The activation and maintenance of Pax2 expression at the mid-hindbrain boundary is controlled by separate enhancers

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SUMMARY

Pax2 is the earliest known gene to be expressed throughout the mid-hindbrain region in late gastrula embryos of the mouse and is essential for the formation of an organizing center at the midbrain-hindbrain boundary (MHB), which controls midbrain and cerebellum development. We have used transgenic analysis to identify three MHB-specific enhancers in the upstream region of the mouse Pax2 gene. A 120 bp enhancer (at –3.7 kb) in cooperation with the endogenous promoter was sufficient to induce transgene expression in the anterior neural plate of late gastrula embryos, while it was already inactivated again at the MHB during somitogenesis. The activity of this early enhancer was severely reduced by mutation of three homeodomain-binding sites, two of which are part of a recognition sequence for POU homeodomain proteins, Oct3/4 (Pou5f1), the mouse ortholog of zebrafish Pou2, efficiently bound to this sequence, suggesting its involvement in the regulation of the early Pax2 enhancer. Starting at the four-somite stage, Pax2 is expressed at the MHB under the control of two enhancers located at –4.1 kb and –2.8 kb. The distal late enhancer contains a 102 bp sequence that is not only highly conserved between the mouse and pufferfish Pax2 genes, but also contributes to the enhancer activity of both genes in transgenic mice. The proximal 410 bp enhancer, which overlaps with a kidney-specific regulatory element, contains a functional Pax2/5/8-binding site and thus maintains Pax2 expression at the MHB under auto- and cross-regulatory control by Pax2/5/8 proteins. Importantly, the early and proximal late enhancers are not only sufficient but also necessary for expression at the MHB in the genomic context of the Pax2 locus, as their specific deletion interfered with correct temporal expression of a large Pax2 BAC transgene. Hence, separate enhancers under the control of distinct transcription factors activate and maintain Pax2 expression at the MHB.

Key words: Pax2, Midbrain-hindbrain boundary, Enhancer, Mouse, Pufferfish, BAC transgenes

INTRODUCTION

The anterior neural plate of the vertebrate embryo is patterned into the forebrain, midbrain and hindbrain under the influence of local organizing centers. One of the best studied organizers is formed during early somitogenesis at the midbrain-hindbrain boundary (MHB, also known as isthmus) and is responsible for the development of the entire midbrain and cerebellum (Wurst and Bally-Cuif, 2001). This MHB organizer was initially discovered by transplantation experiments, as grafting of MHB tissue into the chick diencephalon or hindbrain resulted in the formation of an ectopic midbrain (Martinez et al., 1991) or cerebellum (Marin and Puelles, 1994), respectively. The MHB region secretes the signaling molecule fibroblast growth factor 8 (Fgf8), which is an important mediator of the organizer activity, as it is both necessary and sufficient for inducing midbrain and cerebellum development (Crossley et al., 1996; Meyers et al., 1998; Reifers et al., 1998). Several transcription factors are involved in the establishment and maintenance of the MHB organizer. During gastrulation, Otx2 and Gbx2 are expressed in apposed domains in the anterior and posterior neural plate, respectively, and their expression interface determines the future position of the MHB organizer. Subsequently, the transcription factors Pax2, Pax5 and Pax8, and En1 and En2 are expressed across the Otx2-Gbx2 boundary in the mid-hindbrain region and participate together with the secreted factors Fgf8 and Wnt1 in a cross-regulatory network to maintain the MHB organizer (Wurst and Bally-Cuif, 2001).

Pax2, which codes for a paired domain transcription factor of the Pax2/5/8 subfamily (Dressler et al., 1990), is the earliest known gene to be expressed across the Otx2-Gbx2 boundary in the mouse gastrula embryo (Rowitch and McMahon, 1995). Its expression is initiated at the late primitive streak stage (embryonic day (E) 7.5) in an extended domain corresponding to the prospective mid-hindbrain region. This broad expression of Pax2 is progressively refined to a narrow ring centered at the MHB (Rowitch and McMahon, 1995) during somitogenesis, when expression of the related Pax5 and Pax8 genes is induced
in the same region at 3-4 and 6-7 somites, respectively (Urbánek et al., 1994; Rowitch and McMahon, 1995). This sequential activation of the Pax2/5/8 genes at the MHB is a conserved feature of all vertebrates (Pfeffer et al., 1998; Heller and Brändli, 1999) and critically determines the role of these genes, given the fact that their transcription factors have equivalent biochemical and thus redundant functions in MHB development (Bouchard et al., 2000). Pax2 mutations result in loss of the midbrain and cerebellum both in zebrafish (Brand et al., 1996; Lun and Brand, 1998) and in mice on the C3H/He strain background (Favor et al., 1996; Bouchard et al., 2000). By contrast, mice with an inactivated Pax5 or Pax8 gene exhibit only a mild midline defect (Urbánek et al., 1994) or even normal MHB development (Mansouri et al., 1998). At the molecular level, Pax2 was shown to directly activate Pax5 expression by binding to and regulating the MHB-specific enhancer of Pax5 (Pfeffer et al., 1998; Pfeffer et al., 2000). Moreover, Fgf8 is entirely dependent on Pax2 for its activation at the MHB, as recently shown by gain- and loss-of-function analyses in chick and mouse embryos (Ye et al., 2001). Hence, Pax2 contributes to the formation of the MHB organizer by activating the expression of its key component Fgf8.

The MHB organizer is maintained by a positive feedback loop consisting of complex regulatory interactions between the different MHB-specific factors (Wurst and Bally-Cuif, 2001). Consequently, the MHB organizer is lost upon individual mutation of these regulators, whereas ectopic expression of a single factor activates most of the other components in the regulatory cascade (Nakamura, 2001). Hence, gain- and loss-of-function experiments preclude a detailed analysis of the interactions between the critical players involved in the formation and maintenance of the MHB organizer. To identify direct upstream regulators of Pax2, we have performed an in-depth transgenic analysis to define the MHB-specific enhancers of the mouse Pax2 gene. A starting point was the finding that an 8.5 kb upstream region of mouse Pax2 directs transgene expression in the mid-hindbrain region and developing kidney (Rowitch et al., 1999). We have used the evolutionary conservation of upstream sequences between human, mouse and pufferfish Pax2 genes as a guide to define three functional MHB-specific enhancers and one kidney-specific regulatory element by classical transgenesis. Deletion of these enhancers in a Pax2 BAC transgene revealed that two of these elements are also necessary for directing expression at the MHB in the larger genomic context of the mouse Pax2 locus. A 120 bp early enhancer (at –3.7 kb) under the control of POU homeodomain proteins activates Pax2 in the neural plate of late gastrula embryos. Pax2 transcription is subsequently maintained at the MHB by a 410 bp late enhancer (at –2.8 kb), which is subject to auto- and cross-regulation by Pax2/5/8 proteins. Hence, distinct enhancers control the activation and maintenance of Pax2 expression at the MHB.

MATERIALS AND METHODS

DNA constructs

Construct #1 was generated by inserting a 7.2 kb SacI fragment from the 5′ region of mouse Pax2 into the MscI site of pTrap (Pfeffer et al., 2000). Construct #2 was obtained by cloning a 5-kb BamHI/HindIII fragment from a Fugu Pax2.1 cosmid (Pfeffer et al., 1998) into pTrp. Transgenes #3 to #25 were constructed using strategies involving the restriction sites depicted in the relevant figures. A spontaneous deletion (from position –4738 to –3473) gave rise to constructs #7a and #7b. The conserved region, which is present in tandem copies in transgenes #20 and #21, was PCR-amplified with the primers 5′-aagtc TG-GCTAACATTGGAGGAG-3′ and 5′-aagtcTG-TCTGGTCACATTGGAGGAT-3′. The deletion in transgene #24 was derived from the building vector used to generate the deletion in BAC #33. The intermediate homology sequences of transgene #26 and #27 were PCR-amplified with the primers 5′-accaagctTGTGCTTCTT-ATTCTAAAACAC-3′ and 5′-ggagaagctTGGCTGGGGAGGGAT-3′. Transgenes #15 and #28 were mutated using the QuikChange kit (Stratagene) and ~37 nucleotide long primers containing the mutations (Fig. 4D, Fig. 7D) in their center. The first two TAAT motifs of construct #29 were mutated with one primer pair, and the third TAAT sequence was subsequently mutated by PCR using the downstream primer 5′-GGGTTCTTCTGAATTCGGAATTGACGGTACCTC-3′. The underlined nucleotides indicate the restriction site used for cloning.

BAC modification

The Pax2 BAC clones 468C04 (giving rise to transgene #30) and 55116 (transgene #76) were isolated from a mouse genomic library (Research Genetics, Huntsville, AL) and shown to be ~100 kb in size, as they contained three 30-40 kb NotI fragments. The BAC modification method of Yang et al. (Yang et al., 1997) was used to generate the transgenes #30 and #76 by inserting the eGFP gene, linked to an SV40 poly(A) signal, into Pax2 exon 2 in frame after codon 19 (valine). Deletions were introduced with the same method into BAC #30 by the use of building vectors containing homology boxes flanking the deletion sites. The introduced deletions eliminated the following sequences from the Pax2 upstream region (AF433638): BAC #31, nucleotides 2230-2765 (replaced by a HindIII site) and 3680-4111 (ClaI site); BAC #32, 3680-4111 (ClaI site); BAC #33, 2230-2765 (HindIII site); BAC #34, 2230-3287 (HindIII site). BAC DNA was purified on QIAGEN-500 columns and CsCl/EtBr gradients. The supercoiled DNA was extracted with isomyl-alcohol and extensively dialyzed against 10 mM Tris pH 7.5, 0.1 mM EDTA prior to pronuclear injection.

Transgenic mice

Plasmid-free linearized DNA was injected into pronuclei at 2.5-3 ng/µl and supercoiled BAC DNA at 0.8-1 ng/µl. C57BL/6×CBA F1 mice were used for generating transgenic animals, which were identified by PCR with the lacZ primers 5′-ATACGTCTGTCG-TCCCTCAAACGT-3′ and 5′-TCTCAACCACCGACGATAGAG-3′ or GFP primers 5′-CGAGCGCATGATGACGAC-3′ and 5′-TACGAGCTCCAGCGGACCAT-3′. For embryos younger than E8.5, genotyping was performed on the whole embryo after the staining reaction.

β-Galactosidase staining and GFP visualization

X-gal staining was performed for 1-36 hours as described (Pfeffer et al., 2000). Embryos younger than E9 were washed after postfixation in 50% glycerol/phosphate-buffered saline (PBS) and cleared in 80% glycerol/PBS before photography. For GFP detection, unfixed embryos were photographed with a CCD camera on a Zeiss fluorescence microscope.

EMSA analysis

Whole-cell extracts were prepared from dissected chick embryos as described (Pfeffer et al., 2000). The mouse Pax2b, Pax3, Pax5, Pax6, En1, Gbx2, Oct1, Otx2 and Xenopus HoxD1 cDNAs were cloned into pKWT2, and proteins were synthesized by a coupled in vitro transcription-translation system (TNT, Promega). Binding of in vitro synthesized proteins (1-2 µl) or whole-cell extracts (0.1 µl) to end-
labeled DNA probes was analyzed by EMSA as described (Pfeffer et al., 2000).

Accession numbers
The mouse, human and Fugu rubripes Pax2 gene sequences were submitted to GenBank (AF433638, AF433639 and AF433640, respectively).

RESULTS
Conservation of upstream regulatory sequences of vertebrate Pax2 genes
An 8.5 kb DNA fragment from the 5’ flanking region of the mouse Pax2 gene was previously shown to contain sufficient information for directing transgene expression in the developing kidney and MHB region of the mouse embryo (Rowitch et al., 1999). To facilitate the identification and characterization of enhancer elements within these sequences, we isolated the 5’ region of the Pax2 genes from mouse, human and the pufferfish Fugu rubripes. Comparison of the corresponding human and mouse sequences revealed three blocks of high homology in addition to the conserved promoter region (Fig. 1A). We refer to these conserved sequences as distal (D), intermediate (I) and proximal (P) homology regions, according to their distance from the promoter (Fig. 1A). In addition, the two mammalian Pax2 genes share with the Fugu Pax2.1 gene extensive homology in the promoter (Fig. 1C) and in a 102 bp sequence of the distal homology region, which contains a conserved Pax-binding site (Fig. 1B).

For functional analysis of these conserved sequences, we generated a parental transgene (#1) by inserting a 7.2 kb SacI fragment from the mouse Pax2 locus upstream of the TATA-box and lacZ reporter gene of the transgenic vector pTrap (Pfeffer et al., 2000). Transgene #1 contained 6.9 kb of 5’ flanking sequence as well as the transcription initiation region of Pax2. Mice carrying transgene #1 were generated by pronuclear DNA injection and analyzed for lacZ expression by X-gal staining of transgenic embryos (Fig. 2D,E, Fig. 6B). For comparison, we analyzed the β-galactosidase staining pattern of Pax2+/lacZ embryos that contain an in-frame lacZ insertion in one of the endogenous Pax2 alleles (Bouchard et al., 2000). Expression of transgene #1 was initiated in a broad region of the neural plate during late gastrulation (Fig. 6B) similar to the endogenous Pax2 gene (Rowitch et al., 1999). At the beginning of somitogenesis, this broad expression was refined to a narrow domain at the MHB in the Pax2+/lacZ embryos (Fig. 2B,C), whereas widespread expression throughout the midbrain and hindbrain was maintained in embryos carrying transgene #1 (Fig. 2D,E). As this protracted expression pattern cannot solely be explained by the longevity of the β-galactosidase protein (Fig. 2C), these data point to the absence of regulatory elements from transgene #1 that normally restrict Pax2 expression to a narrow stripe at the MHB of midgestation (E9.5-E10.5) embryos. During kidney development, transgene #1 was expressed in the pronephros, mesonephros and metanephros (Fig. 2D,E) similar to the endogenous Pax2 gene (Fig. 2B,C), but failed to be active in the Pax2 expression domains of the developing eye, ear and spinal cord. Transgene #1 also gave rise to β-galactosidase expression in the branchial
The Pax2 promoter is essential for expression in presomitic embryos

To study the function of the Pax2 promoter, we deleted the first 600 and 1400 bp of 5’ flanking sequences, thereby juxtaposing the upstream region of Pax2 to the minimal promoter of pTrap in transgenes #3 and #4, respectively. In the absence of the Pax2 promoter, the two transgenes were still expressed in the MHB region and kidney of E10.5 embryos (Fig. 2A,I). Interestingly, deletion of the Pax2 promoter resulted in a sharpening of the anterior expression boundary at the MHB due to the loss of ectopic expression in the diencephalon and midbrain (compare Fig. 2E with Fig. 2I). The same effect was also observed with two other pairs of transgenes (#6a/b and #18a/b) that differed by the presence (a) or absence (b) of the Pax2 promoter (Fig. 3A, Fig. 5A). To determine whether the Pax2 promoter itself directs broad lacZ expression in the midbrain-hindbrain region, we generated transgene #5 containing only the first 1400 bp of Pax2 5’ flanking sequences. Notably, β-galactosidase staining was seen in 80% of all transgenic embryos, although never in the same region (Fig. 2A). Hence, the Pax2 promoter, once removed from its endogenous context, is exquisitely sensitive to the action of fortuitous enhancers present at the random transgene integration site. We conclude therefore that the broad ectopic expression of transgene #1 in the midbrain-hindbrain region is not caused by an inherent activity of the Pax2 promoter.

Remarkably however, transgene #3, which lacks the Pax2 promoter, was not expressed in presomitic embryos in contrast to the parental transgene (#1), as its expression was initiated in the MHB region only at the four-somite stage (Fig. 2H,I). These data point to the existence of early and late enhancers in the Pax2 upstream region that control expression initially in the neural plate of late gastrula embryos and subsequently in the MHB region of midgestation embryos. The activity of the early enhancer(s) seems to depend on the presence of the

Fig. 2. Upstream sequences of the mouse and Fugu Pax2 genes direct expression at the MHB. (A) Schematic diagram of the transgenes and statistical overview of the β-galactosidase staining patterns observed in injected founder embryos or permanent transgenic lines. The indicated DNA fragments of the mouse or Fugu Pax2 gene were linked to the minimal promoter and lacZ gene of pTrap. The number of independent transgenic (tg) and β-galactosidase (β-gal)-positive embryos (a), analyzed between E8.5 and E10.5, is shown together with the number of embryos exhibiting β-galactosidase staining at the MHB (b), in the developing kidney (c) or only in ectopic locations (d). B, BsrD1; Ba, BamHI; H, HindIII; K, KpnI; Sa, SacI. (B,C) β-Galactosidase staining of Pax2lacZ/+ embryos. The MHB is indicated by arrowheads in B-G,I. (D,E) Broad expression of transgene #1 throughout the midbrain and hindbrain. X-gal staining was performed for 20 minutes or 4 hours to detect expression in the brain region (D,E) or developing kidney (insert in E). (F,G) Expression of the Fugu transgene #2 at the MHB of transgenic mouse embryos. (H,I) Later onset and more restricted expression of transgene #3 at the MHB. All embryos are shown in lateral view except for D,H (dorsal view). A, anterior; ba, branchial arch; ms, mesonephros; mt, metanephros; nd, nephric duct; os, optic stalk; ov, otic vesicle; P, posterior; pn, pronephros; sc, spinal chord; som, somites.
endogenous Pax2 promoter in contrast to the late enhancer(s), which also functions in the context of a heterologous promoter. Our data furthermore suggest that the broad ectopic expression of transgene #1 in the midbrain-hindbrain region of midgestation embryos is primarily caused by prolonged activity of the early enhancer-promoter module, which is normally curbed by negative regulatory elements absent from transgene #1.

Characterization of MHB- and kidney-specific enhancers in the proximal homology region

The upstream region of Pax2 was next characterized by deletion mutagenesis. Removal of a 2.1 kb DNA fragment upstream of the three conserved homology regions had no effect on the expression pattern of transgene #6 (Fig. 3A). Likewise, transgene #7 lacking the distal and intermediate homology regions was still normally expressed in the MHB and kidney of midgestation embryos (Fig. 3A,B). A series of deletion constructs (#8-11) mapped both activities to a 755-bp PshAI-ApaI fragment encompassing the proximal homology region (Fig. 3A,C). These constructs functioned independently of the endogenous promoter to give rise to strong β-galactosidase staining at the MHB from the four-somite stage until E10.5 as well as to weaker and more variable expression in the developing kidney (Fig. 3C) (data not shown). Moreover, transgene #7a containing the proximal homology region together with the Pax2 promoter failed to be expressed in presomitic embryos (Fig. 3A) (data not shown). Hence, we conclude that the proximal homology region contains a late, but not early MHB-specific enhancer of Pax2.

Further 3’ deletion of the minimal region by 155 bp (up to the XmnI site), which eliminated part of the proximal homology region, prevented expression of transgene #12 at the MHB, while leaving the kidney-specific expression unaffected (Fig. 3A,D). Hence, the kidney- and MHB-specific enhancers can be functionally separated within the proximal homology region. The complementary transgene #13, which contained the 155 bp deleted in construct #12, was also not expressed at the MHB, indicating that the XmnI site must reside within critical sequences of the MHB-specific enhancer (Fig. 3A). Furthermore, a 410 bp NarI-ApaI fragment, which mainly consists of the proximal homology region, gave rise to weak expression of transgene #14 both at the MHB and in the developing kidney (Fig. 3A,E). By characterizing the same kidney-specific enhancer of Pax2, Kuschert et al. (Kuschert et al., 2001) have recently reported that the NarI-ApaI fragment lacks MHB-specific enhancer activity. This discrepancy to our data is most likely explained by the fact that we also scored embryos with weak MHB staining as positive (Fig. 3E). Thus, although the proximal homology region possesses more robust enhancer activity upon addition of flanking sequences (transgenes #10, #11), it is on its own sufficient to direct reporter gene expression in both the MHB region and developing kidney. These data therefore demonstrate that the evolutionary conservation of Pax2 upstream sequences can be used as a guide to identify critical enhancer regions.

We next searched for transcription factors binding to the proximal homology region in protein extracts that were prepared from micro-dissected MHB or trunk tissue of chick embryos at day 2 (~15 somites; HH stage 12) or day 3 (~40 somites; HH stage 20) (Pfeffer et al., 2000). Electrophoretic mobility shift assay (EMSA) with a 0.3-kb Avai-ApaI DNA probe (Fig. 3A, transgene #11) detected a DNA-binding protein that was present in both MHB extracts and co-migrated with in vitro translated Pax2b (Fig. 4A). A second protein, present only in the 3-day MHB extract, migrated with an
elecrophoretic mobility characteristic of Pax5. EMSA analysis with in vitro translated Pax proteins confirmed that Pax2 and Pax5 bound with high affinity, whereas Pax3 and Pax6 failed to interact with the Aval-ApaI DNA probe (Fig. 4B). Inspection of the proximal homology region revealed two potential recognition sites for Pax2/5/8 proteins (Fig. 4D), although only one sequence (S1) proved by EMSA analysis to be a high-affinity Pax-binding site (Fig. 4C). Nucleotide substitutions (S1m) in site 1, which abolished in vitro binding of Pax2 (Fig. 4C, lanes 3,5), were subsequently introduced into construct #10 to produce transgene #15 (Fig. 3A). Embryos transgenic for this mutant construct still expressed the lacZ reporter gene in the developing kidney and branchial arches, whereas the strong MHB-specific expression normally seen with the parental transgene #10 was completely lost (compare Fig. 3F with Fig. 3F). The presence of a functional Pax2/5/8-binding site in the proximal homology region indicates therefore that the late MHB-specific enhancer of Pax2 is under auto- and cross-regulatory control by Pax2/5/8 proteins.

Identification of a second late MHB-specific enhancer of Pax2

Surprisingly, a transgene (#16) lacking the proximal homology region, but retaining more distal S sequences of Pax2 was still expressed in the MHB region of midgestation embryos (Fig. 5A; data not shown). Hence, a second MHB-specific enhancer must reside in the upstream region of Pax2. Transgene #17 mapped this regulatory element to a 1285 bp Scal-PshAI fragment encompassing the distal and intermediate homology regions. β-Galactosidase expression of transgene #17 was first detected in the MHB region at the four-somite stage, was robust at E8.5 and then became diffuse at E10.5 owing to patchy (residual) β-galactosidase activity, suggesting that the second MHB-specific enhancer was already inactive at the last time point analyzed (Fig. 5B,C).

As the only conserved upstream element of mammalian and Fugu Pax2 genes is located in the distal homology region (Fig. 1A,B), we investigated its function by specific deletion in the context of the parental transgenes containing (#1) or lacking (#3) the Pax2 promoter. Transgenes #18a and #18b proved to be indistinguishable from their parental constructs with regard to temporal and tissue-specific expression (Fig. 5A; data not shown), suggesting that the enhancers in the proximal homology region may compensate for the loss of the conserved distal element. The same deletion within the shorter Scal-PshAI fragment was still compatible with strong expression of transgene #19 at the MHB of eight-somite embryos (Fig. 5D). Two days later, β-galactosidase expression was, however, lost in the dorsal region of the MHB (compare Fig. 5E with Fig. 5C), indicating that the conserved element is essential for maintaining the dorsal activity of the second late MHB-specific enhancer. Analogously, a Fugu Pax2 transgene lacking this distal element failed to be expressed at the MHB of transgenic mouse embryos (Fig. 5A). The conserved element had, however, no intrinsic enhancer activity, as multiple copies of its sequence failed to direct expression of transgenes #20 and #21 in the MHB region (Fig. 5A). Together these data indicate that the upstream region of Pax2 contains a second late MHB-specific enhancer whose continued activity depends on the conserved element in the distal homology region.

A 120 bp enhancer activates Pax2 expression in the early neural plate

The two MHB-specific enhancers described so far are active from the four-somite stage onwards in contrast to the parental transgene #1, which is strongly expressed already in the prospective mid-hindbrain region of late gastrula embryos (Fig. 6B) similar to the endogenous Pax2 gene (Rowitch and McMahon, 1995). Time course analyses of permanent transgenic lines indicated that β-galactosidase expression was specifically lost in presomitic embryos upon deletion of either the promoter (transgene #3, Fig. 2H) or distal/intermediate homology region (transgene #7a; data not shown). These two regions together were furthermore sufficient to direct
expression of transgene #23 in presomitic embryos (Fig. 6A). The distal homology region was, however, dispensable for early expression of transgene #24 (Fig. 6A,C). By contrast, a 338-bp deletion eliminating the intermediate homology region prevented expression of transgene #25 in presomitic embryos (Fig. 6D), while later embryos of the same permanent line expressed β-galactosidase activity in the MHB, kidney, and branchial arches (Fig. 6E). The intermediate homology region also fulfilled the sufficiency criterion for an early enhancer, as one or two tandem copies of this element, linked to the Pax2 promoter, directed expression of the transgenes #26 and #27 in the anterior neural plate of late gastrula embryos (Fig. 6F). It is, however, important to note that the expression of these transgenes was extended in the anterior direction compared to the parental construct #1 (Fig. 6B) or endogenous Pax2 expression (Rowitch and McMahon, 1995). Thus, the anterior forebrain was labeled as strongly as the future mid-hindbrain region, suggesting that the activity of the minimal enhancer is normally suppressed in the anterior neural plate by negative elements located outside of the 120 bp enhancer. Importantly, no β-galactosidase activity was detected at the MHB in E10.5 embryos of the same transgenic line (Fig. 6G). Hence, these data unequivocally identified the 120 bp intermediate homology region as the early MHB-specific enhancer of Pax2.

POU homeodomain proteins regulate the activity of the early enhancer

The early enhancer contains four potential binding sites (TAAT) for homeodomain proteins (Gehring et al., 1994) and a recognition sequence for Sp1-like zinc finger proteins (Briggs et al., 1986) (Fig. 7C). The distal two TAAT motifs abut a recognition sequence (GCA T) for the POU-specific domain of POU homeodomain proteins (Herr and Cleary, 1995), whereas the most proximal motif corresponds to a binding site (TAATCC) for Otx-like homeodomain proteins (Gan et al., 1995). EMSA analyses confirmed that Otx2, HoxD1 and the POU proteins Oct1 and Oct3/4 (Pou5f1) were able to bind to the early enhancer in contrast to Gbx2 and En1 (Fig. 7A,B). Moreover, Oct1 and Oct3/4 efficiently bound to the enhancer module consisting of the distal two TAAT motifs (Fig. 7B).

Considering that the minimal early enhancer (transgene #27) is active throughout the anterior neural plate (Fig. 6F) similar to the expression pattern of Otx2 (Simeone et al., 1993), we first mutated the Otx-binding site in the context of the parental transgene #1 (Fig. 7D). Although this mutation resulted in the loss of Otx2 binding in vitro (Fig. 7A, lane 9), the corresponding transgene #28 was expressed in late gastrula embryos with a similar pattern as the parental construct (compare Fig. 7E with Fig. 6B). Hence, Otx2 is unlikely to regulate the early Pax2 enhancer. We next focussed our attention on the homeodomain-binding sites that were efficiently bound by POU proteins. Mutations of the TAAT motifs, which abolished in vitro binding of Oct1 and Oct3/4 (Fig. 7A,B, lanes 7,13,14), were introduced together with the Otx-binding site mutation into the parental transgene #1 (Fig. 7D). Late gastrula embryos carrying the mutant transgene #29 showed a general reduction in β-galactosidase staining both in the prospective mid-hindbrain region and posterior ectopic expression domain (Fig. 7F). The early Pax2 enhancer therefore appears to be controlled by one or more members of the POU protein family.

Function of the MHB-specific enhancers in the genomic context of the Pax2 locus

The classical transgenic approach described above uncovered the complexity of Pax2 regulation in the developing MHB region. Although each of the identified enhancers is sufficient for directing either early or late expression at the MHB, redundancy may exist, thus raising the question about the functional significance of the different enhancers within the entire Pax2 locus. To address this question, we deleted individual enhancers in the context of a large bacterial artificial chromosome (BAC) spanning the Pax2 locus followed by the generation of BAC transgenic mice.
One of the isolated BACs contained the entire Pax2 gene together with at least 30 kb of upstream sequences. Using homologous recombination in E. coli (Yang et al., 1997), a green fluorescent protein (GFP) gene was inserted in frame into Pax2 exon 2, and its temporal expression pattern was analyzed in permanent transgenic lines generated with BAC #30 (Fig. 8A). This BAC transgene completely recapitulated the expression pattern of the endogenous Pax2 gene in the developing MHB, ear, kidney, spinal cord, tail bud and branchial arches with one exception (Fig. 8B-F). GFP expression was never detected in the developing visual system, indicating that the eye-specific enhancer must be located at a considerable distance from the Pax2 gene.

Combined deletion of the proximal and distal homology regions in BAC transgene #31 led to the loss of GFP expression at the MHB without affecting other Pax2 expression domains of E10.5 embryos (Fig. 8H). The same loss of MHB-specific expression was observed upon removal of only the proximal homology region in BAC #32 (Fig. 8I), whereas transgene #33, which contained a specific deletion of the distal homology region, was normally expressed at the MHB (Fig. 8J). These data therefore indicate that the late enhancer in the proximal homology region is absolutely required for Pax2 expression at the MHB of midgestation embryos. Surprisingly, the BAC transgenes #31 and #32 gave rise to GFP expression in the nephric duct and tubules of the developing kidney, despite the absence of the proximal homology region (Fig. 8G-I). These data therefore point to the existence of a second kidney-specific enhancer of Pax2, which must reside outside of the 5' region analyzed.

Although the loss of the distal homology region was still compatible with strong GFP expression in the mid-hindbrain region of late gastrula and early somitic embryos (Fig. 8K), additional deletion of the intermediate homology region abrogated early expression of the BAC transgene #34 (Fig. 8A). Expression of this transgene was first detected at the four-somite stage and subsequently reached high levels in the MHB region of eight-somite and later embryos (Fig. 8M,N). We therefore conclude that the MHB-specific enhancer of the intermediate homology region is strictly required for early activation of the Pax2 gene in its proper genomic context.

**DISCUSSION**

**Complex regulation of Pax2 during MHB development**

Pax2 is the earliest known transcription factor to be expressed in the entire mid-hindbrain region (Rowitch and McMahon, 1995) and is required for formation of the MHB organizer in mouse embryos (Favor et al., 1996; Bouchard et al., 2000), where it controls the expression of the organizer signal Fgf8 (Ye et al., 2001). Using conventional transgenic analysis, we have identified three distinct MHB-specific enhancers in the upstream region of mouse Pax2. Two late enhancers present in the proximal and distal homology regions direct expression at the MHB from the four-somite stage onwards, whereas an early enhancer in the intermediate homology region controls Pax2 activation in the prospective mid-hindbrain region of late gastrula embryos. BAC transgenesis revealed that the early element and the proximal late enhancer are not only sufficient but also necessary for directing MHB-specific expression in the context of the Pax2 locus. Hence, separate enhancers control the initial activation and subsequent maintenance of Pax2 expression, thus accounting for the dynamic expression pattern of this transcription factor during MHB development.

**The early Pax2 enhancer is controlled by POU homeodomain proteins**

The early MHB-specific enhancer of Pax2 was mapped by three criteria to the 120 bp intermediate homology region that is highly conserved between human and mouse Pax2 genes. First, deletion of this sequence from the 6.9-kb 5' region of the parental...
Mid-hindbrain-specific enhancers of Pax2 abolished MHB-specific expression in late gastrula embryos (Fig. 6D). Second, two copies of the conserved 120 bp sequence linked to the Pax2 promoter resulted in strong expression in the anterior neural plate of presomitic embryos, but failed to direct expression at the MHB in midgestation embryos (Fig. 6F,G). Third, a BAC transgene lacking the 120 bp sequence in the genomic context of the Pax2 locus was also not expressed at the MHB before the four-somite stage (Fig. 8L), demonstrating that the early enhancer is non-redundant.

Interestingly, the activity of the early Pax2 enhancer is highly promoter specific, as it strictly depends on cooperation with the endogenous Pax2 promoter in contrast to the late MHB-specific enhancers. The early enhancer-promoter module is furthermore subject to negative regulation at two different levels. A transgene (#27) essentially consisting of this regulatory module was expressed in late gastrula embryos throughout the entire anterior neural plate, including the forebrain, whereas the parental transgene (#1) containing 6.9 kb of Pax2 5’ sequence was expressed only in the prospective mid-hindbrain region. Hence, sequences located outside of the early enhancer, but contained within the 5’ region analyzed must contain negative elements that restrict the enhancer activity to the mid-hindbrain region in presomitic embryos. In midgestation embryos, however, the early enhancer-promoter module failed to be inactivated in the context of the parental transgene (#1), giving rise to broad ectopic expression in the entire mid-hindbrain region. Consequently, the activity of the early enhancer is suppressed at late stages by negative regulatory sequences that are absent from the 6.9-kb 5’ region analyzed, but present in BAC transgene #30, which faithfully recapitulated the Pax2 expression pattern at the MHB.

The homeodomain transcription factors Otx2 and Gbx2 are expressed early on in the neural plate at the time when Pax2 expression is initiated in the presumptive mid-hindbrain region (Simeone et al., 1993; Bouillet et al., 1995). Interestingly, the early Pax2 enhancer contains four consensus homeodomain-binding sites. Otx2, in contrast to Gbx2, was able to interact with one of them in vitro, although mutation of this site did not interfere with normal activation of the early Pax2 enhancer. Consequently, Otx2 and Gbx2 are unlikely to regulate the early enhancer, in agreement with the fact that Pax2 expression was still activated in the mid-hindbrain region of Otx2 mutant embryos (Rhinn et al., 1998; Acampora et al., 1998). Mutation of the other three homeodomain-binding sites strongly reduced transgene expression in the neural plate of late gastrula embryos. These TAAT motifs mediated in vitro binding of Hox and POU proteins. Hox proteins are, however, not expressed anterior to rhombomere 2 (Lumsden and Krumlauf, 1996), in contrast to POU transcription factors, which contain, in addition to the homeodomain (POUH), a second independent DNA-binding unit referred to as POU-specific (POU S) domain (Herr and Cleary, 1995; Ryan and Rosenfeld, 1997). The early Pax2 enhancer contains indeed a high-affinity POU-binding site consisting of the POU S recognition sequence GCAT.
flanked by two TAAT motifs (Fig. 7C). Interestingly, the Pax2.1 gene is only weakly activated at the MHB in zebrafish embryos homozygous for the spieg-ohne-grenzen (spg) mutation (Schier et al., 1996). The recent demonstration, that the spg mutation inactivates the Pou2 gene, identified this member of the POU protein family as an upstream regulator of Pax2.1 in MHB development (Belting et al., 2001; Burgess et al., 2002). The Oct3/4 (Pou5f1) gene is not only the mouse ortholog of zebrafish Pou2, but is also expressed in the neural ectoderm until early somitogenesis (Rosner et al., 1990; Schöler et al., 1990). As shown here, the Oct3/4 protein efficiently binds to the functional POU recognition sequence of the mouse Pax2 enhancer. Together these data strongly argue that Oct3/4 plays a critical role in controlling the early MHB-specific enhancer of mammalian Pax2 genes.

**Pax2 expression is maintained at the MHB by two late enhancers**

The second late phase of Pax2 expression commences at four somites under the combined control of two MHB-specific enhancers that are located in the distal and proximal homology regions of the Pax2 5’ region. Although each late enhancer in isolation is able to induce transgene expression, they are, in the context of the endogenous gene, responsible for the continuation and thus maintenance of Pax2 expression at the MHB. The refinement of Pax2 expression at the MHB from a broad domain in late gastrula embryos to a narrow stripe during somitogenesis (Rowitch and McMahon, 1995) is therefore caused by a switch from an early enhancer to spatially more restrictive late enhancers.

While characterizing a kidney-specific enhancer, Kuschert et al. (Kuschert et al., 2001) have recently identified the same MHB-specific enhancer in the proximal homology region of Pax2. We have demonstrated for the first time that this regulatory element is a late enhancer, contains a functional Pax-binding site and is necessary for maintaining expression at the MHB in the larger context of the Pax2 locus. Interestingly, the activity of this late enhancer is entirely dependent on the integrity of its Pax-binding site, which is recognized with high affinity only by members of the Pax2/5/8 family. Pax2 therefore appears to be involved in autoregulatory activation of its late enhancer, which resembles its cross-regulatory role in activating the MHB-specific enhancer of Pax5 at the same embryonic stage (three to four somites) (Pfeffer et al., 2000). Subsequently, the late Pax2 enhancer may also be subject to cross-regulatory control by Pax5 and Pax8. Such feedback and cross-regulatory interactions may provide a mechanism to maintain, sharpen and stabilize the Pax2 expression domain at the MHB in midgestation embryos. However, the same Pax2/5/8-binding site plays no role in the activation of the kidney-specific enhancer of Pax2, demonstrating that the two overlapping enhancers in the

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*Fig. 8.* Functional analyses of the early and late enhancers in the context of the Pax2 locus. (A) Structure of the Pax2 BAC #30, which contains an in-frame GFP insertion in exon 2 and extends at least 30 kb upstream of Pax2. A structurally different Pax2-GFP BAC (#76) gave rise to the same expression pattern as BAC #30. All mutant transgenes (#31-34) were derived from BAC #30. Nucleotide positions relative to the transcription start site indicate the extent of deletion. n.d., not determined. (B-F) Temporal expression pattern of the parental BAC #30. Strong GFP expression was observed in the metanephros (F) at E16.5. (G-K) The proximal but not distal enhancer of Pax2 is essential for late expression at the MHB (arrowhead). An oblique section (G) through the mesonephros of an E10 embryo revealed normal expression of transgene #32 in both the nephric duct (nd) and tubules (tub). (L-N) Before the four-somite stage, the early enhancer in the intermediate homology region is required for Pax2 expression in the MHB region. ad, adrenal gland; nt, neural tube; tb, tail bud.
proximal homology region differ with regard to their regulation by Pax proteins.

A second late MHB-specific enhancer, present in the distal homology region, is transiently active during the maintenance phase of Pax2 expression from the four-somite stage until about E10. This distal enhancer is, however, not essential for maintaining expression of a Pax2 BAC transgene at the MHB, suggesting that it plays an auxiliary role by fine-tuning Pax2 expression levels in the presence of the proximal late enhancer.

**Evolutionary conservation of Pax2 regulatory elements**

The contracted genome of the pufferfish *Fugu rupribes* has successfully been used to identify conserved regulatory elements in vertebrate genes by sequence comparison and transgenic analysis (Aparicio et al., 1995). Surprisingly, however, the 5′ region of the *Fugu Pax2.1* gene does not contain conserved sequences that are homologous to the essential early and proximal late enhancers of mammalian Pax2 genes. Consistent with this finding, a *Fugu Pax2* transgene failed to be expressed at the late gastrula stage in transgenic mouse embryos, indicating that the early enhancer is either species-specific or, more likely, is present at a different location in the *Fugu Pax2.1* locus. The *Fugu Pax2* transgene is, however, expressed at the MHB during the maintenance phase of Pax2 expression. This activity of the *Fugu Pax2* transgene depends on a highly conserved 102 bp sequence in the distal homology region, which is also essential for activating the second late enhancer of the mouse Pax2 gene in the dorsal MHB region. It appears therefore that the distal homology region has maintained its function as a dominant late enhancer in the *Fugu Pax2.1* gene, in contrast to assuming an ancillary role in mammalian Pax2 genes.

*Pax2*, *Pax5* and *Pax8* have arisen by gene duplications from a single ancestral *Pax258* gene at the onset of vertebrate evolution (Pfeffer et al., 1998; Wada et al., 1998; Kozmik et al., 1999). Although each gene has since assumed a unique developmental expression pattern, all three genes are co-expressed in the MHB region of vertebrate embryos (Nornes et al., 1990; Plachov et al., 1990; Adams et al., 1992; Pfeffer et al., 1998). This common expression domain provides a likely explanation for the observation that the MHB-specific enhancer of *Pax5* shares sequence homology with the distal late and early enhancers of *Pax2*. A functional Pax2/5/8-binding site in element E1 of the *Pax5* enhancer (Pfeffer et al., 2000) is highly similar to a conserved Pax recognition sequence in the distal homology region of *Pax2* (Fig. 1B), despite the fact that Pax-binding sites are usually quite divergent (Czerny et al., 1993). This conserved sequence also interacts with Pax2/5/8 proteins (Schwarz et al., 2000; data not shown) similar to the Pax5 element E1 (Pfeffer et al., 2000). More conspicuously, the 3′ part of the early Pax2 enhancer shares considerable homology with element E2 of the *Pax5* enhancer (Fig. 7C), which contains a functional homeodomain recognition sequence adjacent to overlapping binding sites for Otx and Sp1-like zinc finger proteins (Pfeffer et al., 2000). Interestingly, the zebrafish transcription factor Bts1, belonging to the Sp1 protein family, was recently shown to be both necessary and sufficient for inducing the *Pax2.1* gene within the anterior neural plate during zebrafish gastrulation (Tallafuß et al., 2001). It is therefore conceivable that a Bts1-like factor of the mouse activates the early Pax2 enhancer by binding to the conserved zinc finger-binding site.

**Identification of essential control elements by BAC transgenesis**

Owing to their large size, BAC transgenes are more likely to contain all regulatory information to recapitulate the expression pattern of an endogenous gene in a dose-dependent and integration site-independent manner (Yang et al., 1997). Indeed, we have never observed ectopic expression of our Pax2 BAC transgenes in contrast to conventional transgenes. Moreover, two structurally different BACs faithfully regenerated the Pax2 expression pattern with one notable exception. Both BACs failed to direct expression in the optic vesicle and later optic stalk of the developing eye. Hence, the eye-specific enhancer of *Pax2* must be located at a far distance from the promoter, as both BACs contain the entire Pax2 gene, together with at least 30 kb of 5′ flanking sequences. Our data therefore do not confirm the existence of an eye-specific enhancer within the first 9 kb of Pax2 upstream sequences (Schwarz et al., 2000).

The function of an enhancer is most stringently tested by specific deletion from the endogenous gene in the mouse germline. Where performed, such analyses have often uncovered unsuspected redundancies among regulatory elements (Beckers and Duboule, 1998; Song and Joyner, 2000). BAC transgenes in combination with deletion mutagenesis provide more readily the same information, as they also allow for stringent testing of regulatory elements in a large genomic context. In this manner, we could demonstrate that two of the four Pax2 enhancers, identified by classical transgenesis, are redundant in the context of the Pax2 locus. Loss of the redundant MHB-specific enhancer in the distal homology region was compensated for by the proximal late enhancer. Moreover, Pax2 expression in the developing kidney was unaffected by deletion of the nephric enhancer in the proximal homology region, indicating that the essential kidney-specific enhancer of Pax2 still remains to be identified.

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