Development 129, 3227-3239 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV1823

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

Alexander Picker[1,†], Steffen Scholpp[1], Heike Böhli[1], Hiroyuki Takeda[2] and Michael Brand[1,*]

[1]Max-Planck-Institute of Molecular Cell Biology and Genetics (Dresden), Pfotenhauerstr. 108, 01307 Dresden, Germany
[2]Division of Early Embryogenesis, National Institute of Genetics, Mishima 411-8540, Japan
[†]Present address: Lion Bioscience, Waldhofer Strasse 98, 69123 Heidelberg, Germany
*Author for correspondence (e-mail: brand@mpi-cbg.de)

Accepted 8 April 2002

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; Urbanek 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 (Sanyanusin et al., 1995; Torres et al., 1995; Brand et al., 1996; Favor et al., 1996; Schwarz et al., 1997). In zebrafish, *pax2.1* (*pax1a* – 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 a null mutant allele for *pax2.1*, *no isthmus*^{tu29a} *(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 phase), but expression becomes dependent on Pax2.1 during mid-somitogenesis stages (maintenance phase). Likewise, analysis of a zebrafish *fgf8* mutant, *acerebellar (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 *noi*^{tu29a} 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 *cis*-regulatory 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 *noi*^{tu29a}, 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 F_1 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 zebrafish 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 G119-3 (5′-AGCGTATCTCTACCTACTGTACAG-3′) was [γ^{32}-P]ATP 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, transplantations 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 (Fürthauer et al., 1997). The morpholinos MO^{eng2} and MO^{eng3} (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 beta-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 β-gal activity. Control injection of the *lacZ* vector alone gave no β-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. In addition, weak activation was found especially in forebrain (Fig. 2A-C and G,H). The shorter constructs placZ2.4 and placZ0.8 showed very strong ectopic transcriptional activation (Fig. 2D,F). Restricted expression was only detected for placZ2.4 in a stripe-like domain in the hindbrain region (Fig. 2E). None of the constructs showed expression in the optic stalk (Fig. 2I). Around the 24-hour stage, construct placZ4.5 is expressed in the otic vesicle, hindbrain and spinal cord, but not at the MHB and the pronephros (Fig. 2G,I). At the same stage, construct placZ5.3 was expressed in all of these tissues (Fig. 2H,I). Thus, both placZ4.5 and placZ5.3 show equal reporter gene expression at the MHB at the 10- to 12-somite stage, but around 24 hours, placZ5.3 is more reliably expressed (Fig. 2J). Comparison of the total number of expressing cells per embryo with the number of cells expressing β-gal in ‘appropriate’ locations, showed that placZ4.5+ and placZ5.3 were activated 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 F_0 founder fish, PCR-screening of genomic DNA of their F_1 progeny identified four F_0 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 F_4 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).

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

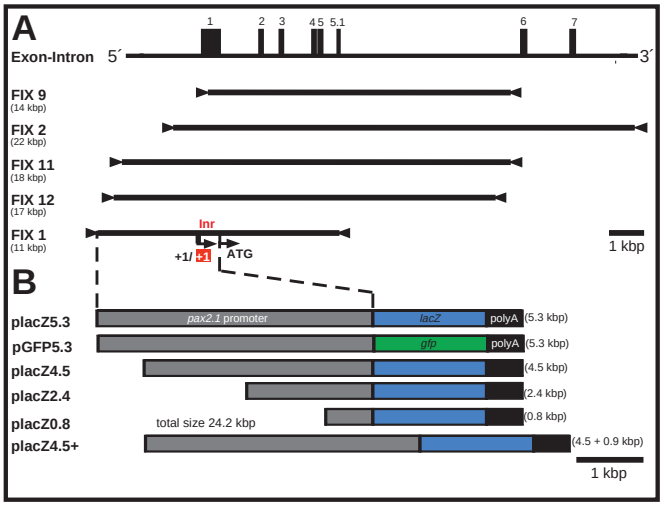

Fig. 1. Structure of *pax2.1*, genomic clones and reporter genes. (A) Five genomic *pax2.1*-clones, which contained 5.3 kb of sequence upstream of the translational start at the 5′ end (FIX1), including at least exon 7 at the 3′ end (FIX2), were isolated by screening a lambda phage library. Orientation 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.

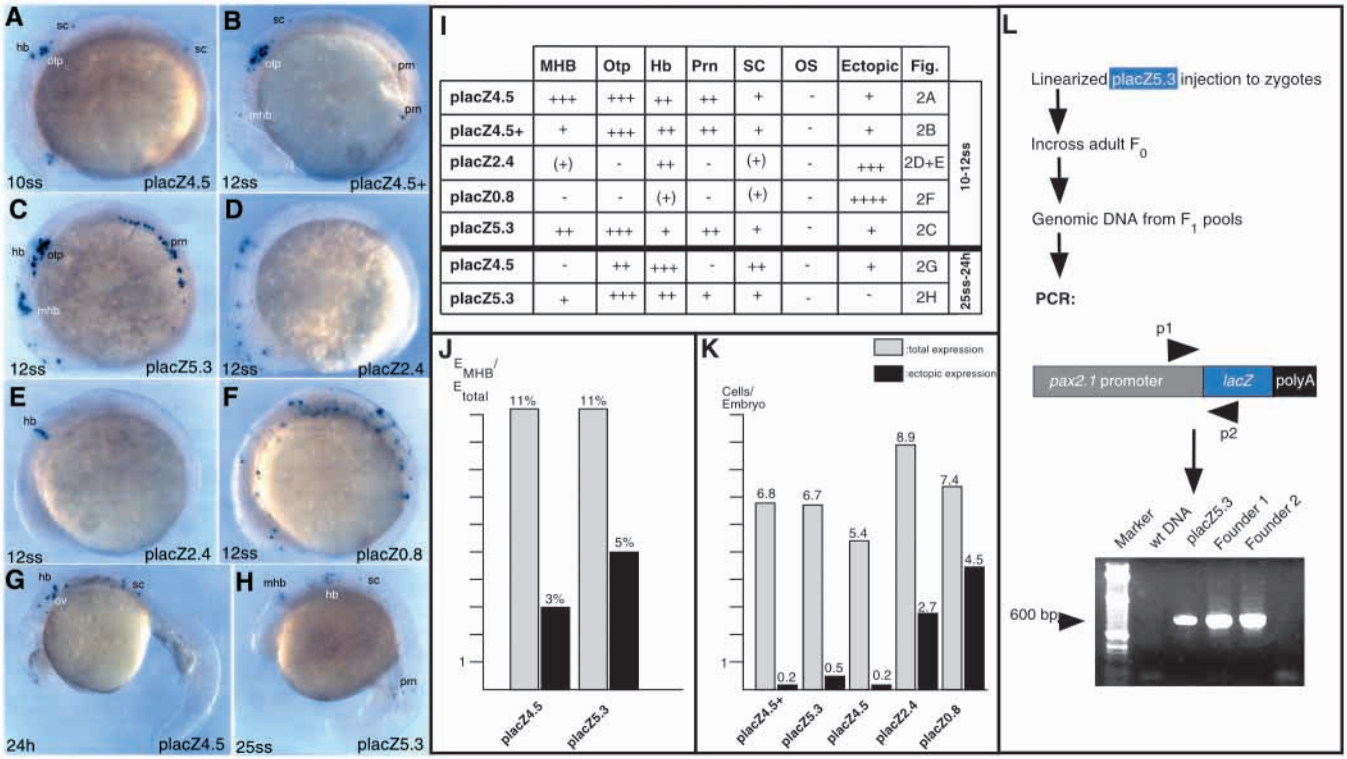

Fig. 2. Transient expression analysis of *pax2.1* promoter/enhancer *lacZ* reporter genes. (A-H) Mosaic β-gal activity of the five constructs between the 10-somite stage and 24 hours after zygotic injection. (A-C,G,H) Constructs placZ4.5, placZ4.5+ and placZ5.3 show transient expression in the MHB, in 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; E_{MHB}/E_{total}, 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.

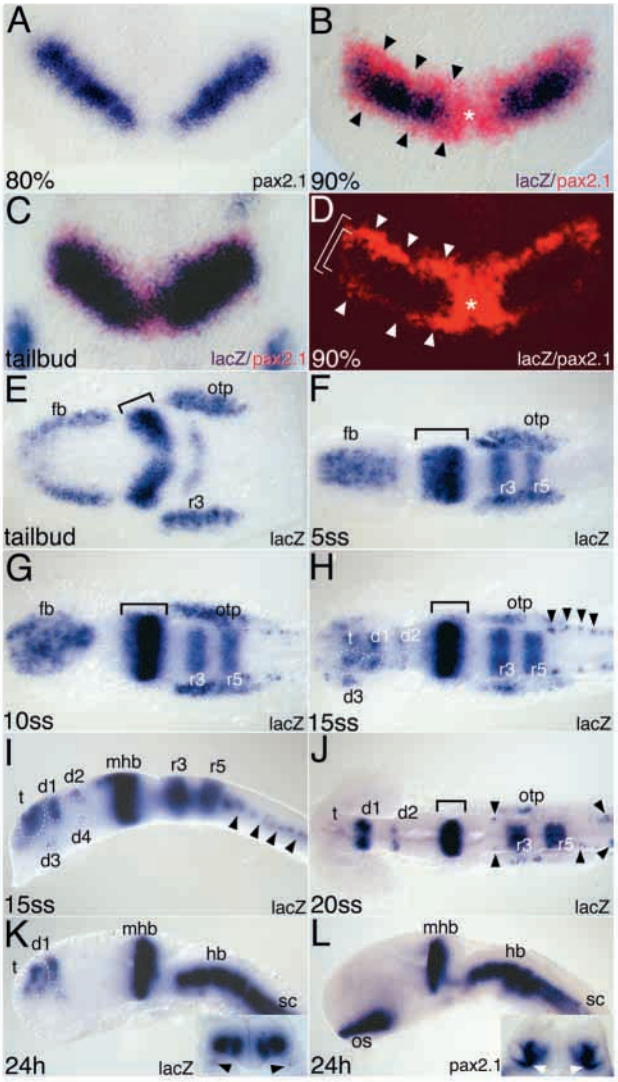

Fig. 3. Stable transgenic expression of placZ5.3 at different stages of development in wild-type embryos. (A) Onset of *pax2.1* expression at late gastrulation, 80% epiboly, at the MHB. (B) Onset of expression of placZ5.3 (blue) and expression of *pax2.1* (red) at 90% epiboly at the MHB determined by 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. (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.

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 expression is unlikely to result from enhancer trapping effects. Instead expression must result from the absence of specific regulatory DNA elements, which are required for regional transcriptional repression.

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).

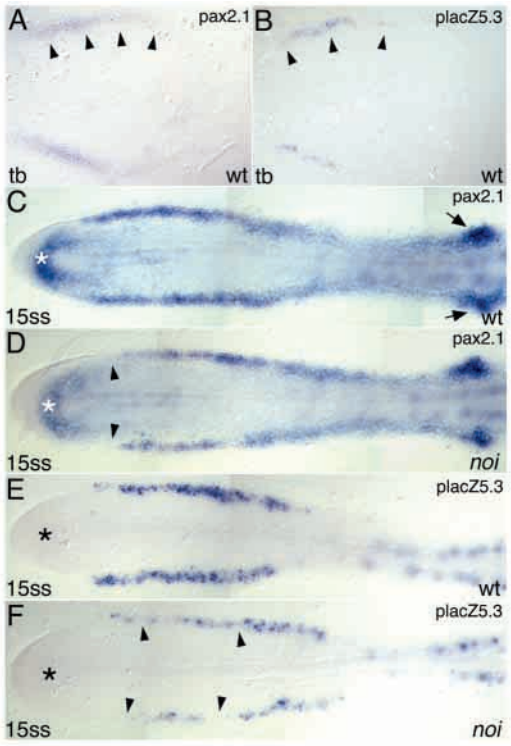

Fig. 4. Expression of placZ5.3 in the developing kidney of wild-type and noi mutant embryos. (A) Expression of *pax2.1* in the anterior portion of the intermediate mesoderm and (B) weaker expression of placZ5.3 in the same region at tailbud stage. (C-F) Expression of *pax2.1* (C,D) and placZ5.3 (E,F) at 15 somites in wild-type (C,E) and *noi* (D,F) embryos. In wild-type embryos placZ5.3 is not expressed in the anterior part of the *pax2.1* domain in the future pronephros (arrows) and in the devloping cloaca (asterisk). In *noi* embryos the same reduction of expression in the posterior pronephric duct is observed for *pax2.1* and placZ5.3.

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

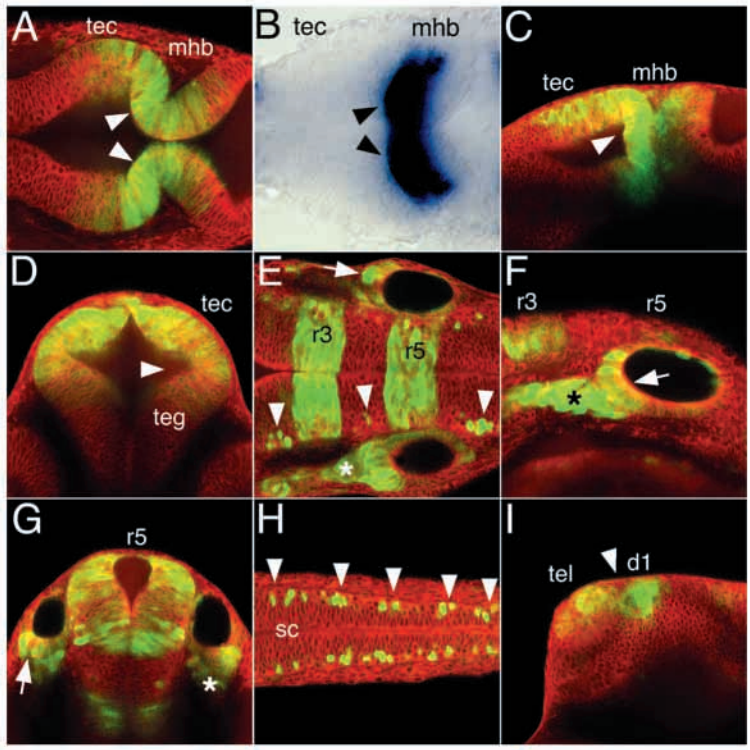

Fig. 5. Expression of pGFP5.3 in living wild-type embryos at 24 hours. (A**)** Horizontal section through the MHB. GFP is found at the MHB, with a peak of expression at the anterior isthmic fold (arrowheads) and less GFP in the midbrain tectum and posterior isthmic fold. (B) GFP ISH at 24 hours, showing restricted transcription of pGFP5.3 at the anterior isthmic fold. Compared to the GFP protein distribution (A) no expression is found in the tectum and posterior isthmic fold. (C) Parasagittal section through the MHB, showing the peak of GFP fluorescence at the anterior isthmic fold (arrowhead). (D) Cross section through the MHB at the level of the anterior isthmic fold, showing GFP expression in the tectum and ventral to the sulcus limitans (arrowhead) in the tegmentum. (E) Horizontal and (F) parasagittal and cross section (G) through the rhombencephalon, showing GFP in isolated neurones (arrowheads), in the ventroanterior otic vesicle (arrow), in the region of the VIIIth ganglion (asterisk) and in the rhombomeres 3 and 5. (H) Horizontal section through the trunk, showing GFP in isolated clusters of cells in the lateral spinal cord (arrowheads). (I) Parasagittal section through the forebrain, showing GFP in the dorsoposterior telencephalon and dorsoanterior diencephalon. All images except B are single optical sections generated with a confocal microscope. Embryos were counterstained with the red dye Bodipy 564/570. (A,B,E,H) Dorsal views with anterior to the left; (C,F,I) lateral views with anterior to the left; (D,G) Cross sections with dorsal up. d1, diencephalic domain, mhb, midbrain-hindbrain boundary; sc, spinal cord; tec, tectum; teg, tegmentum; tel, telencephalic domain; r3, rhombomere 3; r5, rhombomere 5.

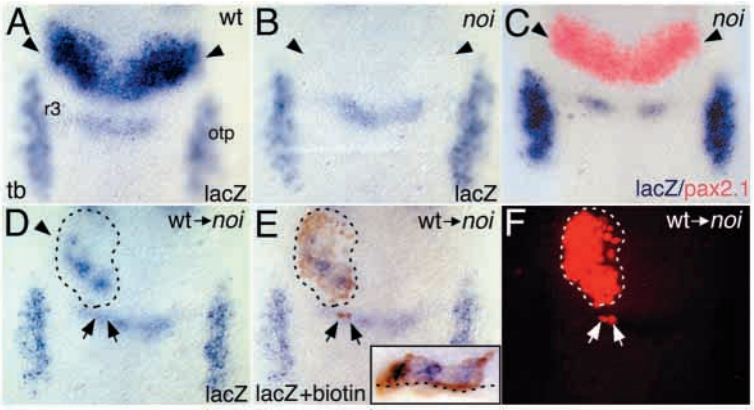

Fig. 6. Requirement of *pax2.1* for the expression of the transgene placZ5.3 at the MHB. Expression of placZ5.3 at the MHB of a wild-type (A) and *noi* mutant (B) embryo at tailbud stage. Normal expression of placZ5.3 is seen in rhombomere 3 and the otic placode but fails at the MHB (arrowheads) in *noi*. (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 transplantion 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 (Fig5E,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^{tu29a})*, 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 Fgf-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

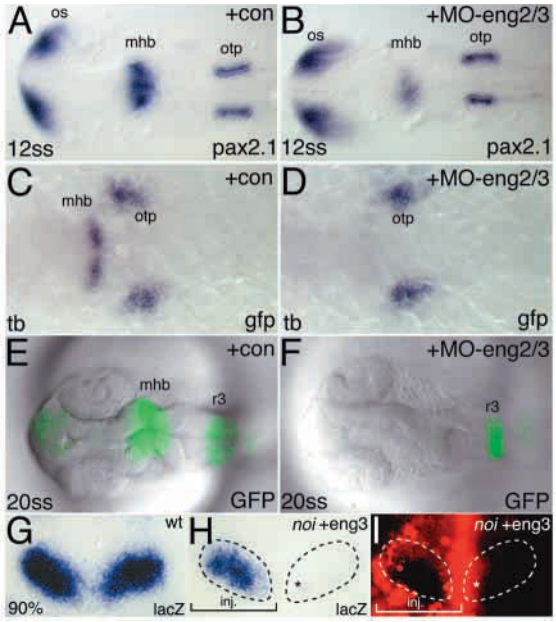

Fig. 7. Positive feedback regulation of *pax2.1* expression at the MHB. Injection of 4 ng antisense morpholino oligos against *eng2* and *eng3* leads to a specific block of translation. At the 12-somite stage there is a strong reduction of *pax2.1* RNA expression (B) compared to the control (A). The expression of pGFP5.3 is absent in morpholino-injected embryos from the onset (D) compared with the control injection (C). In later stages the expression of GFP protein is also absent at the MHB (F) compared with the control (E). (G-I) Expression of pGFP5.3 at the MHB at 90% epiboly in a wild-type embryo (G) and in a *noi*$^{-}$ mutant embryo (H), which had been unilaterally coinjected with *eng3*-mRNA (G,H) or a fluorescent lineage tracer (I). The expression of pGFP5.3 is rescued on the injected side (brackets). A few cells are also pGFP5.3 positive (asterisk) on the non-injected side, where the lineage label is detected close to the midline, possibly due to weak RNA diffusion after injection. A-F are dorsal views with anterior to the left; in G-I anterior is up. mhb, midbrain-hindbrain boundary; inj., injected side; opt, otic placode; os, optic stalk; r3, rhombomere 3; wt, wildtype.

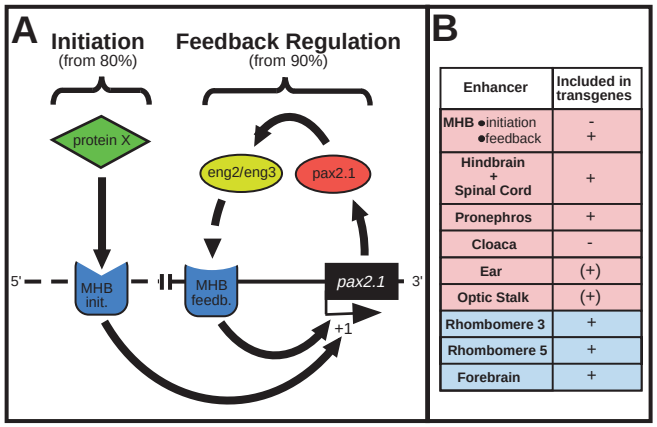

Fig. 8. Transcriptional regulation of *pax2.1* at the zebrafish MHB and promoter elements. Pax2.1 expression in MHB development is controlled by two distinct modes: initiation and feedback regulation. (A) A *cis*-element (MHB feedb.) which governs positive feedback regulation via *pax2.1* (red) and *eng2* and *eng3* (yellow) from 90% epiboly onwards was identified. A second *cis*-element that governs transcriptional initiation of the *pax2.1* gene (black box) at 80% epiboly (MHB init.) by an unknown protein X (green) is not included in the promoter region under study (indicated by a dashed line). Other work indicates that protein X may be Pou2/Oct3/4 (see text). Both modes of regulation overlap during early somitogenesis stages. (B) *cis*-regulatory enhancer elements for *pax2.1* transcription (enhancers that reflect endogenous expression domains are shown in light red and enhancers that regulate ectopic expression are in light blue) at the midbrain-hindbrain boundary (MHB), in the hindbrain, spinal cord and pronephros. The enhancer elements for the embryonic ear and optic stalk are only partially contained (brackets) and cloacal expression of *pax2.1* is not driven by the transgenes. Additional, normally repressed, regulatory elements driving expression in the *pax2.1*-negative rhombomeres 3 and 5 and the forebrain are uncovered in their activity by lack of other parts of the *cis*-regulatory region.

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. 7.E,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

gene constructs and testing them in transgenic zebrafish lines. The transgenes are reproducibly expressed in many of the *pax2.1*-expressing tissues of the zebrafish embryo including the MHB, hindbrain, spinal cord neurones and the intermediate mesoderm. Ectopic spatially restricted expression of the transgenes in rhombomeres 3 and 5, as well as in the forebrain, further shows that specific transcriptional repression plays a crucial role for *pax2.1* regulation. A combination of the transgenic line with cell transplantations, RNA misexpression, morpholino knock-down and mutant analysis reveals a novel regulatory step in MHB development, defined by a positive feedback loop in transcriptional regulation of *pax2.1* during late gastrulation. This feedback loop requires *noi/pax2.1* cell-autonomously, via the *pax2.1* target genes *eng2* and *eng3*, and can be restored in *noi* mutants by reactivating *eng3*.

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 F_4 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*^{tu29a} 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*^{tu29a} 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*$^{-}$ 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 supressor 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; Kuschert 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 – Zebrafish 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; Seitanidou 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 *acerebellar* 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.

We thank the members of the Brand laboratory for stimulating discussions and help, in particular Klaus Lun, Frank Reifers and Claudia Lohs. This work was supported by grants from the Deutsche Forschungsgemeinschaft, the European Union (QLRT 2000-02310), and the Max Planck Society. Hiroyuki Takeda was supported by the Ministry of Agriculture, Forestry and Fisheries of Japan.

REFERENCES

Amsterdam, A., Lin, S. and Hopkins, N. (1995). The Aequorea victoria green fluorescent protein can be used as a reporter in live zebrafish embryos. *Dev. Biol.* **171**, 123-129.

Amsterdam, A., Lin, S., Moss, L. G. and Hopkins, N. (1996). Requirements for green fluorescent protein detection in transgenic zebrafish embryos. *Gene* **173**, 99-103.

Ang, S. L., Jin, O., Rhinn, M., Daigle, N., Stevenson, L. and Rossant, J. (1996). A targeted mouse Otx2 mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain. *Development* **122**, 243-252.

Belting, H. G., Hauptmann, G., Meyer, D., Abdelilah-Seyfried, S., Chitnis, A., Eschbach, C., Soll, I., Thisse, C., Thisse, B., Artinger, K. B., Lunde, K. and Driever, W. (2001). *spiel ohne grenzen/pou2* is required during establishment of the zebrafish midbrain-hindbrain boundary organizer. *Development* **128**, 4165-4176.

Brand, M., Granato, M. and Nüsslein-Volhard, C. (2002). Keeping and raising zebrafish. In *Zebrafish: A Practical Approach* (ed. C. Nüsslein-Volhard and R. Dahm). Oxford: Oxford University Press.

Brand, M., Heisenberg, C.-P., Jiang, Y.-J., Beuchle, D., Lun, K., van Eeden, F. J. M., Furutani-Seiki, M., Granato, M., Haffter, P., Hammerschmidt, M., Kane, D. A., Kelsh, R. N., Mullins, M. C., Odenthal, J. and Nüsslein-Volhard, C. (1996). Mutations in zebrafish genes affecting the formation of the boundary between midbrain and hindbrain. *Development* **123**, 179-190.

Burgess, S., Reim, G., Chen, W., Hopkins, N. and Brand, M. (2002). The zebrafish *spiel-ohne-grenzen* (*spg*) gene encodes the POU homeodomain protein Pou2 related to mammalian Oct4 and is essential for formation of the midbrain-hindbrain territory and early morphogenesis. *Development* **129**, 905-916.

Chavrier, P., Vesque, C., Galliot, B., Vigneron, M., Dolle, P., Duboule, D. and Charnay, P. (1990). The segment-specific gene Krox-20 encodes a transcription factor with binding sites in the promoter region of the Hox-1.4 gene. *EMBO J.* **9**, 1209-1218.

Crossley, P. H., Martinez, S. and Martin, G. R. (1996). Midbrain development induced by FGF8 in the chick embryo. *Nature* **380**, 66-68.

Culp, P., Nusslein-Volhard, C. and Hopkins, N. (1991). High-frequency germ-line transmission of plasmid DNA sequences injected into fertilized zebrafish eggs. *Proc. Natl. Acad. Sci. USA* **88**, 7953-7957.

Czerny, T., Bouchard, M., Kozmik, Z. and Busslinger, M. (1997). The characterization of novel Pax genes of the sea urchin and Drosophila reveal an ancient evolutionary origin of the Pax2/5/8 subfamily. *Mech. Dev.* **67**, 179-192.

Dressler, G., Deutsch, U., Chowdhury, K., Nornes, H. O. and Gruss, P. (1990). Pax2, a new murine paired-box-containing gene and its expression in the developing excretory system. *Development* **109**, 787-795.

Ekker, M., Wegner, J., Akimenko, M. A. and Westerfield, M. (1992). Coordinate embryonic expression of three zebrafish *engrailed* genes. *Development* **116**, 1001-1010.

Favor, J., Sandulache, R., Neuhäuser-Klaus, A., Pretsch, W., Chatterjee, B., Senft, E., Wurst, W., Blanquet, V., Grimes, P., Spörle, R. and Schughart, K. (1996). The mouse Pax21Neu mutation is identical to a human PAX2 mutation in a family with renal-coloboma syndrome and results in developmental defects of the brain, ear, eye and kidney. *Proc. Natl. Acad. Sci. USA* **93**, 13870-13875.

Fjose, A., Njolstad, P.R., Nornes, S., Molven, A. and Krauss, S. (1992). Structure and early embryonic expression of the zebrafish engrailed-2 gene. *Mech. Dev.* **39**, 51-62.

Fürthauer, M., Thisse, C. and Thisse, B. (1997). A role for Fgf-8 in the dorsoventral patterning of the zebrafish gastrula. *Development* **124**, 4253-4264.

Gemel, J., Jacobsen, C. and MacArthur, C. A. (1999). Fibroblast growth factor-8 expression is regulated by intronic Engrailed and Pbx1-binding sites. *J. Biol. Chem.* **274**, 6020-6026.

Haddon, C. and Lewis, J. (1996). Early ear development in the embryo of the zebrafish, Danio rerio. *J. Comp. Neurol.* **365**, 113-128.

Hashimoto, H., Itoh, M., Yamanaka, Y., Yamashita, S., Shimizu, T., Solnica-Krezel, L., Hibi, M. and Hirano, T. (2000). Zebrafish Dkk1 functions in forebrain specification and axial mesendoderm formation. *Dev. Biol.* **217**, 138-152.

Higashijima, S., Hotta, Y. and Okamoto, H. (2000). Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the islet-1 promoter/enhancer. *J. Neurosci.* **20**, 206-218.

Hiromi, Y., Kuroiwa, A. and Gehring, W. J. (1985). Control elements of the Drosophila segmentation gene fushi tarazu. *Cell* **43**, 603-613.

Holland, L. Z., Kene, M., Williams, N. A. and Holland, N. D. (1997). Sequence and embryonic expression of the amphioxus engrailed gene (AmphiEn): the metameric pattern of transcription resembles that of its segment-polarity homolog in *Drosophila*. *Development* **124**, 1723-1732.

Jessen, J. R., Meng, A., McFarlane, R. J., Paw, B. H., Zon, L. I., Smith, G. R. and Lin, S. (1998). Modification of bacterial artificial chromosomes through chi-stimulated homologous recombination and its application in zebrafish transgenesis. *Proc. Natl. Acad. Sci. USA* **95**, 5121-5126.

Joyner, A. L. and Martin, G. R. (1987). En-1 and En-2, two mouse genes with sequence homology to the Drosophila engrailed gene: expression during embryogenesis. *Genes Dev.* **1**, 29-38.

Kim, C. H., Taira, E., Kuo, C. H., Li, B. S., Okamoto, H., Nakahira, K., Ikenaka, K., Higuchi, H. and Miki, N. (1996). Neuron-specific expression of a chicken gicerin cDNA in transient transgenic zebrafish. *Neurochem. Res.* **21**, 231-237.

Koshida, S., Shinya, M., Mizuno, T., Kuroiwa, A. and Takeda, H. (1998). Initial anteroposterior pattern of the zebrafish central nervous system is determined by differential competence of the epiblast. *Development* **125**, 1957-1966.

Krauss, S., Johansen, T., Korzh, V. and Fjose, A. (1991). Expression pattern of zebrafish pax genes suggests a role in early brain regionalization. *Nature* **353**, 267-270.

Kuschert, S., Rowitch, D. H., Haenig, B., McMahon, A. P. and Kispert, A. (2001). Characterization of Pax-2 regulatory sequences that direct transgene expression in the Wolffian duct and its derivatives. *Dev. Biol.* **229**, 128-140.

Liu, A. and Joyner, A. L. (2001). Early anterior/posterior patterning of the midbrain and cerebellum. *Annu. Rev. Neurosci.* **24**, 869-896.

Li Song, D. and Joyner, A. L. (2000). Two Pax2/5/8-binding sites in Engrailed2 are required for proper initiation of endogenous mid-hindbrain expression. *Mech. Dev.* **90**, 155-165.

Long, Q., Meng, A., Wang, H., Jessen, J. R., Farrell, M. J. and Lin, S. (1997). GATA-1 expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene. *Development* **124**, 4105-4111.

Lumsden, A. and Keynes, R. (1989). Segmental patterns of neuronal development in the chick hindbrain. *Nature* **337**, 424-428.

Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-1123.

Lun, K. and Brand, M. (1998). A series of *no isthmus* (*noi*) alleles of the zebrafish *pax2.1* gene reveals multiple signaling events in development of the midbrain-hindbrain boundary. *Development* **125**, 3049-3062.

Majumdar, A., Lun, K., Brand, M. and Drummond, I. A. (2000). Zebrafish *no isthmus* reveals a role for Pax2.1 in tubule differentiation and patterning events in the pronephric primordia. *Development* **127**, 2087-2098.

Mason, P. J., Toriq, E., Wilkinson, D. and Williams, J. G. (1993). Assay of gene transcription in vivo. *In Gene Transcription. A Practical Approach* (ed. B. D. Hames and S. J. Higgins), pp. 5-61. Oxford: IRL Press.

McMahon, A. P., Joyner, A. L., Bradley, A. and McMahon, J. A. (1992). The midbrain-hindbrain phenotype of *Wnt-1*$^{-}$*/Wnt-1*$^{-}$ mice results from stepwise deletion of engrailed-expressing cells by 9.5 days postcoitum. *Cell* **69**, 581-595.

Meng, A., Tang, H., Ong, B. A., Farrell, M. J. and Lin, S. (1997). Promoter analysis in living zebrafish embryos identifies a cis-acting motif required for neuronal expression of GATA-2. *Proc. Natl. Acad. Sci. USA* **94**, 6267-6272.

Mikkola, I., Fjose, A., Kuwada, J. Y., Wilson, S., Guddal, P. H. and Krauss, S. (1992). The paired domain-containing nuclear factor pax[b] is expressed in specific commissural interneurons in zebrafish embryos. *J. Neurobiol.* **23**, 933-946.

Montesinos, M. L., Foucher, I., Conradt, M., Mainguy, G., Robel, L., Prochiantz, A. and Volovitch, M. (2001). The neuronal microtubule-associated protein 1B is under homeoprotein transcriptional control. *J. Neurosci.* **21**, 3350-3359.

Morgan, D. O. (1997). Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annu. Rev. Cell Dev. Biol.* **13**, 261-291.

Muller, F., Chang, B., Albert, S., Fischer, N., Tora, L. and Strahle, U. (1999). Intronic enhancers control expression of zebrafish sonic hedgehog in floor plate and notochord. *Development* **126**, 2103-2116.

Nasevicius, A. and Ekker, S. C. (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* **26**, 216-220.

Nonchev, S., Vesque, C., Maconochie, M., Seitanidou, T., Ariza-McNaughton, L., Frain, M., Marshall, H., Sham, M. H., Krumlauf, R. and Charnay, P. (1996). Segmental expression of Hoxa-2 in the hindbrain is directly regulated by Krox-20. *Development* **122**, 543-554.

Oxtoby, E. and Jowett, T. (1993). Cloning of the zebrafish krox-20 gene (krx-20) and its expression during hindbrain development. *Nucleic Acids Res.* **21**, 1087-1095.

Pfeffer, P. L., Bouchard, M. and Busslinger, M. (2000). Pax2 and homeodomain proteins cooperatively regulate a 435 bp enhancer of the mouse *pax5* gene at the midbrain-hindbrain boundary. *Development* **127**, 1017-1028.

Pfeffer, P.L., Gerster, T., Lun, K., Brand, M. and Busslinger, M. (1998). Characterization of three novel members of the zebrafish *Pax2/5/8* family: dependency of *Pax5* and *Pax8* expression on the *Pax2.1*(*noi*) function. *Development* **125**, 3063-3074.

Pfeffer, P. L., Payer, B., Reim, G., di Magliano, M. P. and Busslinger, M. (2002). The activation and maintenance of Pax2 expression at the mid-hindbrain boundary is controlled by separate enhancers. *Development* **129**, 307-318.

Pinsonneault, J., Florence, B., Vaessin, H. and McGinnis, W. (1997). A model for extradenticle function as a switch that changes HOX proteins from repressors to activators. *EMBO J.* **16**, 2032-2042.

Püschel, A. W., Westerfield, M. and Dressler, G. R. (1992). Comparative analysis of Pax-2 protein distributions during neurulation in mice and zebrafish. *Mech. Dev.* **38**, 197-208.

Reifers, F., Böhli, H., Walsh, E. C., Crossley, P. H., Stainier, D. Y. R. and Brand, M. (1998). Fgf8 is mutated in zebrafish *acerebellar* mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. *Development* **125**, 2381-2395.

Reim, G. and Brand, M. (2002). *spiel-ohne-grenzen*/*pou2* mediates regional competence to respond to Fgf8 during zebrafish early neural development. *Development* **129**, 917-933.

Reinhard, E., Nedivi, E., Wegner, J., Skene, J. H. and Westerfield, M. (1994). Neural selective activation and temporal regulation of a mammalian GAP-43 promoter in zebrafish. *Development* **120**, 1767-1775.

Rhinn, M. and Brand, M. (2001). The midbrain-hindbrain organizer. *Curr. Opin. Neurobiol.* **11**, 34-42.

Rowitch, D. H., Kispert, A. and McMahon, A. P. (1999). Pax-2 regulatory sequences that direct transgene expression in the developing neural plate and external granule cell layer of the cerebellum. *Brain Res. Dev. Brain Res.* **117**, 99-108.

Ryan, G., Steele-Perkins, V., Morris, J. F., Rauscher, F. J., 3rd and Dressler, G. R. (1995). Repression of Pax-2 by WT1 during normal kidney development. *Development* **121**, 867-875.

Sanyanusin, P., Schimmenti, L. A., Noe, L. A., Ward, T. A., Pierpont, M. E. M., Sullivan, M. J., Dobyns, W. B. and Eccles, M. R. (1995). Mutation of the Pax2 gene in a family with optic nerve colobomas, renal abnormalities and vesicouretal reflux. *Nature Genet.* **9**, 358-363.

Scholpp, S. and Brand, M. (2001). Morpholino-induced knock-down of zebrafish engrailed genes *eng2* and *eng3* reveals redundant and unique function in midbrain-hindbrain boundary development. *Genesis* **30**, 129-133.

Schwarz, M., Alvarez Bolado, G., Urbanek, P., Busslinger, M. and Gruss, P. (1997). Conserved biological function between Pax-2 and Pax-5 in midbrain and cerebellum development: evidence from targeted mutations. *Proc. Natl. Acad. Sci. USA* **94**, 14518-14523.

Seitanidou, T., Schneider-Maunoury, S., Desmarquet, C., Wilkinson, D. G. and Charnay, P. (1997). Krox-20 is a key regulator of rhombomere-specific gene expression in the developing hindbrain. *Mech. Dev.* **65**, 31-42.

Smale, S. T. and Baltimore, D. (1989). The 'Initiator' as a transcriptional control element. *Cell* **57**, 103-113.

Song, D. L., Chalepakis, G., Gruss, P. and Joyner, A. L. (1996). Two Pax-binding sites are required for early embryonic brain expression of an Engrailed-2 transgene. *Development* **122**, 627-635.

Stayner, C. K., Cunliffe, H. E., Ward, T. A. and Eccles, M. R. (1998). Cloning and characterization of the human PAX2 promoter. *J. Biol. Chem.* **273**, 25472-25479.

Stuart, G. W., McMurray, J. V. and Westerfield, M. (1988). Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. *Development* **103**, 403-412.

Torres, M., Gómez-Pardo, E., Dressler, G. R. and Gruss, P. (1995). Pax-2 controls multiple steps of urogenital development. *Development* **121**, 4057-4065.

Urbanek, P., Wang, Z. Q., Fetka, I., Wagner, E. F. and Busslinger, M. (1994). Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking *Pax5/BSAP*. *Cell* **79**, 901-912.

Wassarman, K. M., Lewandoski, M., Campbell, K., Joyner, A. L., Rubenstein, J. L., Martinez, S. and Martin, G. R. (1997). Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on Gbx2 gene function. *Development* **124**, 2923-2934.

Westerfield, M. (1994). *The Zebrafish Book.* Edition 2.1. Oregon, University of Oregon Press.

Westerfield, M., Wegner, J., Jegalian, B. G., DeRobertis, E. M. and Puschel, A. W. (1992). Specific activation of mammalian Hox promoters in mosaic transgenic zebrafish. *Genes Dev.* **6**, 591-598.

Wurst, W., Auerbach, A. B. and Joyner, A. L. (1994). Multiple developmental defects in Engrailed-1 mutant mice: an early mid-hindbrain deletion and patterning defects in forelimbs and sternum. *Development* **120**, 2065-2075.

Wurst, W. and Bally-Cuif, L. (2001). Neural plate patterning: upstream and downstream of the isthmic organizer. *Nat. Rev. Neurosci.* **2**, 99-108.