Development 127, 1017-1028 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV2509



# Pax2 and homeodomain proteins cooperatively regulate a 435 bp enhancer of the mouse *Pax5* gene at the midbrain-hindbrain boundary

**Peter L. Pfeffer, Maxime Bouchard and Meinrad Busslinger***

Research Institute of Molecular Pathology, Dr Bohr-Gasse 7, A-1030 Vienna, Austria
*Author for correspondence (e-mail: Busslinger@nt.imp.univie.ac.at)

*Accepted 22 December 1999; published on WWW 8 February 2000*

## SUMMARY

**Pax and homeodomain transcription factors are essential for the formation of an organizing center at the midbrain-hindbrain boundary (mhb) which controls the genesis of the midbrain and cerebellum in the vertebrate embryo. Pax2 and Pax5 are sequentially activated in this brain region, with *Pax2* expression preceding that of *Pax5*. Using a transgenic reporter assay, we have now identified a conserved 435 bp enhancer in the 5′ flanking region of mammalian *Pax5* genes which directs *lacZ* expression in the correct temporal and spatial pattern at the mhb. This minimal enhancer is composed of two distinct elements, as shown by protein-binding assays with mhb-specific extracts. The proximal element contains overlapping consensus binding sites for members of the Pax2/5/8 and POU protein families, whereas a distal element is bound by homeodomain and zinc finger transcription factors. Expression analysis of transgenes carrying specific mutations in these recognition motifs identified the Pax- and homeodomain-binding sites as functional elements which cooperatively control the activity of the mhb enhancer. *lacZ* genes under the control of either the minimal enhancer or the endogenous *Pax5* locus were normally expressed at the mhb in *Pax5* mutant embryos, indicating that this enhancer does not depend on autoregulation by Pax5. In *Pax2* mutant embryos, expression of the endogenous *Pax5* gene was, however, delayed and severely reduced in lateral aspects of the neural plate which, on neural tube closure, becomes the dorsal mhb region. This cross-regulation by Pax2 is mediated by the Pax-binding site of the minimal enhancer which, upon specific mutation, resulted in severely reduced transgene expression in the dorsal part of the mhb. Together these data indicate that Pax2 and homeodomain proteins directly bind to and cooperatively regulate the mhb enhancer of *Pax5*.**

Key words: *Pax*, Gene regulation, Enhancer, *lacZ* transgenes, Mid-hindbrain development, Mouse

## INTRODUCTION

The midbrain and cerebellum develop from an organizing center which is formed at the midbrain-hindbrain boundary (mhb; also known as isthmus) of the vertebrate embryo (Wassef and Joyner, 1997). Genetic studies in mouse and zebrafish revealed that the development and/or activity of this isthmic organizer critically depends on the mhb-specific expression of secreted proteins (Wnt1, Fgf8) and transcription factors (Otx2, Gbx2, En1, En2, Pax2, Pax5). Elucidation of the genetic hierarchy of these regulators is, however, hampered by the fact that targeted inactivation of many of these genes results in a similar phenotype, i.e. in loss of the mhb organizer (reviewed by Wassef and Joyner, 1997). Hence, all these factors appear to participate in a feed-back regulatory network controlling the function of the mhb organizer.

Pax2, Pax5 and Pax8 constitute a distinct class of Pax transcription factors which are all expressed in temporally and spatially overlapping patterns at the mhb of the vertebrate embryo (Nornes et al., 1990; Plachov et al., 1990; Adams et al., 1992; Pfeffer et al., 1998). *Pax2* expression is initiated already during gastrulation in the prospective mhb region of the mouse embryo (Rowitch and McMahon, 1995). *Pax5* transcription is next activated at the 3- to 5-somite stage in the mhb region (Urbánek et al., 1994; Rowitch and McMahon, 1995) followed by *Pax8* expression at approx. 9 somites (Pfeffer et al., 1998). In agreement with this temporal onset of gene expression, a severe, mild or no phenotype was observed in the midbrain and cerebellum of mice lacking *Pax2* (Favor et al., 1996), *Pax5* (Urbánek et al., 1994) or *Pax8* (Mansouri et al., 1998), respectively. The effect of the *Pax2* mutation is, however, strongly influenced by the genetic background of the mouse strain analyzed, ranging from deletion of the posterior midbrain and cerebellum (Favor et al., 1996) or exencephaly (Torres et al., 1996) to almost normal development of these brain structures (Schwarz et al., 1997). In contrast, deletion of the midbrain and cerebellum was consistently observed in *Pax2*, *Pax5* double-mutant embryos, thus demonstrating dosage-dependent cooperation of these transcription factors in the development of the mhb region (Urbánek et al., 1997; Schwarz et al., 1997).

The zebrafish genome contains four members of the *Pax2/5/8* family due to an additional duplication of the *Pax2* gene. All of these zebrafish genes are expressed at the mhb in similar patterns to their mouse orthologues (Pfeffer et al., 1998). Surprisingly however, the *no isthmus* (*noi*) mutation of the *Pax2.1* gene results in consistent deletion of the entire mhb region (Brand et al., 1996), indicating that other members of the *Pax2/5/8* family are unable to compensate for the loss of Pax2.1 function in the fish embryo. In agreement with this finding, the expression of *Pax5* and *Pax8* is never initiated at the mhb of *noi* mutant embryos (Pfeffer et al., 1998). Hence, the Pax2.1 protein is involved, either directly or indirectly, in the regulation of *Pax5* and *Pax8*, thus revealing a genetic hierarchy of the *Pax2/5/8* genes in mhb development.

Here we report the characterization of a 435 bp minimal enhancer of the mouse *Pax5* gene which directs *lacZ* reporter gene expression in the correct temporal and spatial pattern at the mhb of transgenic mouse embryos. This minimal enhancer contains functional binding sites for homeodomain proteins and members of the Pax2/5/8 family. Double mutation of both binding sites abolished enhancer activity, whereas single mutation of either site affected transgene expression most severely in the dorsal mhb region. Similarly, expression of the endogenous *Pax5* gene was initiated only near the midline in *Pax2* mutant embryos, but failed to be expressed in the lateral neural plate which, upon neural tube closure, becomes the dorsal mhb region. Hence, the 435 bp enhancer of *Pax5* is a direct target of Pax2 and requires Pax2 function for correct activation at the mhb of the mouse embryo, indicating that the brain-specific regulation of *Pax5* has been conserved throughout vertebrate evolution.

## MATERIALS AND METHODS

### DNA constructs

**pTrap**: the 3.6 kb *Sma*I fragment of pBGZ40, containing the minimal *β-globin* promoter, *lacZ* gene and SV40 poly(A) signal (Yee and Rigby, 1993), was cloned into the *Sma*I site of pPolyIII-I to generate pTrap. This vector has unique *Sal*I, *Msc*I, *Hin*dIII, *Xba*I, *Kpn*I and *Sph*I sites upstream of the rabbit *β-globin* TATA-box. **25iz**: a 19 kb *Nae*I and 3.6 kb *Nae*I-*Xma*I fragment of *mPax5* cosmid 14 were inserted into pSP65 together with a 3.9 kb *Xma*I-*Xho*I fragment of pGNA-Pax5 containing exon 2 fused in frame to nuclear localization, *lacZ* and SV40 polyadenylation sequences (Urbánek et al., 1994). **In1z**: a 5.9 kb fragment was PCR-amplified from cosmid 14 with primers 5′-AAGATCGATGTAGGAACACCGCGAAACGGGATTC-AT-3′ and 5′-AAGCGGCCGCGAGCGCAGGGCCCTCCGTCTCA-3′ followed by insertion into pTrap. **In2z**: a 5 kb fragment was PCR-amplified from cosmid 14 with primers 5′-ATAGCGGCCGCA-TAGTAGGCGGCGAGCTCGCTTC-3′ and 5′-GAGACTAGTGAT-TCACTCCTCCATGTCCTGAAACAG-3′ and cloned into pBGZ40. **14z**-**1.6z**: these constructs were generated by inserting the relevant fragments (Fig. 1) into pBGZ40. **1.2z**-**0.39z**: the following fragments were cloned into pTrap. **1.2z** contains a 1.2 kb fragment amplified from plasmid 3z with primers 5′-CCACTCGAGTTTCTTTCTCCA-GATGCA-3′ (a) and 5′-CCAGTCGACTTTGGTATCTCATTTCTC-AT-3′ (b). **0.6z**: construct 1.2z was cut with *Sph*I and religated. **0.5z** contains the *Bsa*I-*Ban*II fragment of plasmid 1.2z. **0.43z** contains a PCR fragment generated with 5′-TCTCGTCGACAAAAACCAGC-TCCAAGC-3′ and oligonucleotide 1.2z-b. **0.39z** contains the *Kpn*I-*Pac*I fragment of plasmid 1.2z which was resected with T4 DNA polymerase prior to cloning.

### In vitro mutagenesis

Transgene 0.6z was mutated by using the QuikChange kit (Stratagene) and 33-42 nucleotide long primers containing the mutations (Figs 5A,6A) in their center. The mutated *Sal*I-*Sph*I fragments were recloned into pTrap and verified by DNA sequencing. Construct **0.6z-Δ** was generated by removing the ends of the *Nsi*I site by T4 DNA polymerase treatment.

### Transgenic and mutant mice

C57BL/6×CBA $F_1$ mice were used for the generation of transgenic mice which were identified by PCR analysis with the *lacZ* primers 5′-ATACTGTCGTCGTCCCCTCAAACTG-3′ and 5′-TTCAACCACC-GCACGATAGAGATTC-3′. *Pax5*$^{+/lacZ}$ and *Pax5*$^{+/\Delta}$ mice were maintained on the C57BL/6×129/Sv background (Urbánek et al., 1994; M. Horcher and M. B., unpublished data). *Pax2*$^{-/-}$ mice were generated by in-frame *lacZ* insertion into *Pax2* exon 2 (M. B., unpublished data).

### β-Galactosidase staining and in situ hybridization

Embryos were stained at 30°C for 1-15 hours in 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), postfixed in 4% formaldehyde at 20°C for 1 hour, dehydrated in methanol and then isopropanol for 30 minutes each and cleared in tetrahydronaphthalene prior to photography. Whole-mount in situ hybridization with single-stranded, digoxigenin-UTP-labeled *lacZ* or *Pax5* probes (Adams et al., 1992) was performed as described by Pfeffer et al. (1998).

### Extract preparation

Fertilized chicken eggs were incubated for 2-3 days at 39°C in a humidified incubator. All subsequent steps were carried out on ice. Mhb tissue was dissected by cutting within the posterior third of the midbrain vesicle and near the rhombomere 1/2 constriction. Forebrain tissue was obtained by cutting anterior to the midbrain vesicle. The dissected tissue of 100-200 embryos was resuspended in 2.3 volumes of a buffer containing 0.25 M sucrose, 10 mM Hepes pH 7.6, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 mM Pefabloc, 5 μg/ml aprotinin, pepstatinA, leupeptin, 2 μg/ml antipain, chymostatin and 2 mM benzamidine hydrochloride, in an Eppendorf tube. This mixture was homogenized by hand, using a pellet pestle (Sigma; Z35994-7). NUN buffer of a 5× solution (5 M urea, 1.5 M NaCl, 5% NP40, 125 mM Hepes pH 7.6, 5 mM DTT) was added to a final 1× concentration. This mixture was vortexed for 5 seconds, incubated on ice for 15 minutes and microfuged at full speed for 10 minutes. Glycerol was added to 10%, and the extracts were flash-frozen in liquid nitrogen. For DNase I protection analyses, the extracts were dialysed for 1.5 h in Tube-O-DIALYZER™ tubes (Novus Molecular Inc.) against 2× 500 ml of 25 mM Hepes pH 7.6, 40 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 10% glycerol, 1 mM DTT, 0.1 mM Pefabloc.

### DNase I protection assay

Probe A was subcloned as a blunt-ended *Sfu*I-*Sal*I fragment from plasmid 0.6z into the *Sma*I site of pBluescript. The *Eco*RV-*Bam*HI insert of this plasmid was 3′ end-labeled, and 1 μl of this probe (approx. 30,000 cts/minute, 0.1 pmol) was incubated with protein extracts followed by DNase I digestion and electrophoretic analysis as described by Barberis et al. (1987).

### EMSA analysis

The mouse *Pax2b*, *Pax3*, *Pax5*, *Pax6*, *Oct1*, *Otx2*, *Gbx2*, *En2* and *Xenopus HoxD1* cDNAs were cloned into the expression vector pKW2T, and proteins were synthesized by in vitro transcription-translation. These proteins (0.02-2 μl) or tissue extracts (approx. 0.1 μl) were used for EMSA with end-labeled probes as described by Barberis et al. (1987). The oligonucleotides shown in Figs 5A and 6A were used as competitor DNA at a 100-fold molar excess. The following oligonucleotides were furthermore used: HD, 5′-

AGCTCCAAATTTAATTGAAGAGTG-3′; Sp1, 5′-AATTCGATC-GGGGCGGGGCGAGCG-3′; Oct (*H2A* octamer) 5′-GTCTTTT-GTGCAGCTTATGCAAATGAGGGTAGG-3′; Pit (Pit1-binding site of rat growth hormone gene) 5′-GTCTTTGTGCAGGCCATGAATA-AATGATAGGTG-3′.

### Accession number

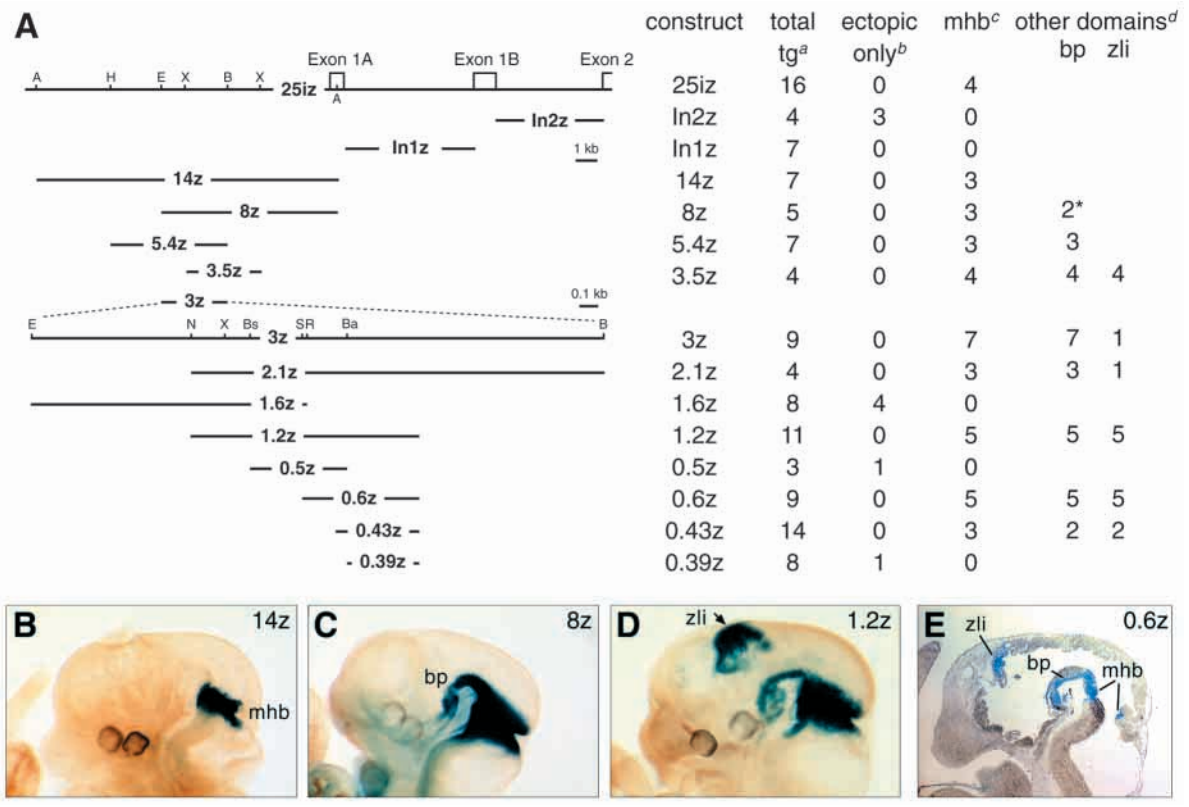
The 5′ sequences of the mouse and human *Pax5* genes were submitted to GenBank (AF222993, AF222994).

## RESULTS

### Identification of a mhb-specific enhancer of *Pax5*

The mouse *Pax5* gene is transcribed from two independent promoters which are separated by 6.5 kb of intervening sequences and give rise to two distinct mRNAs by splicing of alternative 5′ exons (1A or 1B) to the common coding sequences of exon 2-10 (Busslinger et al., 1996) (Fig. 1A). The distal promoter serving exon 1A is predominantly active during B-lymphopoiesis, whereas the proximal promoter directs *Pax5* expression to the embryonic mhb region in addition to the B-lymphoid lineage (Busslinger et al., 1996). Due to the presence of two separate promoters in the *Pax5* locus, we initiated our search for *cis*-acting regulatory elements by fusing 25 kb of the *Pax5* 5′ region in frame in exon 2 to a *lacZ* reporter gene (Fig. 1). Mice carrying this transgene (25iz) were generated by pronuclear DNA injection and then analysed for *lacZ* expression by X-gal staining of transgenic embryos (Fig. 2E-H). Whole-mount in situ hybridization of transgenic embryos was used to compare *Pax5* and *lacZ* expression, which validated the *lacZ* reporter assay for monitoring *Pax5* expression (Fig. 2O,P). Hence, heterozygous *Pax5*$^{+/lacZ}$ embryos, which contained an identical in-frame *lacZ* insertion in the targeted *Pax5* locus (Urbánek et al., 1994), were stained in parallel to reveal the endogenous *Pax5* expression pattern (Fig. 2A-D). These analyses unequivocally demonstrated that the transgene 25iz is correctly initiated in the mhb region of 8.5-day embryos and then continues to faithfully reflect the endogenous *Pax5* expression pattern at the mhb until day 11.5 (Fig. 2A-H). Hence, 25 kb from the *Pax5* 5′ region contain all necessary information to direct early and late expression of *Pax5* at the mhb.

Next, the 25 kb region was systematically resected to determine the number and location of mhb-specific regulatory element(s). For this purpose, we used a transgenic vector consisting of a minimal β-globin promoter linked to a *lacZ* gene which shows little ectopic expression on its own (Yee and Rigby, 1993). The different DNA fragments from the *Pax5* 5′




| construct | total tg[a] | ectopic only[b] | mhb[c] | other domains[d] bp | zli |
|---|---|---|---|---|---|
| 25iz | 16 | 0 | 4 | | |
| In2z | 4 | 3 | 0 | | |
| In1z | 7 | 0 | 0 | | |
| 14z | 7 | 0 | 3 | | |
| 8z | 5 | 0 | 3 | 2* | |
| 5.4z | 7 | 0 | 3 | 3 | |
| 3.5z | 4 | 0 | 4 | 4 | 4 |
| 3z | 9 | 0 | 7 | 7 | 1 |
| 2.1z | 4 | 0 | 3 | 3 | 1 |
| 1.6z | 8 | 4 | 0 | | |
| 1.2z | 11 | 0 | 5 | 5 | 5 |
| 0.5z | 3 | 1 | 0 | | |
| 0.6z | 9 | 0 | 5 | 5 | 5 |
| 0.43z | 14 | 0 | 3 | 2 | 2 |
| 0.39z | 8 | 1 | 0 | | |

**Fig. 1.** Identification of the mhb-specific enhancer of *Pax5*. (A, left) Schematic diagram depicting the *Pax5* 5′ region which was fused in exon 2 to the *lacZ* sequences of transgene 25iz. All other reporter genes contained the indicated DNA fragments upstream of the minimal β-globin promoter of pTrap or pBGZ40 (abbreviated as z) and are referred to by their insert size (in kb). (A, right) Statistical overview of the β-galactosidase staining patterns which were observed in injected founder ($G_0$) embryos or transgenic lines at day 11.5. The number of independent transgenic embryos (*a*) is indicated together with the number of embryos that revealed β-galactosidase expression only in ectopic locations (*b*), specifically at the mhb (*c*) and additionally in the basal plate (bp; *d*) and/or zona limitans intrathalamica (zli; *d*). An asterisk denotes the basal plate expression of those 8z transgenes that contained the *Pax5* 5′ region in opposite orientation to the *lacZ* gene. A, *Apa*I; B, *Bgl*II; Ba, *Ban*II; Bs, *Bsa*I; E, *Eco*RV; H, *Hpa*I; N, *Nco*I; R, *Eco*RI; S, *Sph*I; X, *Xba*I. (B-E) X-Gal staining of 11.5-day embryos carrying the indicated transgenes. A sagittal section is shown in E.

region were inserted upstream of the β-globin TATA-box of this vector, and their activity was directly tested in injected founder ($G_0$) embryos at day 11.5. The transgene 14z containing the 5′ flanking region of *Pax5* was correctly expressed at the mhb (Fig. 1B), whereas intron 1 and 2 sequences were inactive in this assay (Fig. 1A). By analyzing a series of 11 overlapping fragments, the mhb enhancer could be mapped within the 5′ flanking sequences to a minimal region of 435 base pairs (construct 0.43z) which was located 5.6 kb upstream of exon 1A (Fig. 1A). Importantly, this minimal enhancer gave rise to the same temporal and spatial expression pattern at the mhb (Fig. 2I-N) as the endogenous *Pax5* locus (Fig. 2A-D).

Surprisingly, all transgenes containing inserts shorter than 14z were more vigorously expressed at the mhb and additionally showed consistent expression in two ectopic locations, i.e. in the basal plate (extending from rhombomere 1 to the diencephalon) and in a transverse stripe of the forebrain (at the prosomere 2/3 boundary known as zona limitans intrathalamica; Figs 1D,E, 2I-N). These transgenes contained neither the proximal nor distal promoter of *Pax5* with the exception of construct 8z (Fig. 1A). The DNA insert of this transgene was analyzed in both orientations relative to the TATA-box of the *lacZ* gene, indicating that the activity of the mhb enhancer is indeed orientation-independent. Interestingly, the transgene 8z only gave rise to ectopic expression in the basal plate if the exon 1A promoter of *Pax5* was present in opposite direction to the *lacZ* gene and was thus unable to drive β-galactosidase expression (Fig. 1A,C). It is therefore likely that transcription factors binding to the endogenous *Pax5* promoters restrict the activity of the enhancer to the mhb region of the embryo.

### Interaction of proteins from embryonic mhb extracts with the minimal enhancer

The sequence and relative position of the mhb enhancer have been highly conserved in the human and mouse *Pax5* loci (Fig. 3), further indicating an important role of this regulatory element in the control of *Pax5* expression. The minimal enhancer sequences, defined by transgene 0.43z, were 89% identical between the two species. As this high degree of sequence conservation did not allow us to pinpoint potential regulatory elements, we used a biochemical approach to identify transcription factor-binding sites in the enhancer. For this purpose we prepared protein extracts from dissected mhb tissue of early embryos. In the interest of procuring sufficient brain material, we switched to chick embryos, particularly since the temporal and spatial expression pattern of *Pax5* is similar, if not identical, at the mhb in this distantly related vertebrate (Funahashi et al., 1999). Chick embryos at day 3 (HH stage 20), which corresponds approximately to embryonic day 11 of the mouse (Hamburger and Hamilton, 1992), were used for micro-dissection of the mhb region and protein extract preparation. This extract was analyzed by DNase I protection assay for factors binding to three overlapping DNA fragments which spanned the entire enhancer of transgene 0.6z. One of these DNA fragments (probe A; nucleotides 451-666) gave rise to a strong DNase I footprint which mapped to a potential Pax-binding site (Fig. 4A). This finding was confirmed by electrophoretic mobility shift assay (EMSA), using in vitro translated Pax proteins (Fig. 4B). Pax2 and Pax5 bound with high affinity to probe A in contrast to Pax3 and Pax6, which represent two other Pax subfamilies expressed in the developing CNS (Fig. 4B). Hence, members of the Pax2/5/8 family preferentially

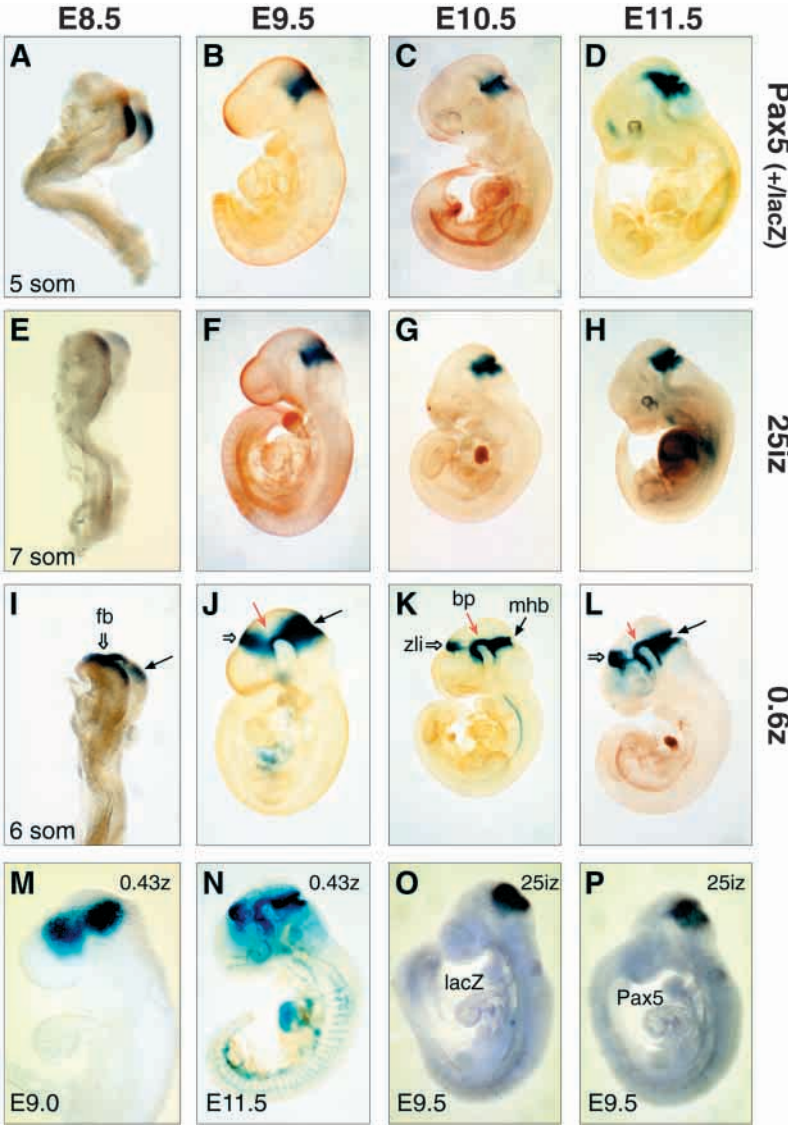



**Fig. 2.** Temporal expression of *Pax5* tansgenes. (A-D) X-Gal staining of *Pax5*$^{+/lacZ}$ embryos reflecting the endogenous *Pax5* expression pattern from day 8.5 (E8.5) to 11.5 (E11.5). (E-H) X-Gal staining of embryos carrying the transgene 25iz (E,F,H-line 16; G-line 17). (I-L) Expression of the transgene 0.6z (I, K, line 6; J, line 4; L, $G_0$ embryo 7). A black arrow points to the mhb, an open arrow to ectopic expression in the forebrain (I) and later zona limitans intrathalamica (zli; J-L) and a red arrow to ectopic staining in the basal plate (bp). (M,N) Expression of the 0.43z transgene ($G_0$ embryos). (O,P) In situ hybridization of transgenic (25iz) embryos with antisense *lacZ* (O) and *Pax5* (P) riboprobes. som, somite.


**Fig. 3.** Conservation of the mhb enhancer sequences of mouse and human *Pax5* genes. Only those nucleotides of the human (h) *PAX5* gene which differ from the corresponding mouse (m) sequence are shown. Dots denote gaps introduced for optimal alignment. Numbers refer to the mouse *Pax5* sequence. The limits of transgenic constructs are indicated, elements 1 (E1) and 2 (E2) are underlined, and the binding sites of homeodomain (HD), POU, Pax and Sp1-like zinc finger (Zn) proteins are boxed.

interact with an enhancer sequence which has been entirely conserved in mammalian *Pax5* genes (Fig. 3).

## Pax2/5 and POU proteins bind to overlapping sites within a proximal enhancer element

EMSA analysis of the 3-day mhb extract with probe A revealed at least five different proteins which bound to the proximal 216 bp sequence of the *Pax5* enhancer (Fig. 5B, lane 4). The formation of these five protein-DNA complexes was prevented in the presence of a 100-fold molar excess of a short oligonucleotide (E1; 465-497) encompassing the Pax-binding site (lane 5). Moreover, oligonucleotide E1, when used as a probe, detected a similar cluster of six protein-DNA complexes in the 3-day mhb extract (lane 16). Hence, a relatively short sequence of the enhancer interacts with multiple proteins and is thus referred to as enhancer element 1.

Sequence inspection of element 1 revealed a good match to the consensus binding site of Pax2/5/8 proteins and identified potential recognition sequences for POU transcription factors (Fig. 5A). Indeed, several members of the POU protein family are expressed in the developing CNS of the mouse embryo and are known to recognize DNA via the bipartite POU domain which consists of two structurally independent DNA-binding units referred to as POU-specific ($POU_S$) and POU homeo ($POU_H$) domains (Herr and Cleary, 1995). The $POU_S$ domain interacts with the sequence ATGC and the $POU_H$ domain with the motif (T/A)AAT (Herr and Cleary, 1995). These two recognition sequences are present in both orientations on enhancer element 1 (Fig. 5A) and may be bound by POU proteins in any possible combination, as the spacing, orientation and positioning of the $POU_S$ and $POU_H$ domains on DNA is highly flexible (Herr and Cleary, 1995). As the POU- and Pax-binding sites overlap (Fig. 5A), we introduced specific mutations into element 1 to identify the different proteins involved in complex formation. The effect of these mutations on protein binding was investigated by using the mutant oligonucleotides either as competitor DNA (Fig. 5B) or end-labeled DNA probes (Fig. 5C) for EMSA analysis with the 3-day mhb extract.

The complexes P1, P2 and P3 were identified by several criteria as Pax2a, Pax2b and Pax5, respectively. First, an excess of the unrelated high-affinity Pax-binding site of the *CD19* gene (Czerny and Busslinger, 1995) prevented the generation of all three complexes (Fig. 5, lane 9). Second, a neutralizing antibody directed against the Pax5 paired domain (Adams et

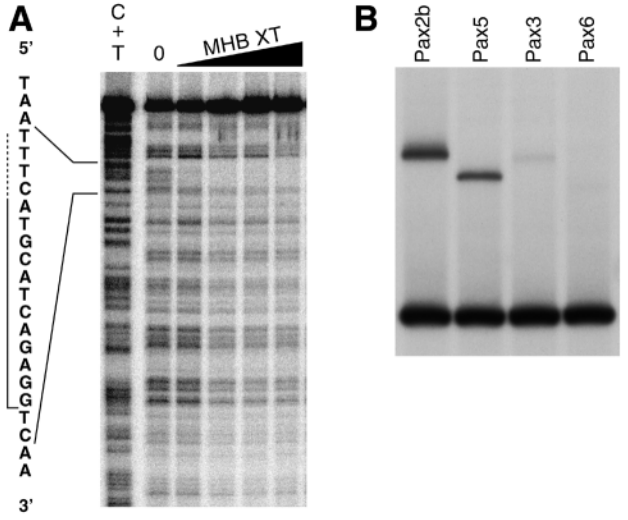



**Fig. 4.** Binding of Pax2/5/8 proteins to the mhb enhancer. (A) DNase I footprint analysis was performed with the 3′ end-labeled probe A (Fig. 3) and increasing amounts (1-8 μl) of an extract prepared from mhb tissue of 3-day chick embryos. Probe A cleaved at pyrimidines was used as a DNA sequence ladder (C+T). (B) Differential binding of Pax proteins to the mhb enhancer. Equimolar amounts of in vitro translated Pax proteins (quantitated by [$^{35}$S]Met incorporation) were analyzed by EMSA for binding to probe A.

al., 1992) interfered with complex formation (lanes 2, 17). Third, deletion (Δ) or substitution (S1) of four base pairs within the Pax recognition sequence abolished binding of all three proteins (lanes 6, 7, 18, 19). Fourth, in vitro translated Pax2b and Pax5 co-migrated with complexes P2 and P3, respectively (lanes 23, 24). Complex P1 is most likely formed by the isoform Pax2a, whose size exceeds that of Pax2b by 23 amino acids (Dressler et al., 1990). Lastly, a mhb extract prepared from earlier, 2-day chick embryos (HH stage 12) only gave rise to complexes P1 and P2 (lane 10) in agreement with the fact that expression of *Pax2* precedes that of *Pax5* during mhb development (Rowitch and McMahon, 1995; Pfeffer et al., 1998).

Likewise, the three complexes referred to as X1, X2 and X3 were generated by POU proteins present in the 3-day mhb extract. First, these complexes were specifically competed away by unrelated octamer and Pit-1 recognition sequences (Fig. 5, lanes 13, 14). Second, an octamer probe generated the same three complexes as enhancer element 1 (lane 29). Third, the in vitro translated POU protein Oct1 also interacted with element 1 (lane 25,30). Fourth, mutation of the $POU_H$-binding sites (S2) abrogated the formation of all three complexes (lanes 7, 20). Finally, mutations (Δ, S1, S3) affecting the recognition motifs of the $POU_S$ domain also interfered with protein binding (lanes 6, 15, 18-20). The effect of substitution S1 differed, however, between the two types of experiments (lanes 7, 18), suggesting that this mutation only reduced, but did not abolish the binding of POU proteins. A recent analysis of comparable mhb extracts prepared from mouse embryos resulted in the identification of three POU proteins with similar electrophoretic mobilities as our complexes X1, X2 and X3, which may therefore be generated by the proteins Brn1, Brn2 and Brn4, respectively (Mihailescu et al., 1999).

In summary, we conclude that members of the Pax2/5/8 and POU protein families bind to overlapping sequences in element 1. These proteins differ, however, in their sequence requirements as the mutations S2 and S3 specifically interfered with binding of the POU proteins (Fig. 5, lanes 8, 15, 27, 28).

## The Pax-binding site is essential for enhancer activity

To assess the in vivo significance of enhancer element 1, we next introduced the same mutations into the Pax- and POU-binding sites of transgene 0.6z. Fig. 7K summarizes the generation and expression analyses of the different transgenic embryos. The mutations Δ and S1, both of which interfere with binding of Pax and POU proteins, resulted in a similar reduction of transgene expression at the mhb (Fig. 7A-D). Both mutations interfered with β-galactosidase expression on the dorsal side of the neural tube, while the effect on ventral

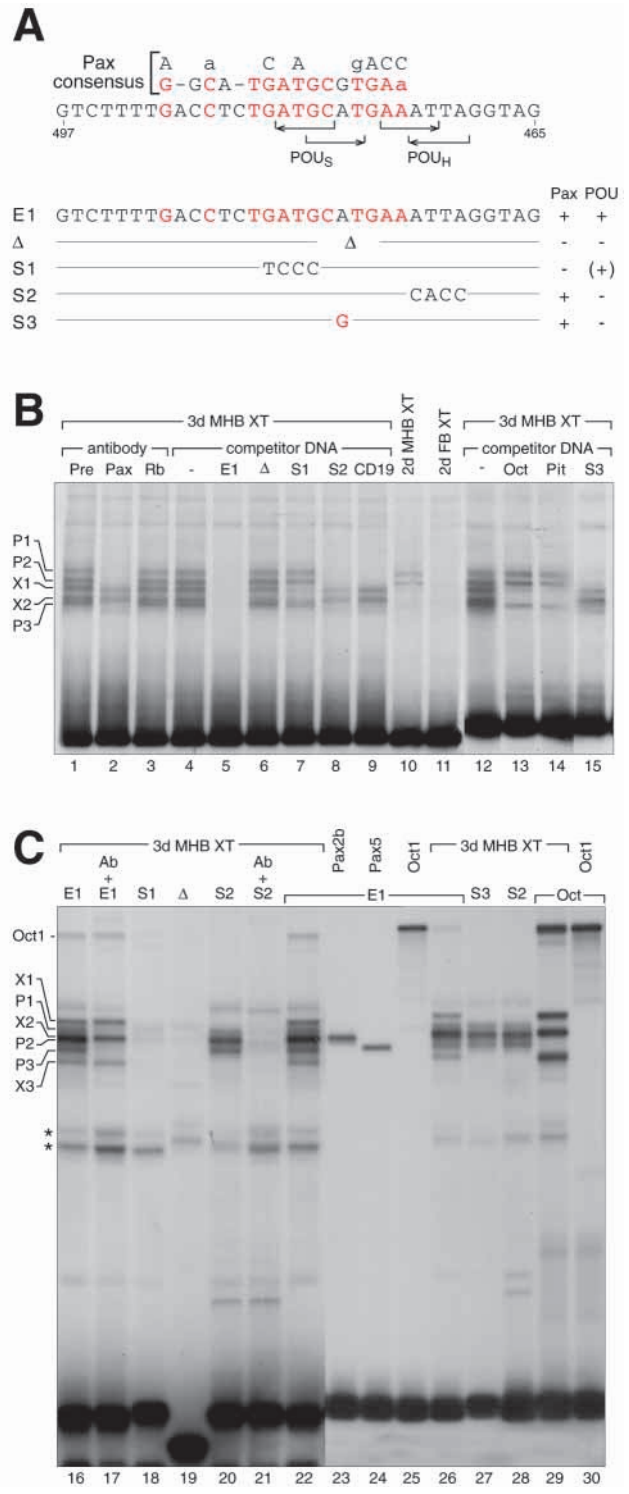



**Fig. 5.** Interaction of Pax and POU proteins with enhancer element 1. (A) Sequence alignment of element 1 (E1; inverted relative to Fig. 3) with the consensus binding sites of Pax2/5/8 (Czerny and Busslinger, 1995) and POU proteins (Herr and Cleary, 1995). The effect of the indicated mutations on protein binding is summarized to the right. (B) Element 1 binds different Pax (P) and POU (X) proteins. Extracts prepared from forebrain (fb) or mhb tissue of 2- and 3-day chick embryos were analyzed by EMSA with probe A (Fig. 3) in the presence of the indicated antibodies (lanes 1-3) or a 100-fold excess of the oligonucleotides (lanes 5-9 and 13-15) shown in A. Oct, Pit and CD19 refer to high-affinity binding sites of Oct1, Pit1 and Pax5 (*CD19* site 1; Czerny and Busslinger, 1995). The anti-Pax antibody was raised against the Pax5 paired domain (Adams et al., 1992). Pre, preimmune serum; Rb, anti-Rb antibody. (C) EMSA analysis with oligonucleotide probes. A mhb extract from 3-day chick embryos and in vitro translated Pax2b, Pax5 and Oct1 proteins were used for EMSA with end-labeled oligonucleotides (A). The complexes P1, P2 and P3 (indicated on the left) are generated by Pax2a, Pax2b and Pax5, respectively. Asterisks indicate potential degradation products. Ab, anti-Pax antibody.

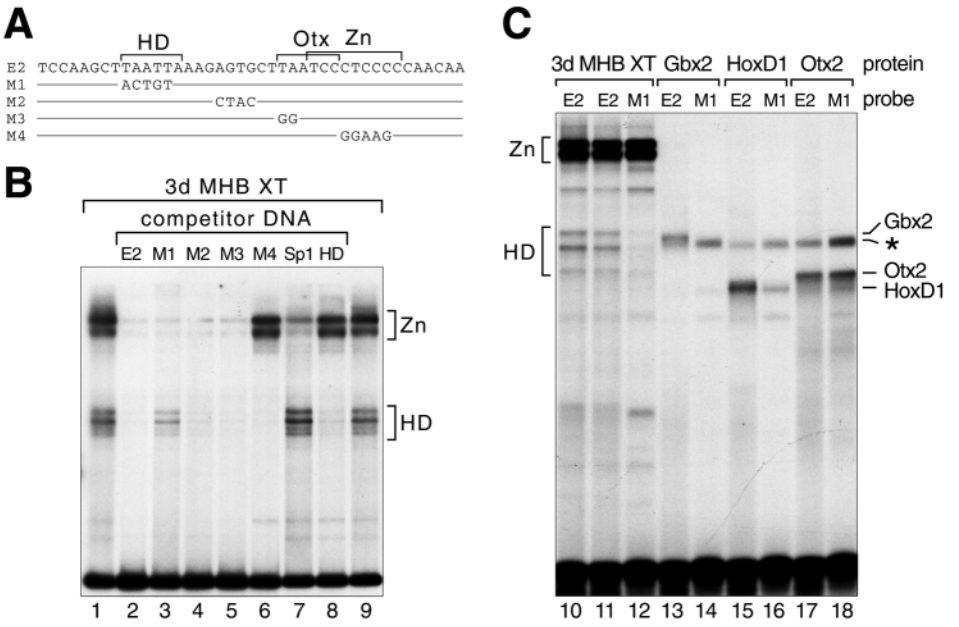



**Fig. 6.** Binding of homeodomain and Zn finger proteins to enhancer element 2. (A) The sequence of element 2 (E2) is shown together with the mutations (M1-M4) introduced into putative binding sites of homeodomain (HD), Otx and zinc finger (Zn) proteins as well as into a conserved nucleotide stretch (M2; Fig. 3). (B) Protein interaction with element 2. A mhb extract prepared from 3-day chick embryos was analyzed by EMSA with probe B (Fig. 3) in the presence of a 100-fold excess of the indicated oligonucleotides (A). HD and Sp1 refer to homeodomain- and Sp1-binding sites. (C) Binding of homeodomain proteins to element 2. A mhb extract from 3-day chick embryos and in vitro translated Gbx2, Otx2 and HoxD1 proteins were analyzed by EMSA with the end-labeled oligonucleotides indicated. An En-specific antiserum (αEnhb-1) (Davis et al., 1991) was added to the binding reaction analyzed in lane 11. The asterisk denotes an unspecific DNA-binding activity.

expression was variable (Fig. 7K). One embryo expressed the transgene only in the basal plate (Fig. 7A), while others showed reduced β-galactosidase expression within the mhb (Fig. 7B-D). In contrast, all embryos carrying the S2 or S3 mutation reproducibly revealed intense β-galactosidase staining at the mhb (Fig. 7E,F,K), as seen with the wild-type 0.6z transgene (Fig. 2L). Hence, the mhb enhancer of *Pax5* is fully functional in the absence of POU protein binding which is selectively abrogated by the A to G mutation S2 or the 4 bp substitution S3 (Fig. 5). These experiments therefore identified the Pax-binding site as the critical regulatory element in the proximal enhancer region.

## Homeodomain and zinc finger proteins interact with a distal enhancer element

The transgene 0.43z contains the smallest enhancer region that was still able to yield mhb expresssion in our transgenic assay (Fig. 2M,N). Further deletion from the 5′ end led to inactivation of the enhancer, indicating that the 45 base pairs missing in transgene 0.39z must contain essential regulatory sequences (Figs 1A, 3). Sequence inspection revealed two overlapping homeodomain-binding sites (TAATTA; Gehring et al., 1994), an Otx-binding site (TAATCC; Gan et al., 1995) and a recognition sequence for Sp1-like zinc finger proteins (Briggs et al., 1986) (Fig. 6A). Indeed, probe B (nucleotides 170-303 in Fig. 3), which encompasses this module of recognition sequences (referred to as element 2), was shown by EMSA analysis to interact with multiple proteins present in the 3-day mhb extract (Fig. 6, lane 1). Moreover, all these proteins bound to sequences within element 2, as an excess of the corresponding oligonucleotide E2 prevented complex formation (lane 2). Conversely, the same oligonucleotide used as a probe also detected the various different proteins (lane 10).

The two slowly migrating complexes are generated by Sp1-like zinc finger proteins as they could not be competed with an excess of the oligonucleotide M4 containing a mutation in the Sp1-binding site (Fig. 6, lane 6). Conversely, the formation of both complexes was specifically inhibited by competition with a consensus Sp1-binding site (lane 7). The second cluster of faster migrating complexes was caused by homeodomain proteins which could be titrated away by an excess of an unrelated homeodomain recognition sequence (lane 8) and failed to bind to oligonucleotide M1 containing a mutation in the homeodomain-binding site of element 2 (lanes 3,12). This finding was confirmed by in vitro translated Gbx2 and HoxD1, two representative homeodomain proteins, which bound the wild-type (E2), but not the mutant (M1) element 2 sequence (lane 13-16). However, in vitro translated En proteins were unable to bind to element 2, nor could an En-specific antiserum (Davis et al., 1991) affect the protein binding pattern in the mhb extract (lane 11, unpublished data). Mutation of the Otx recognition sequence (M3) did also not influence protein binding in the mhb extract (lane 5), although in vitro synthesized Otx2 had the potential to interact with element 2 (lanes 17, 18). It is therefore unlikely that Otx2 binds in vivo to element 2 consistent with the fact that the Otx recognition sequence has not been conserved in the human *PAX5* enhancer (Fig. 3). In conclusion, these EMSA analyses identified a distal enhancer region which contains binding sites for homeodomain and zinc finger proteins.

## The homeodomain- and Pax-binding sites cooperatively regulate the activity of the mhb enhancer

The importance of the homeodomain- and zinc finger-binding sites was next assessed by analyzing the expression of 0.6z transgenes carrying the M1 and M4 mutations, respectively (Fig. 7K). These experiments revealed that the activity of the mhb enhancer is critically dependent on the homeodomain-binding sequence TAATTA, as its mutation (M1) strongly reduced transgene expression at the mbh (Fig. 7G,H). Although the loss of β-galactosidase staining varied among different transgenic embryos, it was in all cases most prominent in the dorsal region of the mhb, similar to the effects observed with Pax-binding site mutations in element 1. In contrast, a transgene carrying the M4 mutation in the zinc

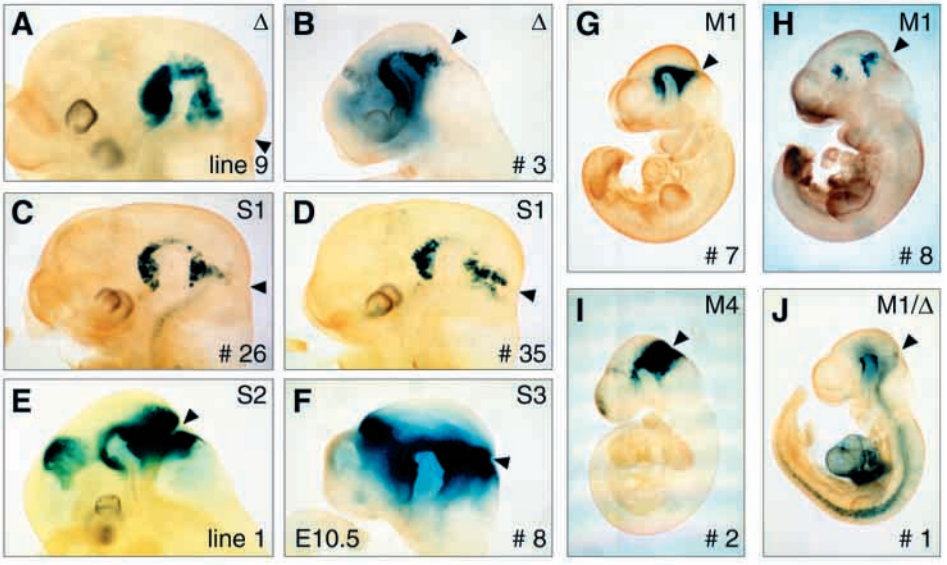
finger recognition sequence was normally expressed at the mhb in all embryos analyzed (Fig. 7I,K). Moreover, the zinc finger recognition sequence did not cooperate with the adjacent homeodomain-binding site, as the variability of transgene expression at the mhb was similar in the presence of the double M1/M4 or single M1 mutation (Fig. 7K). We conclude therefore that the homeodomain-binding site of element 2 is a second regulatory component of the mhb enhancer.

The identification of two functional elements which, upon mutation, similarly affected the activity of the mhb enhancer suggested that the Pax- and homeodomain-binding sites may functionally compensate for each other's loss. To test this hypothesis, we have generated a 0.6z transgene carrying both homeodomain (M1)- and Pax (Δ)-binding site mutations. Although this double mutant transgene was expressed at ectopic locations in 7 different embryos, it was only once found at the mhb (Fig. 7J,K). This embryo showed β-galactosidase staining only in the dorsal region of the mhb where the expression of transgenes with corresponding single mutations was consistently reduced (Fig. 7). Hence, the staining pattern of this embryo did not correlate with the introduced mutations and was also considered to be ectopic due to an intergration site effect. In summary, these data demonstrate that the homeodomain- and Pax-binding sites cooperatively control the activity of the mhb enhancer.

## Cross-regulation of *Pax5* by Pax2 during mhb development

The discovery of an essential Pax2/5/8-binding site in the mhb enhancer of *Pax5* raised the question of which protein of the Pax2/5/8 family is involved in the regulation of this enhancer. All three members of this *Pax* subfamily are known to be expressed at the mhb in a . defined temporal sequence with *Pax2* being activated first followed by *Pax5* and later by *Pax8* (see Pfeffer et al., 1998). In addition, we have recently demonstrated that the Pax2 and Pax5 proteins can substitute for each other and are thus equivalent with regard to their function in mhb development (M. B., unpublished data). The above question can therefore be rephrased to ask whether the initation of *Pax5* expression depends on cross-regulation by Pax2 and/or whether *Pax5* expression is later maintained by autoregulation.

To investigate an autoregulatory role of Pax5, we compared the β-galactosidase staining of heterozygous $Pax5^{+/lacZ}$ and homozygous $Pax5^{lacZ/lacZ}$ embryos which contained a *lacZ* gene insertion in the endogenous, targeted *Pax5* allele (Urbánek et al., 1994). Embryos of the two genotypes failed, however, to reveal any difference in staining pattern at days 10.5 and 12.5 (Fig. 8A; unpublished data). The absence of functional Pax5 protein in homozygous mutant embryos thus indicates that autoregulation does not significantly contribute to *Pax5* expression at the mhb. We next examined a possible dependency of the minimal mhb enhancer on Pax5 function. For this purpose, we crossed the transgene 0.6z into a mouse strain containing a mutant *Pax5* allele which was inactivated by deletion of exon 2 (Δ) rather than by *lacZ*




K

| E2 HD Zn / E1 POU Pax | 0.6z construct | total tg | mhb expression - | w | i | r | n | fb expr. |
|---|---|---|---|---|---|---|---|---|
| | wt | 9 | 0 | 0 | 0 | 0 | 5 | bp, zli |
| | Δ | 9 | 0 | 1 | 3 | 2 | 0 | bp |
| | S1 | 12 | 3 | 0 | 2 | 1 | 0 | bp |
| | S2 | 7 | 0 | 0 | 0 | 0 | 4 | bp, zli |
| | S3 | 6 | 0 | 0 | 0 | 0 | 6 | bp, zli |
| | M1 | 12 | 1 | 2 | 1 | 1 | 0 | bp |
| | M4 | 6 | 0 | 0 | 0 | 0 | 4 | (bp) |
| | M1/M4 | 10 | 4 | 1 | 3 | 1 | 0 | - |
| | M1/Δ | 21 | 7* | 0 | 0 | 0 | 0 | - |

**Fig. 7.** Pax- and homeodomain-binding sites cooperatively regulate the mhb enhancer. The activity of the mhb enhancer depends on a functional Pax-binding site in element 1 (A-F) and homeodomain recognition sequence in element 2 (G-J). The indicated mutations were introduced into transgene 0.6z. Embryos were stained for β-galactosidase activity at day 10.5 (F-J) or 11.5 (A-E). An arrowhead indicates the mhb constriction. Numbers refer to transgenic lines or $G_0$ embryos (#). (K) A statistical overview of the β-galactosidase patterns is shown with a schematic diagram depicting the effects of the different mutations on protein binding. The number of embryos showing β-galactosidase expression at the mhb was classified as weak (w, only ventral staining), intermediate (i), reduced (r, on the dorsal side) or normal (n). Embryos with weak (A,H), intermediate (B-D,G) or normal (E,F,I) staining are shown. Column (-) lists all embryos exhibiting only ectopic β-galactosidase staining. One of seven embryos (asterisk) ectopically expressed the 0.6z-M1/Δ transgene in the dorsal, but not ventral mhb region.

insertion (M. Horcher and M. B., unpublished data). β-Galactosidase staining at days 10.5 and 12.5 also revealed no difference between heterozygous and homozygous mutant embryos (Fig. 8B), indicating that the transgene, like the endogenous *Pax5* locus, is not under autoregulatory control by Pax5.

The initiation of endogenous *Pax5* expression was, however, shown by whole-mount in situ hybridization to be significantly delayed in homozygous *Pax2*$^{-/-}$ embryos compared to heterozygous control embryos (Fig. 8C). In homozygous embryos, *Pax5* expression could be first detected at the 7- to 8-somite stage, whereas *Pax5* transcription is already initiated at 3-5 somites in heterozygous and wild-type embryos (Urbánek et al., 1994; Rowitch and McMahon, 1995). Furthermore, the expression of *Pax5* commences in homozygous embryos near the midline which, on neural tube folding, becomes the ventral region of the mhb (Fig. 8C). At the same stage, *Pax5* expression could not be detected in the lateral (later dorsal) aspect of the neural plate (Fig. 8C). However, the *lacZ* gene inserted in the targeted *Pax2* locus was expressed throughout the entire mhb region in *Pax2*$^{-/-}$ embryos, indicating that the lateral mhb tissue has not been lost at this early stage (Fig. 8E). Moreover, *Pax5* expression increased up to the 12-somite stage (Fig. 8D), but could then not be followed further due to onset of tissue deletion in *Pax2*$^{-/-}$ embryos raised on the C3H/He strain background (M. B., unpublished results). The delayed and medially restricted *Pax5* expression in *Pax2*$^{-/-}$ embryos unequivocally demonstrates cross-regulation of *Pax5* by Pax2 and correlates well with the severe effect of the Pax-binding site mutation on enhancer activity in the dorsal mhb region. Taken together, these data indicated that the Pax recognition sequence in the minimal *Pax5* enhancer mediates cross-regulation by Pax2 rather than autoregulation by Pax5.

## DISCUSSION

The transcription factor Pax5 (BSAP) plays an essential role in B-lymphopoiesis and in the development of the mhb region which gives rise to the midbrain and cerebellum of the vertebrate embryo (Urbánek et al., 1994). Here we have demonstrated that a 25 kb fragment from the 5′ region of the *Pax5* locus contains a 435 bp enhancer which directs gene expression in the correct spatial and temporal pattern at the mhb of transgenic embryos. This 5′ region was, however, unable to activate transgene expression in B-lymphocytes, indicating that the B-cell-specific control region(s) must reside at a more distant location from the *Pax5* promoters. The mhb enhancer of the human and mouse *Pax5* genes has been conserved in sequence and position at −5.6 kb upstream of the distal promoter. Surprisingly however, neither sequence comparison nor transgenic analysis allowed us to identify a mhb enhancer in the 5′ flanking region of the chick and pufferfish *Pax5* genes (unpublished data), although *Pax5* expression is very similar at the mhb in these vertebrates compared to the mouse embryo (Pfeffer et al., 1998; Funahashi et al., 1999). It appears therefore that the function but not the location of the mhb enhancer has been conserved during vertebrate evolution.

Several arguments suggest that the identified enhancer is the only mhb-specific control region of the mammalian *Pax5* locus. First, we have identified only a single mhb enhancer within the 25 kb 5′ region analyzed. Second, this enhancer accurately reflects the early and late phases of *Pax5* expression at the mhb in both wild-type and *Pax5* mutant embryos. Third, the minimal 435 bp enhancer is sufficient to confer the correct mhb-specific activity to a heterologous β-globin promoter. When analyzed outside of the *Pax5* context, the minimal enhancer can give rise to ectopic expression in the forebrain and basal plate of the neural tube, suggesting that communication between the enhancer and the endogenous *Pax5* promoters is important for restricting its activity to the mhb region of the embryo.

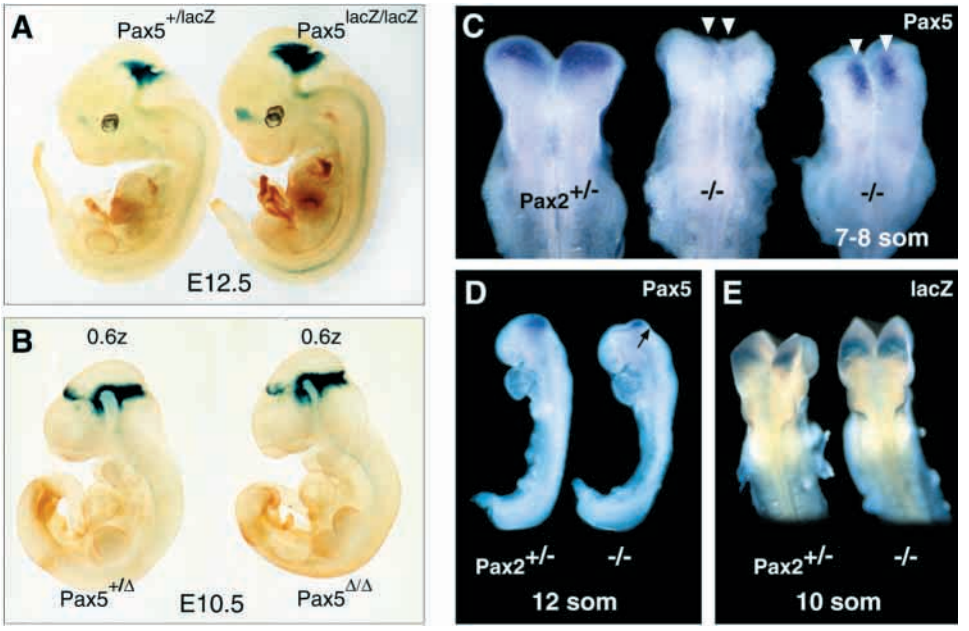



**Fig. 8.** Cross-regulation of *Pax5* by Pax2 during mhb development. (A,B) *Pax5* is not subject to autoregulation. The *lacZ* gene inserted into the *Pax5* locus is normally expressed at the mhb of *Pax5*$^{lacZ/lacZ}$ embryos (A). The transgene 0.6z (line 6) is identically expressed in *Pax5*$^{+/\Delta}$ and *Pax5*$^{\Delta/\Delta}$ embryos (B). The Δ allele lacking *Pax5* exon 2 fails to code for a functional protein (M. Horcher and M. B., unpublished data). (C,D) Delayed initiation of *Pax5* expression in *Pax2*$^{-/-}$ embryos. Whole-mount in situ hybridisation with an antisense *Pax5* probe (Adams et al., 1992) was used to detect endogenous *Pax5* expression (blue) in *Pax2*$^{+/-}$ and *Pax2*$^{-/-}$ embryos of the same litter. At the 7- to 8-somite stage (C), *Pax5* expression is delayed and restricted to the midline of the prospective mhb region (arrowheads) in homozygous embryos (dorsal view). At the 12-somite stage (D), *Pax5* expression has increased, but now the mhb tissue (arrow) starts to be deleted in *Pax2*$^{-/-}$ embryos (lateral view). (E) β-Galactosidase expression throughout the entire mhb region of *Pax2*$^{-/-}$ embryos at the 10-somite stage.

The minimal mhb enhancer of *Pax5* is composed of at least two elements which contain binding sites for homeodomain, zinc finger, POU and Pax proteins. Mutation of individual binding sites revealed that only the homeodomain and Pax recognition sequences are critically involved in the control of the mhb enhancer. Inactivation of either binding site affected the activity of the enhancer in a similar dorsoventral gradient, with the strongest reduction observed in the dorsal mhb region. Simultaneous mutation of both sites resulted in complete inactivation of the enhancer, demonstrating that the homeodomain- and Pax-binding sites cooperatively regulate the activity of the mhb enhancer.

### The mhb enhancer of *Pax5* is a direct target of Pax2

The three members of the *Pax2/5/8* family arose by gene duplications at the onset of vertebrate evolution and have since largely been conserved with regard to their expression patterns (Pfeffer et al., 1998) and DNA-binding specificity (Czerny et al., 1997). The temporal sequence of gene activation at the mhb has also been maintained with *Pax2* expression being initated before *Pax5* and *Pax8* expression (Rowitch and McMahon, 1995; Pfeffer et al., 1998). Our recent expression analysis of *Pax2/5/8* genes in zebrafish embryos revealed that the mhb-specific expression of *Pax5* is entirely dependent on Pax2, as it is never initiated in *noi* mutant embryos lacking the Pax2.1 protein (Pfeffer et al., 1998). In contrast, the absence of Pax2 did not seem to affect expression of the mouse *Pax5* gene at the mhb of *Pax2*$^{-/-}$ embryos (Torres et al., 1996). However, the brain phenotype of the *Pax2* mutation proved to be strongly dependent on the background of the mouse strain analyzed, ranging from almost normal development of the midbrain and cerebellum on the C57BL/6 background (Schwarz et al., 1997) to complete deletion of these brain structures in the C3H/He strain (Favor et al., 1996; M.Bouchard, unpublished data). By analyzing the *Pax2* mutation on the non-permissive C3H/He background, we have now demonstrated that the expression of *Pax5* is delayed at the mhb of *Pax2*$^{-/-}$ embryos and fails to be initiated in the lateral neural plate which, upon neural tube closure, becomes the dorsal region of the mhb. Similarly, mutation of the Pax-binding site in the minimal *Pax5* enhancer affected transgene expression most severely in the dorsal mhb region, suggesting that Pax2 exerts its control by directly interacting with the mhb enhancer. Protein-binding assays indeed demonstrated that the Pax2 and Pax5 proteins of the Pax transcription factor family bind with highest affinity to the Pax recognition sequence of the mhb enhancer. Autoregulatory control of the enhancer by Pax5 is, however, unlikely as *lacZ* genes under the control of the endogenous *Pax5* locus or the minimal enhancer were normally expressed at the mhb of *Pax5* mutant embryos. In summary, our data therefore indicate that the 435 bp enhancer of the mouse *Pax5* gene is a direct target of Pax2 and requires Pax2 function for its initial activation at the mhb. Hence, the *Pax5* gene has been evolutionarily conserved also with regard to its regulation at the mhb. The fish and mouse *Pax5* genes differ only in the degree of their dependency on Pax2 function, as the loss of Pax2 prevents activation of the fish *Pax5* gene at the mhb (Pfeffer et al., 1998), while it is partly compensated for by homeodomain proteins binding to the mouse *Pax5* enhancer (this study).

### Cooperation between Pax2 and homeodomain proteins

Pax2 is already expressed during late gastrulation almost a day before the onset of *Pax5* expression in the mhb region (Rowitch and McMahon, 1995), indicating that Pax2 can activate the *Pax5* enhancer only in cooperation with other regulators. Homeodomain proteins binding to the functional TAATTA sequence of the mhb enhancer are likely candidates for these cooperating transcription factors. Different homeodomain proteins bind, however, promiscuously in vitro to the same TAAT motif which hampers biochemical identification of the particular regulator responsible for a specific in vivo phenotype (Gehring et al., 1994). Normal development of the mhb region depends on the homeodomain proteins Otx2, Gbx2, En1 and En2 (Joyner et al., 1991; Wurst et al., 1994; Matsuo et al., 1995; Wasserman et al., 1997). Of these proteins, only Gbx2 was able to interact with the functional TAAT motif of the *Pax5* enhancer, while Otx2, En1 and En2 failed to bind even in the presence of Pbx1, which is known to enhance the binding of En proteins by cooperative dimerization on DNA (Peltenburg and Murre, 1996). The expression domains of Gbx2 and Otx2 overlap only partially with that of Pax2 in the mhb region (Hidalgo-Sánchez et al., 1999), thus rendering these two proteins less likely candidates for regulating the mhb enhancer. In contrast, the *En1* gene is activated entirely within the *Pax2* expression domain at the 1-somite stage just before the onset of Pax5 expression at 3-5 somites (Rowitch and McMahon, 1995). A direct role of En1 in the activation of the *Pax5* enhancer is, however, excluded both by the inability of En1 to interact with the enhancer and by the fact that En proteins are constitutive repressors of gene transcription (Jiménez et al., 1997). It is therefore likely that a hitherto unknown homeodomain protein with an En1-like expression pattern cooperates with Pax2 in the regulation of the mhb enhancer of *Pax5*.

### Cross-regulation of *Pax* genes

Patterning of the early neural tube critically depends on members of different *Pax* subfamilies. In particular, *Pax6* plays an essential role in the development of the forebrain and spinal cord (Stoykova et al., 1996; Ericson et al., 1997), whereas *Pax2* and *Pax5* are together required for normal morphogenesis of the midbrain and cerebellum (Urbánek et al., 1994; Schwarz et al., 1997, 1999). Negative cross-regulation between *Pax2/5* and *Pax6* has been implicated in the establishment of mutually exclusive expression patterns of these *Pax* genes in the developing CNS (Schwarz et al., 1999). At the molecular level, this hypothesis has been verified in the developing eye where *Pax6* and *Pax2* are expressed in adjacent domains of the neural retina and optic stalk, respectively. In the absence of Pax2, the expression domain of *Pax6* extends into the optic stalk concomitant with a similar expansion of the pigmented neural retina (Torres et al., 1996). Interestingly, the retina-specific enhancer of the *Pax6* gene contains Pax2-binding sites which mediate transcriptional repression of *Pax6* in the *Pax2* expression domain of the optic stalk (Kammandel et al., 1999; P. Gruss, personal communication). Negative cross-regulation between members of different *Pax* subfamilies is therefore used to specify adjacent regions in the developing brain.

In contrast, genes of the same *Pax* subfamily arose by more

recent gene duplications and then assumed non-redundant functions in the same developmental pathway (reviewed by Mansouri et al., 1996). A hierarchical relationship among these genes was frequently established by intercalation of one of the duplicated genes below the other in the same genetic cascade. Here we have presented genetic and biochemical evidence that *Pax2* directly activates the *Pax5* gene during development of the mhb region. A similar situation has recently been described for the duplicated *Drosophila Pax6* genes *toy* and *ey*, both of which play key roles in eye development of the fly (Czerny et al., 1999). Toy was shown to function upstream of *ey* by binding to and directly regulating the eye-specific enhancer of the *ey* gene (Czerny et al., 1999). Hence, positive cross-regulation between members of the same *Pax* subfamily has independently evolved in the insect and vertebrate lineages to diversify the function of Pax proteins within a given developmental pathway.

We thank T. Jenuwein for initial help in generating transgenic mice, M. Horcher for providing $Pax5^{+/\Delta}$ mice, R. Krumlauf for transgenic vectors, G. Martin, S.-L. Ang, W. Wurst and W. Herr for cDNA clones, A. Joyner for anti-En antibodies, U. Schibler for advice on nuclear extract preparation, G. Reim for analysis of the chick and pufferfish *Pax5* genes and G. Schaffner for DNA sequencing. This work was supported by the IMP and the Austrian Science Foundation (grant P11025-MED).

## REFERENCES

**Adams, B., Dörfler, P., Aguzzi, A., Kozmik, Z., Urbánek, P., Maurer-Fogy, I. and Busslinger, M.** (1992). *Pax-5* encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS, and adult testis. *Genes Dev.* **6**, 1589-1607.

**Barberis, A., Superti-Furga, G. and Busslinger, M.** (1987). Mutually exclusive interaction of the CCAAT-binding factor and of a displacement protein with overlapping sequences of a histone gene promoter. *Cell* **50**, 347-359.

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

**Briggs, M. R., Kadonaga, J. T., Bell, S. P. and Tjian, R.** (1986). Purification and biochemical characterization of the promoter-specific transcription factor, Sp1. *Science* **234**, 47-52.

**Busslinger, M., Klix, N., Pfeffer, P., Graninger, P. G. and Kozmik, Z.** (1996). Deregulation of *PAX-5* by translocation of the Eμ enhancer of the *IgH* locus adjacent to two alternative *PAX-5* promoters in a diffuse large-cell lymphoma. *Proc. Natl. Acad. Sci. USA* **93**, 6129-6134.

**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* family. *Mech. Dev.* **67**, 179-192.

**Czerny, T. and Busslinger, M.** (1995). DNA-binding and transactivation properties of Pax-6: three amino acids in the paired domain are responsible for the different sequence recognition of Pax-6 and BSAP (Pax-5). *Mol. Cell. Biol.* **15**, 2858-2871.

**Czerny, T., Halder, G., Kloter, U., Souabni, A., Gehring, W. J. and Busslinger, M.** (1999). *Twin of eyeless*, a second *Pax-6* gene of *Drosophila*, acts upstream of *eyeless* in the control of eye development. *Mol. Cell* **3**, 297-307.

**Davis, C. A., Holmyard, D. P., Millen, K. J. and Joyner, A. L.** (1991). Examining pattern formation in mouse, chicken and frog embryos with an *En*-specific antiserum. *Development* **111**, 287-298.

**Dressler, G. R., 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.

**Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J.** (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-180.

**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 $Pax2^{1Neu}$ 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.

**Funahashi, J.-I., Okafuji, T., Ohuchi, H., Noji, S., Tanaka, H. and Nakamura, H.** (1999). Role of *Pax-5* in the regulation of a mid-hindbrain organizer's activity. *Dev. Growth Differ.* **41**, 59-72.

**Gan, L., Mao, C.-A., Wikramanayake, A., Angerer, L. M., Angerer, R. C. and Klein, W. H.** (1995). An orthodenticle-related protein from *Strongylocentrotus purpuratus*. *Dev. Biol.* **167**, 517-528.

**Gehring, W. J., Qian, Y. Q., Billeter, M., Furukubo-Tokunaga, K., Schier, A. F., Resendez-Perez, D., Affolter, M., Otting, G. and Wüthrich, K.** (1994). Homeodomain-DNA recognition. *Cell* **78**, 211-223.

**Hamburger, V. and Hamilton, H. L.** (1992). A series of normal stages in the development of chick embryos. *Dev. Dyn.* **195**, 231-272.

**Herr, W. and Cleary, M. A.** (1995). The POU domain: versatility in transcriptional regulation by a flexible two-in-one DNA-binding domain. *Genes Dev.* **9**, 1679-1693.

**Hidalgo-Sánchez, M., Millet, S., Simeone, A. and Alvarado-Mallart, R.-M.** (1999). Comparative analysis of *Otx2*, *Gbx2*, *Pax2*, *Fgf8* and *Wnt1* gene expression during the formation of the chick midbrain/hindbrain domain. *Mech. Dev.* **80**, 175-178.

**Jiménez, G., Paroush, Z. and Ish-Horovicz, D.** (1997). Groucho acts as a corepressor for a subset of negative regulators, including Hairy and Engrailed. *Genes Dev.* **11**, 3072-3082.

**Joyner, A. L., Herrup, K., Auerbach, B. A., Davis, C. A. and Rossant, J.** (1991). Subtle cerebellar phenotype in mice homozygous for a targeted deletion in the *En-2* homeobox. *Science* **251**, 1239-1243.

**Kammandel, B., Chowdhury, K., Stoykova, A., Aparicio, S., Brenner, S. and Gruss, P.** (1999). Distinct *cis*-essential modules direct the time-space pattern of the *Pax6* gene activity. *Dev. Biol.* **205**, 79-97.

**Mansouri, A., Chowdhury, K. and Gruss, P.** (1998). Follicular cells of the thyroid gland require *Pax8* gene function. *Nature Genet.* **19**, 87-90.

**Mansouri, A., Hallonet, M. and Gruss, P.** (1996). *Pax* genes and their roles in cell differentiation and development. *Curr. Opin. Cell Biol.* **8**, 851-857.

**Matsuo, I., Kuratani, S., Kimura, C., Takeda, N. and Aizawa, S.** (1995). Mouse *Otx2* functions in the formation and patterning of rostral head. *Genes Dev.* **9**, 2646-2658.

**Mihailescu, D., Küry, P. and Monard, D.** (1999). An octamer-binding site is crucial for the activation of an enhancer active at the embryonic met-/mesencephalic junction. *Mech. Dev.* **84**, 55-67.

**Nornes, H. O., Dressler, G. R., Knapik, E. W., Deutsch, U. and Gruss, P.** (1990). Spatially and temporally restricted expression of Pax2 during murine neurogenesis. *Development* **109**, 797-809.

**Peltenburg, L. T. C. and Murre, C.** (1996). Engrailed and Hox homeodomain proteins contain a related Pbx interaction motif that recognizes a common structure present in Pbx. *EMBO J.* **15**, 3385-3393.

**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.

**Plachov, D., Chowdhury, K., Walther, C., Simon, D., Guenet, J. L. and Gruss, P.** (1990). *Pax8*, a murine paired box gene expressed in the developing excretory system and thyroid gland. *Development* **110**, 643-651.

**Rowitch, D. H. and McMahon, A. P.** (1995). *Pax-2* expression in the murine neural plate precedes and encompasses the expression domains of *Wnt-1* and *En-1*. *Mech. Dev.* **52**, 3-8.

**Schwarz, M., Alvarez-Bolado, G., Dressler, G., Urbánek, P., Busslinger, M. and Gruss, P.** (1999). Pax2/5 and Pax6 subdivide the early neural tube into three domains. *Mech. Dev.* **82**, 29-39.

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

**Stoykova, A., Fritsch, R., Walther, C. and Gruss, P.** (1996). Forebrain patterning defects in *Small eye* mutant mice. *Development* **122**, 3453-3465.

**Torres, M., Gómez-Pardo, E. and Gruss, P.** (1996). *Pax2* contributes to inner ear patterning and optic nerve trajectory. *Development* **122**, 3381-3391.

**Urbánek, P., Fetka, I., Meisler, M. H. and Busslinger, M.** (1997). Cooperation of *Pax2* and *Pax5* in midbrain and cerebellum development. *Proc. Natl. Acad. Sci. USA* **94**, 5703-5708.

**Urbánek, 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.

**Wassef, M. and Joyner, A. L.** (1997). Early mesencephalon/metencephalon patterning and development of the cerebellum. *Perspectives Dev. Neurobiol.* **5**, 3-16.

**Wasserman, K. M., Lewandoski, M., Campbell, K., Joyner, A. L., Rubenstein, J. L. R., 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.

**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.

**Yee, S.-P. and Rigby, R. W. J.** (1993). The regulation of myogenin gene expression during the embryonic development of the mouse. *Genes Dev.* **7**, 1277-1289.