and later from the subcommissural organ (SCO), a cluster of ependymal cells located at the dorsal midline of the diencephalon (for review, Meiniel *et al.*, 1996). We show that *F-spondin2* mRNA is also expressed in the SCO, supporting the idea that F-spondin2 is one of the components of Reissner's fiber.

MATERIALS AND METHODS

Animals

Fish were maintained as described by Westerfield (1994). The *one-eyed pinhead* mutant, *oep*^{m134} (Hammerschmidt *et al.*, 1996; Schier *et al.*, 1996, 1997), was obtained from Dr. Furutani-Seiki and Dr. Nüsslein-Volhard via Dr. Takeda. Embryonic stages are given according to Kimmel *et al.* (1995).

Isolation and Characterization of Zebrafish mindin/F-spondin Family

Routine molecular cloning techniques were carried out according to standard procedures. The zebrafish *mindin/F-spondin* genes were amplified from zebrafish genomic DNA by the polymerase chain reaction (PCR) using two sets of degenerate primers corresponding to an upstream, PSPDW, and a downstream, DAGTD, amino acid sequence. Both of these sequences are located within the FS2 region of rat F-spondin and *Drosophila* M-spondin (Fig. 2B, arrows). PCR products of the expected size were subcloned into pBluescript SK (Stratagene) and sequenced. Four DNA fragments which potentially encoded proteins having an FS2 domain were obtained. They corresponded to *mindin1*, *mindin2*, *F-spondin1*, and an additional DNA fragment the cDNA of which was not subsequently isolated by the cDNA screening described below.

A λgt10 cDNA library made from the poly(A)-rich RNA of 18- to 20-h embryos (Inoue *et al.*, 1994) was screened using a mixture of the four amplified DNA fragments. cDNA clones for *mindin1*, *mindin2*, and *F-spondin1* were obtained. In addition, *F-spondin2* cDNA clones were identified by cross hybridization to the *F-spondin1* probe. Two overlapping *mindin1* cDNA clones were obtained, the 2-kb cMD1a and the 3.3-kb cMD1b, which form a contiguous 3.4-kb cDNA with about 50 bp of 5′ untranslated sequence, about 1 kb of coding sequence, and about 2.4 kb of 3′ untranslated sequence. RACE-PCR suggested that the 5′ terminus of the composite cDNA (contained within cMD1a) is located near the transcriptional start site. On the 3′ side, this cDNA sequence terminates at a poly(A) stretch contained within the 3′ untranslated region of *mindin1* mRNA. The discrepancy between the size (3.4 kb) of the composite cDNA and the estimated size (approximately 4.8 kb) of the full-length cDNA deduced by Northern blot analysis (Fig. 3) suggests that a cDNA fragment (approximately 1.4 kb) corresponding to the 3′-terminal region of *mindin1* mRNA was not recovered in this study. Two *mindin2* cDNA clones were obtained. cMD2a (2.1 kb) includes about 0.7 kb of 5′ untranslated sequences, about 1 kb of coding sequence, and about 0.5 kb of 3′ untranslated sequence, terminating with an internal *Eco*RI site at the 3′ end. Northern blot analysis revealed a major band of 1.8 kb and minor bands around 2.0–2.3 kb, suggesting that cMD2a represents the minor transcripts. The other clone, cMD2b, of about 1 kb in size, is completely contained within cMD2a. In the case of *F-spondin1*, two cDNA clones, the 2.8-kb cSD1a and the 2.2-kb cSD1b, were isolated, but only the longer cSD1a was sequenced. cSD1a was found to lack a 5′-terminal region corresponding to the N-terminal 70 amino acids. The 0.5-kb cSD1r cDNA which encodes an upstream sequence was obtained using RACE-PCR. cSD1a and cSD1r overlap to give a contiguous sequence of 3.2 kb, which includes about 150 bp of 5′ untranslated sequence, about 2.4 kb of coding sequence, and about 0.6 kb of 3′ untranslated sequence. The 3′ end of the composite cDNA sequence terminates at a poly(A) stretch contained within the 3′ untranslated region of *F-spondin1* mRNA. The discrepancy between the size (3.2 kb) of the composite cDNA and the estimated size (approximately 6.2 kb) of the full-length cDNA deduced by Northern blot analysis (Fig. 3) suggested that a cDNA fragment (approximately 3.0 kb) corresponding to the 3′-terminal region of the *F-spondin1* mRNA was not recovered in this study. Two *F-spondin2* cDNA clones, cSD2a of about 3.5 kb in size and cSD2b of about 2.6 kb in size, were isolated, and the longer cSD2a was sequenced. cSD2a includes about 200 bp of 5′ untranslated sequence, about 2.4 kb of coding sequence, and about 1 kb of 3′ untranslated sequence. It also contains a poly(A) tail at its 3′ terminus. RACE-PCR suggested that the 5′ end of cSD2a is

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

located near the transcriptional start site. Northern blot analysis revealed a single major band of 3.6 kb (Fig. 3), nearly identical to the size of cSD2a, indicating that cSD2a is likely to represent the full-length cDNA.

RACE-PCR was carried out using the 5′-AmpliFINDER RACE kit according to the manufacturer's instructions (Clontech). Two gene-specific primers were prepared for each gene (*mindin1*, *F-spondin1*, and *F-spondin2*), and nested PCR was performed. Sequence alignment and calculation of sequence diversity was performed using GeneWorks software (IntelliGenetics).

Northern Blot Analysis

Total RNA was isolated from 28-h embryos using Trizol (GIBCO BRL) and poly(A)$^+$ RNA was prepared using oligo(dT)–latex (Roche). Four micrograms of denatured poly(A)$^+$ RNA was electrophoresed by the dimethylsulfoxide (DMSO)–glyoxal method and blotted onto Hybond-N membrane (Amerciam). The RNA size marker was purchased from GIBCO BRL. Probes were cMD1a (2 kb) for *mindin1*, cMD2a (2.1 kb) for *mindin2*, the 2.4-kb *Cla*I–*Hin*dIII fragment of cSD1a for *F-spondin1*, and the 2.2-kb *Eco*RI–*Xho*I fragment of cSD2a for *F-spondin2*. In all cases, the fragments included most of the coding sequences. Hybridization was carried out by a standard procedure. Final washing conditions were 0.1× SSC, 0.1% SDS at 68°C. The filters were rehybridized with a zebrafish *β-actin* probe, which was isolated by us (S.H., unpublished). The filters were exposed to an imaging plate and analyzed by a BAS-2000 image analyzer (Fuji film).

Generation of Antibodies

Synthetic peptides were prepared for the production of antibodies against F-spondin2 and Mindin1. The two peptides used for F-spondin2 were spo2-1, FKRDTAERHSRSEGGFC (amino acid [aa] position 24–40), and spo2-2, CSEKRKRQEVSDRRRAKQSRE (aa 723–742 except C). The resultant anti-F-spondin2 antibodies (Ab-spo2-1 and Ab-spo2-2) gave identical staining patterns in the spinal cord. Only one antibody, Ab-spo2-1, was positive in the lens, possibly due to posttranscriptional modification of F-spondin2 protein in this tissue. The amino acid sequence of spo2-1 shows no identity with F-spondin1. The amino acid sequence of spo2-2 is also relatively divergent from F-spondin1, i.e., there are not more than two amino acids which are continuously identical. Thus, both Ab-spo2-1 and Ab-spo2-2 were unlikely to crossreact with F-spondin1.

Eight peptides were used for Mindin1 and none of the resultant eight antibodies gave positive signals in immunostaining of the normal developing embryo. At least three of them labeled the basal lamina in those embryos in which Mindin1 was ectopically expressed. The sequences for the three peptides were mdn1-2, CDAGTDSGFTFSSPNF (aa 190–204); mdn1-3, CKRQSRLPVRQQNRLSNH (aa 243–260); and mdn1-6, CTSQKPNHPANSFYYPRLNE (aa 216–234). The amino acid sequence of mdn1-3 is divergent from that of Mindin2. In contrast, the amino acid sequences of mdn1-6 and mdn1-2 show 75 and 100% identities, respectively, to the corresponding regions of Mindin2. Thus, Ab-mdn1-2 and Ab-mdn1-6 may recognize Mindin2. However, staining of the basal lamina by these antibodies in those embryos in which Mindin1 was ectopically expressed was quite likely derived from the ectopically expressed Mindin1, since such staining was absent in uninjected embryos.

For the production of antibodies, the peptides were conjugated to bovine serum albumin (spo2-1, mdn1-2, and mdn1-3) or keyhole limpet hemocyanin (spo2-2 and mdn1-6) using the cysteine residues of the peptides by the *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. Peptides fused to the carrier proteins were used to immunize rabbits to prepare polyclonal antibodies. Serum antibodies obtained were affinity-purified by the corresponding peptides that were coupled to 2-fluoro-1-metylpyridinium toluene-4-sulfonate-activated cellulose (Seikagaku-Kogyo, Japan) through the cysteine residues of the peptides.

RNA and Protein Localization

Whole-mount *in situ* hybridization was performed essentially as previously described (Inoue *et al.*, 1994) with minor modifications. cDNAs of approximately 2 kb in size were used as templates for *in vitro* transcription of all four genes (the same as the fragments used in Northern blotting). Double labeling was conducted essentially according to Hauptmann and Gerster (1994). *krox20* cDNA was obtained by Dr. Jowett via Dr. Ekker (Oxtoby and Jowett, 1993).

Whole-mount immunohistochemistry was performed essentially as described by Westerfield (1994) except that DMSO was omitted from the reaction or washing solution. For signal detection, a Vectastain ABC Elite kit was used (Vector). Cy3-conjugated secondary antibodies (Jackson ImmunoResearch) were also used as controls for signal detection. Primary antibodies used were anti-myc 9E10 (Oncogene Research), anti-Hu (Marusich *et al.*, 1994), anti-acetylated α-tubulin (Sigma), and the anti-F-spondin2 or anti-Mindin1 antibodies described above.

For sectioning of stained embryos in either *in situ* hybridization or immunohistochemistry, durcupan (Fluka) was used as a mounting resin. Embryos were dehydrated by a series of increasing resin concentrations to 100% and mounted according to the manufacturer's instruction. Five-micrometer plastic sections were made.

Construction of mindin1 and mindin2 cDNA with Myc Epitope Tags and Expression in COS Cells

Oligonucleotides encoding the c-myc epitope EQKLISEEDL with suitable cohesive or blunt ends were generated and inserted into the *Hin*cII site of *mindin1* (corresponding to aa position 252) or into the *Bgl*II site of *mindin2* (corresponding to aa position 245). The sites of insertion were located between FS2 and TSR(I) in both cases. *mindin1-myc* and *mindin2-myc* were subcloned into pcDNA1 (Invitrogen) for COS cell transfection. Transfection was carried out using LipofectAMINE (Gibco BRL) according to the manufacturer's instructions. Culture media were recovered after 48 h of transfection and immunoprecipitated with the anti-myc antibody using protein G–Sepharose (Pharmacia). The precipitates were loaded onto SDS–PAGE, transferred to a PVDF membrane (Millipore), and probed with the anti-myc antibody.

Ectopic Expression of Mindin1 in Embryos

The zebrafish *α-actin* (z*α-actin*) promoter was isolated and used to drive expression of transgenes in muscle cells (S.H., unpublished). Briefly, the DNA fragment used (about 3.9 kb) contains about 2.2 kb of upstream sequence, about 40 bp of the first exon, about 1.7 kb of the first intron, and a part of the second exon (5 bp). Structural details of the z*α-actin* promoter will be described elsewhere. An SV40 poly(A) signal was introduced into the z*α-actin* expression vector. The pCS2 vector which contains a simian CMV

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

IE94 (sCMV) promoter was used for ectopic expression of transgenes in the whole zebrafish body (Rupp *et al.*, 1994; Turner and Weintraub, 1994). This vector contains an SV40 poly(A) signal. *mindin1-myc* or *mindin1* was inserted into these expression vectors. *mindin2-myc* was also introduced into the z*α-actin* expression vector.

Approximately 1 nl of linearized plasmid DNA dissolved in water at a concentration of 25 ng/μl was injected into one-cell stage zebrafish embryos according to the method previously described (Westerfield, 1994). Embryos were raised for 1 day, fixed, and stained with the anti-myc antibody or the anti-Mindin1 antibodies.

RESULTS

Isolation and Characterization of the mindin/F-spondin Family in Zebrafish

M-spondin, a *Drosophila* gene expressed mainly in muscle cells, encodes a novel secreted protein (Umemiya *et al.*, 1997). As shown in Fig. 2, M-spondin contains a TSR(I) domain and two novel domains, designated FS1 (Fig. 2A) and FS2 (Fig. 2B), with striking similarity to vertebrate F-spondin (Klar *et al.*, 1992; Ruiz i Altaba *et al.*, 1993). We performed PCR on zebrafish genomic DNA using degenerate primers corresponding to two highly conserved five-amino-acid sequences, PSPDW and DATGD, both of which are present in the FS2 domain (arrows in Fig. 2B). By PCR and subsequent cDNA screening, we identified four zebrafish genes with FS2 domains.

Two of these genes were zebrafish homologs of rat (Klar *et al.*, 1992) or *Xenopus* (Ruiz i Altaba *et al.*, 1993) *F-spondin*, and were called *F-spondin1* and *F-spondin2*. The deduced amino acid sequences of F-spondin1 and F-spondin2 are shown in Fig. 1A. F-spondin1 shows 77% identity, and F-spondin2 68% identity, with rat F-spondin, and this homology extends over the entire length of these proteins, indicating that *F-spondin1* and *F-spondin2* are zebrafish homologs of *F-spondin*. The identity of zebrafish F-spondin1 to rat F-spondin (77%) was higher than that of F-spondin1 to F-spondin2 (72%). It is not clear whether higher vertebrates have more than one F-spondin. Each F-spondin has a hydrophobic signal peptide sequence in the N-terminal region, FS1 and FS2, and six TSR(I) domains (Fig. 2F).

The other two genes encode novel proteins that are closely related to each other (Figs. 1B and 2F). We named these genes *mindin1* and *mindin2*. Although the Mindin1 and Mindin2 proteins are much smaller than *Drosophila* M-spondin, they have a domain organization similar to that of M-spondin, with a hydrophobic signal sequence in the N-terminus, FS1, and FS2 sequences, and one TSR(I) (Fig. 2F). Mindin1/Mindin2 show identity with M-spondin not only in domain organization but also at the amino acid sequence level. Phylogenetic trees derived from an alignment of FS1 or FS2 amino acid sequences indicate that Mindin1 and Mindin2 are more closely related to M-spondin than to F-spondin (Fig. 2D and Table 1). Moreover, in Mindin1, Mindin2, and M-spondin, but not F-spondin, similarity in amino acid sequences extends to downstream of the FS2 site, where 10 of 16 amino acids are conserved (Fig. 1C). Recently, in *Caenorhabditis elegans*, the *F-spondin*-related gene *F10E7.4* was found by the genome project (GenBank Accession No. U41264). The domain organization of F10E.7.4 and the amino acid sequences within the FS1 and FS2 domains of F10E7.4 are more closely related to F-spondin than to Mindin1, Mindin2, or M-spondin (Figs. 2D and 2F and Table 1). These results indicate that Mindin1, Mindin2, and M-spondin constitute a novel subfamily (Mindin subfamily) in the Mindin/F-spondin family. F-spondin and F10E7.4 constitute another subfamily, the F-spondin subfamily. The amino acid sequence identity of FS1 and FS2 among the Mindin/F-spondin family is summarized in Table 1.

Expression Patterns of the mindin/F-spondin Family Genes during Development

To examine the expression of these four novel genes, Northern blotting with poly(A)$^+$ RNA derived from 28-h embryos was performed. Figure 3 shows that each probe hybridized to a single major band of 6.2 kb in *F-spondin1*, 3.6 kb in *F-spondin2*, 4.8 kb in *mindin1*, and 1.8 kb in *mindin2*. In the case of *mindin2*, longer exposure revealed minor transcripts of approximately 2.0–2.3 kb in size. From the intensity of the bands, expression levels of *F-spondin2* and *mindin1* were suggested to be higher than those of *F-spondin1* and *mindin2*. Figure 3 also shows that there was no cross-hybridization under the conditions used in our study.

To determine the spatial and temporal distribution of the four genes, the mRNAs were localized during development by whole-mount *in situ* hybridization in zebrafish embryos. Thin sections of plastic-embedded embryos precisely confirmed the localization of the tissues expressing these genes. The expression patterns of the four genes were similar in some tissues such as the floor plate but distinct in several other tissues (Figs. 4 and 5), indicating that there was no cross-hybridization under the conditions used in our study.

Expression of mindin1

mindin1 expression first appeared in the axial mesodermal region (Figs. 4A and 4B) at about 11 h postfertilization (3-somite stage; Kimmel *et al.*, 1995), but *in situ* staining was not homogeneous at this stage. Cells expressed *mindin1* at different levels (Fig. 4B). Within the next 3 h, the expression in the prospective notochord decreased, whereas cells just dorsal or ventral to the prospective notochord were intensely labeled at this time (Figs. 4C, 4D, 4E, and 4I). Labeled cells located dorsal to the prospective notochord formed a continuous row as development proceeded, and these cells constituted the floor plate cells in the spinal cord. Labeled cells located ventrally were initially present as isolated islands (Fig. 4E). They then became flat elongated cells, forming the hypochord; a single row of cells lying

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

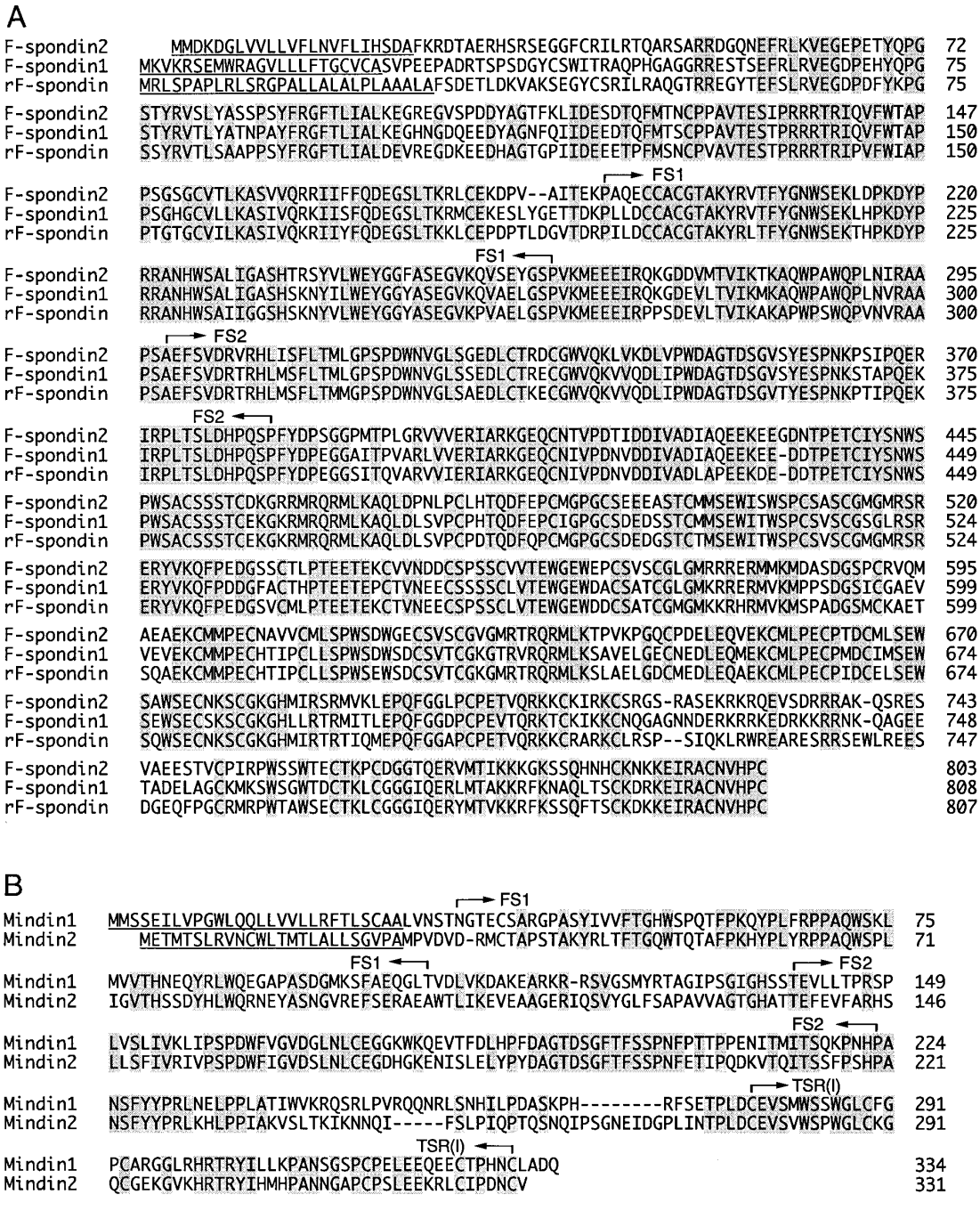


FIG. 1. (A) Alignment of the deduced amino acid sequences of zebrafish F-spondin2 and F-spondin1 with that of rat F-spondin (rF-spondin). Underlined N-terminal residues indicate putative signal sequences. Amino acids present in two of the sequences are shaded. Bent arrows denote the boundaries of the FS1 and FS2 domains. The accession numbers for the cDNA sequences of *F-spondin1* and *F-spondin2* are AB006086 and AB006087, respectively. (B) The deduced amino acid sequences of zebrafish Mindin1 and Mindin2. The residues conserved between Mindin1 and Mindin2 are shaded. Underlined N-terminal residues indicate putative signal sequences. Bent arrows denote the boundaries of FS1, FS2, and TSR(I) domains. From the nucleotide sequence of the *mindin2* cDNA, cMD2a, there are four other candidate initiation ATG codons, all of which are located within 60 bp of the putative initiation methionine. The initiation methionine was adopted on the basis of the length of a signal peptide, which usually consists of 20–30 amino acids, and relative matching of a Kozak consensus sequence (Kozak, 1986). The accession numbers for the cDNA sequences of *mindin1* and *mindin2* are AB006084 and AB006085, respectively.

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

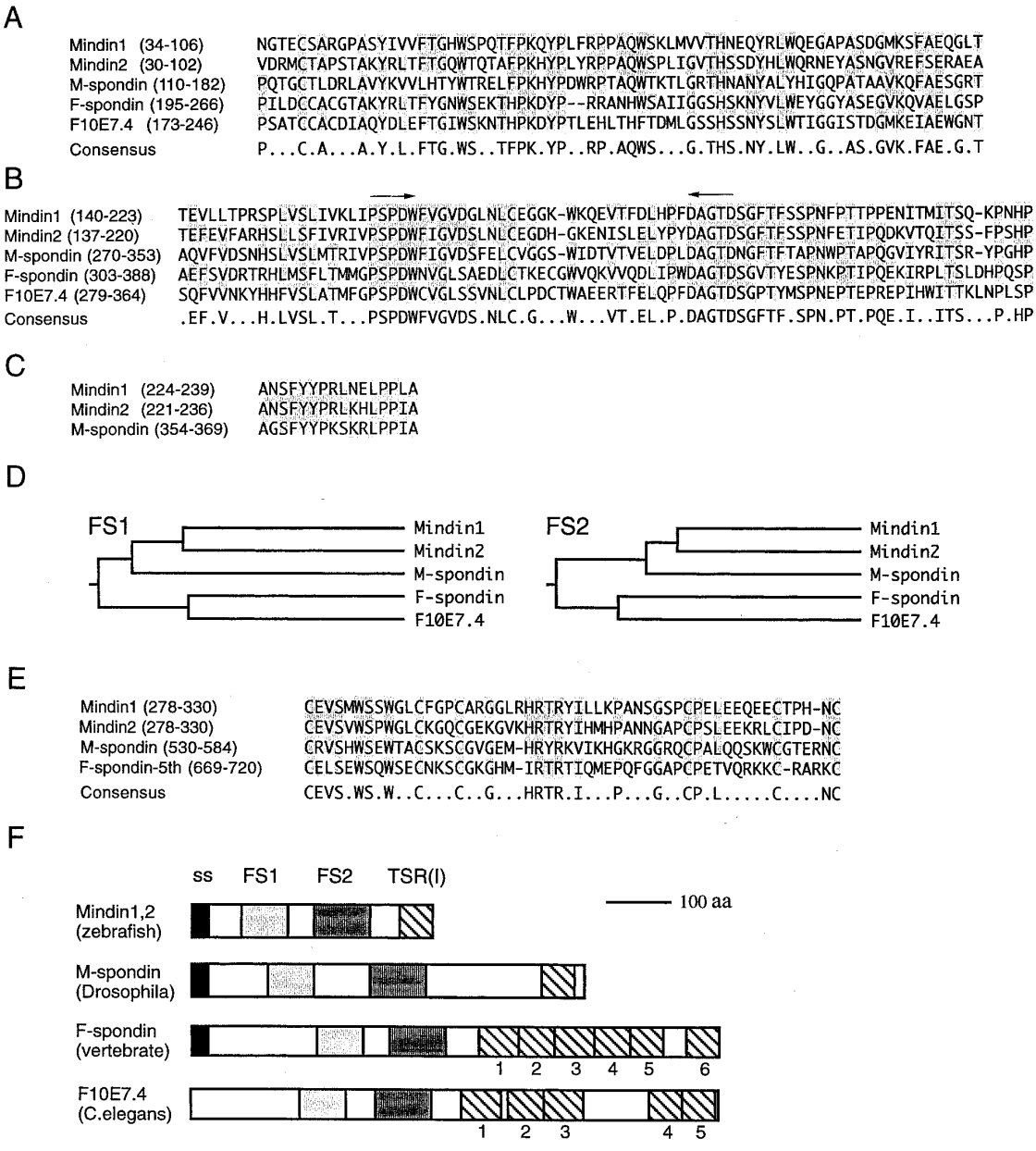


FIG. 2. Comparison of proteins belonging to the Mindin/F-spondin family at the amino acid sequence level. (A and B) Optimal alignments of FS1 (A) and FS2 (B) domains from zebrafish Mindin1 and Mindin2, *Drosophila* M-spondin, rat F-spondin, and *Caenorhabditis elegans* F10E7.4. Amino acids present in three or more of the sequences are shaded. The positions of the first and last amino acid of each domain are shown in parentheses. Straight arrows in (B) denote the amino acid sequences used for designing the degenerate PCR primers. (C) Alignment of amino acid sequences C-terminal to the FS2 domain from Mindin1, Mindin2, and M-spondin. Amino acids present in two or more of the sequences are shaded. (D) Phylogenetic trees of FS1 and FS2 domains. Gene Works software (IntelliGenetics) was used to calculate diversity. (E) Optimal alignment of TSR(I) domains from Mindin1, Mindin2, M-spondin, and the fifth TSR(I) of rat F-spondin, which is the most similar to the TSR(I) of M-spondin among the six TSR(I). Amino acids present in three or more of the sequences are shaded. (F) A schematic diagram showing the domain structure of zebrafish Mindins, *Drosophila* M-spondin, vertebrate (rat, *Xenopus*, and zebrafish-1 and -2) F-spondins, and *C. elegans* F10E7.4. F10E7.4 lacks a signal peptide sequence in its N-terminus, possibly due to missing exon(s). ss, signal peptide sequence.

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

TABLE 1

Amino Acid Identity (%) of FS1 and FS2 Domains among the Mindin/F-spondin Family

	Mindin1	Mindin2	M-spondin	Rat F-spondin
Mindin2	53			
M-spondin	48	45		
Rat F-spondin	42	43	38	
F10E7.4	42	36	39	49

Note. The comparison using *Xenopus* F-spondin, zebrafish F-spondin1, or zebrafish F-spondin2 instead of rat F-spondin gives almost the same results.

beneath the notochord (see below; Hatta and Kimmel, 1993).

In accordance with the decrease of *mindin1* expression in the notochord, the expression of *mindin1* became restricted to the floor plate and hypochord cells (Fig. 4F). From 16 to 40 h, *mindin1* was almost exclusively expressed in these two midline tissues. Figures 4F, 4G, and 4H show the expression of *mindin1* in 17-, 24-, and 33-h embryos, respectively. Two lines of labeled cells are distinctly labeled. Cross sections of 17- and 30-h embryos at the trunk levels showed that only the floor plate and the hypochord, both of which are composed of a row of cells with a single-cell width, expressed *mindin1* (Figs. 4J and 4K). *mindin1* expression in the floor plate extended rostrally up to, but not including, the forebrain (Fig. 6A, arrowhead), while expression in the hypochord extended up to and around the caudal end of the hindbrain (Fig. 6A, arrow). A cross section of the hindbrain showed that *mindin1* expression was restricted to the most ventrally located cell or cells (Fig. 6H).

mindin1 expression reached its peak in the floor plate and hypochord cells at about 18 h, was maintained until 30 h, and then gradually decreased. However, at 40 h, the final stage of our study, the floor plate and hypochord cells still expressed *mindin1* at a high level (data not shown).

Expression of mindin2

mindin2 mRNA was first detected at about 20 h as faint labeling in the floor plate cells (data not shown). As the embryos grew, staining in the floor plate became gradually more intense and additional cells began to express *mindin2*. Figure 4L shows the expression pattern of *mindin2* at about 26 h. A single row of floor plate cells expressed *mindin2*, and in addition, several ventrally located cells expressed *mindin2* (arrows in Figs. 4L and 4M). These labeled cells located at the position corresponding to the anterior part of the somites (Fig. 4L) had a mesenchymal appearance (Fig. 4M). In cross sections, labeled mesenchymal cells were present between the ventral region of the notochord and the myotome or between the ventral region of the myotome and the endodermal cells (arrows in Figs. 4N and 4O). The location and appearance of these labeled cells coincided with those of recently described sclerotomal cells (Morin-Kensicki and Eisen, 1997), and the labeled cells are therefore likely to be sclerotomal cells. *mindin2* was also expressed in a small number of cells in the caudal region of the spinal cord (data not shown). From 24 to 30 h, essentially the same staining patterns were observed.

The expression level of *mindin2* appeared to be significantly lower than that of *mindin1*, as judged by signal intensity upon *in situ* hybridization. This is consistent with the Northern blot data (Fig. 3).

Expression of F-spondin1 and F-spondin2

Expression of *F-spondin1* was first observed at about 16 h as a single row of cells in the floor plate (data not shown). The expression in the early stages was weak but gradually became stronger as development proceeded. Between 18 and 24 h, weak expression was also apparent in the hypochord (Fig. 5A). The expression of *F-spondin* in the hypochord was also reported in *Xenopus* by Ruiz i Altaba *et al.* (1993). Figure 5B shows *F-spondin1* expression at 28 h, which was in the most ventral portion of the spinal cord. A cross section showed that *F-spondin1* was strongly expressed in the floor plate (Fig. 5C, fp). In addition, cells adjacent to the floor plate also appeared to express *F-spondin1* weakly (arrows in Fig. 5C). This is in contrast to *mindin1* expression, which is exclusively restricted to the floor plate in the spinal cord (compare Figs. 4K and 5C).

Expression of *F-spondin2* was first observed at 11–13 h around the axial mesodermal region, like *mindin1* (Fig. 5D). In addition, low levels of *F-spondin2* were also expressed in the somites in this early stage (Fig. 5D). As development proceeded, weak expression in the somites ceased and, in

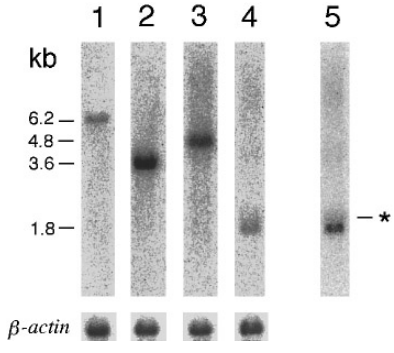


FIG. 3. Northern blot analyses of *F-spondin1*, *F-spondin2*, *mindin1*, and *mindin2*. Four micrograms of poly(A)$^+$ RNA isolated from 28-h embryos was run in each lane. Lane 1, *F-spondin1;* lane 2, *F-spondin2;* lane 3, *mindin1;* and lanes 4 and 5, *mindin2*. Exposure time was the same in lanes 1–4. Lane 5 represents a sixfold-longer exposure of lane 4. Asterisk indicates minor transcripts of *mindin2*, approximately 2.0–2.3 kb in size. *β-actin* indicates patterns of rehybridization with a *β-actin* probe.

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

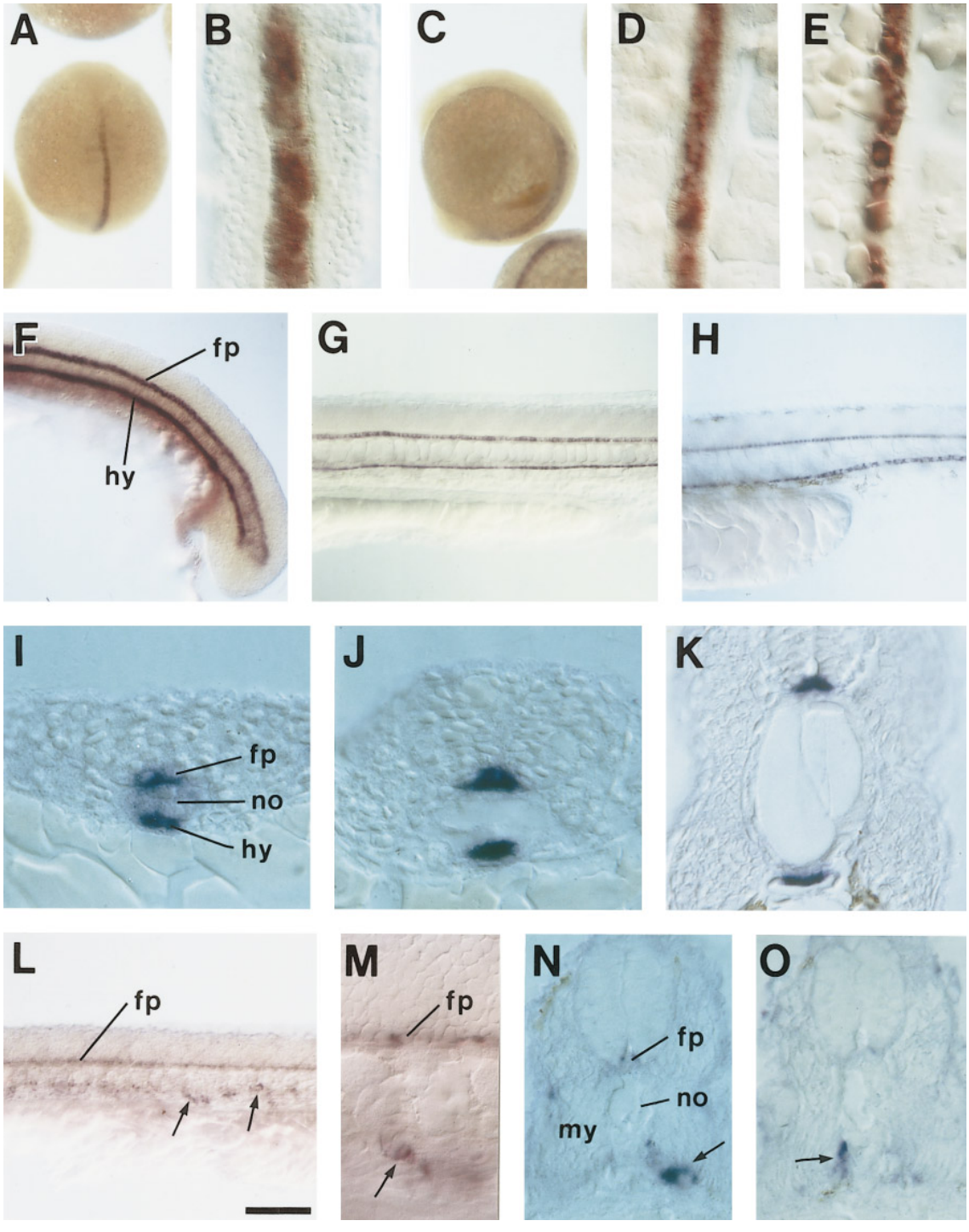


FIG. 4. (A–K) *mindin1* and (L–O) *mindin2* mRNA expression in developing embryos. (A and B) *mindin1* expression in 11-h embryos. Dorsal view. A higher magnification view of a dissected embryo is presented in B. Expression of *mindin1* first appears in the axial mesodermal region. (C) Lateral view of an embryo at 13 h. (D) A dorsal view of a 14-h embryo in higher magnification. The focus is just dorsal to the notochord. A continuous row of cells, which are precursors of the floor plate cells, are labeled. (E) The same embryo shown in D. The focus is just ventral to the notochord. Noncontiguous cells, which are precursors of the hypochordal cells, are labeled. (F–H) Lateral views of 17- (F), 24- (G), and 33-h (H) embryos. Two contiguous rows of cells just dorsal or ventral to the notochord are labeled. The former is the floor plate and the latter is the hypochord. (I–K) Cross sections of 12- (I), 17- (J), and 30-h (K) embryos. In all developmental stages, *mindin1* is expressed in the floor plate and the hypochord, either of which consists of a single row of cells. In the early stage (I),

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

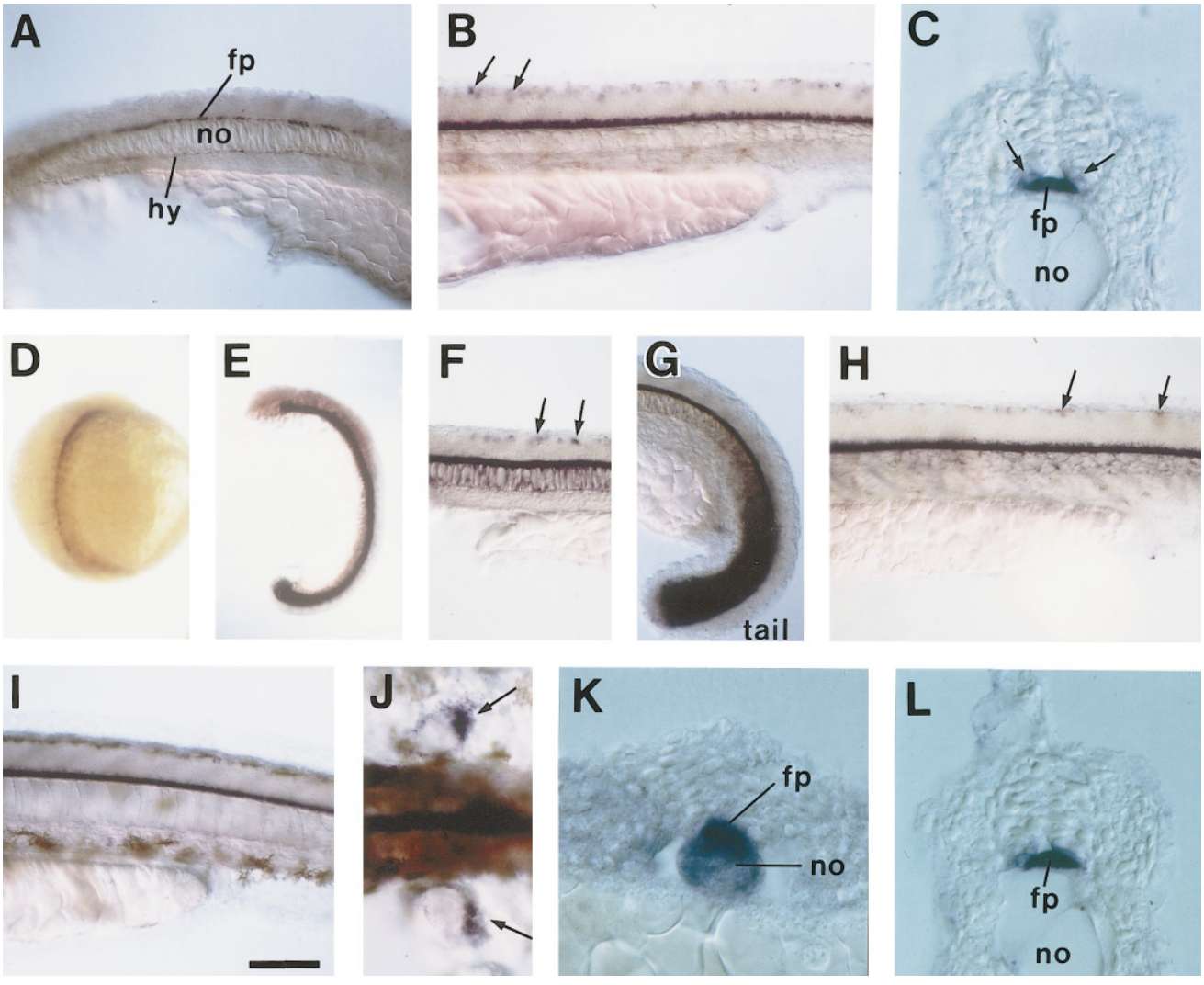


FIG. 5. (A–C) *F-spondin1* and (D–L) *F-spondin2* mRNA expression in developing embryos. (A) Lateral view of a 20-h embryo. *F-spondin1* is clearly expressed in the floor plate and weakly expressed in the hypochord. (B) Lateral view of a 28-h embryo. *F-spondin1* is expressed around the floor plate. In addition, several dorsal cells in the spinal cord express *F-spondin1* (arrows). (C) Cross section of a 28-h embryo. *F-spondin1* is strongly expressed in the floor plate. In addition, *F-spondin1* appears to be weakly expressed in the cells adjacent to the floor plate (arrows). (D) *F-spondin2* expression in a 13-h embryo. Dorsal view. *F-spondin2* is expressed around the axial mesoderm and weakly in the somites. (E) Lateral view of a 17-h embryo. (F and G) Lateral view of 19-h embryos in the trunk (F) and the tail (G). In addition to the expression around the floor plate, *F-spondin2* is also weakly expressed in the notochord and strongly expressed in the tail bud. (H and I) Lateral view of 28- (H) and 36-h (I) embryos. Arrows in (F) and (H) indicate *F-spondin2*-positive cells in the dorsal spinal cord. (J) Dorsal view of a 36-h embryo in the pectoral fin bud region. *F-spondin2* is expressed in the posterior region of the pectoral finbuds (arrows) (K) Cross section of a 15-h embryo. (L) Cross section of a 28-h embryo. fp, floor plate; no, notochord; hy, hypochord. Scale bar, 100 μm in A, B, and F–J; 17 μm in C, K, and L; 250 μm in D; and 160 μm in E.

mindin1 is also expressed weakly in the notochord. (L and M) *mindin2* expression in a 26-h embryo. Lateral view. A higher magnification view of the same embryo shown in L is presented in M. *mindin2* is expressed in the floor plate cells. *mindin2* is also expressed in the mesenchymal cells in the ventral region of the embryo (arrows). (N and O) Cross section of 32-h embryos. *mindin2*-positive mesenchymal cells are located between the ventral region of the myotome and endodermal cells (arrow in N) or between the ventral region of the notochord and the myotome (arrow in O). These cells are likely to be sclerotomal cells. fp, floor plate; no, notochord; hy, hypochord; my, myotome. Scale bar, 270 μm in A and C; 44 μm in B, D, and E; 100 μm in F–H and L; 17 μm in I–K, N, and O; and 33 μm in M.

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

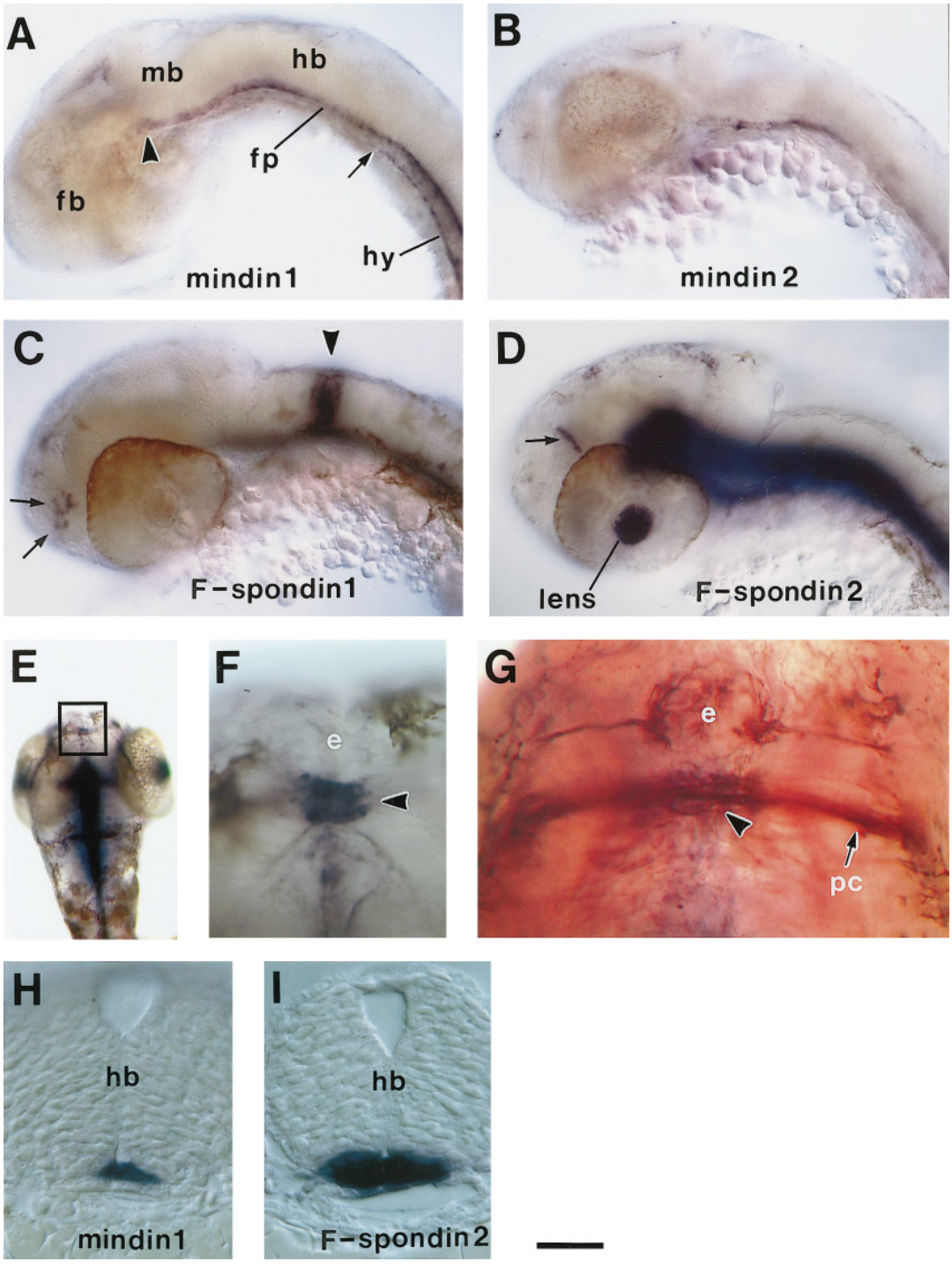


FIG. 6. mRNA expression of *mindin/F-spondin* family in the head region. (A–D) Lateral view of the head region stained with *mindin1* (A), *mindin2* (B), *F-spondin1* (C), and *F-spondin2* (D). Embryos in A and B are 26 h. Embryos in C and D are 28 h. An arrowhead in (A) indicates the rostral end of *mindin1* expression in the floor plate. It extends up to, but not including, the forebrain. The arrow in (A) indicates the rostral end of *mindin1* expression in the hypochord. It extends up to the caudal end of the hindbrain. An arrowhead in (C) indicates *F-spondin1* expression in the rhombomere 4. Arrows in (C) indicate *F-spondin1*-expressing cells in the forebrain. An arrow in (D) indicates *F-spondin2*-expressing cells in the forebrain. (E) Dorsal view of a 36-h embryo stained for *F-spondin2* mRNA. (F) A close-up view of the boxed region in E. *F-spondin2* is expressed in a cluster of cells (arrowhead) just behind the epiphysis. (G) Dorsal view of a 32-h embryo double-stained for *F-spondin2* mRNA and for acetylated α-tubulin. *F-spondin2*-positive signal (arrowhead) is present just beneath the posterior commissure. (H and I) Cross section of the hindbrain region of 28-h embryos stained for *mindin1* mRNA (H) and *F-spondin2* mRNA (I). fp, floor plate; hy, hypochord; fb, forebrain; mb, midbrain; hb, hindbrain; e, epiphysis; pc, posterior commissure. Scale bar, 100 μm in A–D, 180 μm in E, 30 μm in F and G, and 17 μm in H and I.

the midline tissues, intense labeling in the floor plate and weak labeling in the notochord became discernible (Figs. 5E and 5F). A cross section of the embryo at about 15 h clearly showed this mode of expression (Fig. 5K). The expression in the notochord decreased as development proceeded, while the expression in the floor plate remained high (Fig. 5F). During the tail development stage (16–22 h), strong expression of *F-spondin2* was observed in the tail bud region (Fig. 5G). From 24 h, *F-spondin2* showed almost the same expression pattern as *F-spondin1* in the trunk (Figs. 5B and 5H). A cross section of a 28-h embryo showed that *F-spondin2,* like *F-spondin1*, was expressed strongly in the floor plate and weakly in the cells adjacent to the floor plate (Fig. 5L). Expression of *F-spondin1* and *F-spondin2* around the floor plate was maintained at high levels up to 40 h, the final stage of our study. In addition to the ventral region of the spinal cord, *F-spondin1* and *F-spondin2* were also expressed in a small number of cells in the dorsal part of the spinal cord (arrows in Figs. 5B, 5F, and 5H). The expression patterns of *F-spondin1* and *F-spondin2* in zebrafish were consistent with those of *F-spondin* in rat (Klar *et al.*, 1992) and *Xenopus* (Ruiz i Altaba *et al.*, 1993).

Although *F-spondin1* and *F-spondin2* had almost identical expression patterns in the spinal cord, the expression level of *F-spondin2* appeared to be significantly higher than that of *F-spondin1* judging by signal intensity upon *in situ* hybridization, consistent with the Northern blot data (Fig. 3).

Outside the spinal cord, *F-spondin1* and *F-spondin2* showed distinct expression patterns. Both genes were expressed in the ventral part of the CNS in the hindbrain and midbrain, but *F-spondin2* was expressed much more widely than *F-spondin1* (Figs. 6C and 6D). From 26 to 32 h, *F-spondin1* was transiently expressed in the specific rhombomere of the hindbrain (Fig. 6C, arrowhead). Double labeling with *krox20,* which specifically labels rhombomeres 3 and 5 (Oxtoby and Jowett, 1993), revealed that the *F-spondin1*-positive region corresponded to the rhombomere 4 (data not shown). *F-spondin1* was also expressed in a small number of cells in the forebrain (Fig. 6C arrows). From 26 h on, *F-spondin2* was strongly expressed in the lens of the eye (Fig. 6D). *F-spondin2* was expressed in other tissues, including the thin stripes of the forebrain (Fig. 6D, arrow), an unidentified cluster of cells ventral to the notochord at the levels of the third somite, and posterior regions of the pectoral fin buds (Fig. 5J, arrows). *F-spondin2* was also expressed in a cell population located at the dorsal part of the brain (Fig. 6F, arrowhead), which is described further in the next section.

Protein Localization of F-spondin2

To examine the spatial distribution of F-spondin, we raised polyclonal antibodies against synthetic peptides derived from F-spondin2 (see Materials and Methods). Two independent antibodies gave identical staining patterns in the embryonic spinal cord, indicating that these antibodies were very specific.

Figure 7A shows the staining pattern at 26 h. F-spondin2 was localized to a thread-like structure running through the central canal of the spinal cord (Fig. 7A, arrow). Sections of the spinal cord of the stained embryos showed the labeling as a dot in the central canal (Fig. 7B, arrow) that was not fixed at any one location. Rather, the dot was located at various positions of the central canal; adjacent to the floor plate, at the dorsal edge of the central canal, or at a point in between. In the anterior part including the midbrain, the thread-like structure defasciculated (Fig. 7G). The strongest immunoreactivity was observed in the tail region, where the thread-like structure also defasciculated (Fig. 7C). This staining pattern could be followed from 20 through to 40 h, the final stage of our study. The pattern was basically identical at all stages of development (Fig. 7D; 36-h development).

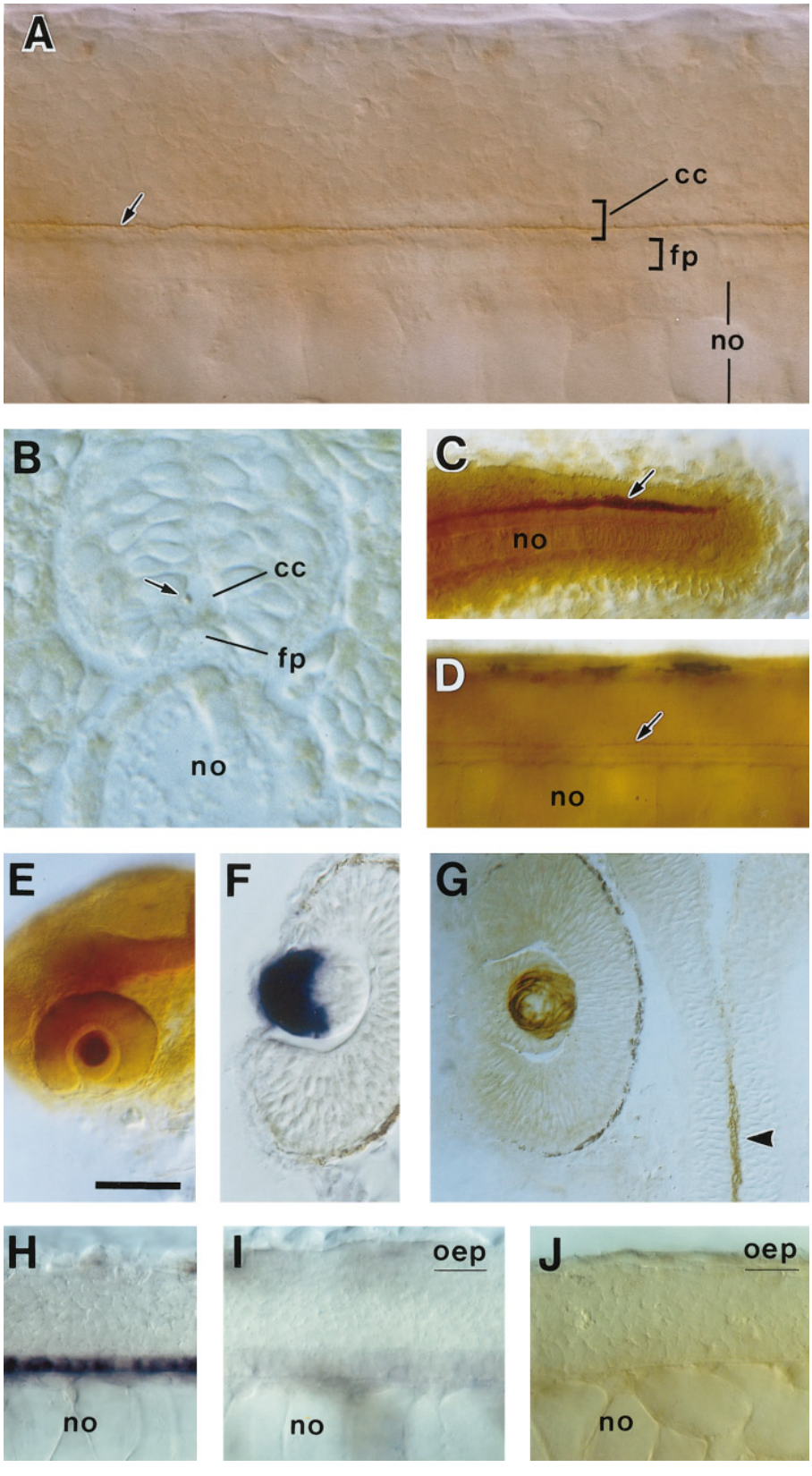
The thread-like immunoreactivity was not observed in the *one-eyed pinhead* mutant (Fig. 7K), in which formation of the floor plate is severely affected (Hammerschmidt *et al.*, 1996; Schier *et al.*, 1996, 1997), and *F-spondin2* mRNA expression was extensively reduced (Fig. 7J). This further supports the specificity of the antibodies.

It has long been known that a thread-like structure, termed Reissner's fiber, is present in the central canal of the spinal cord in the vertebrate phylum (Reissner, 1860; Oksche, 1969). The thread-like structure revealed by our anti-F-spondin2 antibodies is likely to correspond to Reissner's fiber. Thus, the results of immunostaining showed that F-spondin2, produced mainly by the floor plate cells, was secreted into the central canal and selectively deposited on the putative Reissner's fiber.

Previous immunohistochemical analysis suggested that the components of Reissner's fiber are mainly synthesized by the glandular cells facing the lumen of the CNS such as the cells in the subcommissural organ and the floor plate (reviewed by Meiniel *et al.*, 1996). The SCO is a population of cells of ependymal origin located just behind and adjacent to the epiphysis (reviewed by Oksche *et al.*, 1993; Meiniel *et al.*, 1996). We examined whether *F-spondin2* was also expressed in the SCO. Figures 6E and 6F show that *F-spondin2* was clearly expressed in a cluster of cells just behind epiphysis (Fig. 6F, arrowhead). Double labeling with an anti-acetylated α-tubulin which stains most axons (Piperno and Fuller, 1985) showed that the stained cells were located just beneath the posterior commissure (Fig. 6G, arrowhead), confirming that *F-spondin2* was expressed in the SCO. The expression of *F-spondin2* mRNA in the SCO started around 28 h and continued to at least 40 h.

F-spondin2 mRNA was also expressed in the lens of the eye (Fig. 6D), and the lens was selectively stained by one of the antibodies (Fig. 7E). While *F-spondin2* mRNA was mainly expressed in the epithelial layer of the lens (Fig. 7F), F-spondin2 immunoreactivity was observed between the cells in the fiber layer of the lens (Fig. 7G). These results suggest that F-spondin2 is produced in and secreted from

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.



Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

the epithelial layer of the lens, but accumulates between the cells in the fiber layer.

Immunohistochemistry did not detect F-spondin2 in dorsally located cells in the spinal cord, the pectoral fin buds, or the ventral organ in which *F-spondin2* mRNA was detected.

Ectopic Expression of Mindin1

To obtain direct biochemical evidence that Mindin1 and Mindin2 are secreted molecules, COS cells were transfected with myc-epitope-tagged *mindin1* and *mindin2*. The myc-epitope was introduced between FS2 and TSR(I) in both cases (see Materials and Methods). Protein products of the predicted molecular weights, which were absent in the case of mock transfection, were recovered from the culture media in both cases, confirming that Mindin1 and Mindin2 are secreted proteins (data not shown; see Materials and Methods).

To investigate the localization of Mindin proteins in developing embryos, a series of antibodies against peptides derived from Mindin1 was generated (see Materials and Methods). However, none of these detected native Mindin1 in developing embryos, possibly due to low abundance of the antigen. As an alternative approach, we ectopically expressed Mindin1 carrying the myc epitope (Mindin1-myc) by injecting DNA constructs into the cytoplasm of one-cell stage embryos. Cytoplasmic injection of DNA constructs is known to result in mosaic expression of a reporter gene in zebrafish. Populations of cells expressing the reporter differ from one embryo to another (e.g., Amsterdam *et al.*, 1995). The zebrafish muscle-specific *actin* promoter (z*α-actin* promoter) was isolated and used in this study (S.H., unpublished, see Materials and Methods). Figure 8A shows an example of a z*α-actin–mindin1-myc*-injected embryo stained with the anti-myc antibody. Immunoreactivity was observed in a subset of muscle cells as expected. Importantly, strong immunoreactivity was observed at either end of the muscle fibers (Fig. 8A, arrows). A transverse section showed that the surface of the muscle was also intensely immunoreactive (data not shown). These results suggested that Mindin1-myc was secreted from cells and selectively accumulated at the basal lamina located nearby as an ECM protein. Selective accumulation of Mindin1 protein at the basal lamina was also observed when intact Mindin1 was ectopically expressed and detected with an anti-Mindin1 antibody (Fig. 8B). Note that Mindin1 ectopically expressed at increased levels could be detected by some of the anti-Mindin1 antibodies, although these antibodies failed to detect native Mindin1 in developing embryos. Similar results were seen when the Mindin2-myc was ectopically expressed and detected by the anti-myc antibody (data not shown).

We then used the sCMV promoter (pCS2 vector; Rupp *et al.*, 1994; Turner and Weintraub, 1994) that can drive reporter gene expression in virtually all types of cells after midblastula transition (3 h), although actual expression is mosaic. In about 10% of the sCMV–*mindin1-myc*-injected embryos stained with the anti-myc antibody, strong immunoreactivity was localized to almost all the basal lamina in the entire body, in contrast to when Mindin1-myc was driven by the z*α-actin* promoter (Figs. 8C and 8D). This suggested that Mindin1-myc, produced and secreted from a subpopulation of cells at early stages, diffused relatively freely and selectively accumulated at the basal lamina as embryos grew. The same staining pattern was observed in the sCMV–*mindin1*-injected embryos reacted with the anti-Mindin1 antibodies (data not shown).

Taken together, these results indicate that ectopically expressed Mindin1 selectively accumulates at the basal lamina and suggest that the native Mindin1 may also be localized to the basal lamina.

We studied the effect of the ectopic and enhanced expression of Mindin1-myc on neural development using an anti-acetylated α-tubulin which stains most axons (Piperno and Fuller, 1985) or anti-Hu antibody which stains the cell body of early neurons (Marusich *et al.*, 1994). However, no gross abnormality was observed in CNS development under these conditions.

DISCUSSION

Identification of the Mindin/F-spondin Family

M-spondin was identified in *Drosophila*, using enhancer trap screening. It is expressed mainly in muscle cells and encodes a secreted protein (Umemiya *et al.*, 1997). F-spon-

FIG. 7. Expression of F-spondin2 protein. The anti-F-spondin2 antibody Ab-spo2-1 was used for staining. (A) Lateral view of a 26-h embryo. (B) Cross section of a 26-h embryo. (C) Tail region of a 26-h embryo. Lateral view. (D) Lateral view of a 36-h embryo. Arrows in (A–D) indicate F-spondin2 immunoreactivity in the putative Reissner's fiber. (E) Head region of a 28-h embryo. Lateral view. The lens in the eye is specifically labeled. (F) Horizontal section of the eye of a 28-h embryo stained for *F-spondin2* mRNA by *in situ* hybridization. Epithelial layer of the lens is labeled. (G) Horizontal section of the eye of a 28-h embryo stained with the anti-F-spondin2 antibody. In contrast to the pattern of mRNA expression, F-spondin2 immunoreactivity is located in the fiber layer of the lens. An arrowhead shows the signal in the central canal. (H and I) Lateral views of 28-h wild-type (H) and *oep* homozygous (I) embryos stained for *F-spondin2* mRNA by *in situ* hybridization. In the *oep* mutant (I), expression of F-spondin2 in the midline cells of the spinal cord is extensively reduced. (J) Lateral view of a 28-h *oep* homozygous embryo stained with the anti-F-spondin2 antibody. The thread-like immunoreactivity is absent. cc, central canal; fp, floor plate; no, notochord. Scale bar, 25 μm in A; 15 μm in B; 50 μm in C, D, F, and G; 100 μm in E; and 37 μm in H–J.

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

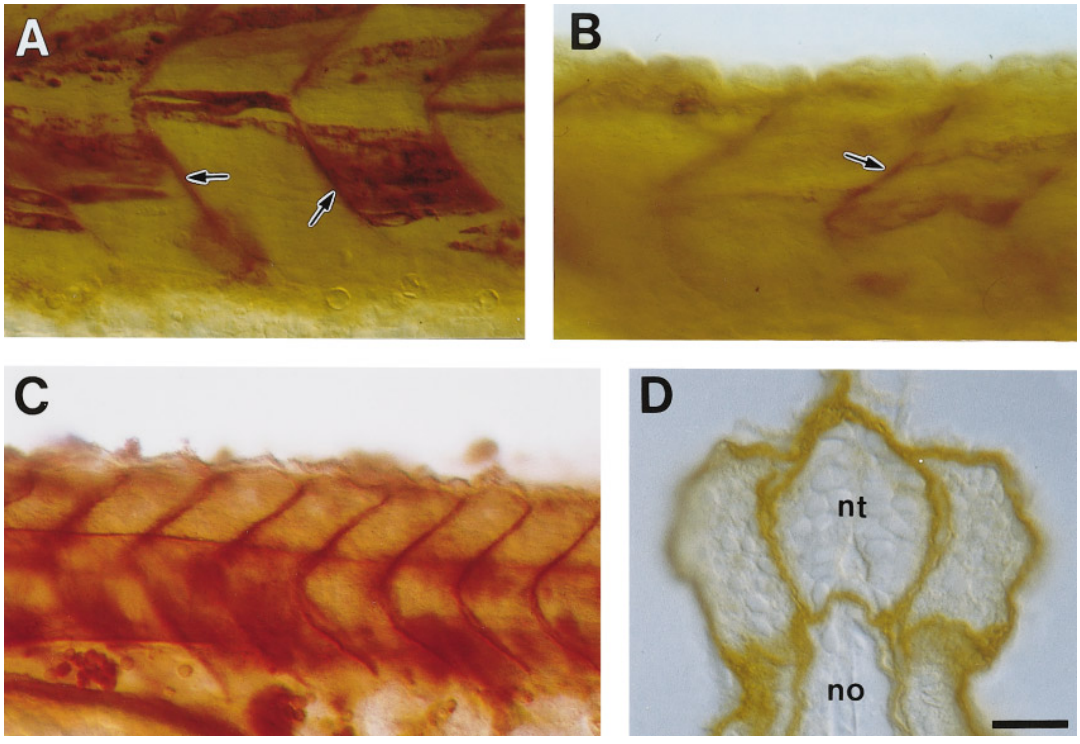


FIG. 8. Localization of ectopically expressed Mindin1 protein. All embryos are at about 24 h of development. (A) Lateral view of the z*α-actin–mindin1-myc*-injected embryo stained with the anti-myc antibody. (B) Lateral view of the z*α-actin–mindin1*-injected embryo stained with one of the anti-Mindin1 antibodies (Ab-mdn1-6). The antibody may recognize Mindin2 (see Materials and Methods). However, the staining pattern was quite likely derived from ectopically expressed Mindin1 in muscle cells, since such staining was absent in uninjected embryos. Arrows in A and B indicate intense immunoreactivity in the basal lamina. (C) Lateral view of the sCMV–*mindin1-myc*-injected embryo stained with the anti-myc antibody. (D) Cross section of the sCMV–*mindin1-myc*-injected embryo stained with the anti-myc antibody. nt, neural tube; no, notochord. Scale bar, 27 μm in A and B, 40 μm in C, and 15 μm in D.

din was originally identified in rat as a secreted molecule expressed at high levels in the floor plate (Klar *et al.*, 1992). Sequence comparison between M-spondin and F-spondin revealed two novel conserved domains, FS1 and FS2, in addition to a TSR(I) domain found in a number of proteins including thrombospondin. Strong phylogenetic conservation led us to expect that FS1 and FS2 along with the TSR(I) domains may be present in many other secreted proteins. Here we have identified four genes encoding secreted proteins with these domains in zebrafish. While two are zebrafish homologs of F-spondin, the other two are novel proteins. All the proteins belonging to this family appear to serve as ECM proteins. Thus we have defined a novel ECM protein family, the Mindin/F-spondin family, in the vertebrate.

Interestingly, all the identified proteins with FS1 and FS2 domains share a similar molecular organization that consists of a signal peptide sequence, FS1 and FS2 regions followed by TSR(I) repeats from N-terminus to C-terminus (Fig. 2F). This also appears to be true in the case of F10E7.4, a *C. elegans* F-spondin-like molecule identified by the genome project, although it lacks a signal peptide sequence possibly due to a missing exon(s). The number of TSR(I) repeats, however, varies in each molecule. F-spondin has six TSR(I)s, F10E7.4 has five TSR(I)s, and both Mindin1 and Mindin2 have only a single TSR(I). *Drosophila* M-spondin also has a single TSR(I). In this respect, Mindin1 and Mindin2 are more similar to M-spondin than to F-spondin or F10E7.4. Furthermore, sequence alignment of FS1 and FS2 domains also indicates that Mindin1 and Mindin2 are more similar to M-spondin than to F-spondin or F10E7.4. A short stretch of amino acid sequences C-terminal to FS2 are also strongly conserved only in Mindin1, Mindin2, and M-spondin. On the other hand, FS1 and FS2 of F-spondin and F10E7.4 are closely related. The Mindin/F-spondin family should therefore be grouped into two subfamilies, an F-spondin subfamily and a Mindin subfamily. F10E7.4 belongs to the former and M-spondin, the latter. This notion is further supported by the finding that *Drosophila* has two other *mindin/F-spondin* family genes that belong to the *F-spondin* subfamily (K. Tatei and A.N., pers. comm.). Given these conditions, it is likely that higher vertebrates have *mindin*-related genes. In addition, there may be additional *mindin*-like genes expressed in muscle cells in vertebrates,

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

since neither *mindin1* nor *mindin2* is expressed in muscle cells in zebrafish although *M-spondin* is found in muscle cells in *Drosophila*. It is possible that the FS1 and FS2 motifs are shared by a number of ECM molecules in both vertebrates and invertebrates. Although the physiological function of FS1 and FS2 remains unclear at present, the high degree of conservation across species strongly suggests that these motifs play an important role.

Localization and Possible Function of F-spondin

The patterns of expression of *F-spondin1* and *F-spondin2* mRNAs in the trunk are similar to those reported in rat (Klar *et al.,* 1992) and *Xenopus* (Ruiz i Altaba *et al.,* 1993). In all cases, *F-spondin* mRNA is expressed at high levels in the developing floor plate cells. Protein localization has not yet been reported. We have shown that zebrafish F-spondin2, the more abundant subtype of the two F-spondin homologs in zebrafish, is secreted from the floor plate cells and deposited in the thread-like structure running longitudinally through the central canal of the spinal cord. The high degree of sequence similarity between F-spondin2 and F-spondin1 suggests that F-spondin1 may also be secreted and deposited in the thread-like structure, although a possibility still remains that F-spondin1 is secreted from the floor plate cells in the opposite direction, i.e., the basal lamina beneath the floor plate.

The observed thread-like structure is likely to be Reissner's fiber known to be present in the vertebrate phylum (Oksche, 1969). Previous immunohistochemical analysis suggested that the components of Reissner's fiber are mainly synthesized by the SCO located just behind the epiphysis in the brain (reviewed in Oksche *et al.,* 1993; Meiniel *et al.,* 1996). The floor plate is also suggested to contribute to the formation of Reissner's fiber (for review, Meiniel *et al.,* 1996). *F-spondin2* mRNA was first expressed in the floor plate and then in the SCO, supporting the idea that F-spondin2 is a component of Reissner's fiber.

The phylogenetic conservation of Reissner's fiber suggests a functional significance, but its exact function remains unclear despite a wide range of studies. Recent *in vitro* analyses have shown that material solubilized from Reissner's fiber supports the survival of neuronal cells (Monnerie *et al.,* 1995). It has also been shown to have an anti-aggregative effect on cultured neuronal cells (Gobron *et al.,* 1996). F-spondin2 may in part be involved in these activities.

In zebrafish, there are a number of mutations that affect floor plate formation including *oep* and *cyclops* (Brand *et al.,* 1996). Embryos with these mutations have sickle-shaped bodies formed by downward curving of the body axis. In these mutants, Reissner's fiber is likely to be absent or reduced, since components of this structure are thought to be derived from the floor plate cells in the embryonic stage (for review, Meiniel *et al.,* 1996). Reissner's fiber is an elastic structure and may have a structural role in the establishment or maintenance of the normal straight body, i.e., an absence of Reissner's fiber is in part responsible for the ventral curvature of the floor-plate-defective mutants. If this is the case, F-spondin2 may function to give structural integrity to Reissner's fiber.

The selective accumulation of F-spondin2 in the putative Reissner's fiber suggests the presence of interacting molecules that are themselves located on the Reissner's fiber. The TSRs in thrombospondin have been shown to interact with a wide range of matrix molecules (Bornstein and Sage, 1994; Bornstein, 1995). Recently, SCO-spondin has been identified as a component of Reissner's fiber secreted from the SCO (Gobron *et al.,* 1996). Structurally, SCO-spondin is different from F-spondin in several aspects but it has TSR(I) repeats like F-spondin. A protein family characterized by TSR(I) motifs may constitute a major part of Reissner's fiber.

The SCO is located in an interesting position, just beneath the point where the axons in the posterior commissure cross the dorsal midline (Chitnis and Kuwada, 1990; Wilson *et al.,* 1990). This position also corresponds to the boundary between the diencephalon and the mesencephalon. This region of brain has been shown to express several regulatory genes (Macdonald *et al.,* 1994) and to be involved in determination of the rostrocaudal polarity of the mesencephalon (Chung and Cooke, 1975; Itasaki *et al.,* 1991; Itasaki and Nakamura, 1992). Our study has revealed that the cells in the SCO and the floor plate both express *F-spondin2* mRNA, suggesting that these tissues have similar physiological activities. Since the main function of the SCO appears to be a secretory one, the SCO could be involved in secreting a number of morphogenetic molecules, including F-spondin2, in this important region and may be involved in regulation of pattern formation or axonal pathfinding.

Expression, Localization, and Possible Function of Mindin Proteins

In *Drosophila, M-spondin* is expressed mainly in muscle cells, and its protein product is secreted and specifically accumulates at the muscle attachment site, a tightly condensed ECM structure which serves as a mechanical link between muscle cells and the epidermis (Umemiya *et al.,* 1997). M-spondin is likely to be present in this ECM structure. Thus, Mindin1 and Mindin2 may likewise be present in the tightly condensed ECM. This is consistent with our observation that ectopically expressed Mindin1, Mindin1-myc, and Mindin2-myc selectively accumulated at the basal lamina, which is also a tightly condensed ECM structure. Given that *mindin1* and *mindin2* mRNA show a restricted expression, the endogenous Mindin proteins may accumulate in the basal lamina near the cells that secrete them. For example, Mindin1 secreted by the floor plate may accumulate at the basal lamina under the floor plate as is seen with S-laminin (Sanes *et al.,* 1990). Alternatively, the endogenous Mindin1 may be secreted in the luminal side of the CNS. In this case, Mindin1 may show similar localization to F-spondin2, which is localized at the putative Reissner's

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

fiber. In addition to the floor plate, *mindin2* appears to be expressed in the sclerotomal cells, the mesenchymal cells which later contribute to cartilage formation. Mindin2 may be one ECM component that is involved in cartilage formation.

The function of the Mindin family in the ECM remains unclear. Mutations of *Drosophila M-spondin* do not result in any obvious defects (Umemiya *et al.*, 1997). *mindin1* and *mindin2* show highly restricted patterns of mRNA expression during development. However, ectopic expression of Mindin proteins in muscle cells or whole embryos did not result in any gross abnormalities in embryonic development. Mindin proteins may act as structural components in specific types of ECM like F-spondin2. Participation of Mindin proteins in the ECM could modulate cell–matrix interaction during development. Future *in vitro* assays may help to clarify the roles of Mindin proteins in morphogenetic processes.

In addition to the floor plate, *mindin1* is expressed in the hypochord. The hypochord is located just beneath the notochord and is one cell wide, like the floor plate. Its developmental function remains unclear. Interestingly, the type II collagen (*col2a1*) gene is expressed in the same manner as *mindin1*, although it is expressed in many other cells in early development (Yan *et al.*, 1995). Collagen is a well-known and abundant ECM molecule. One function of the hypochord may be the secretion of a number of morphogenetic molecules in the ventral part of the embryo, similar to the floor plate in the spinal cord.

mindin1 expression in the floor plate and the hypochord is maintained throughout almost all embryonic stages. *mindin1*, therefore, could be a useful marker for studies of the embryonic axis in zebrafish.

ACKNOWLEDGMENTS

We thank A. Klar, T. Umemiya, and K. Tatei for discussions and the sharing of unpublished data. We also thank C. Nüssllein-Volhard, M. Furutani-Seiki, and H. Takeda for the *oep* strain; T. Jowett and M. Ekker for *krox20* cDNA; M. F. Marusich and J. A. Weston for anti-Hu antibody; D. L. Turner for the pCS2 vector; M. Sugiura for technical assistance; K. Takamatsu, S. Matsubayashi, and Y. Katoh for maintaining our fish colony; and A. Klar and A. Thomson for comments on the manuscript. This work was supported by a Grant-in-Aid (07278246, 08254218, 07279107, and New Program System) from the Ministry of Education, Science and Culture of Japan; a grant from special coordination funds for promoting science and technology from the Agency of Science and Technology of Japan; and a grant from the Japan Science and Technology Corp.

REFERENCES

Amsterdam, A., Lin, S., and Hopkins, N. (1995). The *Aequorea victoria* green fluorescent protein can be used as a reporter in live zebrafish embryos. *Dev. Biol.* **711,** 123–129.

Bornstein, P. (1995). Diversity of function is inherent in matricellular proteins: An appraisal of thrombospondin 1. *J. Cell Biol.* **130,** 503–506.

Bornstein, P., and Sage, E. H. (1994). Thrombospondins. *Methods Enzymol.* **245,** 62–85.

Brand, M., Heisenberg, C-P., Warga, R. M., Pelegri, F., Karlstrom, R. O., Beuchle, D., Picker, A., Jiang, Y-J., Furutani-Seiki, M., van Eeden, F. J. M., Granato, M., Haffter, P., Hammerschmidt, M., Kane, D. A., Kelsh, R. N., Mullins, M. C., Odenthal, J., and Nüsslein-Volhard, C. (1996). Mutation affecting development of the midline and general body shape during zebrafish embryogenesis. *Development* **123,** 129–142.

Chitnis, A. B., and Kuwada, J. Y. (1990). Axonogenesis in the brain of zebrafish embryos. *J. Neurosci.* **10,** 1892–1905.

Chung, S-H., and Cooke, J. (1975). Polarity of structure and of ordered nerve connections in the developing amphibian brain. *Nature* **258,** 126–132.

D'Arcangelo, G., Miao, G. G., Chen, S. C., Soares, H. D., Morgan, J. I., and Curran, T. (1995). A protein related to extracellular matrix proteins deleted in the mouse mutant *reeler*. *Nature* **374,** 719–723.

Eisen, J. S. (1991). Developmental neurobiology of the zebrafish. *J. Neurosci.* **11,** 311–317.

Gobron, S., Monnerie, H., Meiniel, R., Creveaux, I., Lehmann, W., Lamalle, D., Dastugue, B., and Meiniel, A. (1996). SCO-spondin: A new member of the thrombospondin family secreted by the subcommissural organ is a candidate in the modulation of neuronal aggregation. *J. Cell Sci.* **109,** 1053–1061.

Hammerschmidt, M., Pelegri, F., Mullins, M. C., Kane, D. A., Brand, M., van Eeden, F. J. M., Furutani-Seiki, M., Granato, M., Haffter, P., Heisenberg, C-P., Jiang, Y-J., Kelsh, R. N., Odenthal, J., Warga, R. M., and Nüsslein-Volhard, C. (1996). Mutation affecting morphogenesis during gastrulation and tail formation in the zebrafish, *Danio rerio*. *Development* **123,** 143–151.

Hatta, K., and Kimmel, C. B. (1993). Midline structures and central nervous system coordinates in zebrafish. *Perspect. Dev. Neurobiol.* **1,** 257–268.

Hauptmann, G., and Gerster, T. (1994). Two-color whole-mount in situ hybridization to vertebrate and *Drosophila* embryos. *Trends Genet.* **10,** 266.

Inoue, A., Takahashi, M., Hatta, K., Hotta, Y., and Okamoto, H. (1994). Developmental regulation of *Islet-1* mRNA expression during neuronal differentiation in embryonic zebrafish. *Dev. Dyn.* **199,** 1–11.

Itasaki, N., and Nakamura, H. (1992). Rostrocaudal polarity of the tectum in birds: Correlation of *en* gradient and topographic order in retinotectal projection. *Neuron* **8,** 787–798.

Itasaki, N., Ichijo, H., Hama, C., and Nakamura, H. (1991). Establishment of rostrocaudal polarity in the tectal primordium: *engrailed* and subsequent tectal polarity. *Development* **113,** 1133–1144.

Kennedy, T. E., and Tessier-Lavigne, M. (1995). Guidance and induction of branch formation in developing axons by target-derived diffusible factors. *Curr. Opin. Neurobiol.* **5,** 83–90.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203,** 253–310.

Klar, A., Baldassare, M., and Jessell, T. M. (1992). F-spondin: A gene expressed at high levels in the floor plate encodes a secreted protein that promotes neural cell adhesion and neurite extension. *Cell* **69,** 95–110.

Kozak, M. (1986). Point mutations define a sequence flanking the

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44,** 283–292.

Macdonald, R., Xu, Q., Barth, K. A., Mikkola, I., Holder, N., Fjose, A., Krauss, S., and Wilson, S. W. (1994). Regulatory gene expression boundaries demarcate sites of neuronal differentiation in the embryonic zebrafish forebrain. *Neuron* **13,** 1039–1053.

Marusich, M. F., Furneaux, H. M., Henion, P. D., and Weston, J. A. (1994). Hu neuronal proteins are expressed in proliferating neurogenic cells. *J. Neurobiol.* **25,** 143–155.

Meiniel, A., Meiniel, R., Didier, R., Creveaux, I., Gobron, S., Monerie, H., and Dastugue, B. (1996). The subcommissural organ and Reissner's fiber complex: An enigma in the central nervous system? *Prog. Histochem. Cytochem.* **30,** 1–66.

Monnerie, H., Boespflug-Tanbuy, O., Dastugue, B., and Meiniel, A. (1995). Reissner's fiber supports the survival of chick cortical neurons in primary mixed cultures. *Cell Tissue Res.* **282,** 81–91.

Morin-Kensicki, E. M., and Eisen, J. S. (1997). Sclerotome development and peripheral nervous system segmentation in embryonic zebrafish. *Development* **124,** 159–167.

Oksche, A. (1969). The subcommissural organ. *J. Neuro-Visc. Relat. (Suppl.)* **9,** 111–139.

Oksche, A., Rodriguez, E. M., and Fernandez-Llebrez, P. (1993). *In* ''The Subcommissural Organ: An Ependymal Brain Gland'' (A. Oksche, E. M. Rodriguez, and P. Fernandez-Llebrez, Eds.), pp. 1–333. Springer-Verlag, Berlin/Heidelberg/New York.

Oxtoby, E., and Jowett, T. (1993). Cloning of the zebrafish *krox-20* gene (*krx-20*) and its expression during hindbrain development. *Nucleic Acids Res.* **21,** 1087–1095.

Piperno, G., and Fuller, M. T. (1985). Monoclonal antibodies specific for an acetylated form of alpha-tubulin recognize the antigen in cilia and flagella from a variety of organisms. *J. Cell Biol.* **101,** 2085–2094.

Prater, C. A., Plotkin, J., Jaye, D., and Frazier, W. A. (1991). The properdin-like type I repeats of human thrombospondin contain a cell attachment site. *J. Cell Biol.* **112,** 1031–1040.

Reissner, E. (1860). Beitrage zur Kenntnis vom Bau des Rückenmarks von *Petromyzon fluviatilis L. Arch. Anat. Physiol.* **77,** 545–588.

Ruiz i Altaba, A., Cox, T. M., Jessell, T. M., and Klar, A. (1993). Ectopic neural expression of a floor plate marker in frog embryos injected with the midline transcription factor pintallavis. *Proc. Natl. Acad. Sci. USA* **90,** 8268–8272.

Rupp, R. A. W., Snider, L., and Weintraub, H. (1994). *Xenopus* embryos regulate the nuclear localization of XMyoD. *Genes Dev.* **8,** 1311–1323.

Sanes, J. R., Hunter, D. D., Green, T. L., and Merlie, J. P. (1990). S-laminin. *Cold Spring Harbor Symp. Quant. Biol.* **55,** 419–430.

Schier, A. F., Neuhauss, S. C. F., Harvey, M., Malicki, J., Solnica-Krezel, L., Stainier, D. Y. R., Zwartkruis, F., Abdelilah, S., Stemple, D. L., Rangini, Z., Yang, H., and Driever, W. (1996). Mutations affecting the development of the embryonic zebrafish brain. *Development* **123,** 165–178.

Schier, A. F., Neuhauss, S. C. F., Ann Helde, K., Talbot, W. S., and Driever, W. (1997). The *one-eyed pinhead* gene functions in mesoderm and endoderm formation in zebrafish and interacts with *no tail. Development* **124,** 327–342.

Tanabe, Y., and Jessell, T. M. (1996). Diversity and pattern in the developing spinal cord. *Science* **274,** 1115–1123.

Turner, D. L., and Weintraub, H. (1994). Expression of *achaete-scute homolog 3* in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8,** 1434–1447.

Umemiya, T., Takeichi, M., and Nose, A. (1997). M-spondin, a novel ECM protein highly homologous to vertebrate F-spondin, is localized at the muscle attachment sites in the *Drosophila* embryo. *Dev. Biol.* **186,** 165–176.

Westerfield, M. (1994). ''The Zebrafish Book,'' edition 2.1. Univ. Oregon Press, Eugene, OR.

Wilson, S. W., Ross, L. S., Parrett, T., and Easter, S. S. (1991). The development of a simple scaffold of axon tracts in the brain of the embryonic zebrafish, *Brachydanio rerio. Development* **108,** 121–145.

Yan, Y-L., Hatta, K., Riggleman, B., and Postlethwait. (1995). Expression of a type II collagen gene in the zebrafish embryonic axis. *Dev. Dyn.* **203,** 363–376.

Received for publication May 19, 1997
Accepted September 24, 1997

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.