A novel positive transcriptional feedback loop in midbrain-hindbrain boundary development is revealed through analysis of the zebrafish pax2.1 promoter in transgenic lines

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SUMMARY

The pax2.1 gene encodes a paired-box transcription factor that is one of the earliest genes to be specifically activated in development of the midbrain and midbrain-hindbrain boundary (MHB), and is required for the development and organizer activity of this territory. To understand how this spatially restricted transcriptional activity of pax2.1 is achieved, we have isolated and characterized the pax2.1-promoter using a lacZ and a GFP reporter gene in transient injection assays and transgenic lines. Stable transgenic expression of this reporter gene shows that a 5.3-kb fragment of the 5′ region contains most, but not all, elements required for driving pax2.1 expression. The expressing tissues include the MHB, hindbrain, spinal cord, ear and pronephros. Transgene activation in the pronephros and developing ear suggests that these pax2.1-expressing tissues are composed of independently regulated subdomains. In addition, ectopic but spatially restricted activation of the reporter genes in rhombomeres 3 and 5 and in the forebrain, which do not normally express endogenous pax2.1, demonstrates the importance of negative regulation of pax2.1.

Comparison of transgene expression in wild-type and homozygous pax2.1 mutant no isthmus (noi) embryos reveals that the transgene contains control element(s) for a novel, positive transcriptional feedback loop in MHB development. Transcription of endogenous pax2.1 at the MHB is known to be initially Pax2.1 independent, during activation in late gastrulation. In contrast, transgene expression requires the endogenous Pax2.1 function. Transplantations, mRNA injections and morpholino knock-down experiments show that this feedback regulation of pax2.1 transcription occurs cell-autonomously, and that it requires eng2 and eng3 as known targets for Pax2.1 regulation. We suggest that this novel feedback loop may allow continuation of pax2.1 expression, and hence development of the MHB organizer, to become independent of the patterning machinery of the gastrula embryo.

Key words: Pax, Transgenesis, no isthmus, Fgf8, engrailed, Zebrafish, Danio rerio, Cell signaling, CNS, Midbrain, Hindbrain, Isthmus, Organizer, Pattern formation

INTRODUCTION

During pattern formation in vertebrate neural development regional gene expression is integrated and co-ordinated, such that different cell types can arise in specific spatial and temporal domains (Lumsden and Krumlauf, 1996). A key mechanism that governs restricted gene expression is the local transcriptional control by DNA-binding transcription factors. Together with secreted patterning molecules that mediate cell interactions at a distance, they are required to establish and maintain positional information along the axes of the developing embryo. Transcription factors act via cis-regulatory DNA elements on the control of target gene expression. Therefore, in order to advance from the analysis and manipulation of gene expression towards a general understanding of complex gene interactions and epistatic relationships, it is necessary to gain insight into the cis-regulation of the participating genes.

Reporter genes such as the bacterial beta-galactosidase gene (lacZ) or green fluorescent protein (GFP) are useful tools to study promoter activity in transgenic animals, and the construction and analysis of transgenic lines is of prime importance for designing experimental tools. lacZ reporter gene technology has previously been used to study gene expression in zebrafish embryos (Culp et al., 1991; Amsterdam et al., 1995; Amsterdam et al., 1996). However, only a few cases of stable transgene expression driven by tissue-specific, zebrafish promoters have been reported so far (Long et al., 1997; Jessen et al., 1998; Higashijima et al., 2000), and to date, transgenesis has not been exploited during zebrafish mutant analysis. Here, we utilise comparative analysis of reporter gene expression in wild-type and mutant zebrafish embryos, and find
that this approach reveals a novel regulatory step in development of the midbrain-hindbrain boundary region.

The midbrain-hindbrain boundary (MHB) is a region of highly overlapping and dynamic expression of genes encoding the En, Pax2/5/8, Otx and Gbx transcription factors and signaling molecules like Fgf8 and Wnt1 (McMahon et al., 1992; Urbanke et al., 1994; Wurst et al., 1994; Ang et al., 1996; Crossley et al., 1996; Favor et al., 1996; Wassarman et al., 1997; Lun and Brand, 1998; Pfeffer et al., 1998; Reifers et al., 1998). The MHB plays a specialised role during the induction, maintenance and polarization of cell fates in the adjacent midbrain and hindbrain by acting as an embryonic organizer (reviewed by Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001).

The pax2 gene is a key regulator of midbrain, MHB organizer and cerebellar development in several vertebrate species (Sanayusin et al., 1995; Torres et al., 1995; Brand et al., 1996; Favor et al., 1996; Schwarz et al., 1997). In zebrafish, pax2.1 (pax2a – zebrafish Information Network) is the earliest known gene to be specifically activated in this area (Krauss et al., 1991; Lun and Brand, 1998). A closely related gene, pax2.2 is activated only at later stages (Pfeffer et al., 1998). Analysis of pax2.1, no ishmuta29a (noi) showed that downstream gene activation of two zebrafish engrailed-type genes, eng2 and eng3, is dependent on Pax2.1 function at their onset (Lun and Brand, 1998). In contrast, fgf8 and wnt1 activation does not require Pax2.1 activity during gastrulation (establishment stage), but expression becomes dependent on Pax2.1 during mid-somitogenesis stages (maintenance phase). Likewise, analysis of a zebrafish fgf8 mutant, acecerebellar (ace), shows that pax2.1 expression does not initially require Ace/Fgf8 (Reifers et al., 1998), but becomes dependent on it during the maintenance phase. Thus, in zebrafish MHB development, an early establishment phase during gastrulation can be clearly distinguished from a late maintenance phase starting at mid-somitogenesis stages (Rhinn and Brand, 2001).

The factors controlling pax2.1 activation during gastrulation at the MHB are not known, but are likely to involve general mechanisms patterning the gastrula embryo (Koshida et al., 1998; Hashimoto et al., 2000). Since transcription of pax2.1 is activated normally even in the noia29a null mutant background, transcriptional initiation of pax2.1 at the MHB is not dependent on functional Pax2.1 protein (Brand et al., 1996; Lun and Brand, 1998). After gastrulation, however, pax2.1 continues to be specifically expressed in the midbrain and MHB organizer, eventually becoming restricted to the organizer region. During this period, pax2.1 RNA expression is eventually lost from the MHB of noi mutants, raising the possibility that Pax2.1 becomes involved in its own (feedback) regulation. Generally, a switch to feedback regulation is an important transcriptional mechanism for driving irreversible tissue determination (Morgan, 1997), and it is therefore of great interest to examine whether feedback regulation occurs in MHB development.

Here we study the cisulatory basis of pax2.1 gene transcription and relate it to the different phases of MHB development. We isolated genomic clones of the zebrafish pax2.1 gene and its upstream region, and generated promoter fusions with lacZ and GFP reporter genes. These reporter genes were tested initially by transient assays and were subsequently used to generate stable transgenic lines that recapitulate most, but not all aspects of the endogenous pax2.1 expression. Restricted ectopic activation of the reporter gene in the fore- and hindbrain suggests that negative regulation of pax2.1 is also important. We find that discrete cis-regulatory elements are required to drive different temporal and spatial aspects of and within pax2.1 expression domains. Unexpectedly, analysis of reporter gene expression in noi mutants demonstrates the existence of a novel, cell-autonomous, positive feedback loop for pax2.1 transcription at the MHB via its own targets eng2 and eng3, that acts from the end of gastrulation onwards. We suggest that this feedback loop may serve to irreversibly determine midbrain and/or MHB fate.

MATERIALS AND METHODS

Fish strains and whole-mount in situ hybridizations

Zebrafish were raised and kept under standard laboratory conditions at 27°C (Westerfield, 1994; Brand et al., 2002) and heterozygous carriers of noi29a, placZ5.3 and pGFP5.3 were identified by random intercrosses. To obtain homozygous mutants and transgenics, carriers were crossed to each other. Founder fish transmitting the transgenes were identified by PCR on genomic DNA from F1 egg clutches with transgene-specific primers. The offspring of PCR-positive founder fish were then tested for β-gal activity or GFP fluorescence. Both lines were verified by in situ hybridization (ISH) with a DIG-labelled lacZ or gfp RNA probe as described previously (Reifers et al., 1998).

DNA constructs and primer extensions

A library was constructed from genomic DNA of two adult male AB zebras and clones were isolated with a radiolabelled pax2.1 cDNA. Upstream sequences of pax2.1 were subcloned into pCNG301 (Hiromi et al., 1985) which contained the beta-galactosidase coding region and a SV40 polyadenylation. The transcriptional start site of pax2.1 was determined by primer-extension analysis (Mason et al., 1993). The 5’-UTR-specific antisense primer G1139-3’ (5’-ACGGATCTCTACTACTCTGACAG) was 32P-dATP labelled with T4 polynucleotide kinase (Toyobo), hybridized to total zebrafish RNA from 24 hour embryos at 50°C, 55°C or 60°C. From these hybrids cDNA was synthesized with Superscript™ II RNase H Reverse Transcriptase (GIBCO). After removing RNA by RNase A treatment, the extension products were analysed on a standard sequencing gel.

Injections, transplantsations and β-gal assay

For injection, plasmid DNA of the reporter genes was prepared as in Stuart et al. (Stuart et al., 1988). Cytoplasmic DNA injection was done with a pneumatic pico pump (WPI) and thinwall borosilicate glass pipettes (OD=1.0 mm, WPI) at the 1- to 2-cell stage into manually dechorionated embryos at the same stage. Approximately 50-100 pg eng3 RNA was injected as described previously (Reifers et al., 1998). The RNA was injected along with 2.000K tetramethylrhodamine dextran (Molecular Probes) as a lineage label (Führthauer et al., 1997). The morpholinos MOeng1 and MOeng3 (Scholpp and Brand, 2001) (GeneTools) are directed against position 1-25 of the eng2 and eng3 cDNA and 4 ng of each were injected in 25 mmol Hepes into the yolk cell at the 2- to 4-cell stage as described.

For transplantation, donor embryos were injected with 5% biotinylated tetramethylrhodamine dextran (Molecular Probes) in 0.25 M KCl and used for grafts at 30-40% epiboly. Biotin was detected with the Vectastain Kit (Vector Laboratories). Transient expression of the reporter genes was tested by whole-mount staining for β-galactosidase (β-gal) enzyme activity as described previously (Westerfield et al., 1992; Reinhard et al., 1994).
**RESULTS**

**Isolation and characterization of pax2.1 promoter**

Using a pax2.1 cDNA probe we isolated five independent and overlapping genomic pax2.1 clones with a size between 11 and 22 kbp (Fig. 1A). Restriction mapping, hybridization with 5'- and 3'-specific cDNA probes and DNA sequencing confirmed that these clones contain the 5' region of the gene (data not shown). Clone FIX 1 with approximately 5.3 kb contained the largest fragment upstream of the initiation codon and was selected for further analysis. By primer extension analysis, we determined two putative transcriptional start sites 70 bp and 144 bp upstream of the 5'-end of the published cDNA sequence, which contain no TATA boxes but a region with homology to an Initiator (Inr) sequence (Fig. 1 and data not shown). Clone FIX2 contained the 3' region of pax2.1 at least including Exon 7 (Lun and Brand, 1998).

**Construction and transient expression of pax2.1 promoter lacZ reporter genes**

To test the identified 5' fragment of pax2.1 for functional cis-regulatory elements we fused six sub-fragments to a lacZ reporter gene (Fig. 1B). All six constructs contained both putative transcriptional start sites, except for placZ0.8, in which the upstream start site was deleted. The 3' end of the genomic upstream fragment was at the translational start site of pax2.1. All constructs were tested by DNA injection into zygotes and the resulting mosaic zebrafish embryos were analysed for ß-gal activity. Control injection of the lacZ vector alone gave no ß-gal activity. All constructs could drive transient expression, and the resulting mosaic expression was compared at the 10- to 12-somite stage and between the 25-somite and 24-hour stages to the endogenous expression pattern of pax2.1 as determined by in situ hybridization and comparison with morphological landmarks. Judging from this comparison, transient lacZ expression was either designated 'specific' (in pax2.1-expressing regions) or 'ectopic' (in other regions).

pax2.1 is expressed from 80% epiboly onwards in the midbrain and MHB primordium and subsequently in the otic placode, optic stalk, pronephros, cloaca and in hindbrain and spinal cord neurones (Krauss et al., 1991). At the 10- to 12-somite stage, the longer constructs placZ4.5, placZ4.5+ and placZ5.3 were transiently expressed at the MHB, in the otic placode, the hindbrain, spinal cord and pronephros (Fig. 3C). During later development, MHB expression of placZ5.3 becomes restricted along the anterior-posterior (AP) axis of the neural tube as it does for the endogenous pax2.1 gene (Fig. 3E). Until the 15-somite stage placZ5.3 is expressed at the MHB (Fig. 3C). During later development, MHB expression of placZ5.3 becomes restricted along the anterior-posterior (AP) axis of the neural tube as it does for the endogenous pax2.1 gene (Fig. 3E). Until it becomes localized to the isthmic fold at 24 hours (Fig. 3K,L).

From 90% epiboly onwards, pax2.1 is expressed in the anterior portion of the intermediate mesoderm, which is fated to become pronephros (Fig. 4A), and placZ5.3 is expressed in the same region (Fig. 4B). By the 15-somite stage, pax2.1 is expressed in the pronephric duct with a small gap anterior to the domain of the future cloaca, as well as in the cloacal primordium itself. In contrast, the expression of placZ5.3 is restricted to an intermediate domain along the AP axis of the pronephric ducts, with the same posterior limit of expression as pax2.1 but excluding the anterior intermediate mesoderm and the cloaca. (Fig. 4C,E). No expression of placZ5.3 was detected in the pronephros beyond the 20-somite stage although pax2.1 continued to be expressed. Thus, distinct cis-regulatory elements are required for pax2.1 expression at different anterior-posterior levels of the developing kidney and for maintenance of expression into later stages.

In the prospective otic placode transcription of placZ5.3 and pax2.1 is initiated around the tailbud stage, with placZ5.3 slightly preceeding pax2.1 (Fig. 3E). Until the 15-somite stage most specifically (Fig. 2K), whereas placZ0.8 showed the least degree of specificity. We therefore, chose placZ5.3 as the longest and most reliably expressed construct for further analysis (Fig. 2J,K).

**Construction of a transgenic zebrafish line with placZ5.3 and pGFP5.3**

Since transient expression does not allow a detailed analysis of spatial and temporal aspects of transgene expression in the embryos, we generated stable transgenic lines with placZ5.3. Among 44 potential F0 founder fish, PCR-screening of genomic DNA of their F1 progeny identified four F0 founder animals (Fig. 2L). Analysis of the lacZ expression pattern by whole-mount RNA ISH showed that one of these founders produced offspring with ubiquitous expression, two produced offspring without detectable lacZ expression and one with specific lacZ expression in a pax2.1-like pattern. Subsequently, an independent transgenic line was obtained with the identical promoter fragment fused to a GFP reporter gene, which displayed an almost identical pattern of expression as the placZ5.3 transgenic line (see below). Stable expression of these transgenes is now observed into the F4 generation.

**Stable transgenic expression of placZ5.3**

We compared the lacZ expression pattern of the placZ5.3 transgene in wild-type embryos with the expression domains of the endogenous pax2.1 gene by ISH, and found that the transgene recapitulates many, but not all, aspects of pax2.1-expression. During embryonic development, transcription of pax2.1 is first detectable at 80% epiboly in the region of the future MHB (Fig. 3A), placZ5.3 is activated at the MHB approximately one hour later, around 90% epiboly, in a pattern similar to pax2.1. Double ISH at 90% epiboly shows that placZ5.3 is activated within a subregion of the pax2.1 domain (Fig. 3B,D), similar to what is observed for the Pax2.1 target gene eng3 (Lun and Brand, 1998). From tailbud stage onwards, the pax2.1 domain is identical to the placZ5.3 domain at the MHB (Fig. 3C). During later development, MHB expression of placZ5.3 becomes restricted along the anterior-posterior (AP) axis of the neural tube as it does for the endogenous pax2.1 gene (Fig. 3E-K), until it becomes localized to the isthmic fold at 24 hours (Fig. 3K,L).
Fig. 1. Structure of pax2.1, genomic clones and reporter genes. (A) Five genomic pax2.1-clones, which contained 5.3 kb of sequence upstream of the translational start at the 5′ end (FIX1), including at least exon 7 at the 3′ end (FIX2), were isolated by screening a lambda phage library. Orientation relative to the exon-intron-structure of pax2.1 (Lun and Brand, 1998) is shown. FIX1 was used for subsequent subcloning. Two transcriptional start sites (+1) were determined by primer extension. No corresponding TATA sequences were found upstream of these sites but the downstream transcriptional start was found to overlap with an Initiator (Inr) consensus (overlap in red), which can mediate transcriptional initiation in the absence of TATA sequences (Smale and Baltimore, 1989). (B) Structure of the pax2.1 promoter/enhancer reporter genes. Five lacZ constructs and one GFP construct, with different 5′ extent have been constructed from FIX1. The size of the pax2.1 fragment in each construct is given in brackets. placZ5.3 and pGFP5.3 have been analyzed in stable transgenic lines. +1, transcriptional start site; ATG, translational start; polyA, SV40 polyadenylation signal.

Fig. 2. Transient expression analysis of pax2.1 promoter/enhancer lacZ reporter genes. (A-H) Mosaic β-gal activity of the five constructs between the 10-somite stage and 24 hours after zygotic injection. (A-C,G,H) Constructs placZ4.5, placZ4.5+ and placZ5.3 show transient expression in the MHB, in the otic placode, hindbrain, pronephros and spinal cord. This pattern resembles the endogenous pax2.1 pattern. (D-F) Two constructs, with a large 5′ deletion, placZ2.4 and placZ0.8, show less specificity and stronger ectopic activation. placZ2.4 shows confined stripe-like expression in the hindbrain (E). (I) Comparative analysis of the tissue specificity in transient expression between the 10- to 12-somite stages (10-12ss) and the 25-somite to 24-hour stages (25ss-24h). The three largest constructs, placZ4.5, placZ4.5+ and placZ5.3 showed the highest degree of expression in pax2.1 expression domains. For the deleted constructs, placZ2.4 and placZ0.8, no tissue restriction could be determined because of the strong ectopic activation. All endogenous expression domains except the optic stalk are reproduced by the reporter genes. At later stages of development (25ss-24h) placZ5.3 shows a higher degree of expression at the MHB compared to placZ4.5. (J) Expression of placZ5.3 and placZ4.5 at the MHB at 10-12 ss (grey bars) and 25ss-24h (black bars). An average of 11% of lacZ-expressing cells/embryo are found at the MHB at 10-12 somites stage for both constructs. At later stages placZ5.3 shows stronger expression at the MHB (5%) than placZ4.5 (3%). (K) Tissue-specific and ectopic expression of the five constructs. Grey bars show the average total number of lacZ-expressing cells/embryo and black bars show the number of cells in ectopic regions. Tissue restriction is decreasing, ectopic expression increasing, with the degree of 5′-deletions of the construct (left to right). Cells/Embryo, average total number of lacZ-expressing cells/embryo; EEmbryonic, average percentage of lacZ-expressing cells at the MHB relative to total number of lacZ-expressing cells/embryo; Hb, hindbrain; MHB, midbrain-hindbrain boundary; OS, optic stalk; Otp, otic placode; OV, otic vesicle; Prn, pronephros; SC, spinal cord; +, expression; −, no expression. (L) PCR strategy for identification of lacZ transgenic carriers, and identification of two transgenic founder fish by PCR.
placZ5.3 is expressed like pax2.1 in the developing ear (Fig. 3E-I). From the 20-somite stage onwards, little transcription of the transgene is detected in the developing ear, whereas pax2.1 is still strongly transcribed (Fig. 3J-L). Similar to the transgene expression in the embryonic kidney, this suggests two phases of transcriptional regulation: (i) an early phase that is reproduced by the cis-elements included in placZ5.3 and (ii) a late phase, which requires additional cis-elements.

Like pax2.1, the placZ5.3 transgene is expressed in an increasing number of cells in the posterior hindbrain and anterior spinal cord primordia from the 10-somite stage onwards (Fig. 3G). At the 20-somite stage, both the transgene and pax2.1 are expressed in the posterior hindbrain and anterior spinal cord in two rows of cells at distinct dorsoventral positions. In the posterior spinal cord, only one row of interneurones is seen. These interneurone cells are known to express pax2.1 (Fig. 3J-L). Identical domains are seen in a second, independently generated transgenic line with the same promoter/enhancer fragment driving expression of GFP.

In addition to the domains that normally express pax2.1, the placZ5.3 transgene is also expressed in ectopic sites in the fore- and hindbrain. From tailbud stage onwards the transgene is expressed in rhombomere 3, and by the 5-somite stage also in rhombomere 5, similar to the transient expression observed for placZ2.4 (Fig. 3E-K; see Fig. 2E); expression lasts at least until 24 hours (Fig. 3K). Similarly, placZ5.3 is ectopically expressed in the future telencephalon and diencephalon (Fig. 3E-K). At the 15-somite stage, 5 regions of ectopic activation in the forebrain can be distinguished: the dorsal telencephalon, the anterior and posterior dorsal diencephalon, and two small clusters of cells in the ventral diencephalon, one of which is located in the distal optic stalk (Fig. 3H,I). At the 20-somite stage, as well as at 24 hours, only three ectopically expressing regions can be distinguished in the forebrain: (i) the telencephalic domain, (ii) the dorsal-anterior diencephalic domain and (iii) a small expression domain ventral to this (Fig. 3J,K). Since the same ectopic expression is seen in the GFP line carrying the identical promoter/enhancer fragment (t) and four diencephalic domains (d1-d4, fields denoted by dashed lines) and rhombomeres 3 and 5. (L) At 24 hours, pax2.1 is expressed identically to placZ5.3 (K) at the MHB, in the hindbrain and spinal cord. Ectopic expression of placZ5.3 is found in two diencephalic and one telencephalic domain and but no expression is detected in the optic stalk. Expression of placZ5.3 in the ventroanterior wall of the otic vesicle (insets show cross-sections at the hindbrain level) is low compared to pax2.1, which is expressed throughout the ventral vesicle (arrowheads). d1-d4, diencephalic domains; fb, forebrain; hb, hindbrain; mhb, midbrain-hindbrain boundary; os, optic stalk; otp, otic placode; r3, rhombomere 3; r5, rhombomere 5; sc, spinal cord; t, telencephalic domain.

(A-G,I) Dorsal views; (H,J-L) lateral views. For insets in K, L dorsal up. Anterior is to the left for all figures except A-C, where anterior is to the top.

**Fig. 3.** Stable transgenic expression of placZ5.3 at different stages of development in wild-type embryos. (A) Onset of pax2.1 expression at late gastrulation, 80% epiboly, at the MHB. (B) Onset of expression of placZ5.3 (blue) and expression of pax2.1 (red) at 90% epiboly at the MHB determined by double in situ hybridization. (D) Fluorescence image of the embryo in B. The expression domain of placZ5.3 appears nested within the broader domain of pax2.1 (brackets). Cells anterior and posterior to the placZ5.3 domain are expressing pax2.1 (arrowheads). The medial part of the pax2.1 domain is not expressing placZ5.3 (asterisk). (C) From tailbud stages onwards, expression of placZ5.3 (blue) and pax2.1 (red) is congruent at the MHB. (E-K) Expression pattern of placZ5.3 at tailbud (E), 5-somite (F), 10-somite (G), 15-somite (H,I), 20-somite (J) and at 24-hour (K) stages. Expression is found throughout development at the MHB (bracket), otic placode and from the 10-somite stage onwards in hindbrain and spinal cord neurones (G, arrowheads in H-J). Restricted, ectopic expression is found in the forebrain telencephalon (t) and four diencephalic domains (d1-d4, fields denoted by dashed lines) and rhombomeres 3 and 5. (L) At 24 hours, pax2.1 is expressed identically to placZ5.3 (K) at the MHB, in the hindbrain and spinal cord. Ectopic expression of placZ5.3 is found in two diencephalic and one telencephalic domain and but no expression is detected in the optic stalk. Expression of placZ5.3 in the ventroanterior wall of the otic vesicle (insets show cross-sections at the hindbrain level) is low compared to pax2.1, which is expressed throughout the ventral vesicle (arrowheads). d1-d4, diencephalic domains; fb, forebrain; hb, hindbrain; mhb, midbrain-hindbrain boundary; os, optic stalk; otp, otic placode; r3, rhombomere 3; r5, rhombomere 5; sc, spinal cord; t, telencephalic domain.

**In vivo monitoring of pax2.1 promoter activation by pGFP5.3**

To confirm the expression analysis of the lacZ reporter gene placZ5.3 by an independent insertion, and to study pax2.1 promoter activation in living zebrafish embryos, a transgenic zebrafish line for an analogous GFP reporter gene was produced: pGFP5.3 (Fig. 1B). Transcriptional activation of the construct was analysed by monitoring the fluorescence in living embryonic zebrafish with UV light microscopy and confocal laser scanning microscopy and by ISH (Fig. 5).
Overall, pGFP5.3 shows the same expression pattern as placZ5.3 at the level of ISH, but the GFP protein is detected with some delay relative to GFP RNA (not shown). GFP fluorescence is initially detected around the 4-somite stage, but from mid-somitogenesis stage onwards all placZ5.3 expression domains are also identifiable by GFP fluorescence (Fig. 5). As an in vivo counterstain for tissue structures we used the red dye Bodipy 564/570, which stains the yolky content of cells in the zebrafish embryo (Dynes and Ngai, 1998; Cooper et al., 1999) and thus allows a precise localization of GFP expression domains relative to morphological landmarks of the developing embryo (Fig. 5).

At 24 hours, expression of pGFP5.3 is detected in the endogenous pax2.1 expression sites at the MHB, in the ear, in the hindbrain and spinal cord interneurones. GFP distribution at the MHB appears broader compared to the ISH signals of pGFP5.3 (Fig. 5A,B). The expression domain includes the posterior tectum and tegmentum and the anterior cerebellum, although GFP expression in these regions appears weak. The peak of GFP expression at the MHB is localized in the anterior half of the MHB fold, which constitutes the dorsoposterior wall of the tectal ventricle (Fig. 5A,C), corresponding to the region of the strongest transcription of placZ5.3 and pax2.1 at this stage. The broader GFP protein distribution compared with the GFP ISH signal most likely reflects the broad domain of pGFP5.3 transcription at earlier developmental stages, and the higher stability of the GFP protein.

As for placZ5.3, GFP fluorescence in the developing ear is
Feedback regulation in MHB development

Fig. 6. Requirement of pax2.1 for the expression of the transgene placZ5.3 at the MHB. Expression of placZ5.3 at the MHB of a wild-type (A) and noi mutant (B) embryo at tailbud stage. Normal expression of placZ5.3 is seen in rhombomere 3 and the otic placode but fails at the MHB (arrowheads) in noi. (C) Double in situ hybridization at the same stage, showing normal transcription of pax2.1 (red) but no expression of placZ5.3 at the MHB (arrowheads) of a noi mutant. (D-F) Chimeric embryo at tailbud stage, generated by transplantation of wild-type/placZ5.3 cells into the MHB of a noi/placZ5.3 embryo at 50% epiboly. Expression of placZ5.3 in this chimera is restricted to the grafted cells (outlined area), which were visualized by a biotin-avidin assay (brown staining in E) and fluorescence (F). Although the clone is stretching beyond the MHB domain of placZ5.3 at the anterior and posterior (arrows), activation is only seen in a zone corresponding to the MHB. No placZ5.3-expressing cells are seen beyond the boundaries of the transplanted clone (see inset in E; dashed line demarcates the ventral surfaces of the epiblast). All images are dorsal views with anterior up. inj., injected side; otp, otic placode; r3, rhombomere 3; wt, wild type.

particularly strong in the ventroanterior wall of the otic vesicle. Ventroanteriorly to the ear, cells of the forming ganglion of the VIIIth cranial nerve are stained (Fig. 5E-G). This ganglion is formed by projection neurones that have been suggested to delaminate from the overlying otic vesicle (Haddon and Lewis, 1996). Since the ganglion itself does not express GFP RNA (not shown), GFP-positive cells of the VIIIth ganglion must have retained the protein throughout the process of delamination from the otic vesicle, thus providing direct support for this concept. Like placZ5.3, pGFp5.3 is expressed at this stage in hindbrain and spinal cord neurones (Fig. 5E-H). The same sites of ectopic expression were found for pGFp5.3 as for placZ5.3, which include rhombomeres 3 and 5 and the forebrain, where GFP expression at the 24-hour stage is detectable in a telencephalic and three diencephalic domains (Fig. 5I). In addition, a low level of pGFp5.3 expression was detected in the optic stalk (not shown), which probably reflects an earlier expression in the distal optic stalk (compare to Fig. 3E).

Feedback regulation of pax2.1 transcription at the midbrain-hindbrain boundary

Because some or all of the transgene-expression in a wild-type background might reflect auto- or feedback regulation of pax2.1 transcription, we examined placZ5.3 expression in the pax2.1 mutant no isthmus (noi) (Wada et al., 1989), and found evidence for a novel positive transcriptional feedback loop during MHB development (Figs 6-8). In homozygous noi− mutants, endogenous pax2.1 expression is correctly initiated at the MHB at 80% epiboly, indicating that primary transcriptional initiation of pax2.1 at the MHB does not depend on functional Pax2.1 and is thus not dependent on feedback regulation (Fig. 6C). From the 7-somite stage onwards, however, pax2.1 expression at the MHB is reduced in noi− mutants suggesting that feedback regulation could occur at this stage (Brand et al., 1996; Lun and Brand, 1998). Importantly, we find that transcription of the transgene is never activated at the MHB of noi−; placZ5.3/+ embryos, in contrast to wild-type embryos where we find expression of the transgene from 90% epiboly onwards (Fig. 6A-C). The placZ5.3 transgene thus contains a cis element, which mediates feedback regulation of pax2.1 transcription from 90% epiboly onwards. In contrast, it lacks the primary cis-regulatory element, which initiates pax2.1 transcription at 80% epiboly independent of endogenous Pax2.1 protein. A second expression site of pax2.1, which requires Pax2.1 function is the intermediate mesoderm (Fig. 4E,F). In noi− embryos at the 15-somite stage, expression of pax2.1 and placZ5.3 is equally affected in the posterior part of the developing pronephric duct, which probably reflects a general patterning defect, rather than a feedback regulation in this tissue, because transcriptional initiation of the transgene is unaffected in this tissue in noi−. In addition expression of pax2.1 is reduced in the developing cloaca in noi−.

To further analyse the novel feedback loop in MHB development we studied, in transplantation experiments, whether feedback regulation is cell-autonomous and whether it depends on Fgf signaling. First we created chimeras by transplanting cells from wild-type placZ5.3 transgenic donors into noi− placZ5.3/+ hosts at blastula stages. Chimeras were then tested by in situ hybridization for lacZ expression at the MHB at tailbud stage. In noi− mutant embryos carrying wild-type cell clones at the MHB the grafted cells expressed lacZ. In contrast, mutant host cells abutting the wild-type clone never expressed placZ5.3 (Fig. 6D-F). This shows that the requirement for Pax2.1 in feedback regulation at the MHB is cell-autonomous. We further tested for possible non-autonomy by analysing acerebellar (ace)/fgf8 mutant embryos, which initiate pax2.1 normally but fail to maintain expression (Reifers et al., 1998), and in embryos that had been treated with the Fgfr-inhibitor SU5402. In these embryos, early placZ5.3 expression between 90% epiboly and 4 somites at the MHB is not affected, whereas later expression of the transgene is successively reduced from the 10-somite stage onwards, according to the ace-specific or SU5402-induced structural defects during MHB development (data not shown). Thus, Fgf8, which is initially expressed posterior to the pax2.1 domain at the MHB, and Fgfr-signaling in general are not required for pax2.1 feedback regulation, supporting the finding above that this process is cell-autonomous.

The observed feedback regulation of placZ5.3 expression at the MHB could be directly mediated by Pax2.1 or indirectly by Pax2.1-dependent factors. Analysis of the noi mutant has revealed that transcription of eng2 and eng3 is dependent on Pax2.1 (Lun and Brand, 1998). Onset of eng2 and eng3 expression at the MHB is delayed relative to and nested within...
the *pax2.1* domain, similar to what we found for placZ5.3 (Fig. 3A-D). Furthermore, the *pax2.1* promoter/enhancer fragment contains no Pax2.1-binding sites, ruling out direct regulation by Pax2.1 (unpublished data). We therefore sought to test whether the *pax2.1* feedback regulatory loop requires *engrailed* genes for its function. To test this we inactivated *eng2* and *eng3* by injecting 4 ng antisense morpholino oligonucleotides (Nasevicius and Ekker, 2000) which specifically block the translation of these genes (Scholpp and Brand, 2001). A single knock-down of *eng2* or *eng3* had no effect (not shown). In contrast, co-injection of both morpholinos causes a reduction of *pax2.1* expression at the MHB from the 12-somite stage onwards (Fig. 7A,B). After the 12-somite stage *pax2.1* expression at the MHB was no longer detectable in the injected embryos (not shown). *eng2* and *eng3* are therefore necessary to maintain, but not initiate, expression of *pax2.1* at the MHB. At pharyngula stages, the morphological phenotype of *noi*– mutants is nicely phenocopied by the knock-down of *eng2* and *eng3* (Scholpp and Brand, 2001). To determine if *eng2* and *eng3* are required for feedback regulation, we injected *eng2* and *eng3* morpholinos into transgenic pGFP5.3 embryos. Already at the tailbud stage pGFP5.3 expression at the MHB was absent in 58%, or strongly reduced in 27%, of the injected embryos (n=204=100%; Fig. 7C,D). Expression in the otic placode was unaffected, as expected. At later stages neither pGFP5.3 RNA nor GFP protein expression at the MHB recovered (Fig. 7E,F). Therefore, *eng2* and *eng3* are required for activity of the feedback loop detected by expression of the transgenes. We furthermore tested whether *eng3* expression is sufficient to activate feedback loop activity, by injecting *eng3* mRNA at the 2-cell stage into *noi*– mutant embryos; *eng2* was not tested since we expected it to behave identically. We found that *eng3* injection is indeed sufficient to rescue MHB expression of pGFP5.3 in the injected *noi*– embryos (Fig. 7G-I), showing that it is possible to bypass the requirement for *pax2.1* for feedback regulation. We conclude that *eng2* and *eng3* are required and sufficient to mediate positive feedback regulation of *pax2.1* expression at the MHB, and that this regulation only indirectly requires *pax2.1* (Fig. 8).

**DISCUSSION**

We have described the isolation of a functional promoter/enhancer fragment of *pax2.1* by cloning lacZ and GFP reporter
Characterization of the pax2.1 promoter/enhancer region in transgenic embryos

Although the analysis of transient expression patterns of reporter genes is strongly limited by the high degree of mosaicism in the transient expression patterns, as well as by the large numbers of embryos that have to be injected, the analysis can serve as a first functional test for promoter/enhancer fragments (Long et al., 1997; Meng et al., 1997; Muller et al., 1999; Higashijima et al., 2000). On the basis of transient expression patterns we have selected a pax2.1 promoter/enhancer fragment for the generation of stable transgenic lines driving expression of lacZ or GFP that reproduce many aspects of endogenous pax2.1 expression at the MHB, hindbrain and spinal cord neurones and the pronephros. These lines will be useful in future dynamic imaging studies of these tissues. Stable germline transmission of these transgenes occurs into the F4 generation so far, and we have since been able to generate additional promoter fusion constructs which also express reliably and in the correct pattern (unpublished data). Several cases of unstable or mosaic expression of transgenes have been described in zebrafish, mostly using heterospecific promoters of non-zebrafish origin (Westerfield et al., 1992; Reinhard et al., 1994; Kim et al., 1996). Our results add to the short, but growing list of cases where use of a zebrafish promoter has allowed stable and reproducible expression and continued germline transmission over several generations. However, not all transgenic lines we isolated express reliably. As described above, generation of several transgenic lines per construct can circumvent this problem.

Close examination of embryonic transgene expression in comparison with the endogenous pax2.1 gene revealed several subtle differences in the temporal and spatial regulation. Since the same expression pattern is observed in two independently generated lines, with different reporter genes, the observed differences between pax2.1 and reporter gene expression must reflect the presence or absence of regulatory elements, rather than being due to positional ‘enhancer trapping’ effects. These observations provide evidence that several pax2.1 expression domains like the intermediate mesoderm, the embryonic ear and MHB, are composed of independently regulated subdomains.

Initiation and feedback regulation of pax2.1 at the MHB

The presence of different regulatory elements and tissue subdomains is probably of biological importance, as is illustrated by our more detailed analysis of the regulatory logic in MHB development. Our results distinguish two independent regulatory phases of pax2.1 transcription at the MHB, which we will refer to as ‘initiation’ and ‘feedback’ phases (Fig. 8). The initiation phase starts with the transcriptional activation of the endogenous pax2.1 gene around 80% epiboly. Since pax2.1 transcription is unaffected in noi− embryos at this stage, initiation does not require Pax2.1 protein (Brand et al., 1996; Lun and Brand, 1998). The second feedback phase commences around 90% epiboly and is strictly dependent on Pax2.1 protein, as our analysis of transgene expression in noi− mutants clearly demonstrates. Although feedback regulation is corrupted in the mutant noi− embryos, no defect of the endogenous pax2.1 RNA distribution is seen before approximately the 7-somite stage, which may simply reflect pax2.1 RNA stability. More likely, the initiation mechanism can still drive pax2.1 transcription although the feedback mechanism is not working in the noi− background. The two different regulatory mechanisms of pax2.1 transcription at the MHB therefore overlap in time and space, and are partially redundant at stages between 90% epiboly and 7 somites. Decay of pax2.1 transcripts from the 7-somite stage onwards in noi− could then reflect a switch from initiation to feedback regulation. The exact timing of the switch is not clear, since the decay rate of pax2.1 RNA is not known.

Since we did not detect any consensus sites for Pax2/5/8 binding in the analysed upstream sequence (unpublished data), feedback regulation is probably not due to direct binding of Pax2.1 to its own promoter/enhancer region. Alternatively, feedback regulation might be mediated by transcription factor(s) controlled by Pax2.1, such as the Eng homeodomain transcription factors Eng2 and Eng3 (Joyner and Martin, 1987; Ekker et al., 1992; Fjose et al., 1992). In zebrafish, eng2 and eng3 expression at the MHB strictly depends on Pax2.1. In the wild type, expression of the eng2 and eng3 is delayed relative to pax2.1 and nested within the pax2.1 domain, very much like the early expression domain of the transgenes, and indeed Pax2.1 protein binds to Pax2/5/8 consensus binding sites (Lun and Brand, 1998), which are required to drive Pax2-dependent transcription of the mouse En2 promoter (Song et al., 1996; Li Song and Joyner, 2000). This raised the possibility that feedback regulation might be mediated by Engrailed proteins. A morpholino knock-down of eng2 and eng3 phenocopies the noi− phenotype (Scholpp and Brand, 2001). We therefore tested whether morpholino knock-down of eng2 and eng3 also affects expression of the transgene pGFP5.3 at the MHB, and found that this obliterates expression. Moreover, eng3 mRNA injection into noi− embryos restores the feedback regulation of transgene expression at the MHB. Although in this gain-of-function situation it is difficult to rule out a dominant, non-physiological effect, such as interference with another regulator, these results generally are consistent with the more stringent loss-of-function experiments. Together, these experiments indicate that the feedback regulation of pax2.1 transcription is mediated via Engrailed transcription factors (Fig. 8). The detailed mechanism through which feedback regulation occurs remains unclear. Engrailed proteins are typically thought to act as repressor proteins, which is at odds with the loss of positive feedback regulation that we observe after eng2 and eng3 knock-down. Under certain circumstances...
Engrailed proteins can however act as activators, for instance as heterodimers with Pbx1 in regulating Fgf8 (Gemel et al., 1999), or in regulating MAP1b (Montesinos et al., 2001). Interaction with the *Drosophila* Pbx homologue Extradenticle can convert several transcriptional repressor homeodomain proteins into activators (Pinsonneault et al., 1997). We find four potential Eng-binding sites in the analysed pax2.1 promoter/enhancer fragment (unpublished data), suggesting that feedback regulation could also have a direct Engrailed-dependent component.

Based on the analysis of the *noi* and *ace* mutants we have previously proposed that development of the MHB in zebrafish proceeds through at least two phases: (i) an establishment phase during late gastrulation, which is reflected by the early requirement of Pax2.1 for induction of *eng2* and *eng3*, and (ii) a maintenance phase during mid-somitogenesis, which depends on the secreted signaling molecule Fgf8 (Lun and Brand, 1998; Reifers et al., 1998) (for a review, see Rhinn and Brand, 2001). In addition, Wnt1-signaling is required for maintenance of the MHB in mouse embryos (McMahon et al., 1992). Furthermore, it is known that expression of Fgf8 at the MHB as a component of the maintenance phase is lacking from mid-somitogenesis stages onwards in *noi* mutants (Reifers et al., 1998). Thus, the two pathways of establishment and maintenance during MHB development are both affected in the *noi*mut*20a* mutant. This raises a question about the relationship between (feedback) regulation of pax2.1 transcription and these two phases of MHB development.

At later stages, expression of the transgenes is never observed at the MHB in a mutant background; this could be due to a failure to activate the feedback loop cell autonomously, reflecting the establishment phase of MHB development. Alternatively, absence of a non-autonomous pax2.1-activating signal in the mutants, as a component of the MHB maintenance function, could affect feedback regulation. It was therefore important to test whether the apparent absence of feedback regulation in *noi* was due to secondary effects, such as failure to express signaling molecules required for proper maintenance of MHB development. Our cell transplantation revealed however, that wild-type cells are still able to express the transgene if transplanted into the MHB of *noi*mut*20a* mutant embryos. In addition, until about the 10-somite stage, placZ5.3 transgene expression was normal in *acerebellar* mutant embryos, which lack functional Fgf8 for MHB maintenance. This is not due to the presence of other Fgfs that might be unaffected in *acerebellar* mutants, since transgene expression is equally unaffected in embryos which have been treated with a pharmacological inhibitor of Fgf receptor function, SU5402, during late gastrulation (data not shown). This finding also shows independently that initiation of the feedback loop does not require Fgf signaling, and the feedback loop must therefore be distinct from the regulatory situation encountered during the MHB maintenance phase.

The function of the feedback loop is currently unknown. A reasonable assumption is that it may serve to enhance MHB gene expression and thereby maintain regional subdivisions of the neural plate. In addition, cell-autonomous feedback regulation at the MHB could be a prerequisite for the continued and autonomous development of the region beyond gastrulation, where primary signals (planar or vertical) directly induce pax2.1. Furthermore, feedback regulation offers an attractive entry point for non-autonomous signals during the maintenance phase, when the pathways become interdependent. The later loss of the transgene expression from the MHB of homozygous *acerebellar* mutants is consistent with this possibility, and suggests that Fgf8 might impinge on feedback regulation beyond the 10-somite stage. Moreover, since the Fgf8 promoter/enhancer region contains Engrailed binding sites (Gemel et al., 1999), the interactions are likely to be reciprocal, and the feedback loop could therefore serve to maintain the MHB organizer by driving Fgf8 expression.

Regulatory elements controlling MHB expression of murine *Pax2* (Rowitch et al., 1999; Kuschert et al., 2001; Pfeffer et al., 2002) and *Pax5* (Pfeffer et al., 2000) have been described. The relationship between the feedback regulation described here and the elements described for the murine *Pax2* gene (Pfeffer et al., 2002) remains to be clarified. Pfeffer et al. have identified three MHB-specific enhancers in the murine *Pax2* promoter, one early and two later acting (Pfeffer et al., 2002); these enhancers have not yet been tested in *Pax2*mut*20* mutants for primary versus feedback modes of regulation. One early acting enhancer is sufficient to induce transgene expression in the early neural plate, and requires three homeodomain binding sites for its activity. Two of these bound the Pou-type transcription factor Oct3/4 that is orthologous to zebrafish Pou2 (Reim and Brand, 2002); En1 protein did not bind. Consistent with these studies, analysis of the zebrafish MHB mutant *spiel-ohne-grenzen/pou2* showed that Pou2 is a regulator of *pax2.1* initiation (Belting et al., 2001; Burgess et al., 2002; Reim and Brand, 2002). This enhancer is therefore most likely different from the enhancer controlling the maintenance loop we describe here, and is probably responsible for initiating Pax2 expression. The two other mouse enhancers are activated only later, from about the 4-somite stage onwards, but drive expression only transiently. Both are required to maintain late Pax2 expression, and one of them binds Pax2/5/8 proteins to allow cross- or autoregulation similar to that described for the Pax5 enhancer (Pfeffer et al., 2000); En-protein binding was not tested. The Pax5 enhancer similarly activates expression at later stages through Pax2 and Pou homeodomain binding sites. These enhancers may therefore mediate a different mode of regulation than the Engrailed-dependent feedback regulation described here.

**Regulation of pax2.1 in the embryonic kidney**

Studies on developmental gene expression and mutant mouse and zebrafish embryos indicate that early expression of *Pax2* in the intermediate mesoderm is essential for normal kidney development (Dressler et al., 1990; Krauss et al., 1991; Püschel et al., 1992). In zebrafish, analysis of *noi*–mutants has revealed that *pax2.1* is required to maintain differentiation of the pronephric duct epithelium and pronephric tubules (Brand et al., 1996; Majumdar et al., 2000). Similarly, embryonic inactivation or deregulation of mouse *Pax2* leads to severe abnormalities in differentiation of the intermediate mesoderm into the pronephric duct and kidney tubules of the metanephros (Torres et al., 1995; Favor et al., 1996).

Although the embryonic expression pattern and requirement of *Pax2* have been studied in several vertebrates, it remains unclear how the gene is spatially and temporally regulated. The 5’ untranslated regions of the mouse and human *Pax2* genes contains three *cis*-regulatory DNA elements, which are
required for transcriptional repression of Pax2 by the Wilms’
tumor suppressor protein WT1 in cell culture (Ryan et al., 1995; Stayner et al., 1998). In addition, an 8.5-kb fragment of the
Pax2 upstream region includes cis-regulatory elements for
mesonephric duct expression in transgenic mouse embryos
(Rowitch et al., 1999; Kuschi et al., 2001). We find that
different temporal and spatial aspects of the early expression
of zebrafish pax2.1 in the embryonic kidney are probably
regulated via independent cis-regulatory elements. The early
onset of pax2.1 transcription between 90% epiboly and tailbud
stage is not reflected by the expression of the placZ5.3
transgene, and seems independently regulated from the later
phase, which is reproduced by placZ5.3. Furthermore, the
transgene is not expressed beyond the 20-somite stage,
although endogenous pax2.1 expression persists into later
stages of kidney differentiation (Majumdar et al., 2000). Also
in contrast to pax2.1, placZ5.3 is not expressed at mid-
 somitogenesis stages in the anterior-most region of the
developing duct and the cloaca. This suggest independent
mechanisms of cis-regulatory control of pax2.1 transcription at
different anterior-posterior levels of the pronephros and in the
cloaca. In addition, comparison of wild-type and noi mutants
revealed a previously unknown requirement for Pax2.1 protein
in maintaining pax2.1 transcription in the posterior duct at mid-
somitogenesis stages. Thus, the expression of pax2.1 in the
intermediate mesoderm of the embryonic kidney appears to be
governed by independently regulated temporal and spatial
transcriptional control elements, which suggests that multiple
factors interact during the development of different regions
along the AP axis of the pronephros.

Transcriptional repression of pax2.1 in the fore- and
hindbrain

A consideration of the transcriptional control elements
excluded from the described promoter/enhancer fragment of
pax2.1 illustrates the importance of pathways that ultimately
lead to tissue-specific transcriptional repression of pax2.1,
although the function of pax2.1 repression in these tissues is
currently unclear. Examples for this are the ectopic, spatially-
restricted expression domains of placZ5.3 in the forebrain
and in rhombomeres 3 (r3) and 5 (r5) from stages of late
gastrulation onwards.

The zinc-finger transcription factor Krox20 (Egr2 –
Zebrashif Information Network) is transcribed from the end of
gastrulation in r3 and r5 of zebrafish embryos (Oxtoby and
Jowett, 1993) and could therefore mediate regulation of pax2.1
in the hindbrain. Krox20 directly controls expression of Hox
genes, Eph receptors and follistatin in r3 and r5 (Nonchev et
al., 1996; Seitandou et al., 1997), and indeed the pax2.1
upstream sequence contains six potential Krox20 binding sites
(Chavrier et al., 1990). Alternatively, pax2.1 could be activated
independently in r3 and r5 by different factors, or regulated via
a diffusible factor in a non cell-autonomous manner from
adjacent rhombomeres. Although Fgf8 is normally expressed
in r4 (Reifers et al., 1998), it is an unlikely candidate, since
rhombomeric transgene expression in acerbellar mutants is
normal.

The ectopic activation of placZ5.3 in the fore- and hindbrain
in addition to the pax2.1-like domain at the MHB is especially
evident at the 20-somite stage, where the overall expression
pattern has a ‘multiple-stripe’ appearance, akin to a segmental
pattern. The Drosophila Pax-2 orthologue shaven (DPax2), is
at embryonic stages expressed in a segmental pattern in the
developing external sensory organs of the CNS and PNS
(Czerny et al., 1997). There is as yet no direct evidence for a
similar metameric expression pattern of Pax2 orthologues in
other organisms, although it has been previously noted that
pax2.1-expressing interneurones in the hindbrain and spinal
cord form repetitive clusters along the AP axis of the embryo
(Mikkola et al., 1992). However, the Drosophila engrailed
orthologue AmphiEn of the basal chordate Amphioxus is
expressed in ‘metameric’ stripes along the AP axis of the
segmentally organized mesoderm (Holland et al., 1997),
suggesting a relationship between segmentation in protostomes
and deuterostomes. Although it is unclear whether metameric
subdivisions exist in the midbrain and isthmic regions,
metamerism is a well established concept for the vertebrate
hindbrain (Lumsden and Keynes, 1989). An interesting
possibility is therefore that the partially metameric pattern
produced by the pax2.1 promoter/enhancer fragment reflects
an ancestral state of pax2 gene regulation in a ‘metameric’
pattern, which in modern vertebrates, possibly through
evolution of additional silencer elements, became restricted to
one stripe at the MHB.

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