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The Expression Pattern of the Mouse Receptor

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Evolution and Requires *Hoxa-2* for Rhombomere-Specific Expression in Mouse Embryos

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Segmentation of the hindbrain has been conserved throughout the vertebrate species and results in the transient formation of rhombomeres, which are lineage-restricted compartments. Studies on the molecular mechanisms underlying the segmentation process have revealed that rhombomeric boundaries coincide with the expression limits of several evolutionary conserved genes such as the zinc-finger transcription factor *Krox-20* and homeobox genes which are expressed in a specific spatial and temporal order and have been shown to be important regulators of segmental identity. In addition to *Krox-20* and Hox genes, several members of the Eph subfamily of receptor protein tyrosine kinase (RTK) genes are also expressed in a segment-restricted manner in the hindbrain, suggesting that these receptors may act in concert with Hox genes to establish regional identity. In the cascade of regulatory interactions leading to segmental identity, *Krox-20* appears to act ''upstream'' of Hox genes, but the identity of the ''downstream'' effectors has not yet been identified. We report here the isolation of the zebrafish orthologue of the mouse RTK gene *MDK1* which belongs to the Eph receptor subfamily and show that the major expression domains of the mouse and the zebrafish genes have been conserved through evolution. Since the coincident spatial and temporal expression of *Hoxa-2* and *MDK1* in the mouse hindbrain suggested a possible regulatory link between them, we analyzed the expression of the *MDK1* in *Hoxa-2* null mutant embryos. A selective lack of *MDK1* expression in rhombomere 3 of *Hoxa-2* mutant hindbrains together with an overall altered expression pattern in the other rhombomeres was observed, thus demonstrating that *MDK1* lies downstream of *Hoxa-2* in the morphogenetic signaling cascade. © 1996 Academic Press, Inc.

INTRODUCTION

Embryonic development and pattern formation in vertebrates require coordinated growth and cell differentiation. Intercellular communication is crucial for development, and cells respond to many extracellular cues by signal transduction through membrane-bound proteins. One class of such membrane-bound molecules is represented by receptor protein tyrosine kinases (RTKs). Binding of the ligand to these receptors results in the activation of the tyrosine kinase activity localized in the intracellular domain of RTKs, which in turn phosphorylates the receptor itself and several ''downstream'' proteins, triggering a cascade of intracellular signaling (Schlessinger and Ullrich, 1992; Fantl *et al.,* 1993; van der Geer *et al.,* 1994). Some RTKs stimulate cell growth and proliferation, while others promote cell differentiation. For instance, the tyrosine kinase activity of growth factor receptors is critical for signal transduction pathways required for mitogenesis, transformation, and cell differentiation (Ullrich and Schlessinger, 1990; Fantl *et al.,* 1993; van der Geer *et al.,* 1994) and the c-kit receptor tyrosine kinase encoded at the mouse *W* locus (Chabot *et al.,* 1988; Geissler *et al.,* 1988) plays a role in the migration and differentiation properties of primordial germ cells, hematopoietic stem cells, and neural crest progenitor cells. The neurotrophin family which includes Trk RTKs is involved in neuronal differentiation (Snider, 1994).

Based on conserved structural homologies, RTKs have

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been divided into several subfamilies (van der Geer *et al.,* 1994) and, with 13 members, the Eph subfamily is the largest to date. This subfamily is characterized by the presence of a conserved cysteine-rich region, two fibronectin type III repeats (Skorstengaard *et al.,* 1986) in the extracellular domain, and the presence of a cytoplasmic catalytic domain. Several members of this subfamily, namely, *Elk* (Letwin *et al.,* 1988), *Eek* (Chan and Watt, 1991), *Cek-4* (Sajjadi *et al.,* 1991), *Cek-5* (Pasquale, 1991), *Sek-1* (Gilardi-Hebenstreit *et al.,* 1992), *Ehk1/2* (Maisonpierre *et al.,* 1993), and *MDK1/Ebk* (Ciossek *et al.,* 1995; Ellis *et al.,* 1995), are expressed almost exclusively in the developing or adult brain, suggesting a role for these receptors in neural development or function.

Morphogenesis of the hindbrain region of the central nervous system (CNS) involves a segmentation process resulting in the transient formation of rhombomeres (Lumsden and Keynes, 1989; Lumsden, 1990; Guthrie, 1995), which are lineage-restricted compartments. Several members of the Hox gene family are expressed in segment-specific patterns (Murphy *et al.,* 1989; Wilkinson *et al.,* 1989b; Frohman *et al.,* 1990; Sundin and Eichele, 1990; Hunt *et al.,* 1991; Murphy and Hill, 1991) and have been shown to play a role in determining segment identity and patterning neural crest cells (Lufkin *et al.,* 1991; Chisaka *et al.,* 1992; Dollé *et al.,* 1993; Mark *et al.,* 1993; Carpenter *et al.,* 1993; Rijli *et al.,* 1993; Gendron-Maguire *et al.,* 1993). Exposure of vertebrate embryos to an excess of all-*trans* retinoic acid (T-RA) affects the specification of regional identities in the CNS, and this is accompanied by repatterning of the expression domains of homeobox-containing genes in the hindbrain and spinal cord (Conlon, 1995; Conlon and Rossant, 1992; Maden and Holder, 1992; Marshall *et al.,* 1992). Several retinoic acid receptors and retinoic acid binding proteins (Ruberte *et al.,* 1991, 1992; Maden *et al.,* 1991, 1992) also exhibit a segment-specific expression pattern which may indicate the involvement of retinoic acid (RA) in regulating Hox gene expression. Members of the Eph subfamily of RTKs such as *Sek-1, Mek-4, Eck/Sek-2, Nuk/Sek-3,* and *Sek-4* (Becker *et al.,* 1994; Cheng and Flanagan, 1994; Ganju *et al.,* 1994; Henkemeyer *et al.,* 1994; Nieto *et al.,* 1992) are also expressed in characteristic rhombomere-specific patterns, thus suggesting a role for these RTKs in either hindbrain segmentation or establishment of rhombomere-specific properties. Interestingly, the expression of some of these receptors coincides with the zinc-finger transcription factor *Krox-20,* which is expressed in two alternate rhombomeres, 3 and 5, and is crucial for their maintenance (Wilkinson *et al.,* 1989a; Schneider-Maunoury *et al.,* 1993; Swiatek and Gridley, 1993). Little is known concerning the regulatory cascades which ultimately result in positional identity and acquisition of rhombomere-specific properties. Disruption of the *Krox-20* gene in mice results in a loss of *Hoxb-2* expression in r3/r5, thus suggesting that *Hoxb-2* expression is controlled by *Krox-20* (Swiatek and Gridley, 1993; Schneider-Maunoury *et al.,* 1993). Furthermore, *Krox-20* has been shown to regulate the expression of *Hoxb-2* (Sham *et al.,* 1993). However, the mechanism by which Hox genes regulate regional identity is still unclear as virtually nothing is known about the downstream targets of Hox genes in mammals.

We have recently cloned a cDNA sequence corresponding to a gene which is induced in P19 embryonal carcinoma cells by RA treatment (Bouillet *et al.,* 1995; and our unpublished results). This cDNA proved to be identical to that of a novel, recently reported member of the Eph subfamily of RTK genes: the *MDK1/Ebk* gene (Ciossek *et al.,* 1995; Ellis *et al.,* 1995). In the present study, we have cloned *ZDK1,* the zebrafish orthologue of *MDK1,* and shown that the expression patterns of the zebrafish and mouse genes are conserved through evolution. The early onset of *ZDK1* and *MDK1* expression in mouse and zebrafish embryos, prior to rhombomeric segmentation, suggests that these RTKs play a role in hindbrain patterning. In view of the coincident spatial and temporal expression of *Hoxa-2* and *MDK1* in the hindbrain, we have also analyzed the expression of *MDK1* in *Hoxa-2* null embryos (Rijli *et al.,* 1993). Strikingly, these embryos exhibited a selective lack of *MDK1* expression in rhombomere 3, together with an alteration in the normal expression pattern in other rhombomeres. These observations provide the first evidence of a signaling molecule which is a downstream effector of a Hox gene *in vivo.*

MATERIALS AND METHODS

Screening of P19 and zebrafish cDNA libraries. A partial cDNA clone containing a 208-bp fragment (corresponding to nucleotides 101 to 309 of the *MDK1* cDNA; Ciossek *et al.,* 1995) was obtained by differential screening of P19 cells treated with RA (Bouillet *et al.,* 1995). This fragment was used to screen a λZAPII oligo(dT) cDNA library made from P19 cells induced with T-RA for 24 hr, which resulted in the isolation of a full-length cDNA clone. A zebrafish embryo λZAPII oligo(dT) cDNA library (a gift from Catherine Fromental) was screened under low stringency with a 513-bp *Ava*I fragment (containing the conserved tyrosine kinase domain from nucleotides 2573 to 3086 of *MDK1*). Thirty-eight positive plaques were isolated and subjected to secondary screening with the same probe. The filters were stripped and reprobed with a fragment lacking the tyrosine kinase domain. Two plaques which were positive with both conserved and nonconserved domain probes were purified and sequenced on both strands on an Applied Biosystems DNA sequencer.

Whole-mount in situ hybridizations. Whole-mount *in situ* hybridizations on E7.5 to E9.5 mouse embryos were as described (Décimo *et al.,* 1995). The antisense probe corresponded to regions 2855 to 3416 of *MDK1* (Ciossek *et al.,* 1995). For histological analysis, the embryos were dehydrated, embedded in paraffin, and sectioned at 12-μm thickness. Whole-mount *in situ* hybridizations on zebrafish embryos were as described (Thisse *et al.,* 1994). Double labeling whole-mount *in situ* hybridizations were also as described (Hauptmann and Gerster, 1994) except that the dilution of the anti-fluorescein-AP Fab fragments was 1:5000 instead of 1:2000.

Southern blot analysis. Embryos from the *Hoxa-2*$^{+/-}$ intercrosses were genotyped using yolk sac DNA as described previously (Rijli *et al.,* 1993).

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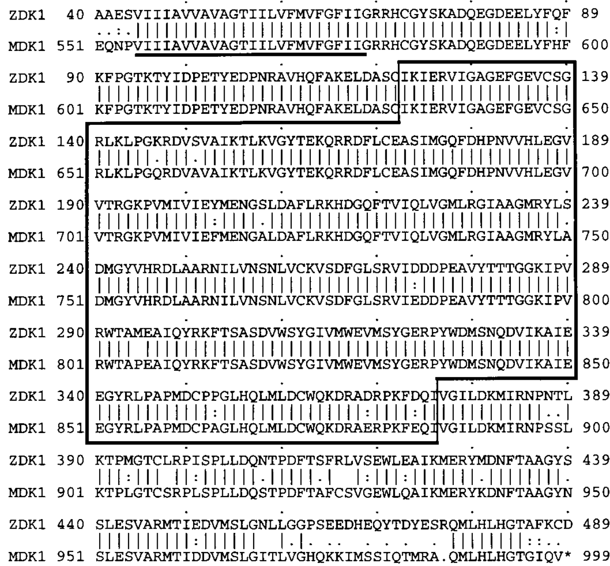

FIG. 1. Alignment of the predicted amino acid sequence of *ZDK1* (starting at position 40) and *MDK1.* Vertical bars show identical amino acids, conserved substitutions are indicated by double dots, and less well-conserved substitutions by single dots. A high degree of identity is seen over the transmembrane domain (underlined) and the catalytic tyrosine kinase domain (boxed). Numbers correspond to the amino acid positions.

RESULTS

Molecular Cloning of MDK1 and Its Zebrafish Orthologue, ZDK1

A differential subtractive hybridization cloning of P19 cell cDNAs has led to the identification of several novel RA-responsive genes (Bouillet *et al.,* 1995), which have been shown to be induced by RA during the course of differentiation of mouse embryonal carcinoma cells (Bouillet *et al.,* 1995; Taneja *et al.,* 1995; Roy *et al.,* 1995). One such partial cDNA clone was used to screen a λZAPII cDNA library prepared from P19 cells treated with RA. The resulting full-length cDNA was identified through database searches as that of a RTK gene, and during the course of the investigation an identical cDNA, *MDK1,* was reported by Ciossek *et al.* (1995). Using a cDNA probe corresponding to the conserved tyrosine kinase domain of *MDK1,* we screened a zebrafish cDNA library and isolated several clones, one of which showed an extensive conservation with *MDK1.* This clone containing a 1.59-kb insert was termed *ZDK1* (for zebrafish developmental kinase 1). Alignment of the predicted amino acid sequence of *ZDK1* with that of *MDK1* indicated a very high conservation over both the transmembrane and the catalytic tyrosine kinase domains (>92% identity; Fig. 1), indicating that *ZDK1* was most probably the zebrafish orthologue of *MDK1.*

Expression Pattern of the Zebrafish ZDK1 Gene

To investigate whether the extensive sequence conservation between *ZDK1* and *MDK1* also reflected an evolutionary conserved expression pattern, zebrafish embryos were analyzed at different stages of development for *ZDK1* expression using whole-mount *in situ* hybridization. *ZDK1* transcripts were first detected in the hypoblast layer (mesendoderm), shortly after the beginning of gastrulation (60% of epiboly, Fig. 2A). *ZDK1* expressing cells correspond to those derived from the organizer region (the central part of the embryonic shield) that express the *goosecoid* gene (Stachel *et al.,* 1993; Schulte-Merker *et al.,* 1994; Thisse *et al.,* 1994) to form the prechordal plate mesendoderm. At 80% epiboly, shortly after midgastrulation, *ZDK1* transcripts were observed in the prechordal plate mesendoderm, but were absent from its most anterior part, the pillow, that later gives rise to the hatching gland (Fig. 2B). At this stage, a new territory of expression appeared in the epiblast layer, in the region corresponding to the presumptive hindbrain (Fig. 2B). *ZDK1* was expressed as two faint transverse bands (Figs. 2C and 2D), forming an angle of about 50–60° with the midline, where it was not expressed. Subsequently, at the end of gastrulation, the two transverse bands corresponding to expression in presumptive rhombomeres (r) 3 and 4 rejoined at the midline (Fig. 2E). At the bud stage, and before the formation of the first somite, another domain of expression of *ZDK1* also appeared as a faintly labeled, bilaterally symmetrical territory localized dorsally in the epiblastic cells in the position of the anterior midbrain and posterior forebrain (Figs. 2D and 2E).

During early somitogenesis *ZDK1* transcripts were observed at the anteriormost tip of the neuroectoderm, and also along the ventral midline of the forebrain (Fig. 3A). The latter expression extended progressively from the ventromedial domain to the dorsomedial part across the anterior tip of the neuroectoderm (not shown). At the same stage, the intensity of the staining increased at the border between the forebrain and the midbrain, forming a dorsoventral gradient of expression (Fig. 3A).

The hindbrain expression of *ZDK1,* first seen at late gastrula, evolved very dynamically, with expression first detected in the regions corresponding to r3 and r4 (Fig. 2E). At the 8 somite stage, a lateral view showed that expression in r3 and r4 was not homogenous, but restricted to the dorsal half of r3 and interrupted in the medial part of r4 with very strong staining observed in the ventralmost part as well as in the dorsal half of this rhombomere (Fig. 3A). This observation was confirmed on a thick section of r4 (Fig. 3B). The demonstration of the precise localization of *ZDK1* expression in the hindbrain was performed using double labeling whole-mount *in situ* hybridization with two other cDNA probes, *krox-20* (Oxtoby and Jowett, 1993) and *Engrailed 2* (Ekker *et al.,* 1992). The *krox-20* gene is expressed in both rhombomere 3 and rhombomere 5, and the *Engrailed 2* gene at the midbrain–hindbrain junction. *ZDK1* expression was observed in r4 and also overlapped dorsally in r3 with *krox-20* (Fig. 3C; see also Fig. 3A).

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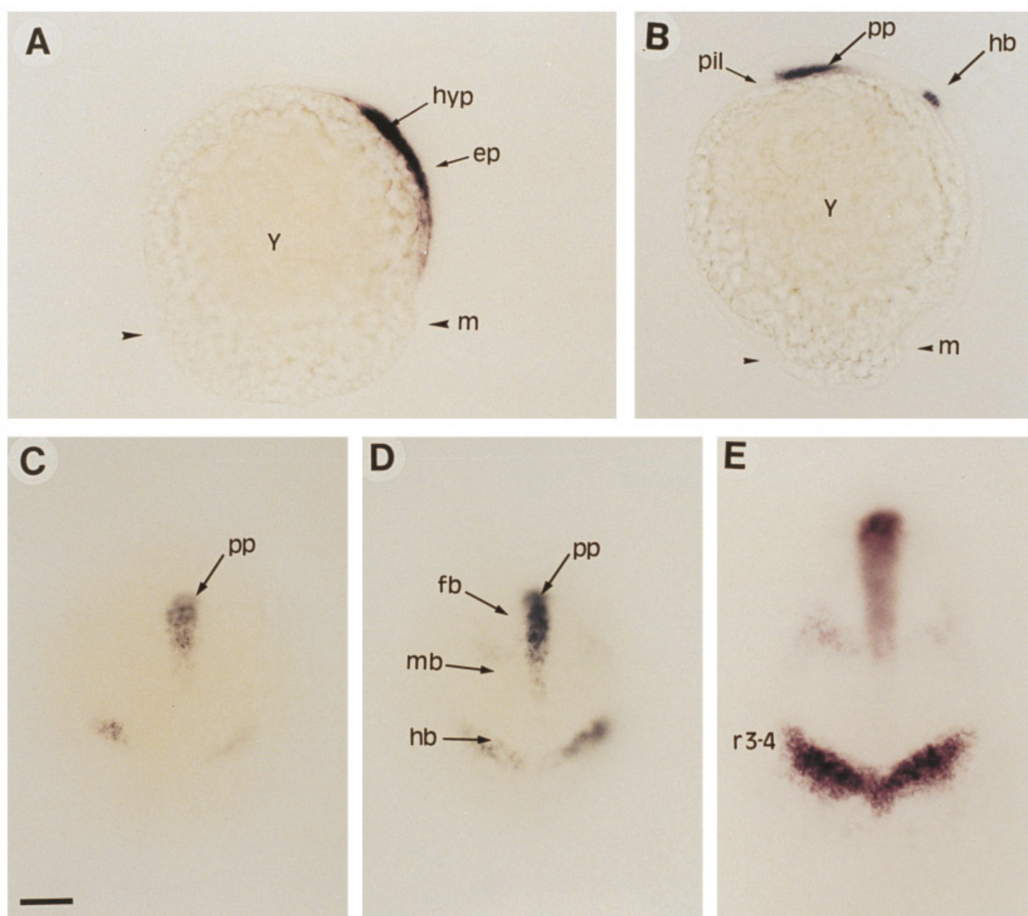

FIG. 2. Early expression pattern of *ZDK1* during gastrulation. (A) At 60% epiboly, expression is seen in the hypoblast but not in the epiblast. (B) At 80% epiboly, additional expression is detected in the presumptive hindbrain region. (C) Dorsal view of embryo shown in B. (D and E) Dorsal views of embryos at 100% epiboly (D) and bud stage (E) showing *ZDK1* expression in hindbrain, in the forebrain–midbrain boundary, and in the prechordal plate mesendoderm which underlies the ventral forebrain. Abbreviations: ep, epiblast; fb, forebrain; hb, hindbrain; hyp, hypoblast; m, margin; mb, midbrain; pil, pillow; pp, prechordal plate; r3-4, rhombomeres 3 and 4; y, yolk. (A and B) Side view, dorsal is to the right, anterior is to the top. (C–E) Dorsal view, anterior is up. Scale bar: A–D, 25 μm; E, 50 μm.

A lateral view (Fig. 3C) clearly showed the interrupted staining in r4. However, viewed dorsally, the labeling appeared homogeneous (Fig. 3D) as also seen on dorsal views at later stages (Figs. 3E–3G).

By the 10 somite stage, *ZDK1* expression appeared in additional rhombomeres. Expression was maximal in r4, but was observed more anteriorly in r3 and r2, as well as more posteriorly in r5 and 6 (Fig. 3E). Expression in r4 decreased by the 20 somite stage, but increased in r3 and r2, as well as posteriorly in r5 and r6 (Fig. 3F). Along the dorsoventral axis, the interruption of the *ZDK1* expression in the medial part of the rhombomere was only observed for r3 and 4, whereas the staining appeared homogeneous in r2, r5, and r6 (data not shown). Weak transcripts were also found in r1 at the 15 somite stage on strongly overstained embryos (data not shown). Subsequently, in 24 hr embryos and up to 48 hr, the hindbrain expression pattern of *ZDK1* was strong in r2 and r3 and followed an anteroposterior gradient from rhombomeres 2 to 6 (Fig. 3G).

In the anterior part of the head, the expression at the midbrain–forebrain boundary which was observed at the beginning of somitogenesis (Fig. 3A) was still observed at later stages (Figs. 3E–3G). At the 10 somite stage, this expression territory was composed of epidermal and neuroectodermal cells. After the 10 somite stage, two domains strongly expressing *ZDK1* were seen in the forebrain–midbrain region. The anterior one was localized in the forebrain between the telencephalon and diencephalon, and the posterior one in the anterior part of the midbrain adjacent to the forebrain–midbrain boundary (Figs. 3F and 3G). In addition, *ZDK1* RNA appeared in the optic vesicle (Figs. 3E–3G). The staining in this territory was restricted to the lateral–

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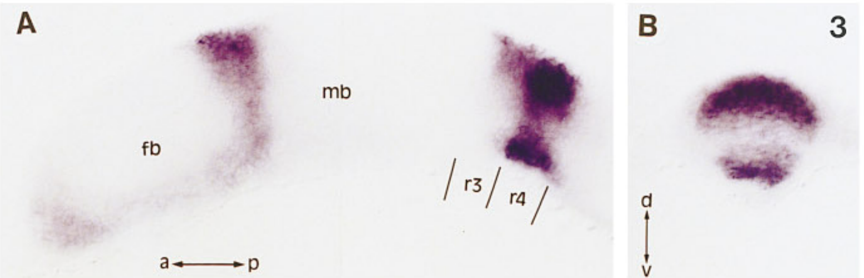

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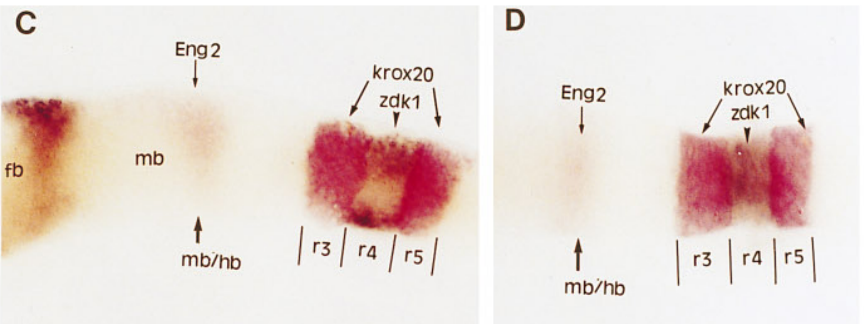

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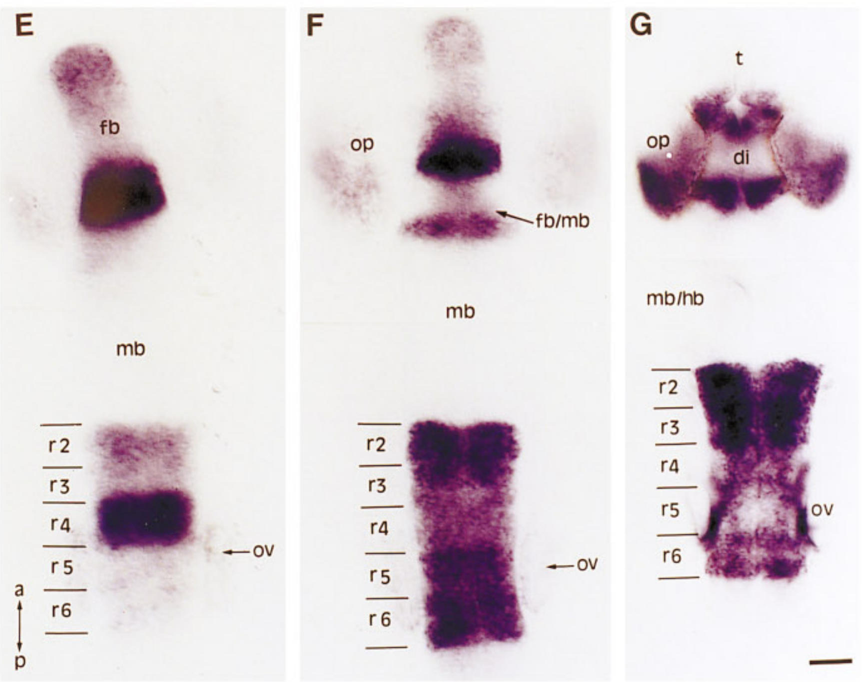

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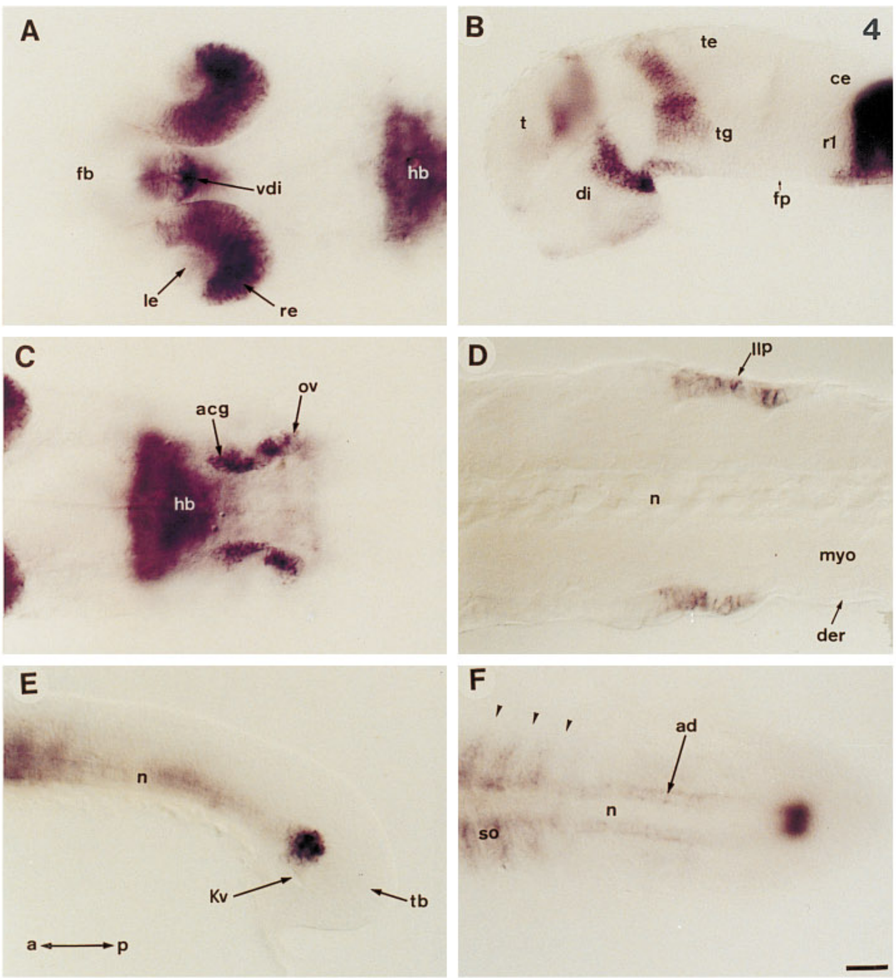

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posterior part of the vesicle that forms the posterior part of the retina (Figs. 3E–3G and 4A). Expression in the posterior part of the retina was detected until 48 hr and cells of the retina adjacent and posterior to the lens were still labeled at this stage (data not shown). Transcripts of *ZDK1* also accumulated in the developing lens after 24 hr of development (Fig. 4A).

Ventrally, the forebrain expression increased strongly after the beginning of somitogenesis and at this stage, *ZDK1* transcripts were located in the telencephalon at the border between telencephalon and diencephalon. *ZDK1* transcripts strongly accumulated in the ventralmost cells of the diencephalon (Figs. 4A and 4B) and additionally extended from this ventral position to the telencephalic domain of expression (Fig. 4B). In the midbrain, at the diencephalon–mesencephalon border, *ZDK1* transcripts were present in a region extending from the floor plate territory to the anterior tectum across the tegmentum (Fig. 4B). Lateral to the hindbrain, the otic vesicle started to accumulate *ZDK1* transcripts after the 10 somite stage (Figs. 3E–3G; see also Fig. 4C). This staining first appeared to be faint and homogeneous in the otic vesicle, but increased in intensity and was progressively restricted to the medioposterior part of the ear (Fig. 4C) where transcripts were still detected at 2 days of development. Anteromedially to the otic vesicle, the acoustic (VIIIth) ganglia showed accumulation of *ZDK1* transcripts after 24 hr of development (Fig. 4C).

ZDK1 expression was also observed in a few other regions, such as in cells located at the tip of the posterior lateral line primordium that migrates at the level of the myoseptum between myomeres and dermis (Fig. 4D). Caudally *ZDK1* transcripts were observed in cells of the axial mesoderm from the beginning to the end of somitogenesis. This expression was strong at the tip of the notochord and decreased rapidly as the notochord cells differentiated (Fig. 4E). A faint staining was also detected in the segmental plate mesoderm which was restricted to the unsegmented adaxial cells, and the labeling disappeared as this teritory became segmented into somites (Fig. 4F). Simultaneously, *ZDK1* transcripts appeared in paraxial mesoderm upon somite differentiation (Fig. 4F). Cells surrounding the anus also contained ZDK1 transcripts after 24 hr of development (not shown). Finally, at 2 days of development some transcripts were also observed in part of pharyngeal arches, especially in cells surrounding the mouth opening (not shown).

Early Expression Pattern of MDK1

The expression pattern of mouse *MDK1* has been described from mid to late gestation in two recent studies (Ellis *et al.,* 1995; Ciossek *et al.,* 1995). Our own *in situ* hybridization studies on E11.5 to E13.5 embryo sections (data not shown) confirm these data. To compare the early expression patterns of *ZDK1* and *MDK1,* we performed whole-mount *in situ* hybridization with *MDK1* at earlier developmental stages (E7.5 to E9.5), which revealed novel or more precise expression domains than previously described (Ciossek *et al.,* 1995; Ellis *et al.,* 1995). *MDK1* transcripts were detected in E7.5 embryos (early primitive streak stage) in the head process ectoderm and mesoderm (Fig. 5A). In slightly older embryos (headfold presomite stage), transcripts were found in the rostral part of the headfolds, both in the neural groove neuroectoderm and in the subjacent head mesoderm (Figs. 5B and 5C) and in more caudal regions of the headfolds, in the most lateral regions of the mesoderm, i.e., in the boundary regions between embryonic and extraembryonic mesoderm (Figs. 5B, 5D, and 5E). Note that there was no transcript signal in the primitive streak and in the adjacent mesoderm (Figs. 5B, 5D, and 5E).

At E8.5, the edges of the forebrain neural folds (Figs. 6A and 6B) were strongly labeled. *MDK1* also showed a dynamic expression pattern in the hindbrain, which appeared to evolve concomitantly to the rhombomeric segmentation process (Fig. 6). *MDK1* expression was initiated before any sign of rhombomeric segmentation in a domain which corresponds to the presumptive r3 (Fig. 6A, embryo with 2–3 somite pairs). *MDK1* expression was clearly restricted to r3 in slightly older embryos, once this rhombomere had segmental boundaries (Fig. 6B, embryo with 6 somite pairs). Soon after, *MDK1* expression extended both rostrally in r2

FIG. 3. Expression of *ZDK1* in the hindbrain and the forebrain. (A) Lateral view of an 8 somite embryo. (B) Thick dorsoventral section at the level of r4 of the same embryo shown in A. (C) Lateral view and (D) dorsal view of an 8 somite embryo, double labeled with *Krox-20* (in red), *Engrailed 2* (*Eng 2,* in red), and *ZDK1* (in brown–black). (E–G) Dorsal views of 10 somite (E) 20 somite (F), and 24 hr (G) embryos. Abbreviations: a, anterior; d, dorsal; di, diencephalon; fb, forebrain; fb/mb, forebrain–midbrain junction; mb, midbrain; mb/hb, midbrain–hindbrain junction; op, optic vesicle; ov, otic vesicle; p, posterior; r2–r6, rhombomeres 2–6; t, telencephalon; v, ventral. Scale bar: A–F, 50 μm; G, 25 μm.

FIG. 4. *ZDK1* expression at later stages of development. (A) 36 hr embryo viewed dorsally showing strong expression in the posterior retina, lens, and ventral forebrain. (B) Lateral view of the same embryo as in A after removing the eye. (C) Posterior domains of expression of the same embryo shown in A. Optical cross section at the level of accoustic ganglia and otic vesicle. (D) Optical, horizontal cross section of a 36 hr embryo at the level of the caudal lateral line primodium. (E and F) Caudal expression in the lateral line primordium at the tip of the notochord of a 14 somite embryo. (E) Lateral view; (F) dorsal view. Abbreviations: a, anterior; acg, accoustic ganglia; ad, adaxial cells; ce, cerebellum; der, dermis; di, diencephalon; fb, ventral forebrain; fp, floor plate; hb, hindbrain; Kv, Kuppfer vesicle; le, lens; llp, lateral line primordium; myo, myotome; n, notochord; ov, otic vesicle; p, posterior; r, rhombomere; re, retina; so, somite; t, telencephalon; te, tectum; tb, tailbud; tg, tegmentum; vdi, ventral diencephalon. Arrowheads indicate the positions of the somitic furrows. Scale bar: A–C, 50 μm; D, 100 μm. Anterior is to the left.

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and caudally in r4 and r5 (Fig. 6C, embryo with 8–10 somite pairs). Note that r3 was labeled much more intensely than any other rhombomeres and that r5 was labeled more intensely than r4 (Figs. 6C and 6F). In older embryos (~12 somite pairs), *MDK1* was strongly up-regulated in r2 (the signal being almost as intense as that in r3), but not in other rhombomeres (data not shown; but see Figs. 6D and 9D). Concomitantly, *MDK1* expression was activated in r6 to end up with the ''final'' rhombomeric expression pattern observed in E9.5 embryos, with strong expression in r2–3 and r5, and weaker expression in r4 and r6 (Figs. 6D, 6E, and 8C). Histological analysis showed that the signal was maximal along the dorsal edges of the rhombomeres and extended more ventrally in cells of the ventricular layer (Figs. 7A and 7B).

In order to verify that our mapping of early rhombomeres was correct, age-matched embryos (8 somite pairs ± 2) were hybridized to a probe for *MDK1* (Fig. 6F), for *Krox-20* (Fig. 6G), or to a mixture of both probes (Fig. 6H). In these embryos, the *MDK1* signal was maximal in r3 and very weak in r5 (Fig. 6F). In contrast, the *Krox-20* signal was much weaker in r3 than in r5 (Fig. 6G), indicating that down-regulation of this gene has already occurred in r3, as previously described (Wilkinson *et al.,* 1989a). A mixture of both probes yielded signals of equal intensities in r3 and r5, thereby confirming that the expression levels of both genes are opposite in r3 and r5 at this developmental stage (Fig. 6H).

MDK1 transcripts were detected at E8.5 in the lateral mesoderm adjacent to the first somites (Figs. 6A and 6B). We did not detect *MDK1* expression in the posterior part of the notochord, in contrast to the expression pattern of the zebrafish gene (see above). *MDK1* expression in the otic vesicle (see Figs. 6D, 6E, and 7B), and later on in the developing cochlea, has been described (Ellis *et al.,* 1995). We note that there was no detectable labeling in the cranial nerve ganglia at E9.5, although *MDK1* transcripts were detected in the trigeminal ganglia at later stages (data not shown; see Ellis *et al.,* 1995). In contrast to this previous study, we did not detect *MDK1* labeling in the developing heart (see, e.g., Figs. 6A, 6E, and 6F).

MDK1 was also expressed within the somitic mesoderm. Its activation clearly followed somite maturation which proceeds in a cranio–caudal direction. Indeed, *MDK1* transcripts were always detected in all somites, except the two to three caudal pairs that were the most recent to segregate (see Figs. 6B and 6C at E8.5 and Fig. 6E at E9.5). Expression was restricted to the most dorsal part of the somites (Figs. 6B and 6E; see also Fig. 7) which was confirmed on histological sections of differentiating somites at E9.5 (Fig. 7C). In rostral somites which are more advanced in their differentiation, *MDK1* transcripts were located in the boundary region between the dermatome and the myotome (Figs. 7C and 7D). In more caudal somites, this expression was clearly restricted to the dorsal edge of the dermamyotomes which have not yet segregated into two distinct structures (Fig. 7E).

Forebrain expression of *MDK1* was seen early in the anterior forebrain neural folds (Fig. 8A; E8.0; 0–2 somites). Later, there were two distinct *MDK-1* expression domains in the E8.5–9.0 developing forebrain. The first one extended from the midbrain–forebrain junction to the optic vesicles and the second one was at the base of the optic vesicles (Fig. 8B). Both domains consisted of very dorsal cells in regions where the forebrain neural folds were about to fuse. At later stages, the *MDK-1* gene was still expressed at and near the dorsal midline in the same forebrain regions (Fig. 8C and Ellis *et al.,* 1995). There was no detectable expression in the optic vesicles during the early steps of their evagination (Fig. 8B).

Altered Expression of MDK-1 in Hoxa-2 Null Mutant Mouse Embryos

Hoxa-1$^{-/-}$ null mutant mouse embryos display an almost complete lack of rhombomeres 4 and 5 (Dollé *et al.,* 1993; Mark *et al.,* 1993; Carpenter *et al.,* 1993), whereas homeotic transformations of the structures derived from the skeletogenic neural crest from the same rhombomeres are associated with the loss of *Hoxa-2* function in mice (Rijli *et al.,* 1993; Gendron-Maguire *et al.,* 1993). The expression pattern of *MDK1* in the hindbrain at different developmental stages is reminiscent of that of Hox genes. A comparison of the *Hoxa-2* and *MDK1* expression patterns in E8.5 WT embryos

FIG. 5. Expression of *MDK1* in presomite stage mouse embryos. (A) Late primitive streak stage embryo (E7.0) hybridized to a *MDK1* digoxigenin-labeled probe. Anterior (head process) is to the left, and posterior (primitive streak) to the right. (B) Early headfold presomite stage embryo (E7.5). The embryo is positioned such that the headfold neural plate is facing the viewer. (C) Transverse histological section through the headfold of the embryo shown in B. Note that there is labeling in the headfold neuroectoderm and mesoderm. (D and E) Serial transverse sections of the same embryo through more caudal levels. There is labeling in the headfold neuroectoderm and in the lateral regions of the mesoderm. In contrast, the posterior part of the embryo (including the primitive streak) is not labeled. Abbreviations: a, anterior; p, posterior; ps, primitive streak; hf, headfold; hp, head process; lm, lateral mesoderm; me, mesoderm; ne, neuroectoderm.

FIG. 6. Expression of *MDK1* in the mouse developing hindbrain. (A) 2–3 somite embryo (E8.5), lateral view. (B) 6 somite embryo (E8.5), dorsal view. (C) 8–10 somite embryo (E8.5), dorsal view. (D) 20 somite embryo (E9.5), dorsal view of the cervical region. (E) 17–18 somite embryo (E9.5), lateral view. A to E were hybridized to a *MDK1* probe. (F) 8–9 somite embryo (E8.5) hybridized to an *MDK1* probe. (G) Age-matched embryo hybridized to a *Krox-20* probe. (H) Age-matched embryo hybridized to a mix of both the *MDK1* and the *Krox-20* probe. Abbreviations: fb, forebrain neural folds; h, heart; hb, hindbrain; lm, lateral mesoderm; ov, otic vesicle; r2–r56, rhombomeres 2–56; so, somitic expression domain.

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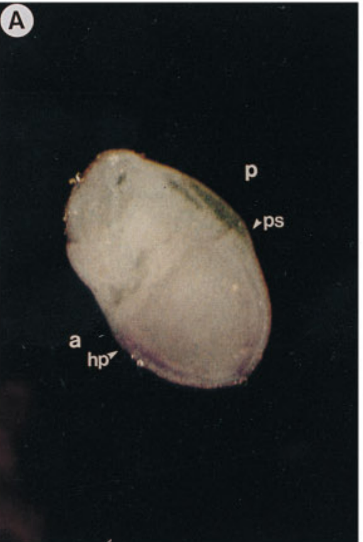
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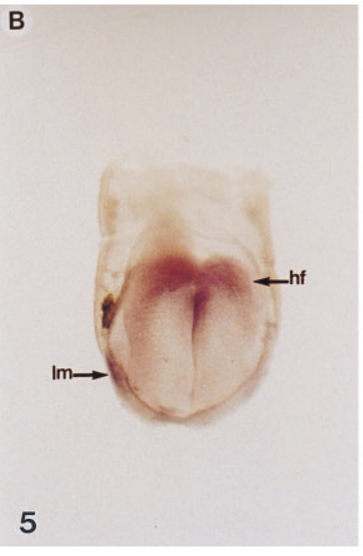
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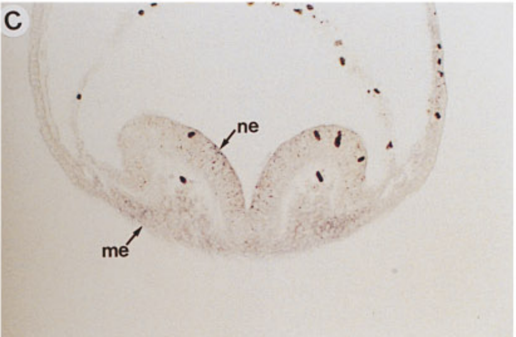
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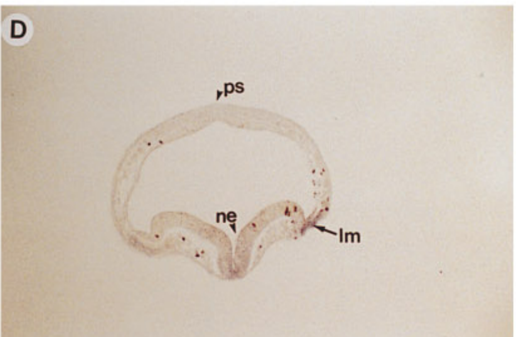
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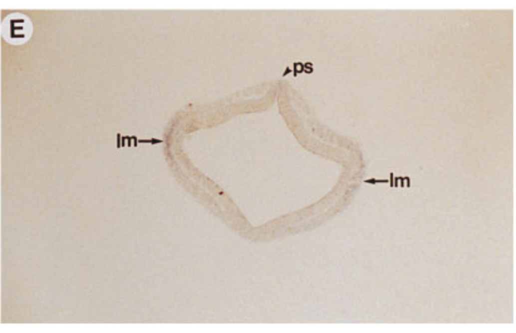
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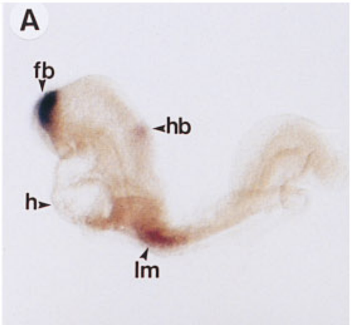
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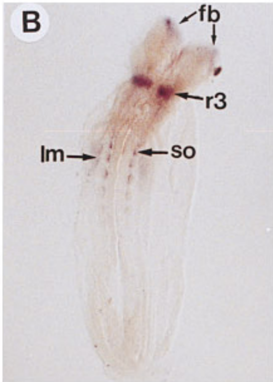
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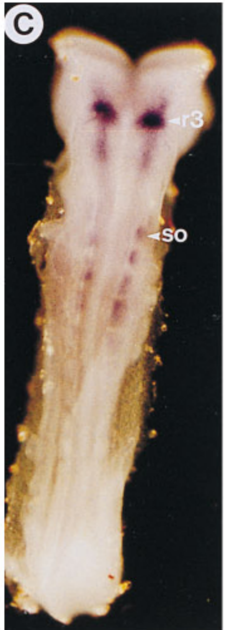
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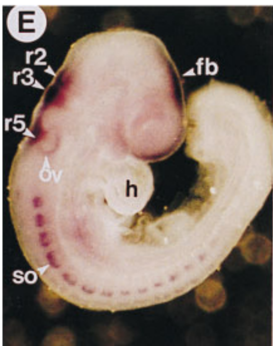
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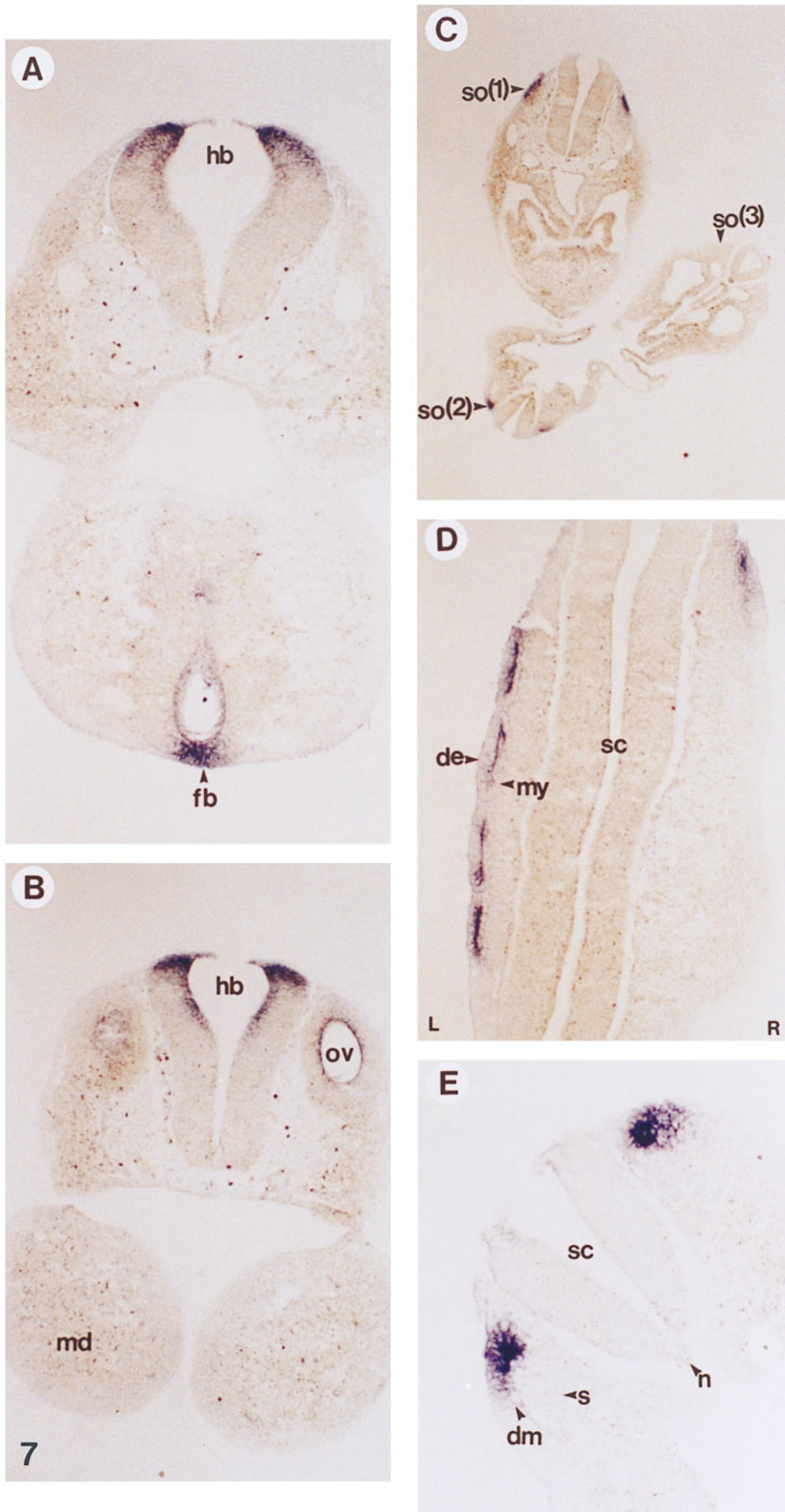
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(compare Figs. 9A and 9B, respectively) revealed striking similarities, as both genes were similarly expressed at the level of r3 and r5. At later stages, *MDK1* was strongly expressed in r3 and r2 in WT embryos, with weaker expression in r5 followed by r4 (Figs. 9D and 9F). A very similar expression pattern was found for the *Hoxa-2* gene (Fig. 9A and data not shown; see also Krumlauf, 1993). We therefore investigated whether part of the rhombomeric expression of *MDK1* might be deregulated in the *Hoxa-2*$^{-/-}$ embryos (Rijli *et al.,* 1993; Gendron-Maguire *et al.,* 1993). Whole-mount *in situ* hybridization was performed on E8.5, E8.75, and E9.0 litters from *Hoxa-2*$^{+/-}$ mutant intercrosses (Figs. 9B–9G). Strikingly, no expression of *MDK1* was detected in r3 of *Hoxa-2* null mutant embryos (Figs. 9C, 9E, and 9G), although *MDK1* was strongly expressed in r3 in WT embryos at equivalent stages (Figs. 9B, 9D, and 9F; see also Fig. 6B). In addition, the r2 expression in the *Hoxa-2* null mutants also appeared to be reduced when compared to the WT counterpart (compare Figs. 9D and 9F). In contrast, r4 appeared more strongly labeled in the *Hoxa-2*$^{-/-}$ mutants than in WT, suggesting that the absence of *Hoxa-2* results in abnormal up-regulation of *MDK1* expression in this rhombomere (compare Figs. 9D to 9E and 9F to 9G, and note that mutant r4 and r2 appeared similarly labeled).

DISCUSSION

Analysis of the RTK genes in *Drosophila* has revealed a critical role for members of this superfamily in pattern formation in the early embryo and the eye (Pawson and Bernstein, 1990). Almost all members of the vertebrate Eph subfamily of RTK genes show a pattern of expression which is restricted to the nervous system. Putative orthologues of some murine members of this subfamily have been identified in chick and human. A putative zebrafish orthologue of *Sek-1* has also been reported on the basis of its sequence identity over the tyrosine kinase domain (Xu *et al.,* 1994) and recently been shown to exhibit a conserved expression pattern in zebrafish and *Xenopus* embryos (Xu *et al.,* 1995). A comparison of transcript tissue-specific distribution patterns of other putative orthologues has shown that they are not strongly conserved between mammals and birds. For instance, *Mek-4* (Sajjadi *et al.,* 1991) is selectively expressed in the brain in adult mice, whereas its putative avian orthologue, *Cek-4* (Sajjadi *et al.,* 1991), is expressed in several additional organs. Similarly, *Ehk* (Maisonpierre *et al.,* 1993) is expressed in the adult mouse brain, whereas its avian counterpart, *Cek-7* (Sajjadi and Pasquale, 1993), is not.

The Zebrafish ZDK1 and the Mouse MDK1 Orthologues Exhibit Evolutionary Conserved Domains of Expression

We have identified here a zebrafish RTK gene, *ZDK1,* which belongs to the Eph subfamily of RTK genes. *ZDK1* appears to be the true orthologue of the mouse RTK gene *MDK1,* on the basis of both a high sequence identity and the evolutionary conservation of most of the major domains of expression during embryonic development. Our present analysis of *ZDK1* gene expression during zebrafish embryogenesis revealed complex and dynamic expression patterns in various germ layer derivatives. *ZDK1* transcripts were first detected during early gastrulation in a part of the hypoblast layer which corresponds to cells derived from the organizer region. This region gives rise to the prechordal plate mesendoderm which was also found to express the *ZDK1* gene. There may be an equivalent expression domain in the mouse, since *MDK1* transcripts are present in the rostral mesoderm of the early head process at E7.5, both in midline cells which correspond to the prechordal plate and in more lateral mesodermal cells.

Similarly, all of the major expression features of *ZDK1* in the developing neuroectoderm are clearly conserved in the mouse embryo. In both species, *ZDK1/MDK1* transcripts appear in a narrow domain in the presumptive hindbrain (compare, e.g., Figs. 2D, 2E, and 6A), as well as in the rostral extremity of the forebrain neuroepithelium (fish) or neural folds (mouse; compare, e.g., Fig. 3A to Fig. 6A). In the mouse, this rostral expression domain becomes restricted to the forebrain dorsal midline and is prominent at the posterior end of the forebrain and the anterior midbrain (which will give rise to the rostral part of the tectum and the subcommisural organ; see also Ellis *et al.,* 1995). Similarly, after an initial phase of ventromedial expression, *ZDK1* transcripts extend to the dorsomedial part of the forebrain epithelium and appear dorsally in the anterior midbrain/posterior forebrain region.

Interestingly, both *ZDK1* and *MDK1* genes display similar and dynamic expression patterns in the developing

FIG. 7. *MDK1* transcript distribution on histological sections of a E9.5 mouse embryo. (A) Transverse section at a level of the hindbrain and rostral forebrain. (B) Transverse section at the level of the hindbrain, otic vesicle, and mandibular arches. (C) Representative section through differentiating somites at three distinct rostrocaudal levels: (1) cervical somites showing labeling at the boundary between dermatome and myotome; (2) trunk somites showing labeling in dorsal part of the dermamyotome; and (3) tail somites where *MDK1* has not (yet) been activated. (D) Section through several trunk somites, showing *MDK1* transcripts along the boundaries between dermatomes and myotomes. Note that the somites to the right are sectioned more ventrally, i.e., outside the region of *MDK1* expression. (E) Transverse section through trunk somites, at a level comparable to level (2) in C, showing maximal labeling at the dorsal edge of the dermamyotome and a gradual decrease in more ventral cells. Abbreviations: de, dermatome; dm, dermamyotome; fb, forebrain; hb, hindbrain; L, left; md, mandibular arch; my, myotome; n, notochord; ov, otic vesicle; R, right; s, sclerotome; sc, spinal cord; so, somites.

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rhombomeres although the initial activation in the hindbrain differs in mouse and zebrafish embryos. Transcripts appear in a defined temporal sequence in specific rhombomeres. In both species, the onset of expression precedes the morphological segmentation and appears to take place in the presumptive domain of rhombomere 3 in the mouse embryos and of rhombomeres 3 and 4 in the fish embryo, thus suggesting that *ZDK1/MDK1* may be required for some early events leading to the segmentation of the hindbrain. We note that the initial expression of *ZDK1* in the hindbrain of zebrafish embryos occurs well before that of the *Krox-20* gene (Oxtoby and Jowett, 1993; our unpublished observations). Although both *MDK1* and *ZDK1* are expressed in rhombomeres 2–6, there are differences in the early activation domains, as well as quantitative differences between *ZDK1* and *MDK1* expression patterns in fish versus mouse rhombomeres.

Several other RTK genes have been shown to exhibit restricted patterns of expression in developing rhombomeres. The various murine *Sek* genes, which belong to the same RTK family as *MDK1,* all display rhombomere-specific expression patterns which tend to be more restricted than that of *MDK1.* Both *Sek-1* and *Sek-4* are expressed only in r3 and r5, while *Sek-3* is expressed in the same rhombomeres and in addition in r2, and *Sek-2* is expressed only in presumptive r4 (Nieto *et al.,* 1992; Becker *et al.,* 1994). In this respect, *ZDK1/MDK1* represents the only RTK gene of the Eph/Elk family that shows an extensive expression in the hindbrain from r2 (or possibly even r1, at least in fish) to r6. Another zebrafish RTK gene, the fibroblast growth factor receptor-4 gene (*FGFR-4*) is expressed in the hindbrain with a pattern that partially overlaps with *ZDK1* (Thisse *et al.,* 1995).

Expression in developing sensory organs, with the exception of the olfactory organs, is a major feature of *ZDK1* expression during late stages. In all cases, this expression is regionally restricted and defines specific domains in the early sensory anlagen. First, after an initial phase of expression in the entire otic vesicle, *ZDK1* transcripts become restricted to the medial–posterior part of the developing ear. Interestingly, *MDK1* is consistently expressed in the otocyst and subsequently in the developing cochlea (this study and Ellis *et al.,* 1995). Second, *ZDK1* is regionally expressed in the optic vesicle and, at a later stage, is specifically expressed in the posterior part of the developing fish retina. Although *MDK1* may be weakly expressed in the early optic evaginations (Ellis *et al.,* 1995), no specific expression of this gene was reported in the developing retina of the mouse (Ellis *et al.,* 1995; Ciossek *et al.,* 1995; and our unpublished observations). Whether these observations reflect a functional difference between both visual systems, or more trivially, a failure to detect a lower level of expression of *MDK1,* remains to be seen. In this respect, note that *ZDK1* is expressed in restricted areas of the diencephalon and mesencephalon which are crossed by afferent axonal tracts originating from the retina and projecting in the dorsal–posterior midbrain (tectum). The specific expression of its murine counterpart, *MDK1,* which occurs in an anterior–posterior gradient in the rostral part of the developing tectum, has been reported (Ellis *et al.,* 1995). This suggests that *ZDK1/MDK1* may be involved in a signaling mechanism which might define the retinal axonal projection across diencephalic and mesencephalic regions. No ligand for *MDK1* has yet been identified. However, ligands for a few members of the Eph family have been recently characterized, which, unlike most other RTK ligands, are membrane bound (Bartley *et al.,* 1994; Beckmann *et al.,* 1994; Cheng and Flanagan, 1994; Davis *et al.,* 1994; Bennett *et al.,* 1995; Bergemann *et al.,* 1995; Kozlosky *et al.,* 1995). Strikingly, some of these receptors and their cognate ligands have been shown to be expressed in gradients in the retinal ganglion cells and the anterior tectum, and one of these ligands has been shown to have an axon repellent activity *in vitro* (Cheng *et al.,* 1995; Drescher *et al.,* 1995). Taken together, these results indicate that a gradient of the ligand on the tectum may provide a positional label recognized by the receptor on the axons, suggesting a role for some of the Eph receptors and ligands in axon guidance. Third, *ZDK1* is also very specifically expressed at the tip (apical region) of the posterior lateral line primordium. Note that another RTK gene, *FGFR-1,* is also expressed in the same territory (C.T. and B.T., unpublished observations). The lateral line is a sensory structure which has no obvious counterpart in mammals. It is derived from an ectodermal placode and migrates between the myotome and the dermis in the posterior part of the embryo. Similarly, during gastrulation, the prechordal plate mesendodermal cells which also express *ZDK1* migrate anteriorly between two layers (the yolk syncytial layer and the neuroectoderm). These observations suggest that *ZDK1* may play a role in the migration of these specific cell populations.

Although the mouse embryo has no structure which is phylogenetically equivalent to the lateral line primordium, it is particularly striking that *MDK1* displays a specific expression domain during early somite differentiation which corresponds to the boundary between the dermatomes and the myotomes, i.e., the region equivalent to that where lateral line primordial cells migrate in the fish embryo. It is also interesting to note that the *Sek-1* gene is expressed slightly before *MDK1* in the early somitic lineages (Nieto *et al.,* 1992). Both genes are expressed in a rostrocaudal gradient. *Sek-1* is transiently expressed in the segmental plate mesoderm prior to somite formation and is down-regulated once the definitive somite is formed. On the other hand, *MDK1* expression occurs shortly after a given somite pair is individualized and appears from the beginning to be restricted to the dorsal compartment of the somite.

MDK1 Is Downstream of Hoxa-2 in the Hindbrain Regulatory Cascade

The zinc-finger transcription factor *Krox-20* has been shown to be ‘‘upstream’’ of Hox genes within the hindbrain regulatory cascade and to control the transcriptional activa-

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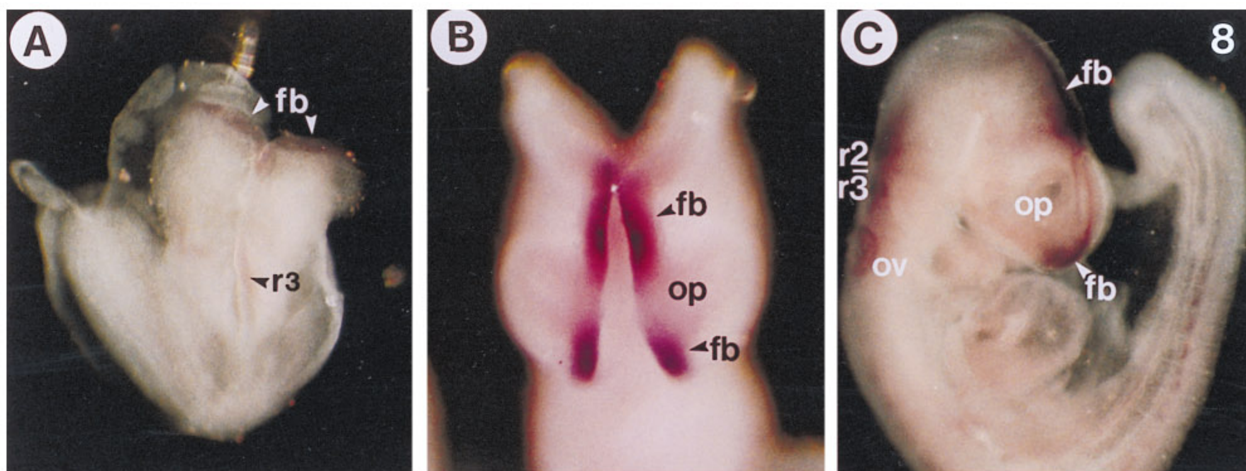
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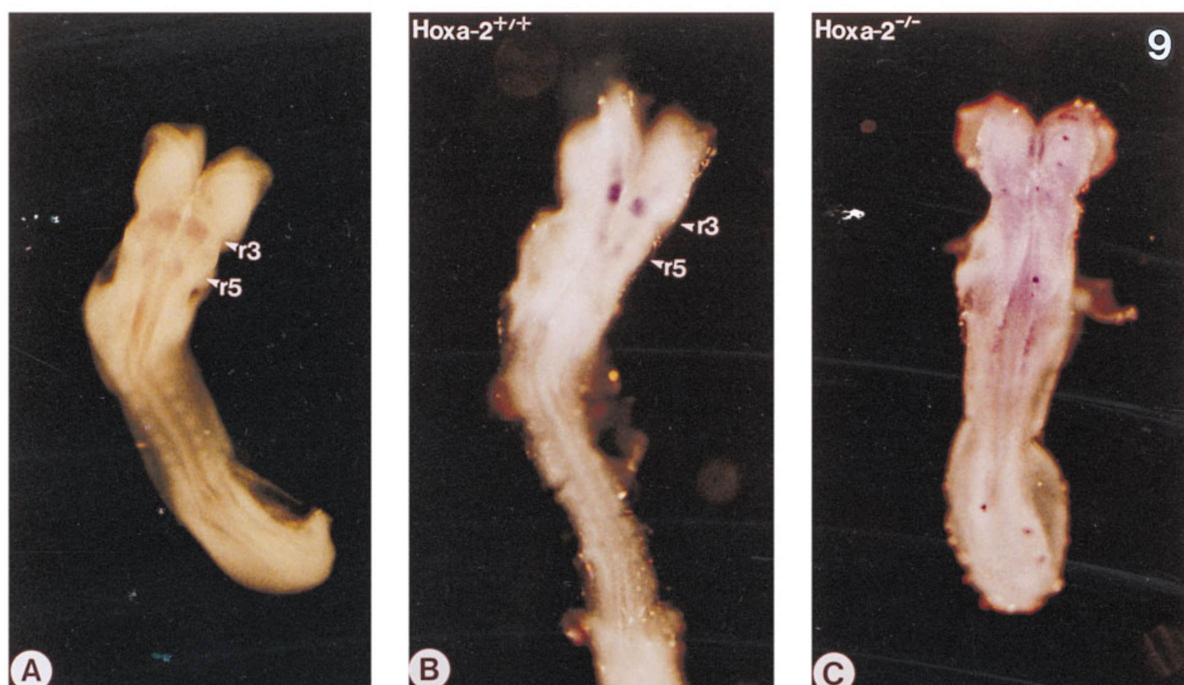
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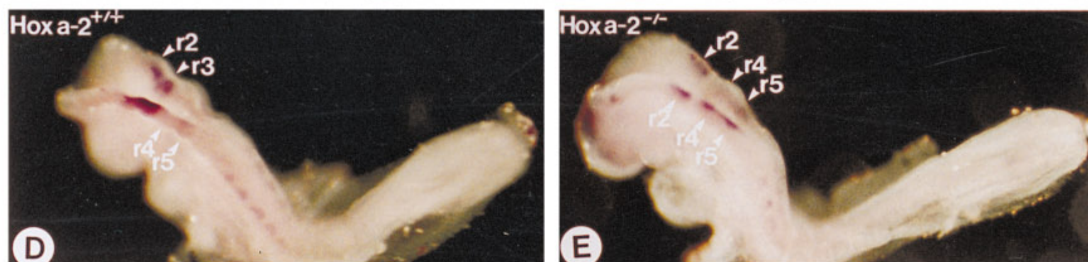
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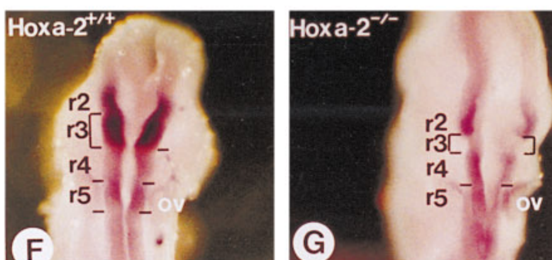
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tion of *Hoxb-2* in r3 and r5 (Sham *et al.,* 1993). However, regulatory proteins which lie ''downstream'' of Hox genes and function in controlling regional identity have not yet been identified. Although there is *in vitro* evidence that Hox genes could control the expression of cell adhesion molecules such as NCAM and cytotactin/tenascin (Jones *et al.,* 1990, 1992, 1993; Hirsch *et al.,* 1990), the identities of such downstream effectors remain to be established *in vivo.*

Strikingly, there is a selective lack of expression of *MDK1* in rhombomere 3 of the *Hoxa-2* null mutants. Furthermore, there is an overall alteration in the pattern of expression of *MDK1,* demonstrating that *Hoxa-2* is required to maintain the hindbrain expression pattern of *MDK1.* Our results do not indicate whether *MDK1* is a direct target of *Hoxa-2.* However, our results show that *Hoxa-2* plays a specific role in *MDK1* expression control in r3, as it cannot be functionally replaced by *Hoxb-2,* which is the only other Hox gene to be expressed in r3 (Krumlauf, 1993) and is normally expressed in *Hoxa-2* null mutants (Rijli *et al.,* 1993). It is thus intriguing that *MDK1* expression is strongly reduced, but not completely abolished in r2, where *Hoxa-2* is the only Hox gene to be expressed (Krumlauf, 1993), which indicates that *MDK1* can be expressed within the hindbrain in the absence of Hox gene expression. It is also clear that although *Krox-20* appears to be an ''upstream'' regulator of Hox gene expression (Sham *et al.,* 1993), the expression of *MDK1* cannot be directly controlled by *Krox-20* alone, since the *Krox-20* r3 expression is not abolished in *Hoxa-2* null mutants (Rijli *et al.,* 1993; Gendron-Maguire *et al.,* 1993). However, it is possible that *Krox-20* may act as a coregulator of *MDK1* expression. Interestingly, the expression of *MDK1* appears to be up-regulated in r4 compared with that in WT embryos, in which the r4 expression is the lowest.

Rhombomeres are lineage-restricted compartments and the formation of rhombomeric boundaries restricts the movement of cells, so that cells from adjacent rhombomeres display only limited intermixing (Fraser *et al.,* 1990; Birgbauer and Fraser, 1994). The presence of different cell-surface molecules on adjacent rhombomeres could be a mechanism involved in restricting movement of cells across boundaries (Guthrie, 1995), thereby resulting in the establishment of rhombomere-specific identities and the spatial restriction of gene expression. *MDK1* may act as a cell adhesion molecule to control cell sorting between rhombomeres. The altered control of expression of *MDK1* following the functional inactivation of *Hoxa-2* might result in improper sorting of cells between rhombomeres, such that some cells of pre-r3 might mix with those of pre-r2 and pre-r4 and eventually be respecified. We note in this regard that the RTK gene *Sek-1* has recently been proposed to play a role in the regulation of rhombomeric cell identities or in restriction of cell movement across rhombomeric boundaries (Xu *et al.,* 1995). The molecular identities of rhombomeres in the hindbrain of the *Hoxa-2* mutants appeared unmodified (Rijli *et al.,* 1993; Gendron-Maguire *et al.,* 1993), although it should be noted that these analyses were performed using ''upstream'' molecular markers, such as *Krox-20, Hoxb-2, Hoxb-1,* and *Hoxa-3.* In addition, the segmentation pattern and the gross morphology of the rhombomeres did not seem to be altered in the mutants (Rijli *et al.,* 1993; Gendron-Maguire *et al.,* 1993), although a fine analysis at the cellular level was not carried out. The aberrant expression pattern of *MDK1* clearly indicates that *Hoxa-2* mutants have a ''molecular'' phenotype. It will be interesting to determine whether this alteration correlates with some changes in cell identities.

Finally, it is noteworthy that, although the overall expression of *MDK1* is altered in the hindbrain of the *Hoxa-2* null mutants, all other domains of *MDK1* expression are not dependent on *Hoxa-2* and therefore are presumably under the control of other genes both rostrally and caudally. Whether RTK genes are differentially regulated by different Hox genes, and hence whether a combinatorial ''RTK'' code might exist to impart positional information for the hindbrain patterning, remains to be investigated.

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FIG. 8. Expression of *MDK1* in the forebrain. (A) E8.0 embryo; (B) E8.5 embryo with unfused neural folds; (C) E9.5 embryo showing expression along the forebrain dorsal midline. Arrowheads in B and C indicate the two domains of forebrain expression. Abbreviations: fb, forebrain; op, optic vesicle; ov, otic vesicle; r, rhombomere.

FIG. 9. Altered expression of *MDK1* in *Hoxa-2* null mutant embryos. (A) Expression of *Hoxa-2* in WT embryos at E8.5 showing labeling in r3 and r5. (B–G) Expression of *MDK1* at equivalent stages (E8.5, E8.75, and E9.0), in WT (*Hoxa-2*$^{+/+}$; B, D, F) and *Hoxa-2* null embryos (*Hoxa-2*$^{-/-}$; C, E, G). Note the lack of r3 expression at all stages in the *Hoxa-2* null mutants. Note also that r3 in *Hoxa-2* null mutants (E and G) appears reduced in size compared with WT (D and F) embryos (compare the size of the bracketed r3s). Abbreviations: r, rhombomere; ov, otic vesicle.

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