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A series of *no isthmus* (*noi*) alleles of the zebrafish *pax2.1* gene reveals multiple signaling events in development of the midbrain-hindbrain boundary

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SUMMARY

Generation of cell diversity in the vertebrate central nervous system starts during gastrulation stages in the ectodermal germ layer and involves specialized cell groups, such as the organizer located at the midbrain-hindbrain boundary (MHB). Mutations in the zebrafish *no isthmus (noi)* gene alter development of the MHB, and affect the *pax2.1* gene (formerly *pax(zf-b)*). Analysis of the structure of *pax2.1* reveals at least 12 normal splice variants. The *noi* alleles can be arranged, by molecular and phenotypic criteria, into a series of five alleles of differing strength, ranging from a null allele to weak alleles. In keeping with a role in development of the MHB organizer, gene expression is already affected in the MHB primordium of the gastrula neural ectoderm in *noi* mutants. *eng3* activation is completely and *eng2* activation is strongly dependent on *noi* function. In contrast, onset of *wnt1*, *fgf8* and *her5* expression occurs normally in the null mutants, but is eliminated later on. Our observations suggest that three signaling pathways, involving *pax2.1*, *wnt1* and *fgf8*, are activated independently in early anterior-posterior patterning of this area. In addition, analysis of the allelic series unexpectedly suggests that *noi* activity is also required during dorsal-ventral patterning of the MHB in somitogenesis stages, and possibly in a later *eng* expression phase. We propose that *noi/pax2.1* participates in sequential signaling processes as a key integrator of midbrain-hindbrain boundary development.

Key words: Midbrain-hindbrain organizer, Neurogenesis, Regionalization, Neural patterning, Splicing, *Pax* genes, *no isthmus*, Zebrafish, *Danio rerio*

INTRODUCTION

In vertebrate embryos, the fate of progenitor cells in the neural tube is determined by their position with respect to cellular sources of inducing signals (Lumsden and Krumlauf, 1996). Transplantation experiments in chicken suggest that the midbrain-hindbrain boundary (MHB; or mesencephalic-metencephalic boundary, isthmus) may contain such a neuroepithelial organizer (Martinez et al., 1991; Marin and Puelles, 1994; see also reviews by Bally-Cuif and Wassef, 1995; Joyner, 1996; Puelles et al., 1996). Several transcription factors are required during development of the MHB, such as En1 and En2 (Wurst et al., 1994; Millen et al., 1994), Pax2 (Torres et al., 1995; Brand et al., 1996; Favor et al., 1996), Pax5 (Urbanek et al., 1994), Otx1 and Otx2 (Ang et al., 1996; Acampora et al., 1997) and Gbx2 (Wassarman et al., 1997). Among the secreted factors, the vertebrate homologue of *Drosophila* Wingless, Wnt1 (McMahon et al., 1992) and Fgf8, a member of the fibroblast growth factor family, are required for MHB development in mice (Crossley et al., 1996; Meyers et al., 1998) and zebrafish (Reifers et al., 1998). In spite of the identification of many factors that function in development of the MHB territory, it is unclear which aspects of development of this area are controlled by the various gene products, how this is linked to the generation of the organizer potential in this region, and when and how exactly the organizer is able to act in cell-fate determination of surrounding cells.

In this study, we focus on the function of zebrafish *pax2.1* (formerly *pax(zf-b)*; Krauss et al., 1991), a member of the family of transcription factors that includes *pax2*, *pax5* and *pax8* (the pax2/5/8 family) in mammals. The proteins in this family share a *paired*-type domain and a partial homeobox as DNA-binding motifs, an octapeptide for protein-protein interaction, and a transactivating/inhibiting domain at the carboxy terminus (Wehr and Gruss, 1996; Dörfler and Busslinger, 1996; Pfeffer et al., 1998). A targeted and a chemically induced null allele of murine *Pax2* have different phenotypes of variable strength, probably due to different genetic backgrounds (Torres et al., 1995; Favor et al., 1996). The phenotype of homozygous mutants ranges from strong defects in development of midbrain, eye, ear and kidney (Favor et al., 1996) to nearly normal development of the MHB (Torres et al., 1995). *Pax5* and *Pax8*, which are expressed in overlapping domains with *Pax2* at the MHB (Nornes et al., 1990; Asano and Gruss, 1992; Plachov et al., 1990) may contribute to this variability. Inactivation of murine Pax5 leads, on its own, only to mildly abnormal development of the posterior midbrain and anterior cerebellum (Urbanek et al., 1994). When both *Pax2* and *Pax5* are inactivated, the resulting phenotype is more severe than in either single mutant,

suggesting that the murine members of this family can functionally replace each other (Urbanek et al., 1997; Schwarz et al., 1997).

In zebrafish, lethal mutations in three genes identified in systematic mutagenesis screens affect development of the MHB. Homozygous *acerebellar* (*ace*; Brand et al., 1996) or *spiel-ohne-grenzen* (*spg*; Schier et al., 1996) embryos lack the MHB and the cerebellum, but retain a midbrain. *acerebellar* is a mutation in the zebrafish *fgf8* gene (Reifers et al., 1998); the gene affected by *spg* is not known. Embryos homozygous mutant for strong *no isthmus (noi)* alleles lack the mid-hindbrain boundary and cerebellum, as well as some or all of the dorsal and ventral midbrain (Brand et al., 1996). One *noi* mutation, *noi*^{th44a}, is genetically linked to the *pax2.1* gene, and in this allele a stop codon interrupts the *pax2.1* reading frame, but leaves a large portion of the molecule intact, suggesting that the *noi*^{th44a} mutation is a hypomorphic allele of *pax2.1* (Brand et al., 1996, and this paper).

A general property of organizer cell populations is that they control cell fate, via gene expression, at a distance. Because organizers are thought to produce morphogens that determine cell fate in a concentration-dependent manner, organizer activity is often sensitive to the functional level of gene products that are involved in controlling organizer function. The availability of six chemically induced alleles of *no isthmus* allowed us to clarify the requirement for *pax2.1* activity, and suggested they might inactivate *pax2.1* to different degrees, thus forming an 'allelic series'. Generally, functional levels, assayed as gene copy number, are critical for several human and murine Pax genes, including *Pax2* (Tassabehji et al., 1992, 1993; Hanson et al., 1994; Sanyanusin et al., 1995; Acampora et al., 1997; Dahl et al., 1997; Schwarz et al., 1997). In this study, we use molecular and phenotypic criteria to establish that the available *no isthmus* alleles form such an allelic series, ranging from a null allele to weak alleles, and examine the expression of potential target genes. Based on their expression, candidate target genes were the *engrailed* genes (*eng1*, *eng2* and *eng3*) (Ekker et al., 1992), *wnt1* (Molven et al., 1991), *fgf8* (Reifers et al., 1998; Fürthauer et al., 1997) and the bHLH transcription factor *her5* (Müller et al., 1996). Our analysis of the various alleles shows that multiple and sequential signalling events must act during development of the zebrafish MHB.

In the accompanying paper, isolation of three new zebrafish *pax2/5/8* genes is described: *pax5*, *pax8* and a second, *pax2*-like gene called *pax2.2* (Pfeffer et al., 1998). While *pax2.1* is already activated in the late gastrula, these additional genes are activated much later, between the 4- and 9-somite stage. Importantly, *pax5* and *pax8* expression at the MHB strictly depends on *noi/pax2.1* function; in this tissue, inactivation of *noi/pax2.1* can therefore be considered as functionally equivalent to triple inactivation of *pax2/5/8* genes. These findings clarify the reasons for the apparent phenotypic differences between mice and zebrafish *pax2(.1)* mutants (Brand et al., 1996; Pfeffer et al., 1998; see also Discussion).

MATERIALS AND METHODS

Fish maintenance

Zebrafish were raised and kept under standard laboratory conditions at about 27°C (Westerfield, 1994). Mutant carriers were identified by random intercrosses. To obtain embryos showing the mutant phenotype, two heterozygous carriers for a mutation were crossed to one another. Typically, the eggs were spawned synchronously at dawn of the next morning, and embryos were collected, sorted, observed and fixed at different times of development at 28.5°C. In addition, morphological features were used to determine the stage of the embryos, as described by Kimmel et al. (1995). In some cases, 0.2 mM phenylthiourea (PTU) was added to prevent melanization. Isolation and initial characterization of *no isthmus* is described in Brand et al. (1996). For survival tests, embryos were dechorionated on the day of birth, kept at low density (approx. 20 per 9 cm dish) and the E2 medium was frequently changed.

Whole-mount in situ hybridization

Digoxigenin- or fluorescein-labelled RNA probes were prepared from linearized templates using an RNA labelling and detection kit (Boehringer). Hybridization and detection with anti-digoxygenin or anti-fluorescein antibodies coupled to alkaline phosphatase (Boehringer) is described in Reifers et al. (1998). 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. Stained embryos were dissected and thick sections prepared with sharpened tungsten needles; these were mounted in glycerol and photographed on a Zeiss axiophot. Composites were assembled with Adobe Photoshop. Probes and wild-type expression patterns are described elsewhere: *Eng1-3*: Ekker et al. (1992); *Pax2.1*: Krauss et al. (1991); *Her5*: Müller et al. (1996); *wnt1*: Molven et al. (1991); *fgf8*: Reifers et al. (1998).

Cloning of *pax2.1* cDNAs

Total RNA was prepared by the hot phenol method (Brown and Kafatos, 1988). cDNA was isolated by RT-PCR with nested primers flanking the coding region in at least two independent amplifications from pools of homozygous *noi* embryos, subcloned into pCRII (Invitrogen) and sequenced using the T7 Sequenase kit (Amersham).

Pax2.1 protein

The wild-type, *noi*^{tm243a} and *noi*^{tu29a} coding regions were cloned into pQE60 or pQE30 (Qiagen). Expression and purification of recombinant proteins was performed according to manufacturer's protocol. Briefly, recombinant fusion proteins contain a 6×histidine tag at the N (pQE30) and C terminus (pQE60), which allowed binding and purification on Ni^{2+}-charged Sepharose resin under denaturing conditions using a stepwise imidazole gradient. Proteins were renaturated by dialysis (8, 6, 4, 2 and 1 M urea) into Z-buffer (25 mM Tris, pH 7.8, 20% glycerol, 12.5 mM MgCl, 0.1 M KCl, 1 mM DTT) at 4°C. Wild-type and mutant proteins were prepared in parallel under identical conditions, and examined on Coomassie blue-stained gels to ensure purity (not shown).

DNA electromobility shift assay

The sequences of the CD19-2(A-ins.) (Kozmik et al., 1992) and BSI+II (Song et al., 1996) *Pax2* binding sites have been described. Double-stranded oligonucleotides (100 pmol) were end-labelled with T4 polynucleotide-kinase and [^{32}P]dATP (6 μCi/μl). Binding reactions were performed in 10 μl at 4°C for 30 minutes and contained an empirically determined amount of affinity-purified *Pax2.1* protein, 100 ng of poly(dI·dC), ^{32}P-labelled probe (40,000-50,000 cpm), 0.5 mg/ml BSA and Z-buffer. For the competition experiments up to 10-fold excess of unlabelled probe (1 nmol) was added to the reaction. Binding reactions were examined on 6% non-denaturing polyacrylamide gels, in 0.5×TBE at 30 mA for 70 minutes.

RESULTS

Morphological strength of the *no isthmus* mutant phenotype

Mutants homozygous for the three strong alleles *noi*^{tu29a}, *noi*^{th44a} and *noi*^{tm243a}, lack the midbrain, MHB and cerebellum, whereas in the two weak alleles, *noi*^{ty31a} and *noi*^{tb21}, some midbrain is still formed (Brand et al., 1996; Fig. 1A-D). In addition, *noi* mutants show defects in formation of the optic stalk, the inner ear and pronephric duct (Brand et al., 1996; Macdonald et al., 1997), all areas that show *pax2.1* expression. The strength of the *noi* phenotype correlates with the ability to survive under optimal conditions: for the strongest allele, 50% of the embryos died at day 7, whereas embryos mutant for the weak alleles survive about 4 days longer (Fig. 1G). On day 7, the mutants show severe oedema of the pericardium and gut epithelium; the heart is malfunctioning and the embryos never feed (Fig. 1E,F).

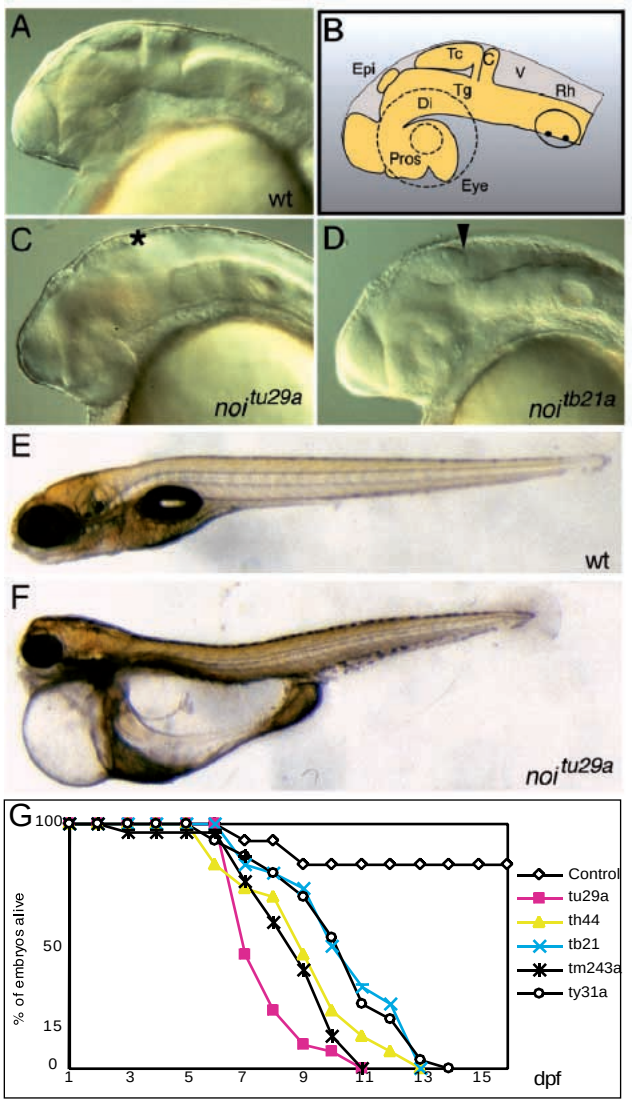


Fig. 1. Lateral views of wild-type and *noi* mutant embryos. (A) Wild-type embryo at 24 hours pf. (B) Schematic drawing of the structures seen in A. (C) Strong *noi*^{tu29a} mutant, which lacks MHB, tectum (asterisk) and cerebellum. (D) Weak *noi*^{tb21} phenotype; a partially formed tectum is observed, the caudal end marked by the arrowhead. (E) A wild-type embryo at day 7. A homozygous mutant for *noi*^{tu29a} of the same age is seen in (F), showing severe oedema of the pericard and gut epithelium, causing delayed development. (G) Survival rates of different *noi* alleles. *n*=30 mutant embryos per allele were analyzed. The difference between the strong *noi*^{tu29a} allele and the weaker alleles is clearly visible. The mutants never feed and die within 2 weeks. c, cerebellum, di, diencephalon, epi, epiphysis, pros, prosencephalon, rh, rhombencephalon, tc, tectum, tg, tegmentum, v, ventricle.

The genomic structure of *pax2.1* is conserved between human and fish

To characterize the mutations affecting the *noi*/*pax2.1* gene, we analyzed the genomic organisation of *pax2.1*. On the basis of the human *pax2* gene structure (Sanyanusin et al., 1996), we designed primers to amplify the exon-intron boundaries by PCR from three overlapping genomic phage clones that cover the complete *pax2.1* locus (A. Picker and M. Brand, unpublished) and sequenced them (accession numbers AF067530 to AF067541 and AF073442 to AF073445). We find that zebrafish *pax2.1* has 12 exons, 10 at the predicted positions and two (5.1 and 7.1) that show no sequence homology to *pax2* exons of other species (Fig. 2A). Exon 7.1 consists of 67 bp and contains a stop codon (Fig. 2F). The predicted truncated protein would lack the transactivating and inhibitory domains (Dörfler and Busslinger, 1996) encoded by exons 8, 9 and 10. Exon 7.1, but without a stop codon, has also been found in Xenopus (Heller and Brändli, 1997).

Multiple splice variants of the zebrafish *pax2.1* gene

While analysing the *no isthmus* mutations by RT-PCR (see below), we found a high number of *pax2.1* cDNA variants. Exons 5.1, 6, 7 and 7.1 are differentially spliced in different combinations, as shown in Fig. 2B. Alternative splicing has been reported for exon 5.1 in zebrafish (Krauss et al., 1991), mouse and human (Sanyanusin et al., 1996), and for exons 6, 7 and 7.1 in Xenopus (Heller and Brändli, 1997). Furthermore, in two variants an alternative 3′ splice acceptor site is located near the 5′-ends of exons 2 and 9 (Fig. 2C,D). For exon 9, usage of the alternative splice acceptor site leads to a frameshift, with an alternative stop codon in the 3′UTR (Fig. 2E) and a protein lacking part of the inhibitory domain which could therefore be constitutively active. Other cDNAs revealed that additional exon(s) are probably located between exons 7 and 8 (not shown), and additional uncharacterized splice products are seen in the RT-PCR (Fig. 4D, arrowheads). We have observed 12 of 64 theoretically possible splice variants. Among these, the two major splice variants are those either containing or lacking exon 5.1 (Fig. 2B(1),(2); Fig. 4D, arrows). The significance of the multiple splice variants is not clear, but may be functionally relevant given the dynamics and complexity of *pax2.1* expression and requirement.

Point mutations in the *no isthmus*/*pax2.1* gene

We cloned and sequenced the coding region of *pax2.1* for all six known *noi* alleles, and found various types of mutations in the different alleles, providing further evidence that the *noi* phenotype results from mutations in the *pax2.1* gene. Due to the mutagen used in the zebrafish screen we expected point mutations, which we found for three alleles, predicting the mutant proteins shown schematically in Fig. 3A.

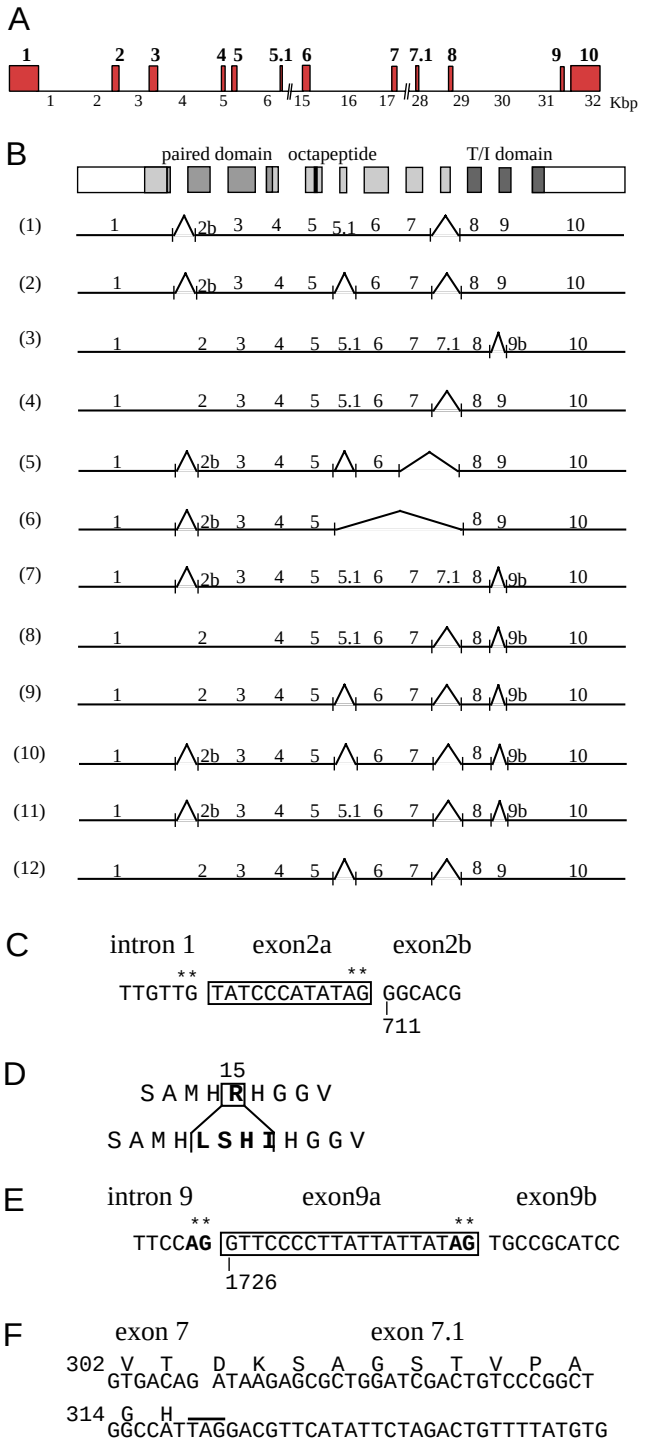


Fig. 2. Genomic organisation and splice variants of the *pax2.1* gene. (A) Genomic structure of *pax2.1*; the complete locus spans approx. 32 kb, with large introns between exons 7 and 7.1 and 5.1 and 6. (B) Splice variants of *pax2.1*; (1) and (2) are the two major forms found. Variants containing exon 7.1 lead to a premature stop within exon 7.1 and to a truncated protein. Splice forms containing exon 9b lead to a frame-shift and to activation of an alternative stop codon in the 3′UTR. (C) Exon 2a is located directly upstream of exon 2b; (D) amino acids introduced by exon 2a. (E) The alternatively spliced exon 9b. (F) The novel exon 7.1. Sequences are numbered according to Krauss et al. (1991). Asterisks in C and E mark the 3′-splice acceptor site.

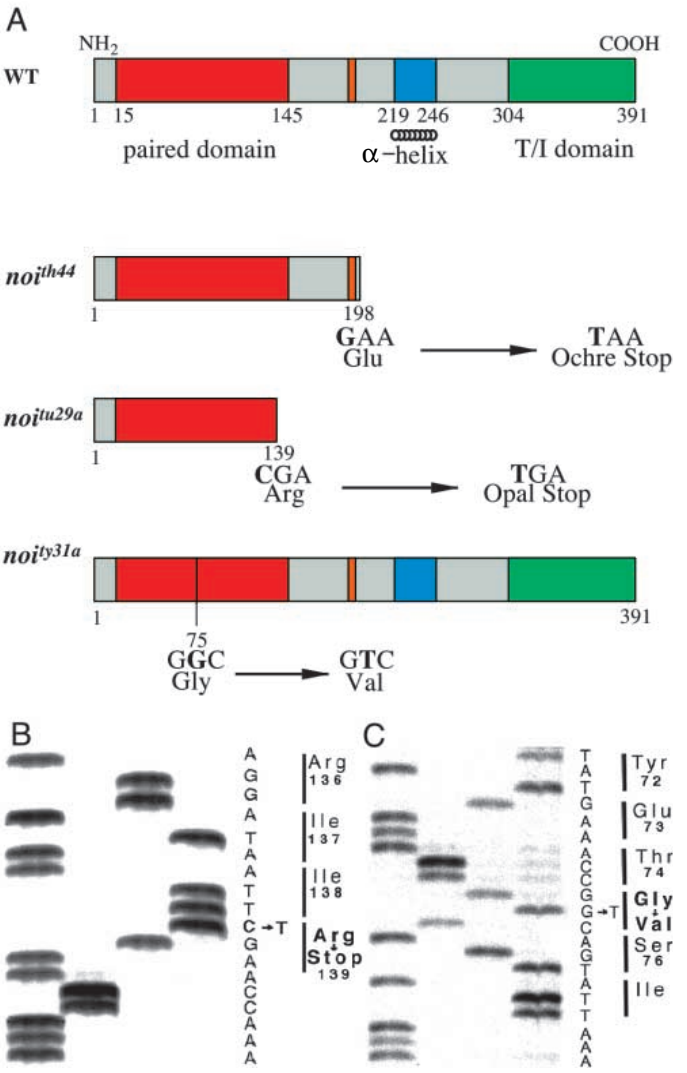


Fig. 3. *noi* mutations causing amino acid transformations. (A) Schematic drawing of the Pax2.1 protein; red, paired domain; green, transactivation/inhibitory domain; orange, octapeptide; blue, α-helical region. In *noi*^{th44a} and *noi*^{tu29a} stop codons cause truncations; in the weak *noi*^{ty31a} allele a conserved glycine is exchanged to valine. (B,C) The sequencing data for *noi*^{tu29a} and *noi*^{ty31a}.

In *noi*^{tu29a}, the C→T transition converts codon 139 into a stop codon (Fig. 3A,B). This leads to a C-terminally truncated protein lacking the transactivation and inhibitory domains (T/I, Dörfler and Busslinger, 1996), and six amino acids of the DNA binding paired domain. The same mutation was found in *noi*^{ty22b}, which is therefore probably a re-isolate of *noi*^{tu29a} (not shown). As shown previously (Brand et al., 1996), in *noi*^{th44a} the G→T transversion produces a stop codon (Fig. 3A), leading to a protein with an intact DNA binding domain that lacks the complete C terminus. In both cases the Pax2 protein is severely affected, consistent with the observed strong phenotype in both alleles. The predicted structure is consistent with the failure of homozygous mutants to stain with an antibody directed against the C-terminal part of the protein (Brand et al., 1996).

The third point mutation (G→T transversion) in the weak *noi*^{ty31a} allele transforms glycine 75 into valine (Fig. 3A,C). Since this highly conserved residue is located in the turn of the DNA recognition helix in the N-terminal helix-turn-helix (HTH) motif within the paired domain (Xu et al., 1995), loss of this amino acid probably impairs the DNA binding activity of the mutant protein.

Deletions are due to point mutations that cause defective splicing

In cDNA from homozygous *noi*^{tm243a} mutants we find an in-frame deletion of 18 bp at the 5′ end of exon 3 (Fig. 4A). This deletion leads to the loss of six amino acids in helix II of the N-terminal paired domain, but leaves the rest of the protein unaffected. As expected, the mutant protein can be detected with the antibody against the C terminus (Brand et al., 1996). Since helix II of the first HTH motif in the paired domain is crucial for the DNA/protein interaction of paired-type transcription factors (Xu et al., 1995), the *noi*^{tm243a} could theoretically cause a complete loss of function, consistent with the observed strong phenotype. Further analysis shows, however, that the deletion is created by splicing that occurs in most, but not all, splicing events. The 18 bp deleted in the cDNA of exon 3 are still present in genomic DNA, which however carries a mutation in the 3′-splice acceptor site preceding exon 3 (Fig. 4B). The highly conserved AG (Padgett et al., 1986) is changed into a TG; this point mutation probably abolishes activity of the original site, and allows usage of a cryptic splice acceptor site in exon 3, thus deleting 18 bp in the cDNA. RT-PCR analysis with primers flanking the region of the deletion shows that the predominant, smaller band diagnostic for transcripts with the deletion is seen only in mutant cDNA and is thus not a normal splice variant. In addition, most, but not all transcripts contain the deletion, explaining the slightly weaker phenotype of this allele (Fig. 4E, arrows).

In homozygous *noi*^{tb21} mutants all *pax2.1* transcripts lacked exon 7, both by sequencing of cDNAs and RT-PCR analysis, suggesting that the deletion is also generated by aberrant splicing (Fig. 4C,D). The weak phenotype of *noi*^{tb21} suggests that the mutated Pax2.1 protein retains some activity.

noi^{tu29a} is a null allele of *pax2.1*

To understand the requirement for *pax2.1* in development it is

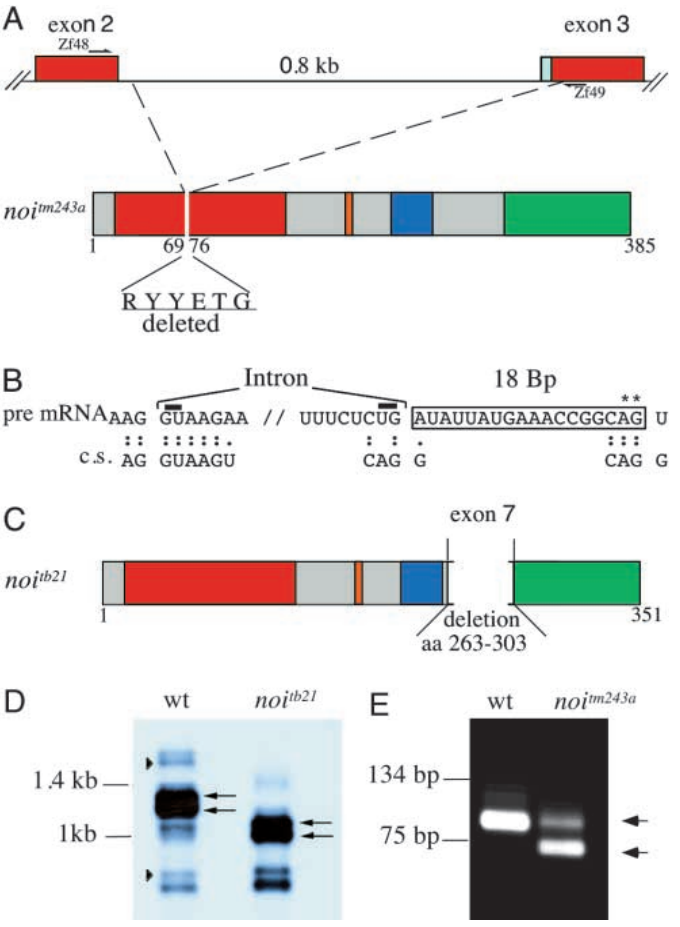


Fig. 4. *noi* mutations with deletions caused by aberrant splicing. (A,B) Structure of *noi*^{tm243a}. In the conserved GT-intron-AG the AG is changed into TG, thus weakening this 3′-splice acceptor site and activating a cryptic splice site in exon 3. This deletes the first 18 bp of exon 3, as found in the *pax2.1* cDNA of *noi*^{tm243a}. (C) Structure of *Noi*^{tb21}, lacking amino acids encoded by exon 7. (D) Southern blot analysis of RT-PCR products in *noi*^{tb21} mutants: lack of exon 7 causes a shift of the two major splice products containing or lacking exon 5.1 (arrows; see Fig. 2B). Bands that are not shifted are splice products without exon 7, e.g. variant 6 (arrowhead). (E) RT-PCR with primers Zf48 and Zf49 with wild type and *noi*^{tm243a} mutant cDNA. Lower arrow: the band corresponding to the 18 bp deletion is specific to the mutant. Upper arrow: residual wild-type transcript.

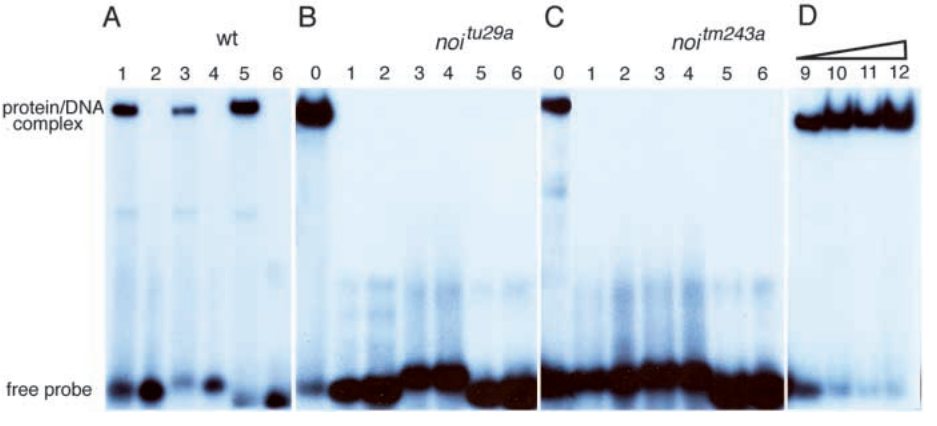
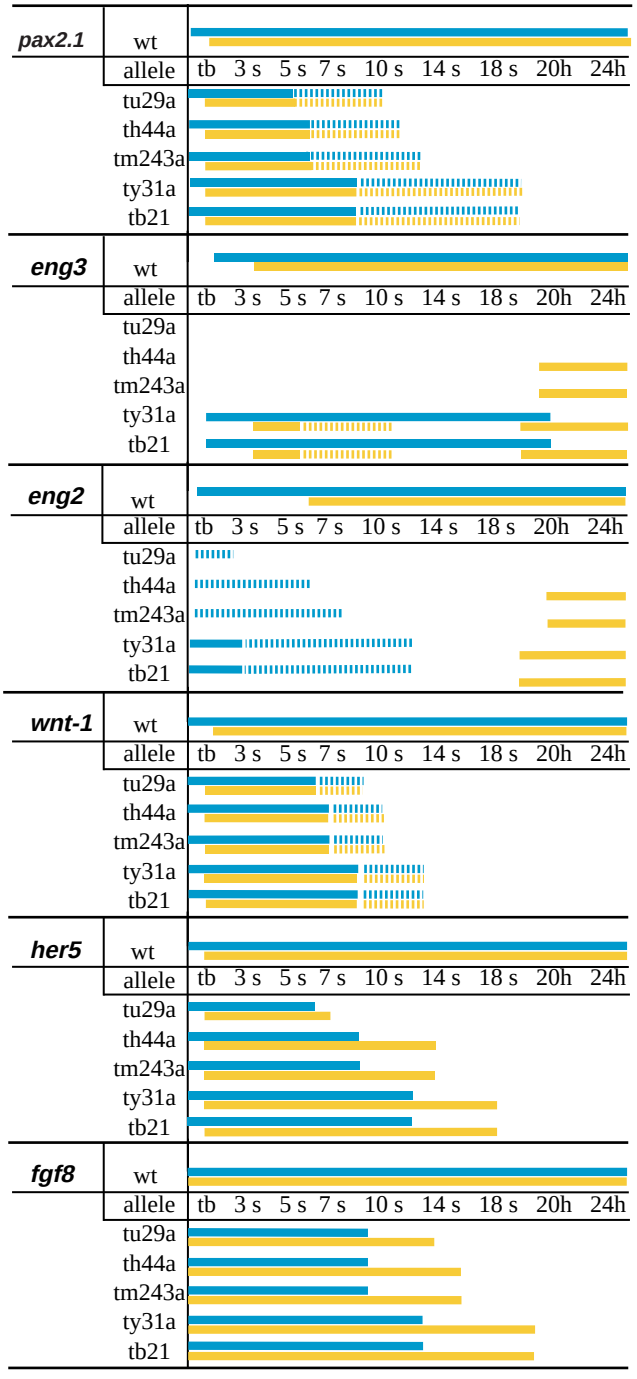


Fig. 5. Band-shift assays. (A) Wild-type Pax2.1 protein binds to known Pax2 binding sites, whereas Noi^{tu29a} (B) and Noi^{tm243a} (C) do not. Lanes 1+2, BSI; 3+4, BSII of the mouse EN2 promoter (Song et al., 1996); 5+6, optimized CD-19(A-ins.) Pax2 binding site (Kozmik et al., 1992). In even numbered lanes, a tenfold excess of unlabelled oligonucleotide competes binding. Lane 0, positive control with wild-type Pax2.1 and BSI. (D) No competition with binding of Pax2.1 to the high-affinity BSI is seen after addition of a 5-, 50- or 100-fold molar excess of Noi^{tu29a} protein; the slight increase in bound probe with higher protein concentrations is also seen with BSA and most likely reflects increased protein stability.

Table 1. Summary of marker gene expression studies for the wild type (wt) and the various *noi* alleles, ordered by severity, at the stages indicated



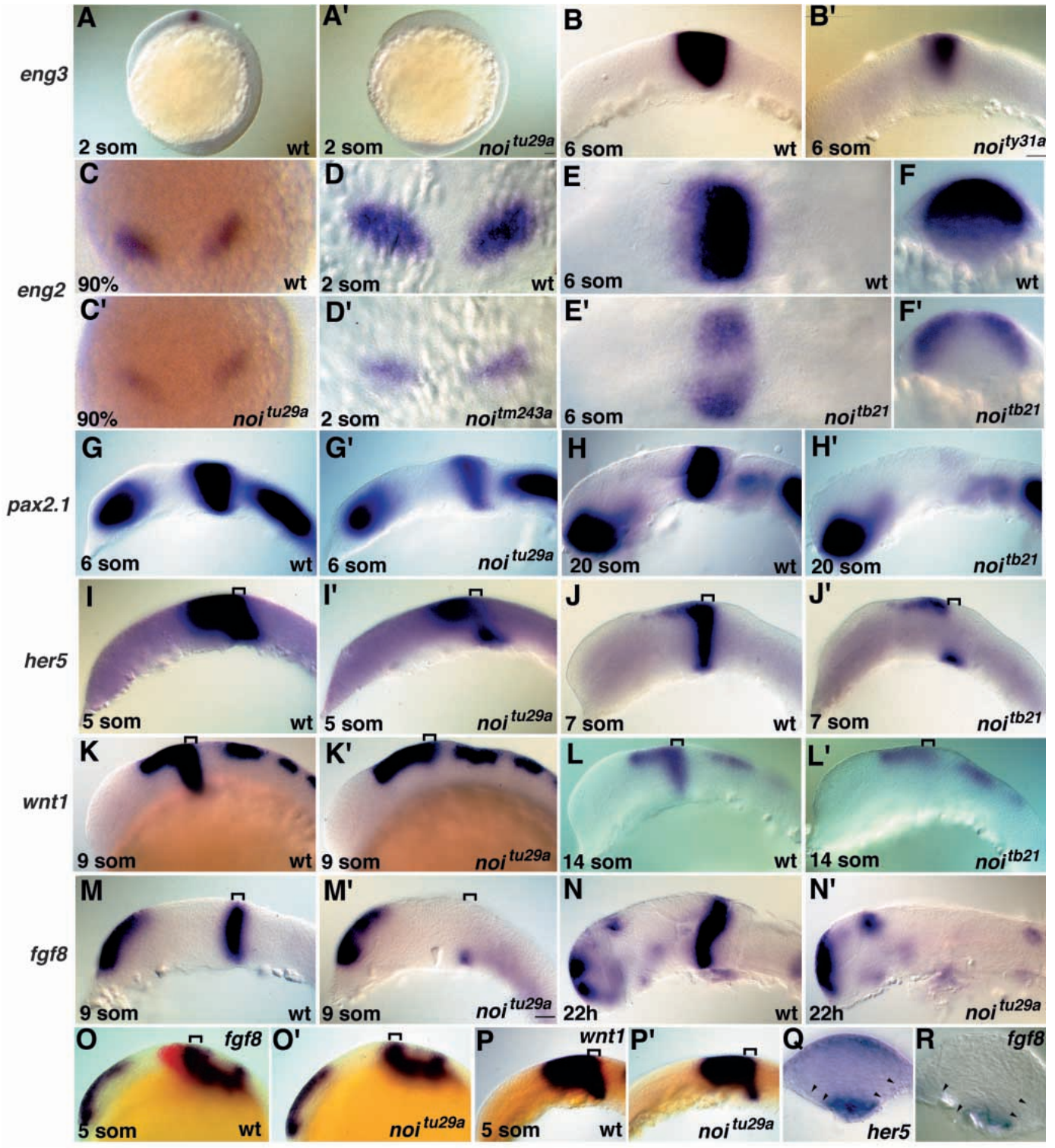
The duration of gene expression in the dorsal and ventral portion of the MHB is shown as two separate bars, blue and yellow, respectively, when they could be distinguished. A dashed line denotes reduced expression levels. Note that *eng2* and *eng3* are affected in similar ways, as are *wnt1*, *her5* and *fgf8*.

Fig. 6. Requirement for *Pax2.1* in early MHB development. Whole-mount ISH; embryos shown in one row are stained with the marker depicted on the left side. Stages and genotypes are noted; shown are lateral views, anterior to the left, except where indicated. A prime (′) indicates mutants. (A) *eng3* expression is not activated in $noi^{tu29a.}$ (B) In weak noi^{ty31a} mutants expression fades away from ventral to dorsal. (C,D) Weak transient *eng2* expression in the MHB primordia, dorsal views, anterior to the top; in noi^{tu29a} (C) the expression is very faint and lost at the end of epiboly; in strong noi^{tm243a} mutants (D) expression initiates normally, fades away around 2 somites, and is lost later in development. (E) Dorsal views and (F) optical cross sections through the *eng2* domain of wild-type embryo and weak noi^{tb21} mutants. Expression persists dorsally. (G,H) Expression of *pax2.1* as a marker for MHB development is initiated normally in noi^{tu29a} mutants, and is gradually eliminated during somitogenesis stages. (G) *pax2.1* expression in noi^{tu29a} is smaller in its a/p extent than in the wild type; later deletion is complete in the weak noi^{tb21} allele (H). (I) At the 5-somite stage, *her5* expression fades away from the MHB, except for a ventral patch in the neural tube (Q: cross section, arrowheads outline the neural keel). (J) In weak mutants, the midbrain primordium still expresses *her5*, also to later stages. (K) The dorsal midbrain expression of *wnt1* is seen at 9 somites in noi^{tu29a}, whereas MHB expression is lost, similar to the weak noi^{tb21} allele at later stages (L). (M) Following normal initiation, *fgf8* expression is absent from the MHB except in the ventral portion (R: cross section; arrowheads outline the neural keel), that has disappeared by 22 hours (N). (O,P) At the 5-somite stage, MHB cells are present in noi^{tu29a} mutants and express *fgf8* overlapping with *eng3* (red) (O) and *wnt1* (P). Brackets mark the MHB.

essential to determine its null phenotype, particularly in view of the variable phenotype of mouse *pax2* mutants. Critical for the biological function of a transcription factor is its ability to specifically bind DNA and interact with the transcriptional machinery. Our above analysis suggested noi^{tu29a} and noi^{tm243a} as the best candidates for null alleles, since both have deletions in the DNA binding paired domain; in particular noi^{tu29a} protein was likely to be function-less since the T/I domains are deleted as well.

We have used DNA electrophoretic mobility shift assays (EMSA) with purified recombinant wild-type and mutant proteins to test whether noi^{tu29a} and noi^{tm243a} could be null alleles. As binding sites we used known Pax2 binding sites, such as BSI and BSII in the murine *En2* promoter (Song et al., 1996) and an optimized Pax2 binding site CD19-2(A-ins.) (Kozmik et al., 1992). All sites are bound by wild-type protein (Fig. 5A), but not by the Noi^{tm243a} and Noi^{tu29a} mutant proteins (Fig. 5B,C). It remained a formal possibility that the Noi^{tu29a} protein fragment is able to interfere with binding of wild-type protein, thus exerting a dominant negative effect. We therefore added, in a competition experiment, up to a 100-fold molar excess of noi^{tu29a} protein to a wild-type Pax2.1 binding reaction to the high-affinity BSI site. Presence of the mutant protein does not interfere with DNA binding of the wild-type protein, showing that the noi^{tu29a} protein fragment is unlikely to act in a dominant negative way (Fig. 5D). Our results show that in both mutant proteins the DNA binding activity is completely lost. As shown above, however, some functional *Pax2.1* transcripts are present in the noi^{tm243a} mutants, consistent with a weaker phenotype in the marker gene expression analysis than in noi^{tu29a} (see below).

In conclusion, our molecular data show that noi^{tu29a} is a null allele (now referred to as $noi^{-/-}$); noi^{tm243a} and noi^{th44a} are strong alleles, but weaker than the null, and noi^{tb21} and noi^{ty31a} have weaker molecular defects.



pax2.1 functions during establishment of the MHB region

Expression of *eng2*, *eng3*, *wnt1*, *fgf8* and *her5* occurs in the early MHB primordium with a similar time course to *pax2.1*, suggesting that *pax2.1* might regulate their expression. We used the allelic series to examine in detail the onset of expression of these genes in wild-type and in *noi* mutant embryos, and find that they are differentially regulated. *eng3* expression normally starts at the 1-somite stage, and is never activated in $noi^{-/-}$ mutants (Fig. 6A,B; Table 1). *eng2* is expressed from 90% epiboly onwards in the wild type, and expression is detectable at a strongly reduced level in $noi^{-/-}$ mutants, in a subpopulation of the wild-type domain that fades away with the appearance of the first somite (Fig. 6C).

eng1 expression is seen in few cells of the dorsal MHB of wild-type embryos from the 15-somite stage onwards, and is likewise eliminated in *noi*$^{-/-}$ (Fig. 8J,K). In mutants homozygous for weak alleles, *eng3* and *eng2* are both activated normally (not shown). From the 6-somite stage onwards, however, the expression narrows, persisting only in the dorsal part of the normal expression domain (Fig. 6B,E,F). These findings show that *noi* functions in establishment of *eng2* and *eng3* gene expression in the midbrain and MHB primordium.

Failure to express *eng* genes at the MHB of *noi*$^{-/-}$ mutants could theoretically be due to absence of these cells in the mutants. We used expression of *pax2.1* RNA and double ISH in *noi* mutant embryos to determine how long MHB cells persist in the various alleles (Fig. 6G,H,O,P). Onset of *pax2.1* expression occurs during late gastrulation (80% epiboly) and is unaffected by all *noi* alleles (not shown); *pax2.1* is therefore not initially required for its own expression. The MHB expression in *noi*$^{-/-}$ mutants is lost between the 6- and 9-somite stages (Fig. 6G,H). In strong mutants, expression becomes undetectable by the 12-somite stage, and in weak mutants, by the 20-somite stage (Fig. 6H). Within the mutants, *pax2.1* RNA levels decrease uniformly, without a particular bias along the dorsoventral axis (Fig. 6G).

noi-independent initiation of *wnt1*, *fgf8* and *her5* expression

In wild-type embryos, *her5* and *wnt1* are initially expressed throughout the midbrain and MHB primordium, and maintenance, rather than initiation, of MHB expression is affected in *noi*$^{-/-}$ mutants. During somitogenesis stages of the wild type, *her5* and *wnt1* expression becomes gradually restricted from the midbrain towards the MHB; *wnt1* retains a dorsal expression stripe in the midbrain. In *noi*$^{-/-}$ mutants (identified by absence of *eng3* staining), expression is initiated normally, but maintenance of expression becomes abnormal from the 6-somite stage onwards (Fig. 6K,L,P). Similarly, expression of *fgf8* in *noi*$^{-/-}$ mutants is initiated normally and disappears from the MHB by the 9-somite stage (Fig. 6M,N,O; Reifers et al., 1998). We conclude that *noi* function is not required for initiating, but for maintaining expression of *her5*, *wnt1*, *fgf8* and *pax2.1* itself at the MHB.

Anteroposterior and dorsoventral differences in *noi* sensitivity

Interestingly, the midbrain and the MHB primordium of the mutants differ in the kinetics with which gene expression disappears (summarized in Table 1). *her5* expression in the developing midbrain is still normal at a time when MHB expression is already partially reduced in *noi*$^{-/-}$ mutants at the 5-somite stage (Fig. 6I). Homozygotes for weak alleles show the same phenomenon at slightly later stages (Fig. 6J). Likewise, MHB expression of *wnt1* is missing in *noi*$^{-/-}$ embryos from about the 6-somite stage onwards, whereas the dorsal stripe persists (Fig. 6K, bracket). For intermediate and weak *noi* alleles, MHB expression of *wnt1* persists longer but is also eliminated eventually (Fig. 6L). We conclude that during the maintenance phase, the sensitivity towards missing *noi* function appears to be higher at the MHB proper than in the adjacent midbrain.

Within the MHB of the mutants, dorsoventral differences in sensitivity can be observed that differ for the marker gene considered. For instance, *her5* expression is first lost from the medial, then the dorsal part of the MHB of *noi*$^{-/-}$ mutants, and

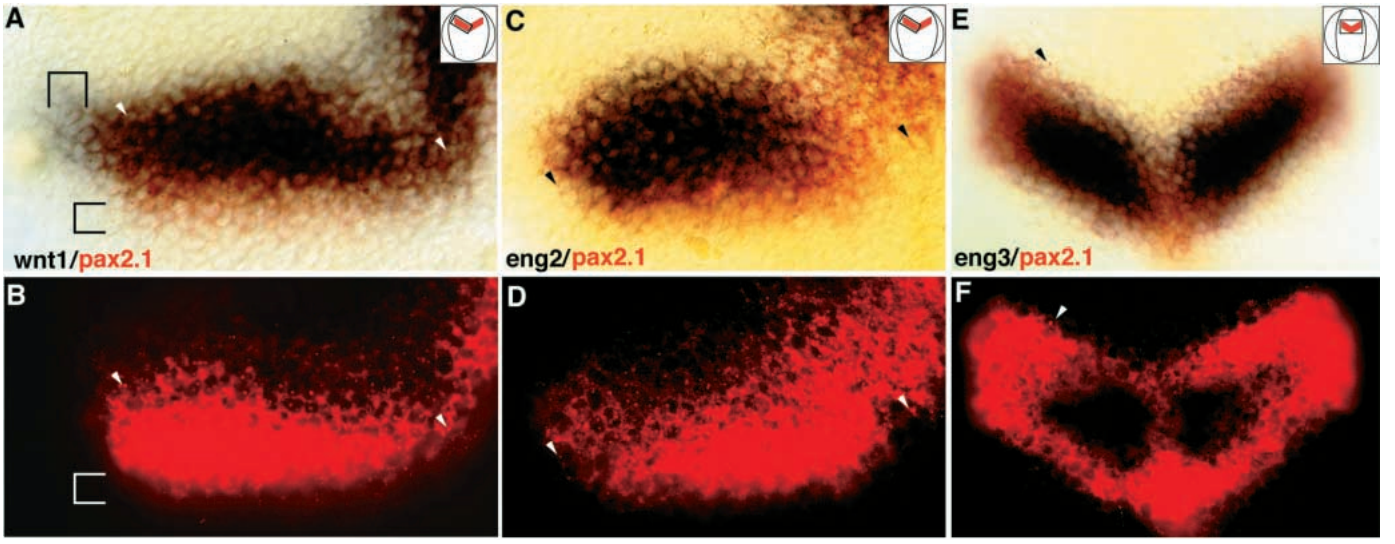


Fig. 7. Localization of *eng3* and *wnt1* (blue) relative to *pax2.1* (red/fluorescent) in double in situ hybridizations. All embryos are oriented anterior to the top; insets give the area shown in detail, and arrowheads point to identical landmark cells in corresponding panels. (A) 90% epiboly stage embryo stained for *wnt1*/*pax2.1*. The two brackets mark the area where only *pax2.1* or *wnt1* are expressed. The *wnt1* domain extends further laterally than *pax2.1*, whereas *pax2.1* extends further posteriorly; at this stage the anterior boundaries of *wnt1* and *pax2.1* coincide. (B) Fluorescence image of A. FastRed fluorescence is quenched in the overlapping part by the *wnt1* signal, but not posteriorly. (C) 90% epiboly stage embryo stained for *eng2*/*pax2.1*. The *eng2* domain lies within the *pax2.1* domain. (D) The fluorescent image of C, clearly showing that the *eng2* cells lie within the *pax2.1* domain. (E,F) 1-somite embryo stained for *eng3*/*pax2.1*; the initial *eng3* expression domain lies within the *pax2.1* domain; shortly after, the two domains become coincident (not shown).

then the expression is reduced to a ventral spot (Fig. 6I) that is eventually eliminated (Fig. 8); for weak alleles, the ventral spot persists to later stages (Fig. 6J). Expression of *fgf8* in *noi*$^{-/-}$ mutants also persists longest in ventral MHB tissue (Fig. 6M,N). In contrast, *eng2* and *eng3* expression persists longest in the dorsal MHB (Fig. 6B,E,F; see above). We conclude that gene expression at the MHB is sensitive to the level of *noi* function during midsomitogenesis stages, and that the requirement differs, unexpectedly, along the dorsoventral axis.

Topology of early gene expression domains

To further understand the differences in genetic requirement for *pax2.1*, we compared the distribution of *pax2.1* with *eng2*, *eng3* and *wnt1*, using a double ISH procedure that provides nearly cellular resolution (Fig. 7). At its onset in late gastrulation (90% epiboly), *eng2* expression coincides with *pax2.1* expression (Fig. 7C,D). *eng3* expression is initiated at the 1-somite stage within the *pax2.1* domain, and slightly later, is expressed coincident with *pax2.1* (Fig. 7E,F and not shown). The coexpression in the same cells is consistent with a role for *pax2.1* in regulating these genes. In contrast, the domain of *wnt1* expression resembles, but is not identical to the *pax2.1* domain: the anterior *wnt1* border coincides with the anterior *pax2.1* border at this stage, but *pax2.1* extends further posteriorly than *wnt1*, and *wnt1* extends further laterally than *pax2.1* (Fig. 7A,B; brackets denote non-overlapping regions). A separate study showed that the domain of *fgf8* expression during gastrulation is located posterior to the *pax2.1* domain, with a significant overlap becoming apparent only during mid-somitogenesis stages (Reifers et al., 1998). These findings complement our observations that expression of *wnt1*, *fgf8* and *her5* does not depend on *noi* function. Together, they show that multiple signaling pathways become activated in parallel during early development of the MHB territory.

Late MHB development in *noi* mutants

Up to 48 hours of development, *eng* expression is not observed in *noi*$^{-/-}$ embryos, presumably due to the absence of midbrain and MHB cells at this stage (Fig. 8). Analysis of the weaker alleles of the series suggests, however, that *pax2.1* may also be required for later expression of *eng2* and *eng3*. As described above, *eng2* and *eng3* are not expressed in strong *noi*^{tm243a} and *noi*^{th44a} mutants at midsomitogenesis. From the 20-somite stage onwards, however, *eng2* is detected in a small patch of ventral neural tube cells in strong but not in null mutants (Fig. 8F-I), and this increases in *noi*^{tb21} and *noi*^{ty31a} (Fig. 8H,I). Later expression is also seen for *eng3*: apart from the dorsal patch of cells that persists until 20 hours of development (Fig. 6B), an additional *eng3*-positive domain is detected in the ventral neural tube (Fig. 8D,E). Again, expression in this ventral domain is stronger in the two weak alleles than in the strong alleles. Mutants for the strong alleles have not expressed *eng3* during earlier neuroepithelial development; hence, late *eng3* re-expression must occur independently of the earlier neuroepithelial expression. We do not observe re-expression for *eng1*, *her5* or *fgf8* in *noi* mutants at 24 hours of development (Fig. 8J-M, Fig. 6N). Several differentiated neurons later express *eng* genes in this area in zebrafish and chicken (Hatta et al., 1991; Millet and Alvarado Mallart, 1995) and the expression we observe may

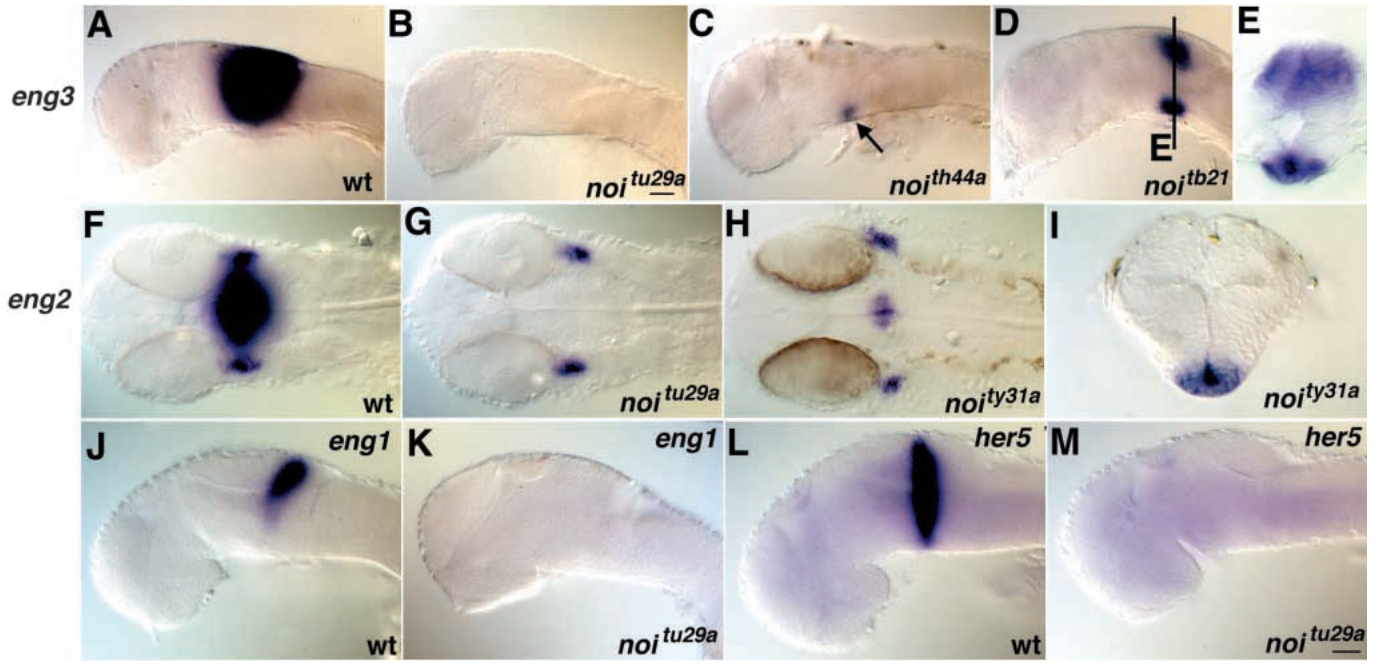


Fig. 8. Late marker gene expression during pharyngula stages in *noi* mutant embryos. Shown are lateral views, anterior to the left (except F-H: dorsal views). (A,B) no *eng3* is seen in null mutants at 22 hours, whereas in the strong allele *noi*^{th44a} (C) a ventral *eng3*-positive spot can still be detected, and both ventral and dorsal expression is visible in weak *noi*^{tb21} mutants (D,E); E, transverse section. (F-H) 24 hours. *eng2* expression in the neural tube is absent in *noi*^{tu29a}; lateral expression in presumptive eye muscles is not affected. In the weak *noi*^{ty31a} allele ventral expression is seen, and is located, similar to the *eng3* expression, in the ventral neural tube (I; transverse section). (J-M) *Eng1* and *her5* expression is completely absent at 26 hours.

be in precursors for these neurons. Notably, this cell population is highly sensitive to the level of functional Pax2.1, which may therefore be required also for the re-expression phase; alternatively, the cells may simply be able to persist to later stages in weak *noi* mutants.

DISCUSSION

We have examined the requirement for *no isthmus/pax2.1* in development of the zebrafish midbrain hindbrain boundary. In addition to the previously characterized *noi*^{th44a} (Brand et al., 1996), we describe four additional mutations in the *pax2.1* gene. By molecular and phenotypic criteria, the *noi* alleles form an 'allelic series' in which *pax2.1* function is probably inactivated to different degrees, in the following order: null (*noi*^{tu29a}) > strong (*noi*^{tm243a}, *noi*^{th44a}) > weak (*noi*^{ty31a,} *noi*^{tb21}), and have presented evidence that *noi*^{tu29a} is a null allele (now called *noi*$^{-/-}$). Analysis of the *noi*$^{-/-}$ mutants allowed subdividing genes expressed during early MHB development into those that require *noi* function already at the end of gastrulation (*eng2*, *eng3*) and those that are activated independently (*fgf8*, *wnt1*, *her5* and *pax2.1* itself), but require it for maintenance during mid-somitogenesis. Together with other data, this argues that multiple signaling pathways operate in early MHB development. Analysis of the intermediate and weak alleles of the allelic series suggests an additional requirement for *pax2.1* during dorsoventral patterning, and possibly in a later phase of *engrailed* gene expression during MHB development.

Structure and activity of *pax2.1*

The *pax2.1* genomic structure shows overall conservation of the exon/intron structure between mammals and zebrafish, but also some differences. Of the 12 exons, 10 show sequence homology to other *pax2/5/8* sequences, whereas exons 5.1 and 7.1 encode zebrafish-specific sequences. Such species-specific exons have also been reported for murine, human and Xenopus *Pax2* (Sanyanusin et al., 1996; Heller and Brändli, 1997; Tavassoli et al., 1997), suggesting that functional specializations have occured between orthologues in different vertebrates. As shown in the accompanying paper, zebrafish *pax2.1* and mouse *Pax2* differ for instance in their requirement for controlling transcription of *pax5* and *pax8* at the MHB, which depend on *pax2.1* function in zebrafish, but which may act in parallel in mice (Pfeffer et al., 1998). Multiple functions of zebrafish *pax2.1* may be reflected in the high number of splice variants we found, which remain to be tested for functional differences.

Molecular defects of *noi* alleles

For functional studies, and given the variable phenotype of mouse *Pax2*$^{-/-}$ mutants, it was crucial to determine to what extent *pax2.1* function is impaired by *noi* mutations. In addition to the previously characterized *noi*^{th44a} allele, we found molecular aberrations for the remaining five *noi* alleles in *pax2.1*. Together with our previous data on genetic linkage and protein expression (Brand et al., 1996), this provides further evidence that the genetically defined *no isthmus* gene is identical with *pax2.1*.

The following observations argue that *noi*^{tu29a} is a null allele: (1) the mutation creates a stop codon in the paired domain, in an exon common to all splice variants; (2) the predicted mutant protein lacks the C terminus with the transactivating and inhibiting domains, as well as 6 amino acids of the paired domain, which are crucial for DNA recognition (Adams et al., 1992); (3) the proposed structure of the mutant protein is consistent with the absence of a C-terminal Pax2.1 protein epitope in *noi*^{tu29a} mutants (Brand et al., 1996), and the protein size when the mutant protein is expressed in bacteria (data not shown); (4) our DNA-binding assays show that the *noi*^{tu29a} protein fragment does not bind to known Pax2 binding sites, including two sites required for Pax2-dependent activity of the mouse *En2* promoter (Song et al., 1996); (5) we observe no dominant negative effect in DNA-binding assays, even at a 100-fold molar excess to wild-type protein; (6) expression of *eng3* as a likely downstream target is abolished in *noi*^{tu29a} homozygotes.

Other *noi* alleles partially reduce *pax2.1* activity. The morphologically strong alleles show distinct differences in their molecular phenotype compared to *noi*^{tu29a}. The truncated *noi*^{th44a} protein contains an intact paired domain and octapeptide (Fig. 3A), and shows a weaker phenotype in our marker gene expression studies; this protein may therefore be able to activate transcription at a low level (see also Nutt et al., 1998). The postulated Noi^{tm243a} protein lacks six amino acids in the N-terminal region of the paired domain that mediate protein/DNA contacts (Xu et al., 1995) due to a mutated splice acceptor site. Although DNA binding activity of this protein is completely abolished, mutants for this allele display a slightly weaker phenotype in the expression of the marker genes than *noi*^{tu29a}, probably because a small amount of normally spliced mRNA is still present. Similar splice acceptor mutations in the globin genes cause thalassemia (Treisman et al., 1983), and the same substitution in a splice acceptor site of *Pax3* activates cryptic splice sites within the following exon (Epstein et al., 1993). In these cases, the mutated splice acceptor sites continue to function with low efficiency, and we propose that this is also the case for *noi*^{tm243a}.

The deleted exon 7 in mRNA from *noi*^{tb21} homozygotes may also be due to aberrant splicing. The occurence of deletions in two of five *noi* alleles by aberrant splicing could be chance, but might also reflect easier detectability of deletion phenotypes in the morphological screens. The *noi*^{tb21} mutation effectively forces creation of the naturally occuring major splice form lacking exon 7. Since *noi*^{tb21} retains partial activity, the natural splice variant may also be functional. Alternatively, a small amount of normally spliced transcripts or a minor splice form unaffected in *noi*^{tb21} could provide the residual function. Reconstitution of the various splice forms into a *noi*$^{-/-}$ background can now be used to address their function.

Requirement for *pax2/5/8* genes in MHB development

The MHB phenotype of *noi*$^{-/-}$ mutants is more constantly severe than that of murine *Pax2* mutants, and we previously hypothesized that either only a single *pax2/5/8* gene exists in zebrafish, or that *pax2.1* has become functionally predominant (Brand et al., 1996). The isolation of three additional zebrafish *pax2/5/8* genes reported in the accompanying paper (Pfeffer et al., 1998) supports the latter possibility, since *pax5* and *pax8* are critically dependent on *noi/pax2.1* function at the MHB.

Between its onset at 80% of epiboly and the 4-somite stage, *pax2.1* is the only known *pax2/5/8* gene expressed at the MHB. *pax2.2*, though expressed in *noi* mutants from the 5-somite stage, appears unable to replace $noi^{-/-}$ function, perhaps because its MHB activation occurs too late (see also the discussion in Pfeffer et al., 1998). In other tissues, e.g. in the optic stalk (Macdonald et al., 1997), *pax2.2* may partially compensate the missing *noi* function.

Multiple pathways in early MHB development

A key finding in our study is that, through the availability of the null allele noi^{tu29a}, we were able to subdivide genes expressed in the early midbrain-hindbrain primordium according to their requirement for *pax2.1*. *pax2.1* is activated before neural plate formation, prior to and in an overlapping expression pattern with *wnt1*, *eng1*, *eng2* and *eng3*, whereas *her5* and *fgf8* are activated slightly earlier. Expression of *eng2* and *eng3* already clearly requires *pax2.1* function during late gastrulation. In contrast, the normal onset and expression of *wnt1*, *fgf8*, *her5* and *pax2.1* itself up to approximately the 4- to 5-somite stages suggests that additional, *pax2.1*-independent pathway(s) operate upstream and/or in parallel to induce gene expression in the MHB primordium.

Alternatively, *wnt1* or *fgf8* could simply be upstream of *pax2.1*, and would thus not be affected in $noi^{-/-}$ mutants. For *wnt1* this is unlikely, since *En1*, *En2* and *Pax2* are activated normally in mouse $wnt1^{-/-}$ mutants (McMahon et al., 1992; Rowitch and McMahon, 1995). In zebrafish, a *wnt1* mutant is not yet known, but since *wnt1* and *pax2.1* are activated in different, only partially overlapping domains (Fig. 7), and *wnt1* misexpression in zebrafish does not activate *pax2.1* (Kelly and Moon, 1995), it is very likely that the two genes are activated independently of each other in zebrafish as well. Expression of *fgf8* in $noi^{-/-}$ mutants is unaffected for a different reason: during gastrulation, *fgf8* is expressed in the anterior hindbrain, posterior to the domains of *pax2.1* and *wnt1*; moreover, *fgf8* misexpression does not alter the anterior-posterior extent of *pax2.1* expression (Reifers et al., 1998). Importantly, in the zebrafish *fgf8* mutant *acerebellar*, which lacks MHB and cerebellum but has a midbrain, expression of MHB marker genes is initiated normally but not maintained (Reifers et al., 1998). However, *fgf8* inactivation in mice also causes midbrain defects (Meyers et al., 1998), perhaps as a secondary consequence of gastrulation defects which are less severe in *acerebellar* mutants (see discussion in Reifers et al., 1998).

Together, our findings in zebrafish suggest that *pax2.1*, *wnt1* and *fgf8* are initiated independently of each other in late gastrulation, and only later in somitogenesis come to interact (Reifers et al., 1998; this paper; Fig. 9). Evidence for multiple pathways in MHB formation also comes from *otx* gene dosage studies (Acampora et al., 1997). In $otx2^{-/-}$, $otx1^{+/-}$ animals, ectopic anterior expression of *fgf8* and later formation of an ectopic MHB are observed. At the early neural plate stage, however, expression of *En2* and *wnt1* are initiated at their normal location, and only in midsomitogenesis is the expression recruited to the ectopic, anterior position. As in zebrafish, these studies therefore distinguish at least two pathways, one positioning *fgf8* (controlled via *otx* dosage), and a second pathway activating *En* and *wnt1* expression, independent of *otx*.

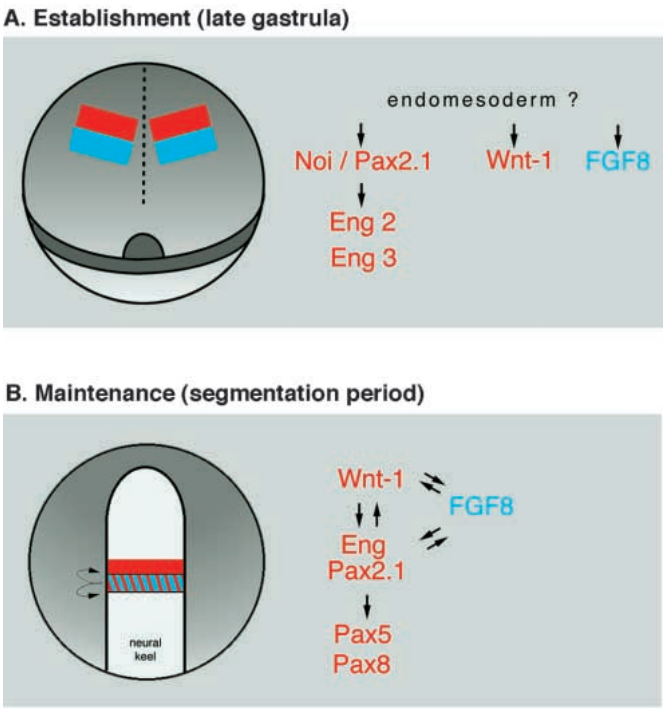


Fig. 9. Stepwise development of the zebrafish MHB. We postulate that during the establishment phase in the late gastrula (A), three parallel pathways are activated in similar, but not identical domains in the primordia of the early midbrain and MHB (red, *pax2.1* and *wnt1*) and anterior hindbrain (blue, *fgf8*). Noi/Pax2.1 is required to activate *engrailed* gene expression. The activating signals are unknown, but may derive from the endomesodermal germ ring (grey ring). (B) During the maintenance phase, expression overlaps at the MHB organizer, which secretes Wnt1 and Fgf8 as signaling molecules. At this stage, the pathways become mutually dependent, and control downstream gene expression in the MHB organizer (Pax5, Pax8) and the surrounding neural plate (arrows).

pax2.1 as an *engrailed* regulator

In $noi^{-/-}$ embryos, *eng3* expression is completely abolished and *eng2* is expressed transiently at a low level; similarly, the later activation of *eng1* is not seen in $noi^{-/-}$ mutants. *noi/pax2.1* is therefore a crucial upstream component in the pathway that activates *eng2* and *eng3* in the midbrain and MHB primordia during late gastrulation, consistent with the colocalization of the expression domains (Fig. 7C-D). The *noi*-independent component of *eng2* expression demands, however, an additional mechanism for activating *eng2*. Generally, expression of *eng* genes is under the control of both *pax2(.1)* (this paper; Song et al., 1996) and *wnt1* (McMahon et al., 1992; Danielian and McMahon, 1996), and can be activated by *fgf8* misexpression (Crossley et al., 1996; Lee et al., 1997; Shimamura and Rubenstein, 1997). Importantly, although *wnt1* and *fgf8* are activated normally in $noi^{-/-}$ embryos during late gastrulation, they are unable to drive *eng3* or normal *eng2* expression in the mutants. Thus, although *wnt1* and *fgf8* are necessary to maintain *eng* expression (McMahon et al., 1992; Reifers et al., 1998), they are not sufficient, with the possible exception of the weak transient *eng2* expression.

In mice, Pax2 directly regulates *En2* promotor activity via *Pax2/5/8* binding sites (Song et al., 1996). As in mice, Pax2.1

may directly activate *eng* genes by binding to the *eng* promoters. These remain to be characterized in zebrafish to explain the differences in regulation of the *engrailed* genes we have observed, and to distinguish direct from indirect regulation. Given the ongoing morphogenetic movements and the successive restriction of *pax2/5/8* and *eng2/3* genes towards the MHB constriction, the regulatory relationship between *pax2.1* and *engrailed* genes is likely to be important for later midbrain development, e.g. for retinotectal projection into the midbrain tectum, which is thought to require an Engrailed protein gradient (Rétaux and Harris, 1996).

Establishment and maintenance of the MHB

Organizing centers often establish concentration gradients of signaling molecules that pattern the adjacent tissue, and are thus sensitive to the functional level of components of the signaling pathway. Our comparison of the molecular and phenotypic strength within the *noi* allelic series suggests that the functional level of *pax2.1* is critical for MHB development. By analogy with similar studies in *Drosophila* (Anderson et al., 1985), we assume that 'functional level' means here 'level of Pax2.1 protein activity', though this remains to be proved. The failure to maintain expression of *her5, fgf8, wnt1* and other markers in $noi^{-/-}$ mutants may mean that, for continued expression, the MHB organizer needs to be successfully established, producing, for instance, *wnt1* and *fgf8* as signals important for maintenance. When this occurs in development is currently unknown; our data point to a critical period around the 5-somite stage. Interestingly, the requirement for *noi* function at this stage is higher at the MHB than in the midbrain primordium, since this is where gene expression is affected first in the mutants. Differential effects on the midbrain and MHB expression of *wnt1* are also observed in *Gbx2, Otx2* and *fgf8* mutants (Acampora et al., 1997; Wassarman et al., 1997; Reifers et al., 1998; Takada et al., 1994). The maintenance requirement we observe in *noi* mutants is not necessarily direct: it could reflect an inability to form a functional MHB organizer in $noi^{-/-}$ mutants, or inability to respond to signals from the organizer, or both; further studies are needed to distinguish these possibilities.

Taken together, our data support a model where establishment of the zebrafish MHB occurs in several steps (Fig. 9). During the establishment phase, at least two pathways are activated in the MHB primordium in late gastrulation: one pathway involves Pax2.1 and leads to proper activation of *eng* genes (with its downstream consequences for development of the retinotectal map). Independently, a second pathway is activated that employs the secreted Wnt1 molecule. Very likely, a third independent pathway employing Fgf8 is activated in the posteriorly abutting anterior hindbrain. What signal(s) in turn activate these pathways is not yet known; they may derive at least in part from the endomesoderm (Ang and Rossant, 1993; Woo and Fraser, 1997; Ang, 1996; Miyagawa et al., 1996).

During the second or maintenance phase in early to mid-somitogenesis, gene expression at the MHB is maintained by reciprocal interactions, as is also suggest by the functional requirement for *wnt1* and *En1* in mice (McMahon et al., 1992; Wurst et al., 1994). During this period, significant overlap between *pax2.1, wnt1* and *fgf8* expression is established in the future MHB, which may be crucial in generating its organizing properties. During this period, *noi/pax2.1* continues to be required for expression of *eng* and other genes, and directly or indirectly regulates downstream gene expression in the MHB and around it. Also during the maintenance period, patterning occurs along the dorsoventral axis in the MHB organizer in a manner that still requires *pax2.1* activity.

Requirement for *pax2.1* in dorsoventral patterning of the MHB

So far, *pax2.1* has mainly been considered as a component of the patterning machinery along the anteroposterior (a/p) axis of the brain. Analysis of the allelic series of *noi* alleles unexpectedly revealed different sensitivity of MHB cells to *pax2.1* functional levels along the dorsoventral (d/v) axis, which differs for the gene considered. Expression of *pax2.1* itself and *wnt1* disappears homogeneously, probably simply reflecting loss of the cells normally destined to form MHB tissue. With increasing strength of the *noi* allele examined, the MHB loses *her5* and *fgf8* gene expression from dorsal to ventral, such that the highest level of *pax2.1* is required in the dorsal area. In contrast, *eng* gene expression disappears from ventral to dorsal with increasing allele strength, as has also been observed in for En2 in En1 mutants (Wurst et al., 1994). Thus, these genes respond to d/v positional information, but in a way that is critically dependent on the functional level of *pax2.1*, and in different ways for the various marker genes. We have no indication that, in wild-type development, *pax2.1* itself is distributed in an asymmetric way along the d/v axis of the MHB (although this view does not take the complexity of alternative splice forms into account), and the requirement could be indirect.

Why then are differences observed, depending on the allele and the marker gene studied? Probably, MHB cells continue to require *pax2.1* during the maintenance phase. In this view, *pax2.1* would serve as an integrator for both a/p and d/v determining signals at sequential stages of development. Sequential assignment of a/p and d/v positional values is also seen in rhombomere 4 of the chick hindbrain (Simon et al., 1995). Likely candidates for d/v signals are signaling molecules like *sonic hedgehog* (*shh*) from the ventral side, and BMPs from the dorsal side (Tanabe and Jessel, 1996). Indeed, *pax2.1* and *shh* are functionally linked in patterning of the optic vesicle and optic chiasm in zebrafish (Macdonald et al., 1995, 1997). More recently, a medaka homologue of *Drosophila spalt*, a target gene for Hedgehog signaling in *Drosophila*, was found to be expressed in MHB development and to respond to *shh* RNA injection specifically at the MHB (Köster et al., 1997).

Evolutionary considerations

The nervous systems of invertebrates and vertebrates share a common origin, reflected in many conserved interactions within the patterning processes (DeRobertis and Sasai, 1996). In *Drosophila*, Engrailed, Wingless and Hedgehog are involved in a feedback loop that functions in boundary formation in the embryo and the imaginal discs; a *paired*-type gene is involved in establishing this loop (Martinez-Arias, 1993). Of their vertebrate homologues, *eng* genes, *pax2(.1)* and *wnt1* are clearly required for a/p patterning of the MHB, whereas *shh* is not, and it has been problematic to understand the 'missing' *shh* involvement. Our observations suggest a possible explanation: the circuitry of genes including *shh* may originally

have been maintained for their function within the MHB organizer proper, and may only later in evolution have become adapted for the a/p patterning function of the organizer. Vertebrates have an elaborated midbrain that presumably requires presence of the a/p patterning function, whereas more basal chordates do not (Butler and Hodos, 1996). Interestingly, the protochordate ascidian *Halocynthia roretzi* has a single archetypal *pax2/5/8* gene, which is expressed in the 'neck' region, and has therefore been suggested to be related to the midbrain and MHB of vertebrates (Wada et al., 1998). Ascidian *pax2/5/8* is expressed posteriorly adjacent to ascidian *otx*, whereas *otx2* and *Pax2* expression overlap in the midbrain of vertebrates (Acampora et al., 1997), including zebrafish (unpublished observations). A speculative possibility is therefore that the ascidian *pax2/5/8* expression domain is related only to the MHB, but not the midbrain portion of *pax2* expression, and that the organizing potential of the MHB for the surrounding midbrain and cerebellar primordia was secondarily acquired in vertebrates.

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