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Isolation and Expression of the Homeobox Gene *Gbx1* During Mouse Development

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In zebrafish, *gbx1* and *otx2* are among the earliest genes expressed in the neuroectoderm, dividing it into an anterior and a posterior domain with a common border that marks the midbrain–hindbrain boundary (MHB) primordium. Here, we describe the sequence and expression pattern of *Gbx1* in mouse. The first transcripts are found at embryonic day 7.75 in the hindbrain. Later on, expression of *Gbx1* is detectable in the hindbrain (rhombomeres 2 to 7), spinal cord, optic vesicles, and in the ventral telencephalon. In mouse, *Gbx1* expression is not observed at the MHB as is the case during early zebrafish development. We suggest that an evolutionary switch occurred: in mouse *Gbx2* is involved in the early specification of the MHB primordium, whereas in zebrafish, *gbx1* is required instead of *gbx2*. *Developmental Dynamics 229:334–339, 2004.* © 2004 Wiley-Liss, Inc.

Key words: *Gbx1*; *Gbx2*; MHB; isthmus; organizer; hindbrain; rhombomere; mouse; zebrafish

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INTRODUCTION

Previous studies in mouse, chicken, and *Xenopus* have shown that development of the midbrain and hindbrain requires two homeobox genes, *Otx2* and *Gbx2* (reviewed in Joyner et al., 2000; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). In mouse, they are both expressed at the headfold stage in the anterior and posterior neuroectoderm, respectively (Simeone et al., 1993; Ang et al., 1994; Bouillet et al., 1995; Wassarman et al., 1997). Recent studies in mouse and chicken showed that ectopic juxtaposition of *Gbx2* and *Otx2* expression can induce midbrain–hindbrain boundary (MHB) marker expression (Hidalgo-Sanchez, 1999; Katahira et al., 2000). Misexpression of *Gbx2* represses *Otx2* expression in the posterior midbrain (Millet et al., 1999; Tour et al., 2002). Similarly, misexpression of *Otx2* in the anterior hindbrain represses *Gbx2* expression in the metencephalon (Broccoli et al., 1999; Katahira et al., 2000). In both cases, the expression domain of the MHB markers are shifted and situated at the level of the new *Otx2-Gbx2* interface. These results indicate that the region where *Otx2* and *Gbx2* abut might demarcate the primordium of the MHB.

Gbx genes are related to the *Drosophila unplugged* gene, which functions in development of the tracheal system and perhaps specific neuroblast sublineages (Chiang et al., 1995; Cui and Doe, 1995). Recently, we reported that in zebrafish *gbx2* is activated too late to fulfill a primary role in the establishment of the primordium of the MHB (Rhinn et al., 2003). In contrast, the high similarity of the early expression pattern of the zebrafish *gbx1* gene with the early *Gbx2* expression in other species suggested that the functional requirement may be distributed differently in zebrafish: *gbx1* instead of *gbx2* may be required for the correct specification of the MHB primordium in zebrafish (Rhinn et al., 2003). Little is known about amniote *Gbx1* genes; potential *Gbx1* homologues have been cloned partially in mouse (Frohman et al., 1993), chicken (Fainsod and Greunbaum, 1989; Obinata et al., 2001), human (Matsui et al., 1993), and carp (Stroband et al.,

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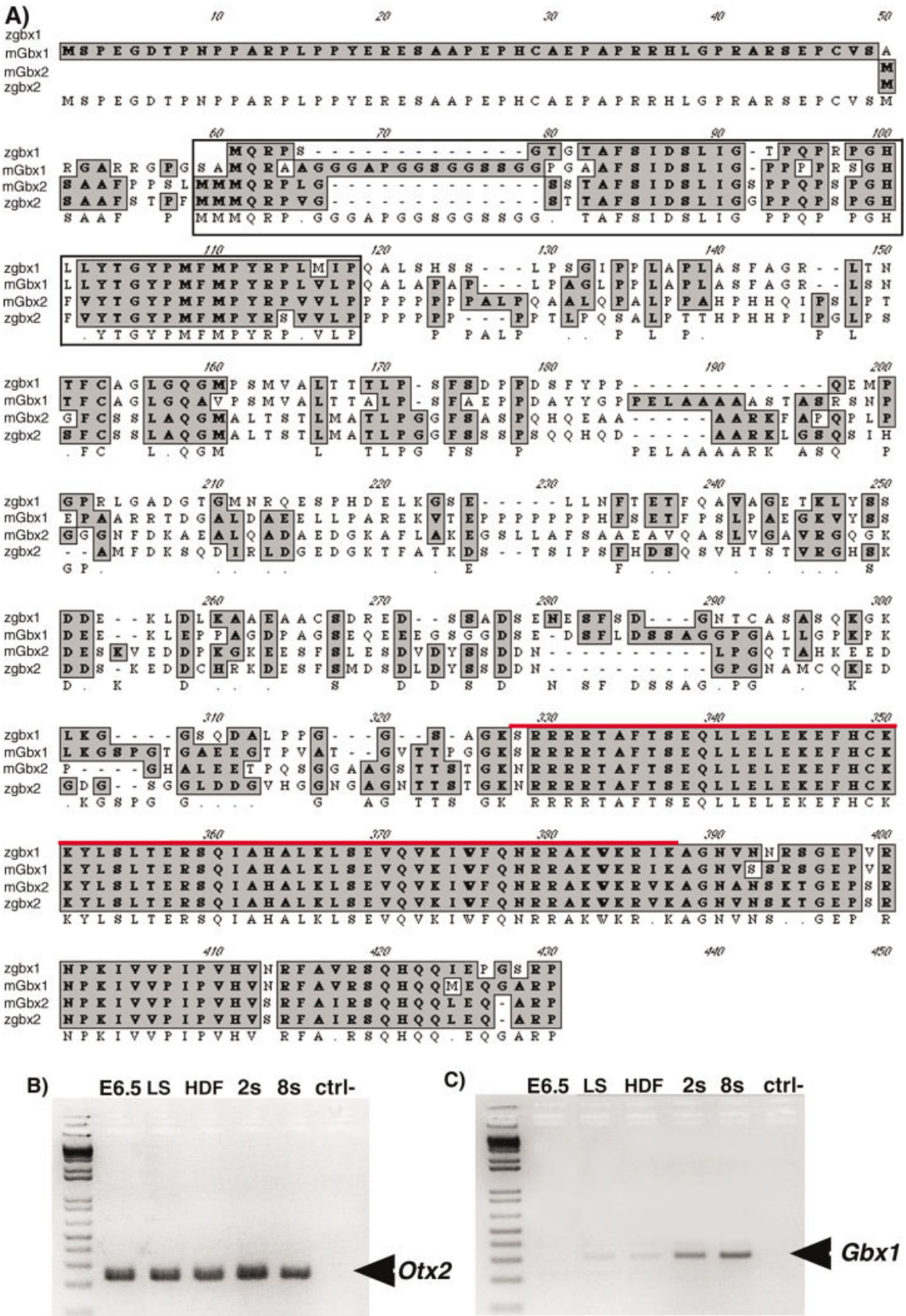


Fig. 1. **A**: Multiple sequence alignment of mouse Gbx1 protein sequence with zebrafish gbx1 protein sequence, mouse and zebrafish Gbx2 protein sequences; the black box outlines the N-terminal conserved region, which is enriched in prolines. The checkered bar marks the homeobox. **B**: *Otx2* expression was examined by reverse transcriptase-polymerase chain reaction (RT-PCR) in the same cDNA samples. **C**: *Gbx1* expression was examined by RT-PCR using the P1 primers. B,C: Unmarked lane, molecular weight marker; E6.5, embryonic day (E) 6.5 embryos; LS, late streak stage embryos; HDF, headfold stage embryos; 2s, two-somite stage embryos; 8s, eight-somite stage embryos; ctrl-, water control. (Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.)

1998) and entirely in zebrafish (Rhinn et al., 2003). To date, it has been reported that *Gbx1* is expressed at the embryonic day (E) E11.5 in mouse forebrain (Frohmann et al., 1993) and also in adult rat brain nuclei in the basal forebrain (Asbreuk et al., 2002). Here, we describe the identification and expression pattern of the *Gbx1* gene during mouse development.

RESULTS AND DISCUSSION

To isolate a full-length cDNA for mouse *Gbx1*, we used a *Gbx1* genomic fragment containing 200-bp coding region followed by 376-bp 3′-untranslated cDNA (the sequence was kindly provided by M.A. Frohman). Screening of an embryonic cDNA library allowed identification of a fragment covering the second exon and the 3′-UTR. Blast searches of the Celera and Ensembl databases allowed us to find the first exon (GenBank accession no. AY319256). Alignments of the available *Gbx* sequences show that the *Gbx* genes can be subdivided into two classes based on diagnostic amino acid substitutions at positions 1 and 59 within the Gbx homeodomain. Residue 1 is either a serine or an asparagine and residue 59 is either an isoleucine or valine (Chapman and Rathjen, 1995), in the *Gbx1* and *Gbx2* class, respectively (Fig. 1A). The homeodomain sequence of the newly isolated clone contains a serine in position 1 and an isoleucine at position 59, identifying it as a *Gbx1* class gene (Fig. 1A).

Furthermore, we observed a conserved N-terminal portion between the zebrafish Gbx1 protein and the Gbx2 proteins (Rhinn et al., 2003). The mouse *Gbx1* gene contains an amino acid sequence showing 68% identity to the zebrafish Gbx-Box (Fig. 1A). We used a BLAST search to determine whether this conserved N-terminal portion of the zebrafish Gbx1 and Gbx2 proteins is also present in the human genome and have identified a human amino acid sequence showing 84% identity to the zebrafish Gbx-Box (Rhinn et al., 2003). This homologous region localises to the position on chromosome 7, where the human *Gbx1* gene maps. These findings suggest that *gbx* genes might generally share a distinctive N-terminal Gbx-Box that is conserved between the two family members. Both genes occupy areas of long-range synteny between the zebrafish and mammalian genomes, thus supporting the assignment of the *gbx1* and *gbx2* orthologies (Rhinn et al., 2003).

To determine the onset of *Gbx1* expression during embryonic development, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) on cDNA from mouse embryos at different stages. With primers amplifying a 300-bp fragment (P1, see Experimental Procedures section) we detect weak expression at E7.5 (late streak stage) and stronger expression at the be-

Fig. 2. Expression analysis of *Gbx1* during gastrulation and somitogenesis using whole-mount in situ hybridization and comparison with the early *Gbx2*, *Krox-20*, and *Hoxb1* expression. A–I: Lateral views. C′,K′: Ventral views. E′–G,J: Dorsal views. **A:** Expression of *Gbx1* at embryonic day (E) 7.75. *Gbx1* is expressed in the posterior part of the embryo. **B:** Expression of *Gbx2* at E7.75. *Gbx2* is expressed in the posterior part of the embryo. Its expression is adjacent to the presumptive midbrain (Wassarman et al., 1997; Millet et al., 1999). **C:** At the four-somite (4 som) stage, *Gbx1* is seen in the hindbrain. **C′:** A faint expression is observed in the anterior neural plate. **D:** At the four-somite stage, *Gbx2* is expressed with a sharp anterior border at the midbrain–hindbrain (MHB) boundary (arrowhead). No expression in this area is observed with *Gbx1*. *Gbx2* is also expressed in the foregut at the level of the first pharyngeal pocket (Fg) and in the posterior region of the embryo (Po). **E,E′:** Close-up view of the embryo shown in C. *Gbx1* is expressed in a transverse stripe in the hindbrain corresponding to prospective rhombomere (rh) 3, 4, and 5 (brackets). **F,F′:** Expression of *Otx2* and *Krox-20* at the four-somite stage. *Otx2* is expressed in the forebrain and midbrain. The posterior border of its expression domain corresponds to the MHB (arrowhead). *Krox-20* is expressed in rh3 and 5. **G,G′:** Expression of *Otx2* and *Hoxb1* at the four-somite stage. The arrowhead indicates again the posterior border of the *Otx2* expression domain. *Hoxb1* is expressed in rh4. **H:** At the eight-somite (8 som) stage, *Gbx1* is seen in the hindbrain. Expression is also seen in the optic vesicle (arrow). A faint expression can also be observed below the heart (arrowhead). **I:** At the eight-somite stage, *Gbx2* is expressed in comparable domains as at the four-somite stage. *Gbx2* is expressed with a sharp border at the MHB (arrowhead). *Gbx2* is also expressed in the foregut at the level of the first pharyngeal pocket (Fg) and in the posterior region of the embryo (Po). **J:** The anterior limit of expression of *Gbx1* aligns with the preotic sulcus (arrowhead), which corresponds to rh3. The arrow indicates the faint domain below the heart. **K, K′:** Ventral view showing the expression in pericardio–peritoneal canal region (K) and a section at the heart level showing this expression domain (arrowhead, K′).

Fig. 3. Expression of *Gbx1* during the midgestational period and comparison with *Gbx2*, *Krox-20*, and *Hoxb1* expression. A–E: Lateral views. C′–E′: Dorsal views. **A:** Expression of *Gbx1* at embryonic day (E) 9.5. *Gbx1* is expressed in the distal part of the optic vesicle (arrow), in rhombomere (rh) 2–7 (hb, hindbrain) and in the spinal cord (sc), except the most posterior end. The dotted area indicates the otic vesicle where *Gbx1* is not expressed. Inset: Dorsal view showing somite expression. **B:** Expression of *Gbx2* at E9.5. All domains described in Bouillet et al. (1995) and Wassarman et al. (1997) are visible. *Gbx2* is expressed in the pharyngeal arches, at the level of the midbrain–hindbrain (MHB, arrowhead), in the rhombomeres (except rh5 and 6), the otic vesicle, and the entire spinal cord. **C,C′:** Close-up view of the embryo shown in A. *Gbx1* is expressed in rh2 to 7, with a stronger expression in rh2, 3, and 5. The arrow indicates the expression in the optic vesicle in (C); arrowheads indicate the neural crest cells from rh4 and 6 in C′. **D,D′:** Expression of *Otx2* and *Krox-20* at E9.5. *Otx2* is expressed in the prosencephalon, diencephalon, and mesencephalon. The posterior border of its expression domain corresponds to the MHB (arrowhead). *Krox-20* is expressed in rh3 and 5. **E,E′:** Expression of *Otx2* and *Hoxb1* at E 9.5. The arrowhead indicates the posterior border of the *Otx2* expression domain. *Hoxb1* is expressed in rh4. The dotted area indicates the otic vesicle (ov) in all figures.

Fig. 4. Expression of *Gbx1* at embryonic day (E) 10.5 and E12.5 of development and comparison with *Gbx2* expression. A,E: Lateral views. B,F: Medial views. Expression of *Gbx1* **(A)** and of *Gbx2* **(E)** at E10.5 in whole embryos. Expression of *Gbx1* **(B)** and of *Gbx2* **(F)** at E10.5 in the medial ganglionic eminence. *Gbx2* is expressed in additional domains like the corpus striatum and the thalamus. Transverse section through the neural tube of an embryo stained for *Gbx1* **(C)** and for *Gbx2* **(G)**. Distribution of transcripts for *Gbx1* **(D)** and *Gbx2* **(H)** on frontal sections through he forebrain of E12.5 embryos. lge, lateral ganglionic eminence; mge, medial ganglionic eminence; Th, thalamus; cs, corpus striatum; cer, cerebellum.

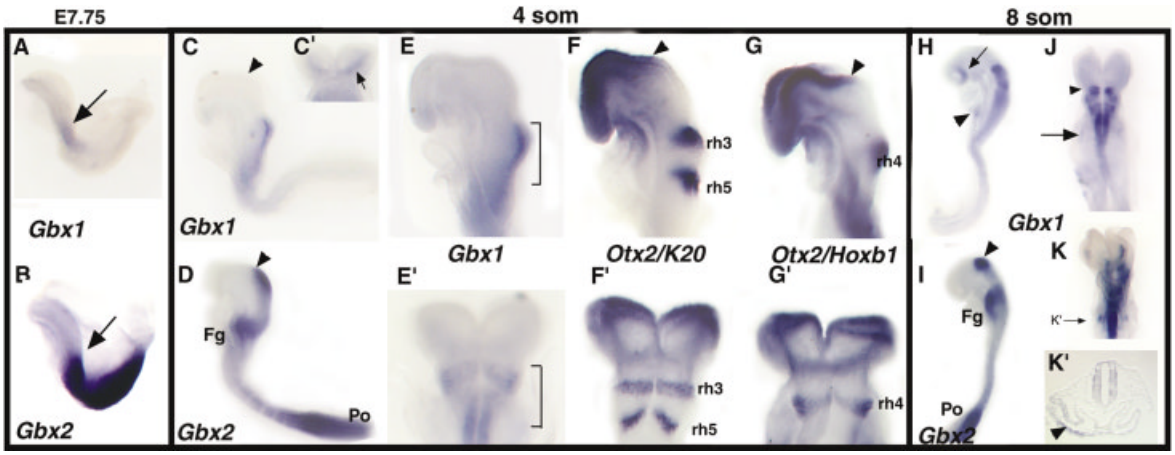
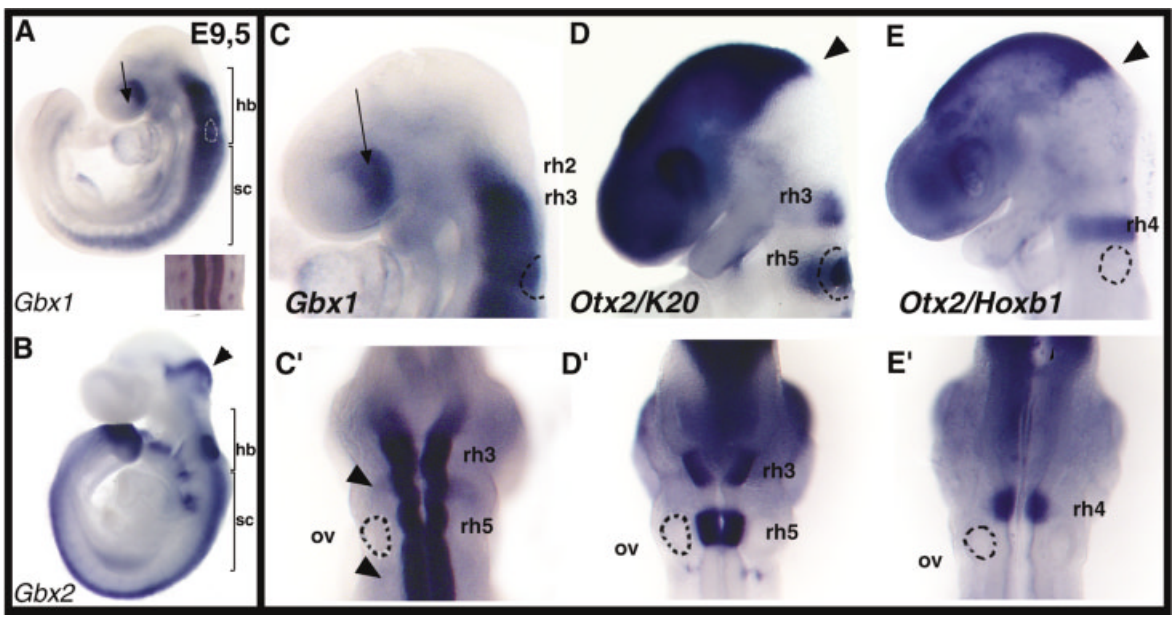


Fig. 2



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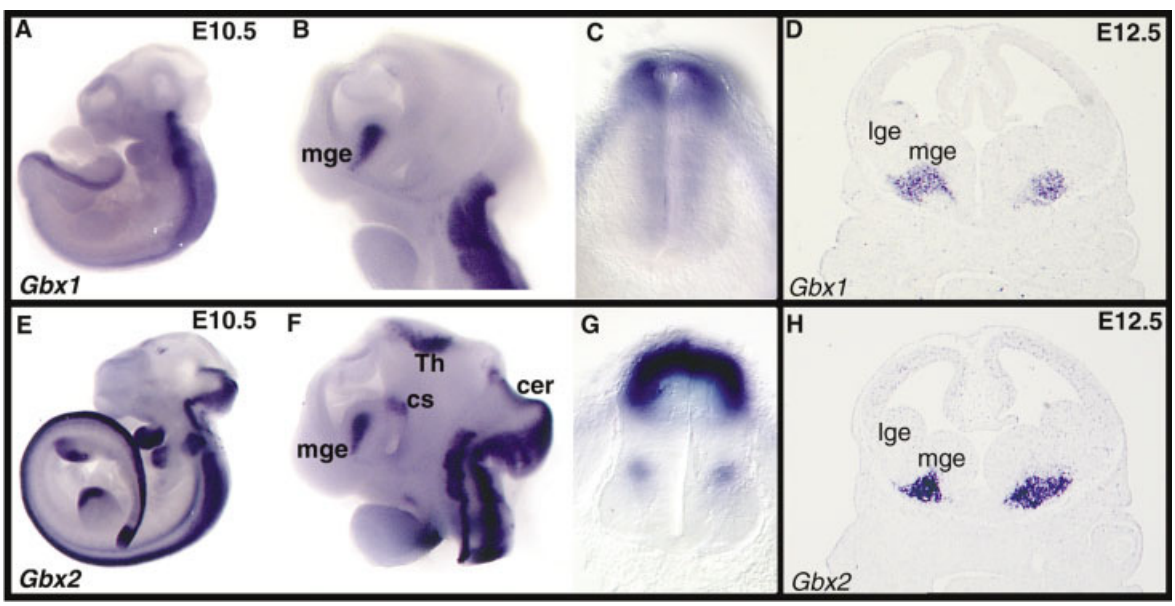

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ginning of somitogenesis (Fig. 1C). In control RT-PCRs, *Otx2* transcripts are strongly detected at late streak stage (Ang et al., 1994; Suda et al., 1999; Fig. 1B). Whole-mount in situ hybridisation first detects *Gbx1* expression at E7.75 (headfold stage) in the hindbrain, and this expression is excluded from the headfolds (Fig. 2A). At this stage, *Gbx2* transcripts are detected in a wide posterior region with its anterior expression domain laying at the presumptive MHB (Bouillet et al., 1995; Wassarman et al., 1997; Fig. 2B). In contrast, *Gbx1* expression domain does not extend to the same level anteriorly and its anterior limit is situated slightly posterior to the *Gbx2* anterior limit (compare Fig. 2A,B,arrows). Expression levels increase during somitogenesis, and at the four-somite stage, the expression is restricted to the nervous system, where a faint domain is observed in the anterior neural plate (Fig. 2C′) and a stronger domain in the hindbrain (Fig. 2C,E,E′). Comparison with other hindbrain markers like *Krox-20,* which is specifically expressed in rhombomeres (rh) 3 and 5 (Fig. 2F,F′), and *Hoxb1* which is expressed in rh4 (Fig. 2G,G′), shows that *Gbx1* is expressed at this stage in a diffuse domain covering the prospective rh3 and all of rh4 and part of rh5. Unlike *gbx1* and *gbx2* in zebrafish (Rhinn et al., 2003) or *Gbx2* in mouse (Bouillet et al., 1995; Wassarman et al., 1997; Fig. 2D), *Gbx1* is not expressed at the level of the MHB at this stage (compare Fig. 2C and D). The posterior border of the *Otx2* expression domain demarcates the posterior border of the midbrain (Fig. 2F,F′,G,G′). *Gbx1* is not expressed at this level, confirming the absence of *Gbx1* expression at the isthmus. As development proceeds, *Gbx1* expression becomes stronger and stays confined to the nervous system. At the eight-somite stage, the anterior limit of expression of *Gbx1* aligns with the preotic sulcus (Fig. 2H, arrowhead), an indentation in the hindbrain that delineates the rh2/rh3 boundary (Trainor and Tam, 1995) and coincides with the anterior limit of *Krox-20* (Barrow et al., 2000). *Gbx1* is strongly expressed in rh3 and rh5 and more weakly along the hindbrain (Fig. 2H,J). Faint expression is also observed below the heart level, which corresponds to the pericardio–peritoneal canal region (Fig. 2J arrow, K,K′). No expression is detected at the MHB at this stage (compare Fig. 2H and I arrowhead) or later in development (compare Fig. 3A and B arrowhead). *Gbx1* expression domain is visible in the distal part of the optic vesicle (Fig. 2H, arrow), and this expression persists at E9.5 (Fig. 3A,C). At E 9.5, *Gbx1* is strongly expressed in rh3 and in rh5 (Fig. 3C,C′), the two rhombomeres expressing *Krox-20* (Fig. 3D,D′). Only weak expression is observed rh2 (Fig. 3A,C) and in rh4 that is also labeled with *Hoxb1* (Fig. 3E,E′). Rh5 and rh6 juxtapose with the otic vesicle; *Gbx1* is weakly expressed in rh6 and rh7 (Fig. 3A,C) and more posteriorly in the spinal cord (Fig. 3A). *Gbx1* is also expressed in neural crest migrating from rh4 and rh6 (Fig. 3C′) and in the dorsal part of the somites (Fig. 3A). At E10.5, *Gbx1* is still expressed in the hindbrain and spinal cord (Fig. 4A) and starts to be expressed in more anterior region like the medial ganglionic eminence of the forebrain (Fig. 4B), where it overlaps with the *Gbx2* expression domain (Fig. 4F). A section through the neural tube shows *Gbx1* expression in the ventricular zone (Fig. 4C), where it overlaps with *Gbx2* expression in the most dorsal part (compare with Fig. 4G). At this stage, *Gbx1* is not expressed in the limb, otic vesicle, and branchial arches as is *Gbx2* (compare Fig. 4A,B). At E12.5, both genes are expressed in the basal telencephalon in the mantle zone of the medial ganglionic eminence that forms the basal forebrain cholinergic system (Fig. 4D,H).

In conclusion, we analysed the spatiotemporal expression pattern of the mouse *Gbx1* gene. Sequence comparison shows that mouse *Gbx1* is a member of the *Gbx1* class of genes (Fig. 1A). Surprisingly, the spatially restricted expression of *Gbx1* in mouse is seen only at the end of gastrulation, whereas in zebrafish, carp (Stroband et al., 1998), and chicken (Fainsod and Gruenbaum, 1989) such expression is already seen during early gastrulation. Mouse *Gbx1* expression, however, shares common features with the later *gbx1* expression in zebrafish (Rhinn et al., 2003): at early somite stages *gbx1* in zebrafish is also expressed in the hindbrain primordium, with a strong expression in rh4. At the 7-somite stage, *gbx1* expression fades away from the MHB and its expression is up-regulated in more anterior rhombomeres (except rh1) and starting at the 15-somite stage expression in rh4 is weaker relative to other rhombomeres. *gbx1* in zebrafish is also expressed in the spinal cord, and at 30 hr of development, *gbx1* is expressed also in the basal telencephalon just dorsal to the optic recess. This finding suggests that *Gbx1* is conserved in protein sequence but only partially in expression, and presumably biological function. We propose that the functional requirement during gastrulation may be distributed differently in zebrafish compared with mouse, in that *Gbx2* in mouse is required for the earliest steps of MHB development instead of *gbx1* in zebrafish. During submission of this work, Waters et al. (2003) described independently the isolation and expression of mouse *Gbx1*.

EXPERIMENTAL PROCEDURES

cDNA synthesis with SuperScriptII reverse transcriptase (GibcoBRL) was performed according to the manufacturer's instructions. In control RT-PCR, *Otx2* transcripts are detected by using the primers described in Suda et al. (1999). The following PCR primer pairs are used: P1, 5′-CCCCCGGACGCCTACTAC-3′ (sense) and 5′-AGGAAGCTGTCCTCGCTGT-3′. P1 primers amplify a 300-bp fragment (Fig. 1C).

Whole-mount RNA in situ hybridisations were performed by using the protocol described previously by Conlon and Hermann (1993). Section RNA in situ hybridisations were performed as described in Casarosa et al. (1999). Probes and wild-type expression patterns are described in for *Krox-20* (Wilkinson et al., 1989a), *Otx2* (Ang et al., 1994), *Gbx2* (Bouil-

let et al., 1995), and *Hoxb1* (Wilkinson et al., 1989b).

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