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Fgf8 is mutated in zebrafish *acerebellar* (*ace*) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis

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SUMMARY

We describe the isolation of zebrafish *Fgf8* and its expression during gastrulation, somitogenesis, fin bud and early brain development. By demonstrating genetic linkage and by analysing the structure of the *Fgf8* gene, we show that *acerebellar* is a zebrafish *Fgf8* mutation that may inactivate *Fgf8* function. Homozygous *acerebellar* embryos lack a cerebellum and the midbrain-hindbrain boundary organizer. *Fgf8* function is required to maintain, but not initiate, expression of *Pax2.1* and other marker genes in this area. We show that *Fgf8* and *Pax2.1* are activated in adjacent domains that only later become overlapping, and activation of *Fgf8* occurs normally in *no isthmus* embryos that are mutant for *Pax2.1*. These findings suggest that multiple signaling pathways are independently activated in the midbrain-hindbrain boundary primordium during gastrulation, and that Fgf8 functions later during somitogenesis to polarize the midbrain. *Fgf8* is also expressed in a dorsoventral gradient during gastrulation and ectopically expressed *Fgf8* can dorsalize embryos. Nevertheless, *acerebellar* mutants show only mild dorsoventral patterning defects. Also, in spite of the prominent role suggested for *Fgf8* in limb development, the pectoral fins are largely unaffected in the mutants. *Fgf8* is therefore required in development of several important signaling centers in the zebrafish embryo, but may be redundant or dispensable for others.

Key words: Neurogenesis, Regionalization, *Fgf8*, *acerebellar*, *Pax* genes, Midbrain, Hindbrain, Organizer, Zebrafish, Somitogenesis, Axis specification, *no isthmus*, *Danio rerio*, Splicing

INTRODUCTION

Generation of the large number of different cell types in the nervous system requires cell intrinsic programs and coordination between neighbouring cells. Work in recent years has established that designated cell populations exist in the neural plate that influence cell fate in surrounding neural plate cells. One such population is located at the boundary between midbrain and hindbrain (MHB), also referred to as isthmus (Alvarado-Mallart, 1993; Nakamura et al., 1994, Marin and Puelles, 1994; for review: Bally-Cuif and Wassef, 1995; Joyner, 1996; Lumsden and Krumlauf, 1996).

The midbrain derives from the mesencephalic neural plate and includes as major derivatives the optic tectum and the tegmentum. When MHB tissue is transplanted into the caudal forebrain primordium, midbrain-hindbrain markers are not only expressed in the transplanted tissue, but also in the surrounding forebrain tissue. When such transplants are allowed to develop, the induced cells show a midbrain-like character (Gardner and Barald, 1991; Martinez et al., 1991; Bally-Cuif et al., 1992). Substructures in the induced midbrain and MHB tissues are arranged in the normal sequence relative to each other, but are inverted with respect to the endogenous midbrain and cerebellum (Marin and Puelles, 1994). Similarly, transplantation of the MHB cells into the dorsal spinal chord leads to induction of a second cerebellum (Martinez et al., 1995). These experiments identify the MHB region as an important organizing center with a role in midbrain and cerebellar induction and patterning.

At the MHB, several molecules are expressed that have been implicated in cellular interaction processes and could mediate the activity of the MHB organizer. *Wnt1* is a cognate of the secreted *wingless* gene product in *Drosophila*, and is expressed from the early neural plate stage onwards at the MHB of mouse and other vertebrate embryos (Wilkinson et al., 1987). Targeted inactivation of *wnt1* has demonstrated its requirement during maintenance, but not initiation, of midbrain and cerebellar development of mouse embryos (Thomas and Capecchi, 1990; McMahon et al., 1992). In *Drosophila*, *wingless* cooperates with the Engrailed transcription factor in several cellular interaction processes, and its vertebrate homologues *En1* and *En2* are likewise expressed during and required for maintaining early MHB development (Wurst et al., 1994; Millen et al., 1994). Indeed, a major role for *wnt1* during MHB development

is to maintain expression of *En1* (Danielian and McMahon, 1996). Apart from *wnt1*, *engrailed* expression also requires the activity of the paired box gene *Pax2.1* (formerly *pax-b*, Pfeffer et al., 1998) in zebrafish and mice (Brand et al., 1996; Favor et al., 1996; Lun and Brand, unpublished data), consistent with the presence of binding sites for paired box proteins in the *En2* promoter (Song et al., 1996).

Members of the family of secreted fibroblast growth factors (*Fgfs*) signal through receptor-tyrosine kinases (Fgfrs 1 to 4) to activate ras signaling (Basilico and Moscatelli, 1992). *Fgfs* play important roles during growth and patterning in the embryo. For instance, injection of dominant negative Fgf receptor constructs in *Xenopus* and zebrafish leads to posterior truncation, demonstrating that Fgf signaling is required during gastrulation and mesoderm development (Kroll and Amaya, 1996; Griffin et al., 1995).

In this study, we describe the isolation and expression of zebrafish *Fgf8*, and its functional requirement in embryonic development. *Fgf8* was originally isolated as an androgen-induced growth factor (AIGF, Tanaka et al., 1992). *Fgf8* is expressed in chicken and mouse from early somitogenesis onwards at the MHB and in a number of other cell groups with signaling properties (Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin, 1995; Mahmood et al., 1995). Implantation of beads soaked in Fgf8 or Fgf4 in chicken induces midbrain or cerebellar tissue in a manner analogous to cells of the MHB organizer, and *Fgf8*, *En2* and *wnt1* are activated by the implantation (Crossley et al., 1996a). These experiments strongly suggested that *Fgf8* or a similar *Fgf* is an important component of MHB organizer function. Consistently, the receptors that Fgf8 and Fgf4 bind to in vitro are expressed during MHB development in mouse and zebrafish (MacArthur et al., 1995, and references therein; Thisse et al., 1995; Ornitz et al., 1996; Blunt et al., 1997).

Apart from the MHB, *Fgf8* has been suggested to be a key signaling molecule in development of the limb bud (review: Cohn and Tickle, 1996), forebrain (Shimamura and Rubenstein, 1997), tooth (Neubüser et al., 1997), among others. In the limb bud, *Fgf8* and *Fgf4* are expressed in the apical ectodermal ridge (AER), which directs outgrowth of the limb. Mesenchymal cells of the zone of polarizing activity (ZPA) impose anteroposterior pattern on the limb bud, an activity that is mimicked by Sonic hedgehog (Shh) (Riddle et al., 1993), and Fgf8 and Fgf4 are thought to act in a feedback loop controlling Shh in the ZPA. Fgf beads or Fgf-expressing cells for *Fgf1*, *Fgf2*, *Fgf4*, *Fgf8* and *Fgf10* are all able to induce an additional limb from the flank of chick embryos (review: Cohn and Tickle, 1996; Ohuchi et al., 1997a), raising questions about the relative role of the various Fgfs. Both *Fgf8* and *Fgf10* are expressed early enough in the mesenchyme that is thought to induce the limb bud. In two chicken mutants, however, limb buds are established independently of *Fgf8* expression (Niswander, 1997), and *Fgf8* may therefore mimic the action of *Fgf10* in limb induction (Ohuchi et al., 1997a).

Loss-of-function mutations for several *Fgf*s often display weaker phenotypes than anticipated from their expression patterns or misexpression experiments. Although *Fgf3* and *Fgf5* are expressed from gastrulation onwards, targeted inactivation of *Fgf3* leads only to later defects in morphogenesis and differentiation of the inner ear and somites (Mansour et al., 1993) and *Fgf5* mutants have fur alterations (Hebert et al., 1994). Others show very severe phenotypes: *Fgf4* mutants die shortly after implantation (Feldman et al., 1995). Likewise, inactivation of *Fgfr1* leads to absence of somites and expansion of notochord, suggesting that these embryos cannot respond to an unidentified, organizer-derived signal required to pattern the gastrula embryo (Deng et al., 1994; Yamaguchi et al., 1994). Also, heterozygous mutations in human *Fgfr1* to *Fgfr3* cause dominant defects in craniofacial development, vertebrae and limbs, indicative of functions in later development (review: Yamaguchi and Rossant, 1995). The effects of loss of *Fgf8* function have not been described yet in zebrafish, but a recent study in mice indicates that *Fgf8* is required in gastrulation and brain development (Meyers et al., 1998).

As in other species, zebrafish *Fgf8* is expressed during gastrulation, in mesodermal tissue, during early MHB development and several other sites in the nervous system. We show here that *Fgf8* is mutated in *acerebellar* (*ace*). A single recessive *acerebellar* allele exists, and homozygous mutant embryos lack a MHB and a cerebellum (Brand et al., 1996). We analyze the requirement for *Fgf8* in the mutants, compare the effects of misexpressing wild-type and mutant *Fgf8* transcripts, and examine *Fgf8* dependence in *no isthmus* mutants which inactivate *Pax2.1* (Brand et al., 1996; Lun and Brand, unpublished data).

MATERIALS AND METHODS

Zebrafish were raised and kept under standard conditions at about 27˚C (Westerfield, 1994). Mutant carriers were identified by random intercrosses. To obtain mutant embryos, heterozygous carriers were intercrossed. Time of development at 28.5˚C and morphological features were used to stage the embryos (Kimmel et al., 1995). Occasionally, 0.2 mM phenylthiourea (PTU) was added to prevent melanization. Histology is described in Kuwada et al. (1990).

Whole-mount in situ hybridisation

Digoxigenin- or fluorescein-labelled RNA probes were prepared from linearized templates using an RNA labelling and detection kit (Boehringer). Hybridisation and detection with anti-digoxigenin or anti-fluorescein antibodies coupled to alkaline phosphatase (Boehringer) was modified from Thisse et al. (1994). Hybridisation was at 68.5˚C, and Boehringer DIG blocking agent was used during detection as specified by the supplier. To determine overlap in double stains with BM purple and FastRed fluorescent substrate (Boehringer), the BM purple reaction was allowed to proceed until it quenched but did not obliterate the fluorescent FastRed signal. Antibodies were preabsorbed against fixed embryo powder. Stained embryos were dissected and thick sections were prepared with sharpened tungsten needles, mounted in glycerol, photographed on a Zeiss Axioskop and assembled using Adobe Photoshop.

Isolation of *Fgf8* cDNA

Fgf8 was isolated from a λgt11 library (kindly provided by Kai Zinn) using as probe the coding sequence of mouse *Fgf8* variant 4 during the initial screen, and a chicken *Fgf8* cDNA during rescreen (Crossley et al., 1996b). Candidates were subcloned into pCRII and sequenced (accession number AF051365). One additional zebrafish gene of uncertain relationship resembles *Fgf8*, but also other Fgfs; in contrast to the *Fgf8* gene reported here, this gene is expressed much later in development than *Fgf8* (S. Schulte-Merker, personal communication, and F. R. and M.B., unpublished data).

Molecular analysis of acerebellar

To determine linkage, heterozygous carriers for *acerebellar* (induced in the Tübingen strain) were crossed to AB wild-type strain. Carriers were identified in F_1 and intercrossed. Embryos from such crosses were separated into homozygous *acerebellar* mutants (*n*=100 and 108 for two independent experiments) and their siblings (*n*=100), and DNA and cDNA was prepared from each pool. cDNA synthesis with SuperScriptII reverse transcriptase (GibcoBRL) was according to manufacturers instructions. Intron sequences between exons 1 and 2 (1.6 kb) were amplified from both pools and from Tübingen and AB strains, assuming that exon/intron structure would be conserved relative to mouse *Fgf8* (Crossley and Martin, 1995). This assumption was confirmed by our results, and by sequencing of the amplified introns (not shown). Amplified fragments were digested with *Bgl*II, which detects a restriction fragment length polymorphism (RFLP) between the Tübingen and AB strains; the resulting gel was blotted and probed with a fragment containing only intron sequences to confirm that the fragments are derived from the *Fgf8* locus (not shown). Linkage was also observed for a second RFLP (not shown). Equivalent amplifications were carried out to obtain and sequence the exon 2/3 intron. cDNA was isolated by RT-PCR with nested primers flanking the coding region in two independent amplifications from cDNA pools of homozygous Tübingen wild-type and *acerebellar* embryos, and was subcloned and sequenced on an ALF sequenator. RT-PCR to detect presence of exon2 was carried out on cDNA from wild-type and *acerebellar* embryos under standard PCR conditions.

Injections

Wild-type and *acerebellar* mutant versions of *Fgf8* were subcloned into pCS2+ (Rupp et al., 1994) and transcribed using the SP6 message machine kit (Ambion). The amount of RNA injected was estimated from the concentration and volume of a sphere of RNA injected into oil at the same pressure settings. Typically, about 25 pg of *Fgf8* RNA were injected; higher concentrations cause more severe dorsalizations that lead to rupture of the embryos during somitogenesis (not shown). RNA was dissolved in 0.25 M KCl with 0.2% of phenol red and backloaded into borosilicate capillaries prepared on a Sutter puller. During injection, RNA was deposited into the cytoplasm of 1- to 8-cell-stage embryos; in embryos after the first cleavage, the RNA usually stays in the progeny of the injected blastomere, as judged from the unilateral distribution of control *lacZ* RNA, as detected with anti-β-gal antibody (Promega, 1:500) after ISH (Dornseifer et al., 1997).

RESULTS

Cloning and expression of zebrafish *Fgf8*

We have isolated zebrafish *Fgf8* from an embryonic cDNA library. The aminoacid sequence of zebrafish *Fgf8* is 79% identical to mouse and human *Fgf8*, and 84% identical to chicken *Fgf8* (Fig. 1). Amino acids encoded by exon 2 are diagnostic for *Fgf8* relative to other *Fgf* family members (Lorenzi et al., 1995); here the identity is 83% to mouse and human, and 91% to chicken *Fgf8*, with other similarities being much lower (e.g. *Fgf7*, 37% and *Fgf4*, 29%).

Expression during gastrulation

To study possible functions of *Fgf8*, we examined expression in wild-type embryos using whole-mount in situ hybridisation (ISH; Fig. 2). Expression becomes detectable at 30% epiboly in the marginal zone, and develops at 50% epiboly into a gradient with a highpoint in the dorsal embryonic shield, the zebrafish equivalent of Spemann's organizer (Fig. 2A-C). During gastrulation, dorsoventrally graded expression continues in the marginal zone. At 70% epiboly, expression starts in two transverse stripes in the anterior hindbrain primordium (Fig. 2D,E), and towards the end of epiboly at the anterior margin of the forebrain primordium (Fig. 2F).

Somitogenesis

During somitogenesis, expression continues in the prospective MHB (see below) and the tailbud, and is initiated in presomitic mesoderm in segmental expression domains (Fig. 2H,J). Expression is found throughout newly formed somites (Fig. 2J), but eventually becomes confined to the anterolateral margin of the maturing somite (Figs 2K, 10R). Transient expression occurs in the floorplate as it emerges from the tailbud (not shown). Posterior to the MHB, three additional stripes are detected in the hindbrain neural keel during early somitogenesis (Fig. 2H). In the forebrain primordium, a dorsomedian stripe is observed in the presumptive telencephalon with an anterior high point of intensity (Fig. 2H,I).

Expression in the brain

In the brain of pharyngula stage embryos (24-48 hours), expression is still prominent in the MHB, excluding the floorplate (Fig. 2O), and in the optic stalks, retina, a pair of paramedian telencephalic stripes that forms the commissural plate and in the dorsal diencephalon (Fig. 2N-R). Additional expression is seen in the dorsal hypothalamus ventral to the optic recess, in the area where the postoptic commissure will form (Fig. 2P). Around 36 hours, expression is detected in addition near the ventral midline of the hypothalamus in the hypophysis and infundibulum (Fig. 2P,Q), and in the nasal placodes (Fig. 2S). Expression continues in these tissues until 48 hours, the latest stage that we have examined (not shown).

Outside of the brain, expression is found in tissues of the

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zebrafish   MRLIPSRLSY LFLHLFAFCY YAQVTIQSPP NFTQHVSEQS KVTDRVSRRL   50
 chicken    -DPCS-LF-- V-M---VL-L Q----V---- ------R--- L---QL----
  mouse     -GSPR-AL-C -L---LVL-L Q----V---- ------R--- L---QL----
  human     -GSPR-AL-C -L---LVL-L Q----V---- ------R--- L---QL----

                                                       ↓
zebrafish   IRTYQLYSRT SGKHVQVLAN KKINAMAEDG DVHAKLIVET DTFGSRVRIK  100
 chicken    V--------- ------I-D- ---------- ---------- ----------
  mouse     ---------- ---------- -R-------- -PF------- --------VR
  human     ---------- ---------- -R-------- -PF------- --------VR

                                ↓
zebrafish   GAETGFYICM NRRGKLIGKK NGLGKDCIFT EIVLENNYTA LQNVKYEGWY  150
 chicken    --A------- -KK------S --K----V-- ---------- ---A------
  mouse     -----L---- -KK----A-S --K----V-- ---------- ---A------
  human     -----L---- -KK----A-S --K----V-- ---------- ---A------

zebrafish   MAFTRKGRPR KGSKTRQHQR EVHFMKRLPK GHQIAE_HRPF DFINYPFNRR 200
 chicken    ---------- ---------- ---------- ---TT-P--R- E-L-------
  mouse     ---------- ---------- ---------R --HTT-QSLR- E-L---PFT-
  human     ---------- ---------- ---------R --HTT-QSLR- E-L---PFT-

zebrafish   TKRTRYSGER       210
 chicken    S----N-SASLRP    214
  mouse     SL-GSQRTWAPEPR   215
  human     SL-GSQRTWAPEPR   215
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Fig. 1. Sequence comparison between the predicted amino acid sequences of zebrafish, chicken, mouse and human Fgf8 proteins. Horizontal bars indicate identical residues; arrows mark exon boundaries; consensus N-linked glycosylation sites are shaded.

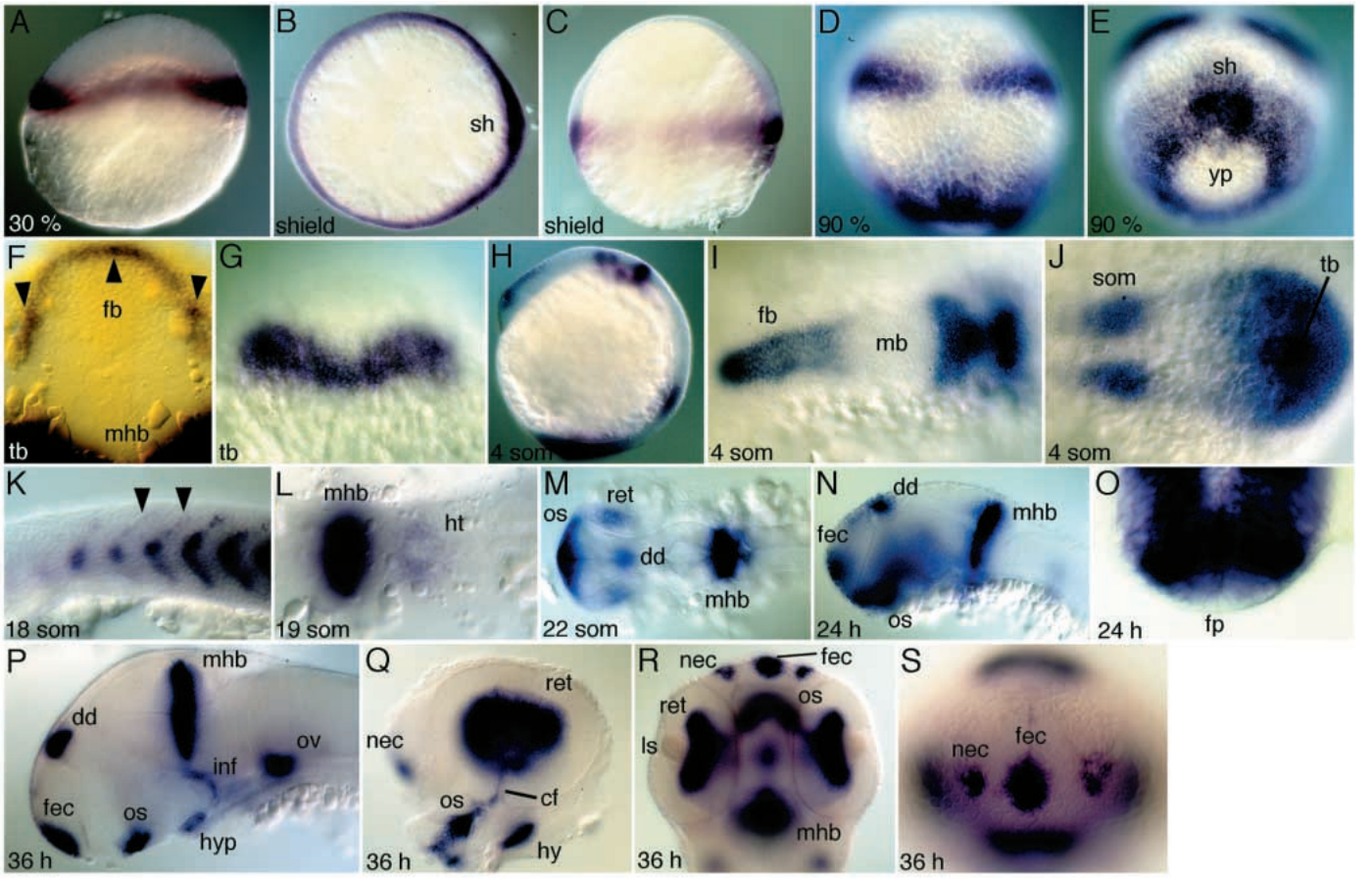

Fig. 2. Expression of *Fgf8* in wild-type embryos. (A) *Fgf8* in the blastoderm margin at 30% epiboly. (B,C) At shield stage, *Fgf8* is expressed in a dorsoventral gradient in the germ ring with a high point of expression in the shield (B, vegetal pole view; C, lateral view). (D,E) Graded expression persists in the margin of the blastoderm at 90% epiboly. Prospective anterior hindbrain expresses *Fgf8* (D, dorsal view; slightly tilted in E). (F) Forebrain expression in a tailbud stage embryo (arrowheads point to high points of expression). (G) Prospective MHB domains fuse at the midline at tailbud stage. (H) 4-somite stage, lateral view. Expression in forebrain, mid-hindbrain region, segmental plate and tailbud. (I,J) Flat mount of H, depicting anterior and posterior expression domains. (K) *Fgf8* expression at the anterior somite border (arrowheads). (L) Flat-mounted 19-somite embryo. Expression in the heart ring posterior to the MHB. (M) Flat mount at 22 somites; expression in the brain is detected at the MHB, dorsal diencephalon, retina and optic stalks. (N) Lateral view of a 24 hours embryo. Additional expression occurs in the facial ectoderm. (O) Thick cross section through the MHB demonstrating absence of expression in floorplate. (P) Lateral view of a dissected brain at 36 hours of development. Additional expression in the infundibulum, hypophysis and otic vesicle (eyes are removed). (Q) Details of expression in the retina, choroid fissure and the optic stalks. Additional expression is detected in nasal ectoderm and the hyoid. (R) Ventral view of head at 36 hours demonstrating expression in the retinal epithelium, but not in the lens. (S) Frontal view, expression in the facial and nasal ectoderm. cf, choroid fissure; dd, dorsal diencephalon; fb, forebrain; fec, facial ectoderm; fp, floorplate; ht, heart; hy, hyoid; hyp, hypophysis; inf, infundibulum; ls, lens; mb, midbrain; mhb, mid-hindbrain boundary; nec, nasal ectoderm; os, optic stalks; ov, otic vesicle; ret, retina; sh, shield; som, somites; tb, tailbud, yp, yolk plug.

developing head, such as the hyoid, heart, inner ear (Fig. 2L, P-S) and the fin buds (Fig. 11).

Fgf8 expression at the MHB

Because of its possible patterning function in development of the MHB territory, we have examined the expression in this area in more detail. *Fgf8* activation is seen initially as a bilateral stripe at 70% epiboly (Fig. 3A-D). Relative to Krox-20, a marker for rhombomeres 3 and 5 (Oxtoby and Jowett, 1993; Fig. 3A,B), this stripe encompasses by tailbud stage the anterior hindbrain up to and including rhombomere 4. At 5 somites, this domain has become subdivided into several stripes lying at the MHB, in rhombomeres 1 and 4 and ventral rhombomere 2 (Figs 3C-D, 2H). In double stainings with the midbrain marker *Pax2.1*, *Fgf8* expression (Fig. 3E-H) is localized posterior to the domain of *Pax2.1* expression at 90% epiboly, with very little, if any, overlap. At 6 somites, however, the MHB stripe is completely contained within the posterior part of the *Pax2.1* domain (Fig. 3I,J).

Fgf8 is mutated in acerebellar

Fgf8 expression occurs in several tissues that are defective in *acerebellar* mutant embryos. Mutant larvae older than 2 days are retarded and eventually die with severe oedemas (not shown), but develop without significant retardation during the first 48 hours of development. In particular, homozygous *acerebellar* mutants lack a MHB and a cerebellum (see below). By testing candidate genes, we found that *Fgf8* is linked to the

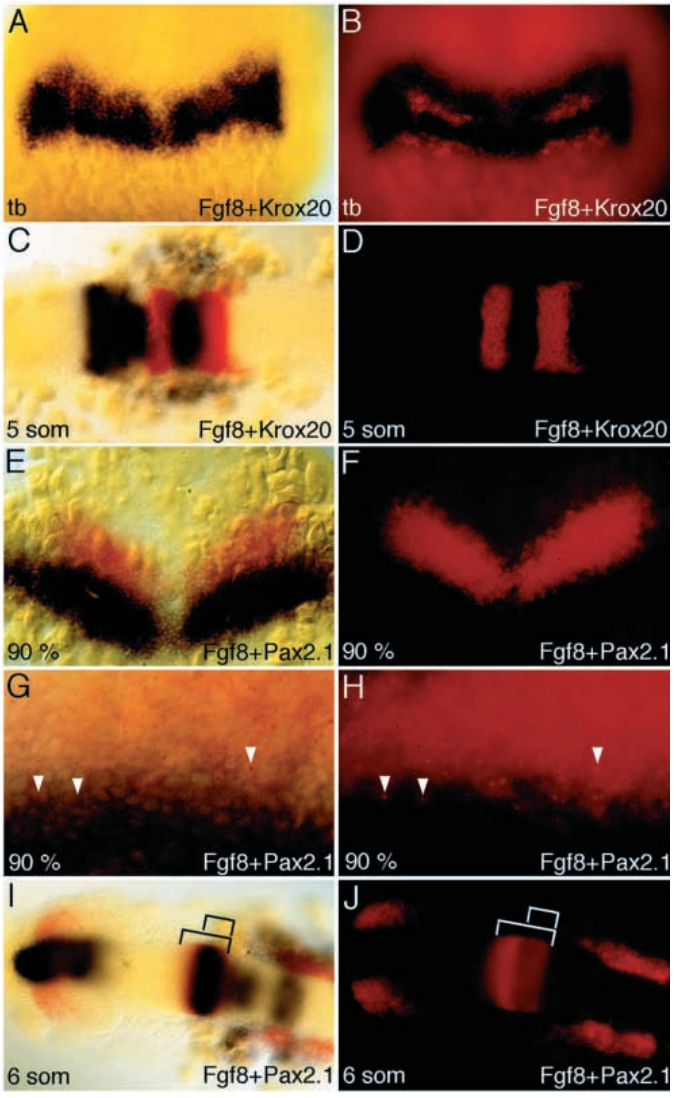

Fig. 3. *Fgf8* expression in early midbrain and anterior hindbrain development. (A-D) Double ISH with *Fgf8* (blue) and *Krox20* (red, fluorescent) of wild-type embryo at tailbud stage (A,B) and 5 somites (C,D). At tailbud stage, *Fgf8* expression extends throughout the anterior hindbrain incl. rhombomere 4 (r4) posteriorly. At 5 somites, expression of *Fgf8* is detected at the MHB, in r1, r4 and in ventral r2 (see also Fig. 2H). (E-J) Double ISH with *Fgf8* (blue) and *Pax2.1* (red, fluorescent) at 90% epiboly (E-H) and 6 somites (I,J). At 90% epiboly, the *Fgf8* expression domain is located posterior to the *Pax2.1* domain with very little overlap (E,F; higher magnification: G,H), while at 6 somites the *Fgf8* domain at the MHB is completely included in the *Pax2.1* expression domain (visible as quenching of the fluorescent *Pax2.1* signal). Embryos in C-J are flat mounted, A,C,E,G and I show bright field, B,D,F,H and J show fluorescent images of the same embryos.

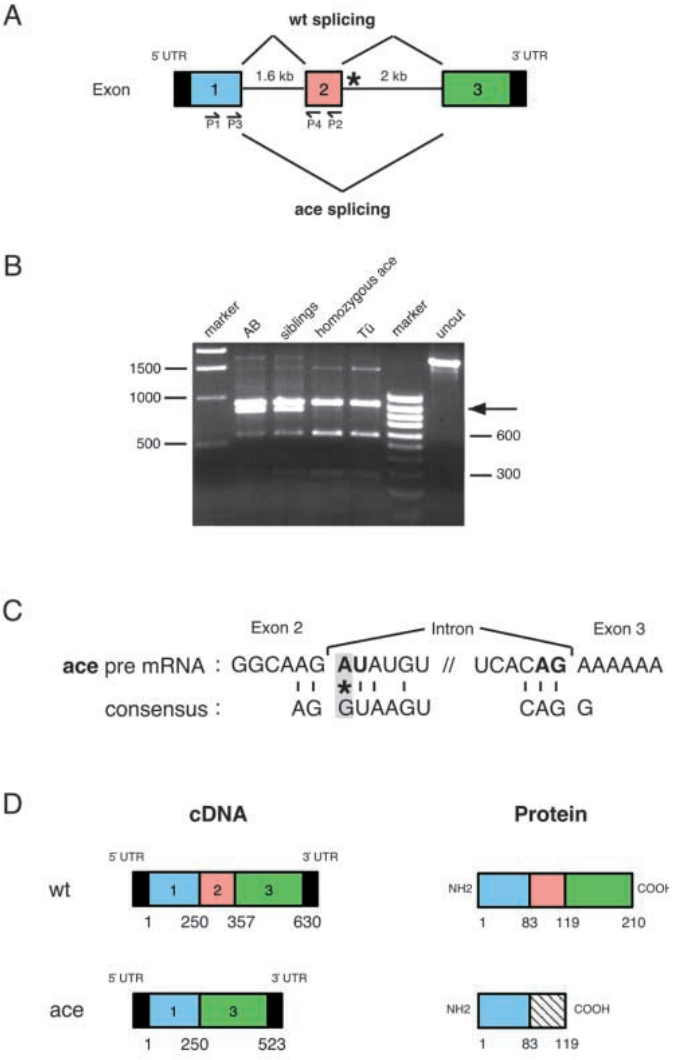

Fig. 4. *Fgf8* is mutated in *acerebellar*. (A) Genomic structure of the zebrafish *Fgf8* gene and possible splicing variations in wild-type and *acerebellar* embryos (asterisk depicts mutated 5′ splice site). (B) The intron between exon 1 and 2 was amplified and digested with *Bgl*II. An RFLP was identified for AB versus 'Tübingen' strain zebrafish (compare lane 'AB' with lane 'Tü'; arrow points to the polymorphic band). The homozygous *acerebellar* mutation was induced in a Tü strain and shows the Tü restriction pattern (compare lane 'homozygous ace' with lane 'Tü'), while their siblings show the AB pattern (compare lane 'siblings' with lane 'AB'). Therefore the *acerebellar* phenotype is linked to the *Fgf8* gene. (C) A 100% conserved G in the 5′ splice site following exon 2 is changed to an A, leading to skipping of exon 2. (D) cDNA from *acerebellar* embryos lacks exon 2 (red). This causes a frame shift in the open reading frame, leading to altered amino acids (hatched) and a premature stop in translation.

acerebellar mutation. In a test cross with two segregating RFLPs of *Fgf8*, the RFLP characteristic for the 'Tübingen' strain (in which *acerebellar* was induced) is linked to the *acerebellar* phenotype (Fig. 4). This RFLP was located in intron one and was lost during subsequent generations. However, by RT-PCR with single embryos, we found that the *acerebellar* phenotype and the lesion in *Fgf8* (see below) could not be separated in 101 embryos representing 202 meiotic events (0±0.5 cM; Fig. 5D).

acerebellar mutant transcripts lack exon 2

Through characterizing the *Fgf8* gene in *acerebellar* embryos, we found that *acerebellar* is a mutation that strongly or completely inactivates the *Fgf8* gene. We used RT-PCR to

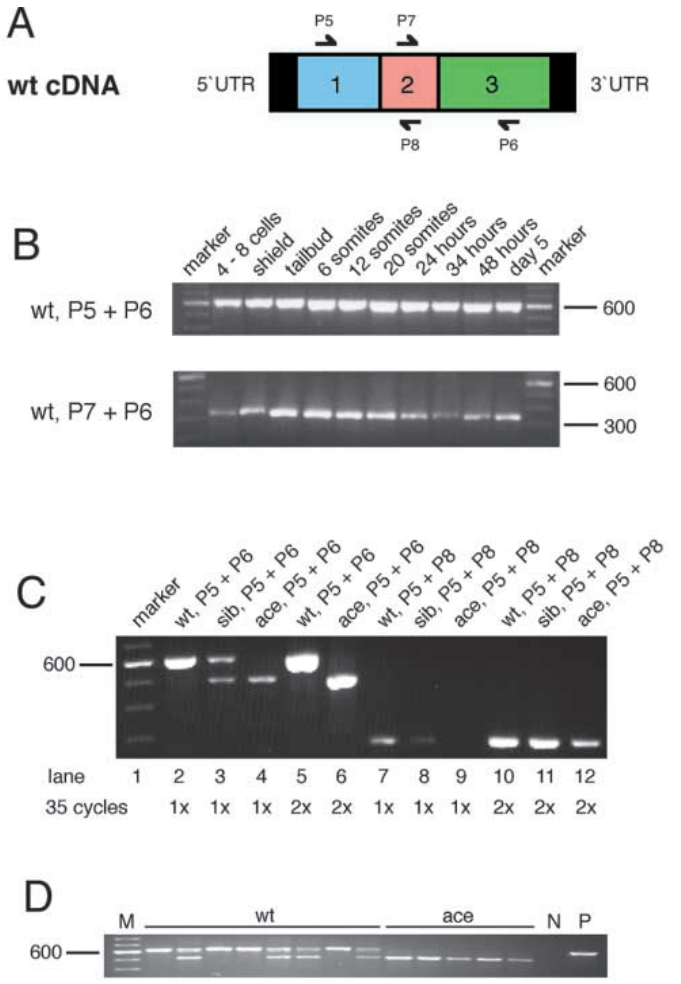

Fig. 5. RT-PCR analysis of *Fgf8* transcripts in wild-type and *acerebellar* embryos. (A) Structure of wild-type cDNA and placement of primers. (B) Only transcripts containing exon 2 are detected in wild type throughout development either with primers spanning exon 2 (P5 + P6) or with one primer located in exon 2 (P7 + P6). (C) Exon 2 is missing in *Fgf8* transcripts of *acerebellar* embryos. RT-PCR with primers spanning exon 2 (P5 + P6) yields a single band of 612 bp in wild-type embryos. In *acerebellar* embryos, no transcripts of wild-type size are detected; instead only transcripts without exon 2 are seen, which are shorter by 107 bases (compare lanes 2 and 4, and lane 5 and 6). In heterozygous siblings, bands of both sizes are detectable (lane 3). With one primer located in exon 2 (P5 + P8), amplification is only possible in *acerebellar* embryos after reamplification (compare lane 9 and 12), while in wild-type embryos and siblings one round of amplification is sufficient (lanes 7, 8), demonstrating the low abundance of exon 2 containing transcripts in *acerebellar* embryos. (D) *acerebellar* mutant embryos and their siblings were sorted by phenotype, indicated by bars. Single embryo RT-PCR with primers spanning exon 2 (P5 + P6) was performed for 101 *acerebellar* embryos and 13 siblings. Phenotypically wild-type siblings show either a wild-type or a heterozygous pattern. In all phenotypically *acerebellar* embryos tested, only exon 2-less transcript is detected, confirming genetic linkage (0 ± 0.5 cM).

amplify *Fgf8* cDNAs from homozygous *acerebellar* embryos and compared them to wild-type cDNA (Fig. 4). In two independent amplifications of the coding region, we find a deletion of 107 bases exactly corresponding to exon 2 in the mutant cDNAs; no other amino acid changes were detected. In order to study how this deletion is generated, we examined genomic DNA of *acerebellar* embryos and found that exon 2 is both present and of normal sequence. Upon sequencing of the 1.6 kb intron between exons 1 and 2, no conspicuous changes were detected and, in particular, the splicing consensus sequences were found to be intact (not shown). However, in the 5′ splice donor site following exon 2, a G residue was mutated to an A (Fig. 4C). Since this G is 100% conserved in all 5′ splice donor sites (Padgett et al., 1986), this mutation may inactivate this splice site, leading to skipping of exon 2 in the mutants.

As a result of skipping exon 2, the open reading frame runs into a premature stop codon (Fig. 4D). The predicted protein fragment in *acerebellar* embryos therefore lacks the aminoacids encoded in exons 2 and 3, which are required to activate the receptor and which are conserved between different *Fgf8*s and other *Fgf* family members (Lorenzi et al., 1995). The protein fragment in *acerebellar* mutants is therefore presumably non-functional, a notion that is confirmed by our injection experiments (see below).

Exon 1, but not exon 2, is alternatively spliced in murine *Fgf8* (Crossley and Martin, 1995; MacArthur et al., 1995). We find no evidence for differential splicing of exon 2 of *Fgf8* in zebrafish : in a timecourse up to day 5, we detect in wild-type embryos a single transcript of the size predicted for transcripts containing exon 2 (Fig. 5). This transcript is also detected at the 4- to 8-cell stage, i.e. prior to activation of zygotic transcription (Kane and Kimmel, 1993), showing that maternal *Fgf8* message is present in these embryos (Fig. 5B). We could not, however, detect any maternal RNA by in situ hybridisation (not shown), suggesting that these RNAs are rare.

To assess the strength of the *acerebellar* allele, it was crucial to determine if any wild-type *Fgf8* transcript is present in the mutants. We therefore performed RT-PCR on cDNA from *acerebellar* embryos. With primers flanking exon2, we detect a single band of the size predicted for transcripts lacking exon 2, but no transcripts of wild-type size (Fig. 5C). With one primer in exon 2 and another in the flanking exons, exon-2-containing transcripts can be detected in *acerebellar* embryos, but only after two rounds of amplification (Fig. 5C). These transcripts could be of maternal origin (see above), or they could be due to partially spliced mRNA in our cDNA pools; although partially spliced transcripts are usually unstable and confined to the nucleus (Padgett et al., 1986; Khoury et al., 1979). We can at present not distinguish between these possibilities. In either case, wild-type transcripts containing exon 2 must be rare, since they are not detectable with primers flanking exon 2 or by in situ hybridisation. We conclude that *acerebellar* partially or completely inactivates the *Fgf8* gene (see Discussion).

ace mutant Fgf8 is unable to dorsalize embryos

In order to determine whether the Fgf8 protein left in *acerebellar* mutants has any functional properties, we developed a functional assay for *Fgf8* activity. When wild-type *Fgf8* RNA is injected into developing embryos, we observe dorsalization and axis duplication (Fig. 6A-F; Table 1). We monitored adaxial and somitic development with *myoD* probe, and the location of the injected cells by coinjection of *lacZ* RNA. Embryos with a secondary axis formed in 12% of the

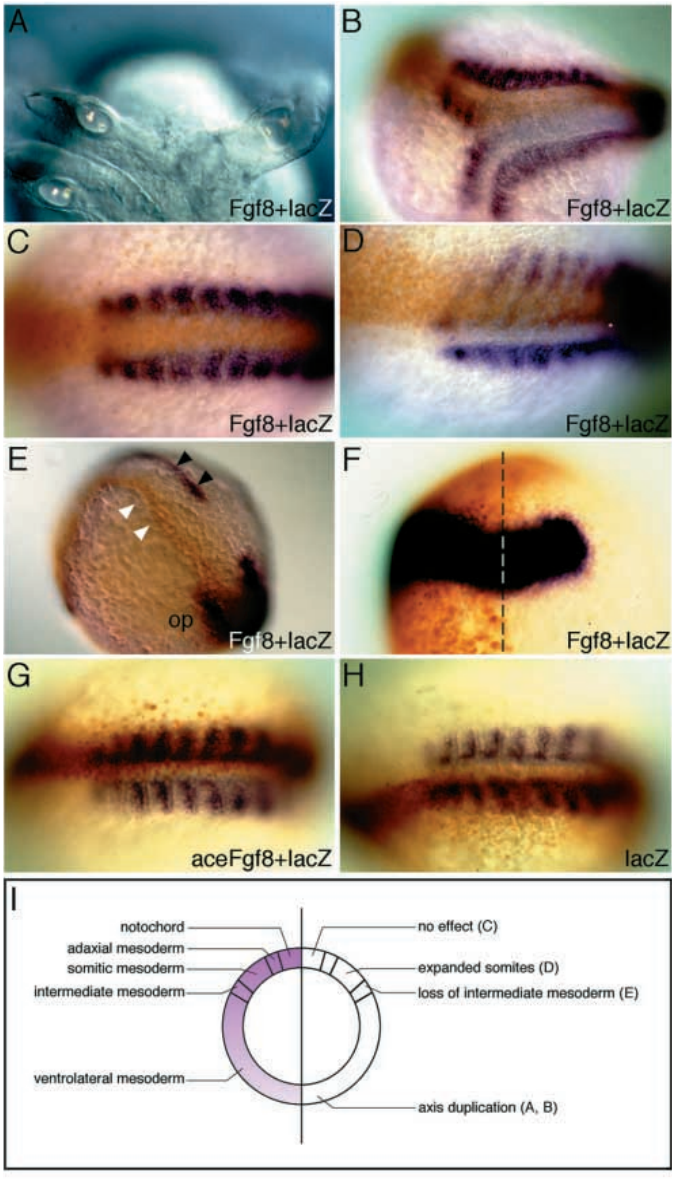

Fig. 6. Function of wild-type and mutant *Fgf8* in dorsoventral patterning of the gastrula. Adaxial and somitic mesoderm is visualized with *myoD* (blue), or intermediate mesoderm and MHB with *Pax2.1*, and location of the *lacZ* co-injected cells with an antibody to β-gal (brown). RNA distribution is mostly restricted to one side, allowing comparison with the contralateral side as a control. (A-F) Misexpression of *Fgf8* in wild-type embryos by RNA injection. (A,B) Live 28 h embryo with axis duplication (A) and stained for *myoD* (B). (C) Axial location of the injected RNA yields no obvious defect in mesoderm patterning. (D) Expansion of somitic mesoderm on the injected side of embryo. (E) *Pax2.1*-positive intermediate mesoderm (black arrowheads) is missing on the injected side (white arrowheads). (F) *Fgf8* misexpression alters the d/v, but not the a/p extent of the *Pax2.1* expression domain on the left, injected side (midline is given by a dashed line; op, otic placode). (G) No effect is seen after injection of the *acerebellar* version (lacking exon2) of *Fgf8* RNA, showing that the *acerebellar* transcript is inactive in vivo. (H) *lacZ* control injections had no effect. (I) Summary of the effects observed after injection of *Fgf8*. Left: schematic fate map of a gastrula, and the *Fgf8* gradient in the germ ring. Right: consequence of misexpressing *Fgf8* in the respective area. All embryos shown are at early to midsomitogenesis stages; B-D, G, H show *myoD* in situ stainings of injected embryos, E and F show *Pax2.1* in situ stainings; β-gal was detected by antibody staining.

cases (Table 1). Invariably, the cells of the secondary axis were the injected cells (Fig. 6B), and failed to form a head, notochord and adaxial cells. Exclusively dorsal location of the injected cells in the notochord primordium has little or no effect, suggesting that this tissue is not competent to respond (Fig. 6C). In dorsolateral levels, *Fgf8* misexpression causes severe expansion of the somites to ventral levels (Fig. 6D), which in some cases encircle the embryo (not shown). Consistent with the expansion of dorsal cell fates, *Pax2.1* expression in the intermediate mesoderm is suppressed or shifted to more ventral levels (Fig. 6E). Notably, although MHB expression of *Pax2.1* is expanded ventrally, it is *not* expanded along the anteroposterior axis (Fig. 6F), showing that *Fgf8* is not sufficient to induce *Pax2.1* expression. These findings are summarized in Fig. 6I. Our misexpression studies do not necessarily imply that Fgf8 normally functions in dorsoventral patterning, but they do provide a sensitive assay for functional activity of ectopically expressed *Fgf8* transcripts. In contrast to the severe effects of misexpressing wild-type *Fgf8*, injections of the *ace* mutant *Fgf8* at the same or a tenfold higher concentration causes no effect (Fig. 6G; Table 1). We conclude that the *Fgf8* transcript lacking exon 2 is inactive.

Requirement for *Fgf8* in MHB development

Examination of living embryos and of histological sections shows that the MHB fold and the cerebellum are absent in *acerebellar* embryos (Fig. 7). In living embryos, the MHB fold and the posteriorly adjacent cerebellar primordium are missing (Fig. 7A,B). In histological sections, the MHB tissue is

Table 1. Summary of Fgf8 and aceFgf8 overexpression

Experiment	Injected RNA	Normal	Dorsoventral effect	Double axis	Necrotic/ disorganized	Σ embryos (n = 100%)
Fgf8 overexpression	25 pg Fgf8	35%	29%	15%	21%	34
	25 pg Fgf8 + 250 pg *lacZ*	27%	49%	12%	12%	41
	250 pg *lacZ*	60%	0%	4%	36%	25
aceFgf8 overexpression	25 pg aceFgf8 + 500 pg *lacZ*	65%	0%	1%	34%	168
	500 pg *lacZ*	78%	0%	0%	22%	77
	250 pg aceFgf8 + 500 pg *lacZ*	83%	0%	0%	17%	59
	25 pg aceFgf8 + 500 pg *lacZ*	79%	0%	2%	19%	53

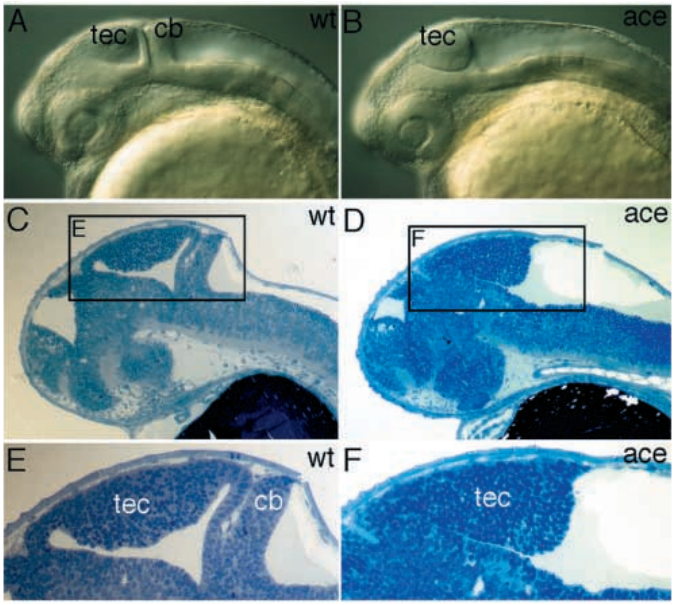

Fig. 7. Brain phenotype of *acerebellar* embryos. (A,B) At pharyngula stage, mutant embryos lack a cerebellum and the mid-hindbrain fold, but show an enlarged tectum (lateral view of living embryos). (C,D) Sagittal section of 36 hour embryos. (E,F) High magnification view of area depicted in C and D, showing the mid-hindbrain phenotype in more detail. cb, cerebellum; tec, tectum.

eliminated posterior to the tectum opticum, which itself often appears slightly enlarged (Fig. 7C-F). The MHB can be subdivided into an anterior portion (probably still part of the midbrain) and a posterior portion, which is thought to give rise to cerebellum (Kimmel et al., 1995). Both portions are absent in *acerebellar* mutants, showing that the defect is not restricted to the cerebellar primordium. We do not know the fate of the prospective fold tissue in *acerebellar* mutants but, since we have not detected cell death in this region previously (Brand et al., 1996), the apparent increase in the tectal tissue could reflect a transformation to a midbrain fate.

Our above results show that cerebellar and MHB development fail during embryonic stages in *acerebellar* mutants. We therefore examined the expression of marker genes of this area in a detailed time course (Fig. 8, Table 2). Like *Fgf8* itself, *wnt1*, *Pax2.1*, *Eng1*, *Eng2*, *Eng3* and *Her5* are expressed during normal development of the MHB primordium (Krauss et al., 1991; Molven et al., 1991; Ekker et al., 1992; Müller et al., 1996). After initially widespread expression in the midbrain primordium, expression of these genes is restricted during mid-somitogenesis towards the posterior midbrain and MHB. Expression of all marker genes that we examined is initiated normally in *acerebellar* mutant embryos. During early to mid-somitogenesis stages, however, expression gradually fades in *acerebellar* mutants, but continues in wild-type siblings. The expression domains of all markers gradually narrow and seem to persist longest in dorsoposterior parts of the MHB; eventually, expression is completely eliminated in the mutants (Fig. 8, Table 2). The earliest defect is seen for *Her5* at the 5-somite stage (Fig. 8G,J). These results show that maintenance, but not initiation of gene expression at the MHB, is affected in *acerebellar* mutant embryos.

Early *Fgf8* expression does not require *Pax2.1*

To examine if establishment of *Fgf8* signalling is dependent on *Pax2.1*, a gene required for early midbrain development (Brand et al., 1996; Lun and Brand, unpublished data), we examined *Fgf8* expression in *no isthmus* (*noi*) mutant embryos for noi^{tu29a}, which lack a functional *Pax2.1* gene (Fig. 9). Up until the 10 somites stage, we observe no difference in *Fgf8* expression between wild-type and *noi* embryos (Fig. 9A). At 18 somites, expression is eliminated at the MHB, but not in several other tissues (Fig. 9B,C). Since *noi* mutants lack the isthmus at this stage (Brand et al., 1996), this is most likely a secondary consequence of elimination of the tissue. We conclude that *Fgf8* signalling in the early MHB primordium is activated independently of *Pax2.1*/Noi.

Requirement during dorsoventral patterning and mesodermal development

To study whether *Fgf8* functions during development of the mesodermal derivatives that it is expressed in, we examined *ace* mutants with marker genes for axial, paraxial and intermediate mesoderm, and found defects that are probably due to weakly abnormal dorsoventral patterning (Fig. 10). *myoD* is expressed in adaxial cells lateral to the notochord from

Table 2. Marker gene expression at MHB in ace

Expression of markers at the MHB during shown time course of zebrafish development.
Black bar shows normal expression at MHB, while dashed bar indicates decreasing expression domain as compared to wild type.
*Eng1 transcripts could be detected only from 12 somites onwards in wild type and *ace* embryos using non-radioactive in situ hybridization.
MHB, mid-hindbrain boundary; som, somites; nd, not detectable.

80% epiboly onwards, and later spreads to the forming somites in the paraxial mesoderm (Weinberg et al., 1996). *myoD* expression in adaxial cells of *acerebellar* mutants is strongly reduced at 80% to tailbud stages, and is interrupted during somitogenesis stages (Fig. 10A-F).

Since *Fgf8* is not yet expressed in adaxial cells or notochord between 80% and tailbud, the early failure to express *myoD* could reflect a weak requirement for *Fgf8* in dorsoventral patterning. To test a possible requirement in dorsoventral patterning, we examined expression of *eve1*, *fkd3* and *BMP4* as markers for ventral and dorsal cell fates (Joly et al., 1993; Chen et al., 1997; J. Odenthal, unpublished) and found no difference at 50%, 80% and tailbud stages (not shown). Likewise, *Pax2.1* expression in the intermediate mesoderm is normal at 7 somites (Fig. 10I,J). The tailbud is viewed as a site of continuing gastrulation and patterning (Gont et al., 1993) and expresses *Fgf8*. We find *snail1* expression (Hammerschmidt and Nüsslein-Volhard, 1993) to be absent in the vicinity of the tailbud, possibly reflecting another weak function for *Fgf8* in gastrulation or patterning (Fig. 10K,L). We conclude that early defects of *myoD* and *snail1* expression in *acerebellar* mutants could reflect a weak requirement in dorsoventral patterning. Notably, this requirement is most apparent in the future adaxial and somitic mesoderm, close to the site of highest *Fgf8* expression on the dorsal side.

Following activation throughout the somites and adaxial cells, *Fgf8* expression is successively confined to anterior-lateral cells of wild-type somites (Fig. 10Q,R). Both somite and adaxial cell development is affected in *acerebellar* mutants. During midsomitogenesis, *myoD* and *snail1* are expressed in condensing somites of the wild type, but are reduced and patchy in *acerebellar* mutants (Fig. 10E,F,M,N). Towards the end of the segmentation period, wild-type somites assume a distinct chevron shape and continue to express *myoD* and *snail1* (Fig. 10G,O). In *acerebellar* mutants, the somites appear more block-shaped, and have strongly reduced levels of *myoD* and *snail1* (Fig. 10H,P).

To examine development of adaxial cells, we studied expression of *Eng* in muscle pioneers that are derived from a subset of adaxial cells (Devoto et al., 1996). Expression of *Eng1* (not shown) and *Eng2* is reduced in *acerebellar* mutants at 24 hours (Fig. 10S,T). Adaxial cells themselves depend on signals from the notochord (Halpern et al., 1993). Brachyury/T expression as a marker for notochord (Schulte-Merker et al., 1992) was however unaffected at 80%, tailbud and 5-somite stages (not shown). In zebrafish *you-too* mutants, adaxial cells are missing, without affecting overall *myoD* expression in the early somites (van Eeden et al., 1996). In contrast, *acerebellar* mutants are defective both in early adaxial cell development (Fig. 10A-D) and in somitic expression of *myoD* and *snail1* (see above). *Fgf8* may therefore function independently in development of both cell types; alternatively, the defect could be an indirect consequence of the earlier abnormal expression of paraxial mesoderm genes like *myoD* and *snail1*. Given its anteriorly restricted expression pattern at later stages, Fgf8 might also function in polarization of somites along the craniocaudal axis (Hrabe de Angelis et al., 1997). However, in mutant somites, *Fgf8* itself is still expressed anteriorly, and *snail1* is more highly expressed posteriorly as in wild type, suggesting that the mutant somites are still patterned along the rostrocaudal axis (not shown).

Fgf8 in pectoral fin development

Teleost pectoral fins are homologous to tetrapod forelimbs (Sordino et al., 1995). Fgf8 is discussed as an important regulator of limb development, possibly by maintaining or inducing expression of *sonic hedgehog* (*shh*) in the zone of polarizing

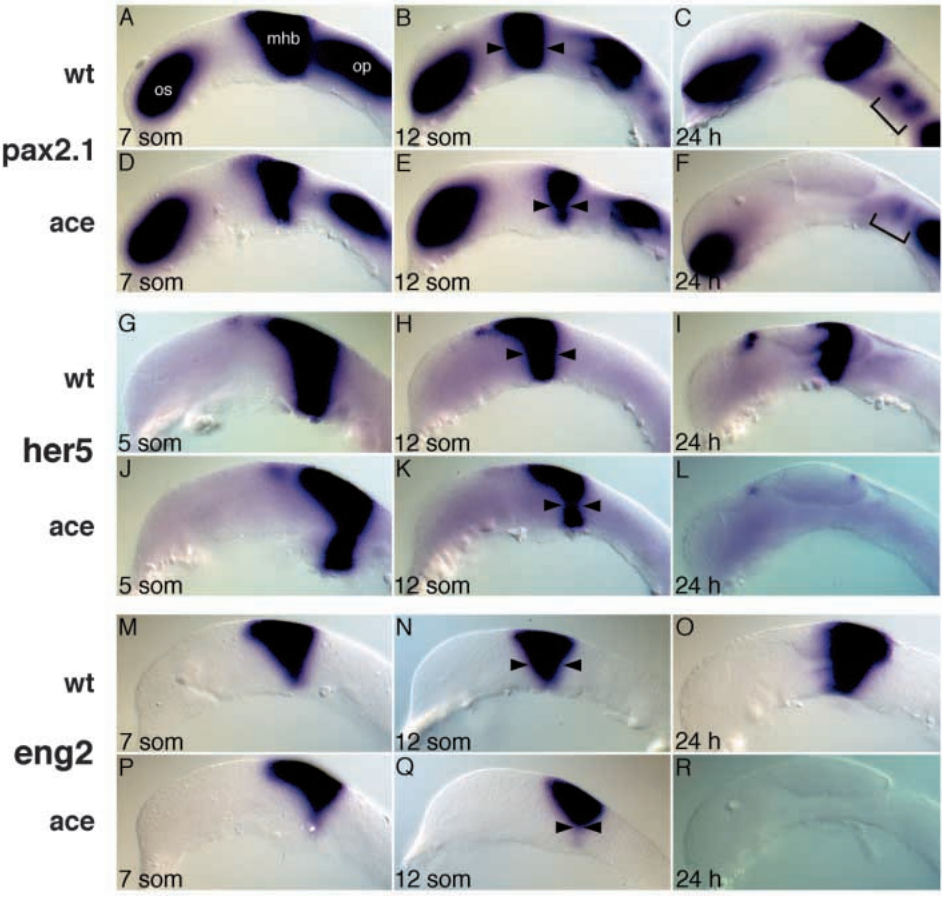

Fig. 8. *Fgf8* is required for the maintenance of MHB marker genes. Lateral views of dissected brain primordia. Stages and markers as indicated. (A-F) Expression of *Pax2.1* in wild-type (A-C) and *acerebellar* (D-F) embryos. Notice the gradual reduction in width at the MHB in B, E, and the reduction of otic placode (A,D), optic stalk and anterior hindbrain expression in C versus F. (G-L) Expression of *Her5* in wild-type (G-I) and *acerebellar* (J-L) embryos. Expression of *Eng2* in wild-type (M-O) and *acerebellar* (P-R) embryos. Arrowheads depict the width of the MHB, brackets mark anterior hindbrain. op, otic placode.

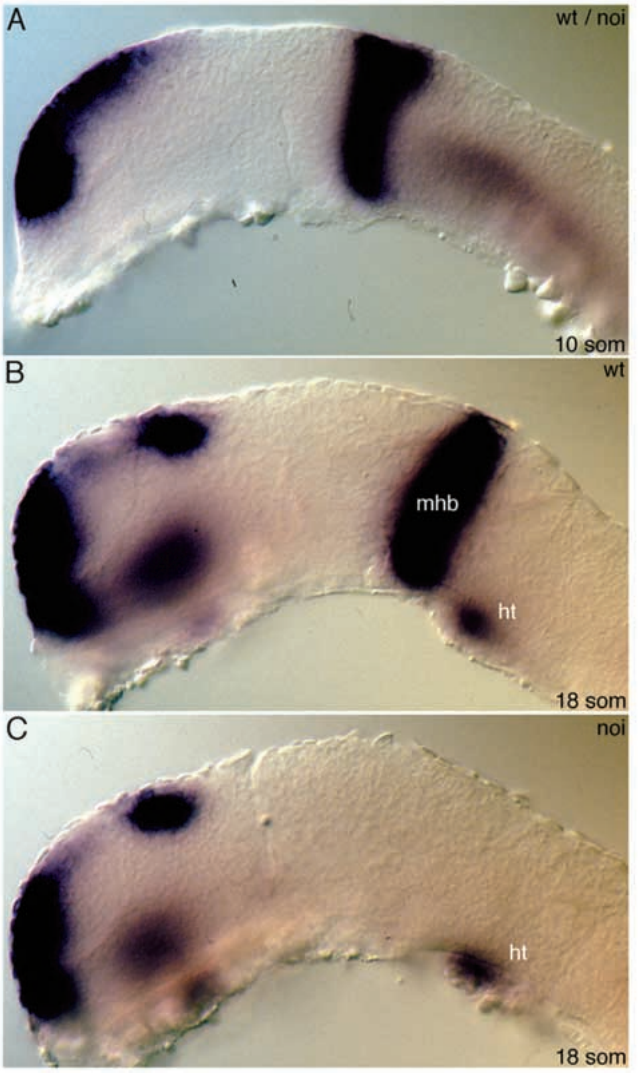

Fig. 9. *Fgf8* expression at the MHB is independent of *Pax2.1*. (A) Expression of *Fgf8* at the MHB in *noi*/*Pax2.1* mutant embryos cannot be distinguished from their wild-type siblings at the 10-somite stage. (B,C) At 18 somites, the expression of *Fgf8* is absent from the MHB in *noi*/*Pax2.1* mutant embryos, due to the loss of MHB territory. A-C show lateral views of *Fgf8* in situ-stained embryos. ht, heart; mhb, mid-hindbrain boundary.

activity (ZPA; see Cohn and Tickle, 1996, for review). In contrast to chicken and mice, *Fgf8* expression starts in the pectoral fin bud ectoderm only after initial fin bud formation, at 36 hours (Fig. 11B). At 48 hours, *Fgf8* is confined to the distal-most ridge of the developing fin (Fig. 11C,M), the equivalent of the apical ectodermal ridge of other vertebrates (Wood, 1982). *eng1* and *shh* are activated earlier than *Fgf8* in the fin bud of wild-type embryos (Hatta et al., 1991; Krauss et al., 1993), and *Fgf8* is therefore probably not involved in the induction and early patterning of the fin bud in zebrafish (Fig. 11A,E,H). To examine if *Fgf8* could function in later stages of fin development, we analysed *shh* expression in the ZPA, and *eng1* expression in the ventral-anterior fin bud, but could not detect an effect in *acerebellar* embryos (Fig. 11F,G,I,J). Furthermore, the overall structure of fins on day 5 of development appears normal in living *acerebellar* larvae (Fig. 11K,L). We do, however, consistently observe a slight increase in *Fgf8* expression in the mutants at 48 hours, possibly reflecting a later function for *Fgf8* (Fig. 11D). Although there are no obvious defects, our data do not exclude a late function for Fgf8 in development of the fin bud, since other Fgfs could compensate for missing Fgf8 activity, as reported for other vertebrates (Cohn et al., 1995; Crossley et al., 1996b; Ohuchi et al., 1997a).

DISCUSSION

We have described the isolation and expression pattern of zebrafish *Fgf8* and have shown that *acerebellar* is a mutation in *Fgf8*. Unexpectedly, our analysis demonstrates a requirement for *Fgf8* in maintenance, but not initiation, of MHB development. In addition, *Fgf8* is weakly required for normal dorsoventral patterning and in somite development. Other tissues, like the pectoral fins, apparently require *Fgf8* to a lesser degree. Maternally supplied *Fgf8* or other *Fgfs* may compensate for the loss of *Fgf8*.

Cloning and expression

Within the *Fgf* family, zebrafish *Fgf8* is most closely related, by sequence, expression pattern and genomic structure, to the *Fgf8* subgroup. During development, *Fgf8* is expressed in many cell populations that are known to be important signaling centers, such as the shield (Spemann's organizer), the anterior edge of the neural plate, the MHB and the limb bud. A similar association of *Fgf8* expression with signaling centers occurs in other vertebrates, often combined with expression of other *Fgfs* (Heikinheimo et al., 1994; Crossley and Martin, 1995; Mahmood et al., 1995; Bueno et al., 1996; Neubüser et al., 1997). Our detailed analysis of *Fgf8* expression at the MHB shows that even within a given domain expression can be very dynamic.

Strength of the *acerebellar* allele

The combined data of our linkage studies, analysis of the *Fgf8* locus in *acerebellar* mutants, and of our phenotypic studies show that *acerebellar* is mutated in *Fgf8*. A key issue is to what extent *Fgf8* activity is inactivated in *acerebellar*. Our analysis shows that mutation of a 100% conserved residue in a splice donor site following exon 2 leads to skipping of exon 2 in mutant embryos (Fig. 4). In a direct comparison of the prevalence of transcripts containing or lacking exon 2 with flanking primers, only transcripts lacking this exon are detectable in *acerebellar* mutants (Fig. 5). In the more sensitive assay where one primer is located in exon 2, also transcripts containing exon 2 are detectable, but only after two rounds of PCR amplification; such transcripts are therefore probably rare. They could be maternal transcripts, or transcripts resulting from incomplete inactivation of the mutated splice site. Absence of exon 2 results in a frameshift and premature chain termination of the predicted mutant protein. The conserved amino acids encoded by exons 2 and 3 that are thought to be important for Fgf function (Basilico and Moscatelli, 1992) are absent, and the resulting truncated protein is therefore probably inactive. This prediction is confirmed by the results of our injection experiments with RNA encoding the *ace* mutant version of *Fgf8*, which even at 10-fold higher RNA concentration does not have a biological effect (Fig. 6G). While these results show that transcripts lacking exon 2 do not

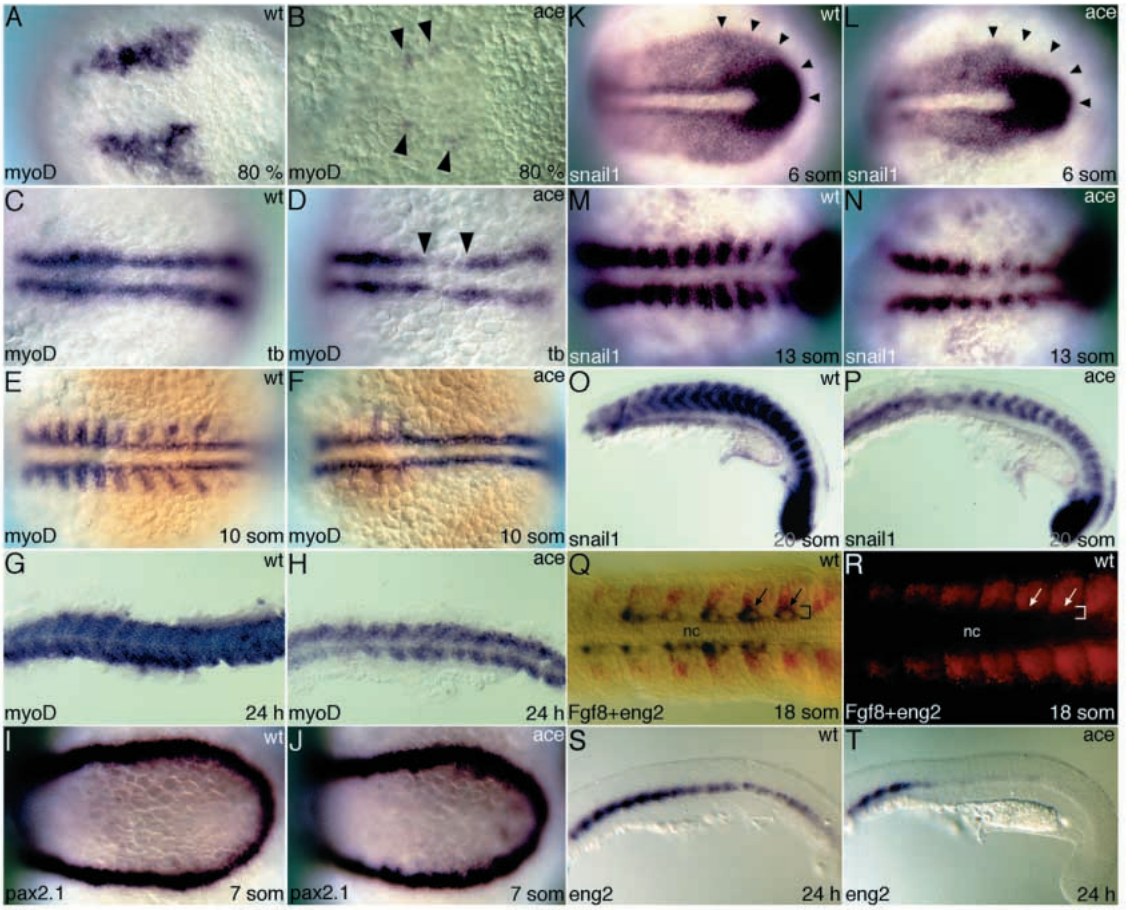

Fig. 10. *Fgf8* is involved in mesoderm and somite patterning. (A,B) Expression of *myoD* is strongly reduced in adaxial mesoderm of *acerebellar* embryos at 80% epiboly (arrowheads point to remnants of expression). (C,D) At tailbud stage, *myoD* expression in adaxial mesoderm is interrupted in *acerebellar* (arrowheads). (E,F) *myoD* staining in the somitic mesoderm is strongly reduced in mutants at the 10-somite stage. (G,H) At 24 hours, the expression of *myoD* is weak in the smaller and less-well-differentiated somites of *acerebellar* embryos. (I,J) No obvious difference could be detected between wild-type and *acerebellar* embryos in formation of intermediate mesoderm, shown here with *Pax2.1* staining at the 7-somite stage. (K,L) Expression of *snail1* is reduced in *acerebellar* embryos in the region around the tailbud at 6-somite stage (arrowheads point to the wild-type border of expression). (M,N) At the 13-somite stage and (O,P) 20-somite stage, snail1 transcripts are strongly reduced in the somites of mutant embryos. (Q,R) Dorsal view of wild-type embryo stained for *eng2* (blue) and *Fgf8* (red, fluorescent) showing partial overlap of these expression domains at an early stage of somite development (arrows). Note the restriction of *Fgf8* expression to anterolateral cells of the somites over time (anterior is to the left; brackets depict adaxial cells; nc, notochord). (S,T) Muscle pioneers are reduced in *acerebellar* embryos, as shown here for 24 hours embryos with *Eng2* staining.

produce functional Fgf8 protein, the fact that we do observe a minor amount of wild-type message containing exon 2 means that *acerebellar* may not cause complete inactivation of Fgf8. Elimination of the maternal component and isolation of further alleles of *Fgf8* can be used to address this issue.

Fgf8 function in dorsoventral patterning

In contrast to the drastic effect of misexpressing *Fgf8* on patterning of the gastrula, *acerebellar* mutants display a surprisingly mild phenotype. One possibility is that *ace* is not a null allele. A stronger phenotype was recently described for mouse *Fgf8* mutants: homozygous null *Fgf8* mutants fail to gastrulate and have no mesodermal derivatives, whereas weaker alleles display phenotypes more akin to what is seen in *acerebellar* mutants, including deletions of the posterior midbrain and cerebellum (Meyers et al., 1998).

Other explanations are, however, also possible for the weaker phenotype of *acerebellar* mutants. The maternal *Fgf8* RNA that we have observed (which contains exon 2, Fig. 5) could partially ameliorate the phenotype of *acerebellar* mutants, thus 'masking' a requirement for *Fgf8* in zebrafish, but not in mice, which have little maternal cytoplasm. We do not consider this possibility very likely: in contrast to zygotic RNA, maternal RNA is only detectable using the much more sensitive PCR assay, but not by in situ hybridisation, and maternal RNA may not be localized.

A more likely possibility is offered by the observation that *Fgf8* is often coexpressed with other members of the *Fgf* family in gastrulation (reviewed by Yamaguchi and Rossant, 1995). Prior to and during mouse gastrulation, *Fgf3*, *Fgf4*, *Fgf5* and *Fgf8* are expressed in distinct but overlapping patterns in the primitive streak: whereas *Fgf5* is found throughout the gastrula ectoderm, *Fgf3* is found in future mesodermal cells in the streak, and *Fgf4* is at the anterior end of the streak. While the expression patterns of these genes are suggestive, only *Fgf4* is required during gastrulation, whereas *Fgf3* and *Fgf5* are not (Feldman et al.,

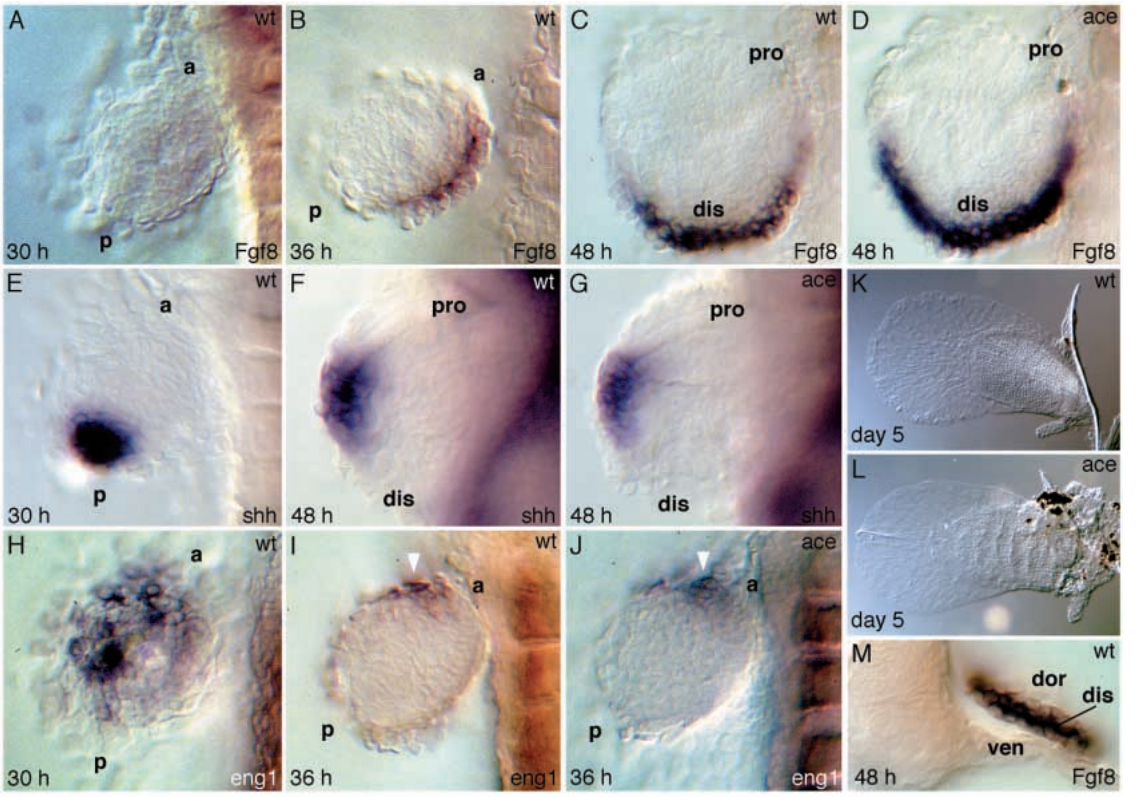

Fig. 11. *acerebellar* embryos show no severe defects in pectoral fin development. (A-D,M) *Fgf8* expression in the finbud. (A-C) Wild type. (D) *acerebellar*. No *Fgf8* expression is detected at 30 hours of development. (E-G) *shh* expression in the ZPA precedes *Fgf8* expression and is not affected in *acerebellar.* (E,F) Wild type. (G) *acerebellar*. (H-J) *eng1* (arrowheads) in the ventral fin bud precedes *Fgf8* and is normal in *acerebellar*. (H,I) Wild type. (J) *acerebellar*. (K,L) Fins of wild-type (K) and *acerebellar* (L) embryos on day 5 of development are of similar size and shape. (M) *Fgf8* expression in the distalmost ridge, AER, of the developing fin at 48 hours of development (viewed from posterior). a, anterior; dis, distal; dor, dorsal; p, posterior; pro, proximal; ven, ventral.

1995; Mansour et al., 1993; Hebert et al., 1994). An alternative explanation for the absence of severe gastrulation defects in *ace* mutants is therefore that other Fgfs can compensate for lack of Fgf8, or that Fgf8 has only a weak function.

Inactivation of *Fgfr1* causes absence of somites, expanded notochords and primitive streak defects. Fgfr1 was therefore proposed to be the receptor for an organizer-derived signal that patterns paraxial mesoderm (Yamaguchi et al., 1994; Deng et al., 1994). Based on its expression pattern and the phenotypes seen after misexpression and loss of function, Fgf8 or a similar Fgf could be this signal. Similar observations on the effects of *Fgf8* misexpression were made by M. Fürthauer, C. Thisse and B. Thisse, who also showed that *Fgf8* misexpression alters the distribution of the Bmp4 morphogen (Fürthauer et al., 1997). We note that the mild defect in activation and overall expression of *myoD* in the *acerebellar* mutants is consistent with a function of *Fgf8* in dorsoventral patterning, in keeping with the stronger gastrulation defect of the mouse mutant (Meyers et al., 1998).

Fgf8 in MHB development

The dynamic pattern of expression of *Fgf8* at the MHB is compatible with the functional requirement that we have observed. In chicken, beads containing Fgf8 or Fgf4 protein placed into the posterior forebrain or alar hindbrain primordium are able to induce ectopic isthmic, midbrain and cerebellar structures, strongly suggesting a role for Fgfs in MHB development (Crossley et al., 1996a). Our analysis of *Fgf8* requirement is generally compatible with these results. However, the bead experiments have raised the possibility that Fgf8 is the endogenous molecule which *induces* the midbrain, a notion that is not supported by several observations. (i) At the time *Fgf8* is activated at late gastrulation stages, it clearly marks the anterior hindbrain (Fig. 3). Posteriorly, its expression extends to the rhombomere 4/5 boundary and, anteriorly, it abuts the *Pax2.1* expression domain. Since the *Fgf8* and *Pax2.1* domains are largely non-overlapping at this stage, the early expression of *Fgf8* is clearly not sufficient to induce MHB markers such as *Pax2.1*. (ii) Secreted Fgf8 might act on the anteriorly adjacent cells at a distance to induce midbrain fate. We have observed, however, that misexpression of *Fgf8* leads to severe expansion of *Pax2.1* only along the d/v direction during gastrulation (as a consequence of altered dorsoventral patterning, see Fig. 6), but not to an expansion along the anteroposterior axis as would be expected if anterior cells could respond to *Fgf8*. Similarly, delocalized *Fgf8* expression does not alter early *En1* and *wnt1* midbrain expression in mice with altered *Otx* gene dosage; instead, the later restriction of *wnt1* and *Fgf8* to the posterior midbrain is affected (Acampora et al., 1997), similar to our findings in *acerebellar*. (iii) We find that *Fgf8* and *Pax2.1* expression come to gradually depend on each other only during mid-somitogenesis, after the time when

the anterior-most *Fgf8* subdomain in the MHB region is fully contained within the posterior *Pax2.1* domain. We speculate that this time may coincide with the establishment of the isthmus organizer in the region of overlap. (iv) In mice, *Fgf8* activation at the MHB occurs only at the 3- to 4-somite stage, and is thus preceded by activation of *wnt1* and *En1* as midbrain markers (Crossley and Martin, 1995; Mahmood et al., 1995). Taken together, Fgf8 is unlikely to act as the endogenous inducer of midbrain development in zebrafish and mice.

What could be the real inducer? Experimental manipulations in mouse, zebrafish and chicken have provided evidence for a vertical signal in late gastrula stages from mesendoderm to overlying ectoderm to activate expression of some, but not all midbrain markers (Ang and Rossant, 1993; Miyagawa et al., 1996; Darnell and Schoenwolf, 1997). To explain the ability of Fgf8 beads to induce midbrain, Crossley et al. (1996a) proposed that in normal development *Fgf8* expression in cardiogenic mesoderm underlying the MHB could provide, by a vertical path, the inductive signal. In zebrafish, cardiogenic precursors have been fate mapped throughout development. They derive from ventrolateral levels of the germ ring and migrate during gastrulation into a longitudinal domain which moves closer to the axis during early somitogenesis (Stainier and Fishman, 1992). Because we have not seen any *Fgf8* expression in these cells during gastrulation and because the shape and orientation of the cardiogenic domain (longitudinal) versus the *Fgf8* ectodermal domains (transverse) are very different, cardiogenic precursors are unlikely to provide the inductive signal for the ectodermal expression at the MHB in zebrafish gastrulae. We cannot rule out, however, that part of these cell populations are adjacent to each other at some stage during development and that signaling may occur between them.

An alternative source for the midbrain-inducing signal is the germ ring from which mesendodermal tissues derive. Fate-mapping studies in zebrafish have shown that a midbrain primordium is already separately established at late gastrulation stages (Woo and Fraser, 1995). Transplantation studies have suggested that an unknown signal responsible for hindbrain induction is present in the germ ring. This signal is not mimicked by bFgf beads and is absent from the dorsal shield (which has high levels of *Fgf8*), and may therefore not be a member of the *Fgf* family (Woo and Fraser, 1997). During gastrulation, *Fgf8*-expressing and *Pax2.1*-expressing domains in the neural primordium look quite similar in shape and width. By analogy to the hindbrain, the germ ring could therefore also provide the signal responsible for midbrain induction.

Establishment and maintenance phases

On the basis of the evidence presented here, we suggest that early MHB development occurs in at least two phases. During the establishment phase in late gastrulation, midbrain and hindbrain primordia are set up independently, in a process that does not require *Fgf8*. Given that *Fgf8* and *pax2.1* are activated independently of each other, at least two independent signalling pathways must act in parallel during early MHB development. The establishment phase is followed during early somitogenesis by a maintenance phase during which gene expression in the midbrain depends on signal(s) from the MHB. The gain- and loss-of-function experiments in chicken and fish together suggest that *Fgf8* is required for the maintenance phase, possibly in combination with *wnt1*. The beginning of the maintenance phase may be coincident with establishment of the isthmus organizer at the interface between mid- and hindbrain territories.

Why then is *Fgf8* on its own, when misexpressed, sufficient to reprogram posterior forebrain to midbrain and/or MHB development? Many of the genes expressed in the maintenance phase in the isthmus (*Pax2.1*, *wnt1*, *Eng2*, *Eng3*, *Fgf8*, *Her5*) are also active earlier during the establishment phase in the midbrain. We have shown here that MHB expression of *Eng* genes, *wnt1*, *Her5* and *Pax2.1*, all require *Fgf8* activity during the maintenance phase, since they all start to fail in their expression around early to midsomitogenesis and are eventually eliminated in *acerebellar* mutants. It is thus likely that *Fgf8* can impinge on their regulation. Thus, misexpressing *Fgf8* probably ectopically activates the complement of genes that also acts during establishment of midbrain development. Indeed, at least *Fgf8*, *wnt1* and *En2* are ectopically activated following the Fgf8 bead insertion into neural plate tissue (Crossley et al., 1996a). Moreover, ectopic *Fgf8* expression in embryos with altered *otx* gene dosage recruits *En1* and *wnt1* to the ectopic position only after some delay (Acampora et al., 1997). Once re-established in an ectopic position, the gene program could then develop accordingly.

What would be the normal function of Fgf8 during the maintenance phase? During this phase, expression of many marker genes is restricted to the posterior part of the midbrain primordium, towards the zone of overlap between *Fgf8* and *Pax2.1* at the isthmus. A crucial function for *Fgf8*, and possibly for the isthmus organizer in general, may therefore be to ensure polarized expression of midbrain markers, rather than initial induction. In keeping with this possibility, we find that all posterior midbrain markers we examined are absent from the midbrain of *acerebellar* mutants at later stages.

A distinction between establishment and maintenance functions for *Fgfs* has also been made for development of the chick limb bud (for a review, see Cohn and Tickle, 1996; Niswander, 1997). Similar to the situation at the MHB and as with other Fgfs, Fgf8 bead implantation is able to activate the full limb development program ectopically (Crossley et al., 1996b; Vogel et al., 1996). The earliest signal to establish limb development is thought to derived from the mesenchyme of the prospective limb bud. *Fgf10* is expressed in the mesenchyme at the right time, preceding *Fgf8* expression, and is able to induce complete limbs (Ohuchi et al., 1997a), so Fgf8 could mimic the limb-inducing action of Fgf10. Consistent with this possibility, in two chicken mutants, *limbless* and *wingless*, limb buds are established independently of *Fgf8* expression (Grieshammer et al., 1996; Ros et al., 1996; Ohuchi et al., 1997b), and Fgf8 is only weakly required in mouse limb bud development (Meyers et al., 1998). In zebrafish, *Fgf8* is neither expressed nor required during pectoral fin bud formation (Fig. 11), arguing that also in fish Fgf8 is not involved in fin bud establishment.

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