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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 *Sac*I fragment from the 5′ region of mouse *Pax2* into the *Msc*I site of pTrap (Pfeffer et al., 2000). Construct #2 was obtained by cloning a 3-kb *Bam*HI/*Hin*dIII fragment from a *Fugu Pax2.1* cosmid (Pfeffer et al., 1998) into pTrap. 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′-aagtctAGAAAGGGAGAGCGCGAGGA-3′ and 5′-aagtctagaTTCTGGTCACATTGGAGGAT-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′-acccaagctTGTCCCTTCATTCTAAACAC-3′ and 5′-gagaagcttAGCTCTGGGGAGGGGAT-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′-GGGTCTTCGAAATTCCGAAGTGAAGCGTACCTC-3′. The underlined nucleotides indicate the restriction site used for cloning.

BAC modification

The *Pax2* BAC clones 468C04 (giving rise to transgene #30) and 551I16 (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 *Not*I 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 *Hin*dIII site) and 3680-4111 (*Cla*I site); BAC #32, 3680-4111 (*Cla*I site); BAC #33, 2230-2765 (*Hin*dIII site); BAC #34, 2230-3287 (*Hin*dIII site). BAC DNA was purified on QIAGEN-500 columns and CsCl/EtBr gradients. The supercoiled DNA was extracted with isoamyl-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 F_1 mice were used for generating transgenic animals, which were identified by PCR with the *lacZ* primers 5′-ATACTGTCGTCGTCCCCTCAAACTG-3′ and 5′-TTCAACCACCGCACGATAGAGATTC-3′ or *GFP* primers 5′-CCGACCACATGAAGCAGCACGAC-3′ and 5′-TCACGAACTCCAGCAGGACCAT-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 pKW2T, 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 *Sac*I 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

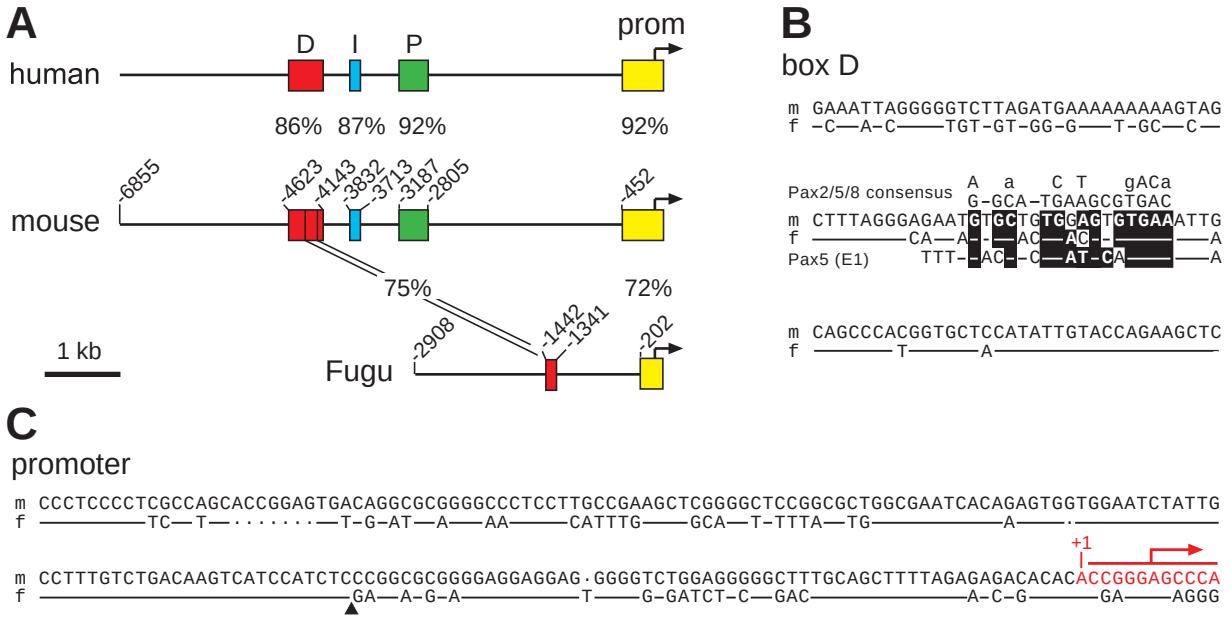

Fig. 1. Conservation of upstream sequences between vertebrate *Pax2* genes. (A) The promoter and proximal (P), intermediate (I) and distal (D) homology regions of the human, mouse and *Fugu rubripes Pax2* genes are shown with their degree of sequence identity and nucleotide positions relative to the transcription start sites. The *Fugu* gene was identified as *Pax2.1* by comparison of its exon 1 with those of the two zebrafish *Pax2* genes (Pfeffer et al., 1998). (B,C) Conservation of the box D (B) and promoter of *Pax2* (C). Only those nucleotides of the *Fugu* (f) *Pax2.1* gene that differ from the mouse (m) sequence are shown. A high-affinity Pax-binding site in box D is aligned with the Pax2/5/8 consensus sequence (Czerny and Busslinger, 1995) and with element E1 of the MHB-specific enhancer of *Pax5* (Pfeffer et al., 2000). An arrowhead points to the insertion of 26 nucleotides in the *Fugu* promoter, and a red arrow indicates the region of heterogeneous transcription initiation of mouse *Pax2* (Ryan et al., 1995).

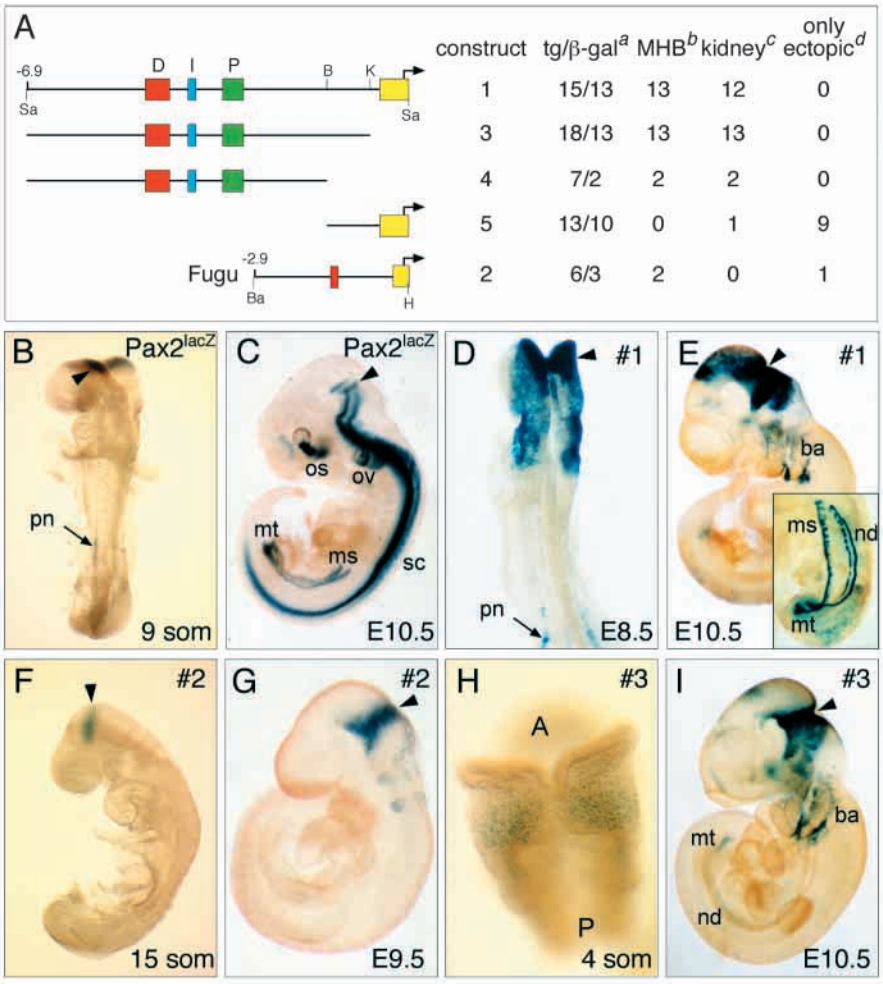

Fig. 2. Upstream sequences of the mouse and *Fugu Pax2* genes direct expression at the MHB. (A) Schematic diagram of the transgenes and statistical overview of the β-galactosidase staining patterns observed in injected founder embryos or permanent transgenic lines. The indicated DNA fragments of the 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, *Bsr*D1; Ba, *Bam*HI; H, *Hind*III; K, *Kpn*I; Sa, *Sac*I. (B,C) β-Galactosidase staining of *Pax2*$^{lacZ/+}$ 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.

arches (Fig. 2E), where expression of the endogenous *Pax2* gene was transiently detected in *Pax2*$^{+/lacZ}$ embryos at E9.5 (data not shown).

We next investigated whether 2.9 kb of upstream sequences of the *Fugu Pax2.1* gene (including the two conserved sequence blocks) were able to direct *lacZ* expression in transgenic mouse embryos. Indeed, two permanent lines carrying transgene #2 expressed β-galactosidase activity at the MHB (Fig. 2F,G). Time course analyses revealed transgene expression at the MHB from the five-somite stage onwards until at least E10.5, but failed to detect β-galactosidase activity in presomitic embryos and in the developing kidney (Fig. 2F,G) (data not shown). These data suggest that the conserved promoter and/or distal homology region of *Pax2* may be involved in controlling MHB-specific expression from early somitogenesis onwards.

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

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 *Psh*AI-*Apa*I 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 *Xmn*I 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 *Xmn*I site must reside within critical sequences of the MHB-specific enhancer (Fig. 3A). Furthermore, a 410 bp *Nar*I-*Apa*I 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 *Nar*I-*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 *Ava*I-*Apa*I 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

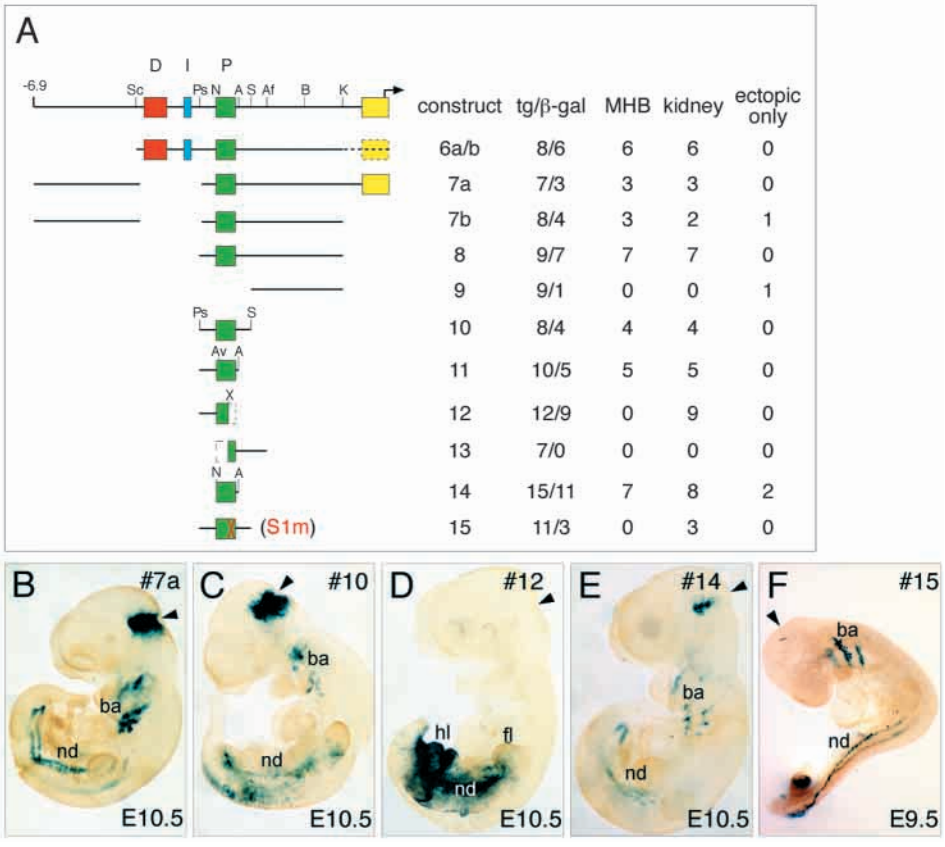

Fig. 3. Characterization of the proximal homology region of *Pax2*. (A) Constructs and statistical overview of transgenic embryos. Transgene #6 was expressed in the MHB region and kidney of midgestation embryos both in the presence (#6a) or absence (#6b) of the *Pax2* promoter (broken lines). A, *Apa*I; Af, *Afl*II; Av, *Ava*I; B, *Bsr*D1; K, *Kpn*I; N, *Nar*I; S, *Sph*I; Sc, *Sca*I; Ps, *Psh*AI; X, *Xmn*I. (B-F) X-Gal staining of representative transgenic embryos. Strong staining was observed at the MHB for all transgenes up to construct #11 (B,C), whereas the minimal 410 bp *Nar*I-*Apa*I fragment of transgene #14 (E) gave rise to reduced and more variable staining at the MHB. The activity of the MHB-specific enhancer was lost upon removal of the Pax2/5/8-binding site (S1) by either 3′ deletion (D) or point mutation (F). Ectopic staining was frequently observed in the hindlimb (hl) and mesenchymal tissue of the trunk. fl, forelimb.

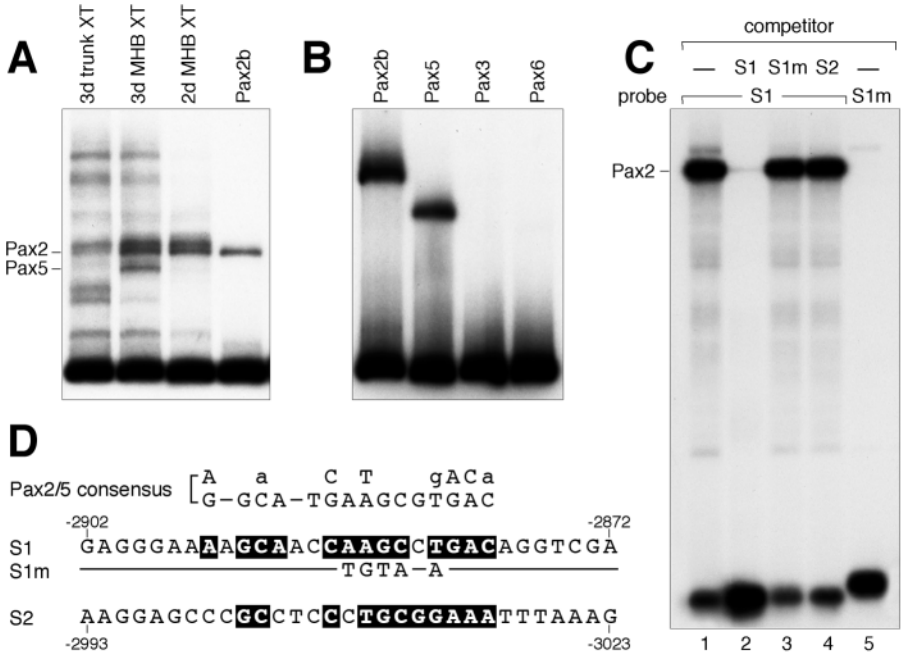

Fig. 4. Binding of Pax2/5/8 proteins to the proximal enhancer. (A) EMSA analysis of extracts prepared from dissected trunk or MHB tissue of 2-day-old and 3-day-old chick embryos (Pfeffer et al., 2000) with a 0.3 kb *Ava*I-*Apa*I probe of transgene #11 (Fig. 3A). In vitro synthesized Pax2b was used as a control. (B) Preferential binding of Pax2 and Pax5 to the proximal enhancer. Equimolar amounts of in vitro translated Pax proteins (quantitated by [^{35}S]Met incorporation) were analyzed by EMSA for binding to the 0.3 kb *Ava*I-*Apa*I probe. (C) Identification of a high-affinity Pax-binding site. Binding of Pax2b to the end-labeled S1 and S1m oligonucleotides was investigated by EMSA in the absence or presence of a 100-fold molar excess of the indicated oligonucleotides. (D) Alignment of the proximal enhancer sequences S1 and S2 with the consensus Pax2/5/8-binding site (Czerny and Busslinger, 1995). The S1m mutations are indicated.

electrophoretic 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 *Ava*I-*Apa*I 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. 3C 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 5′ sequences of *Pax2* was still expressed in the MHB region of midgestation embryos (Fig. 5A; data not shown). Hence, a second late MHB-specific enhancer must reside in the upstream region of *Pax2*. Transgene #17 mapped this regulatory element to a 1285 bp *Sca*I-*Psh*AI 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 *Sca*I-*Psh*AI 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

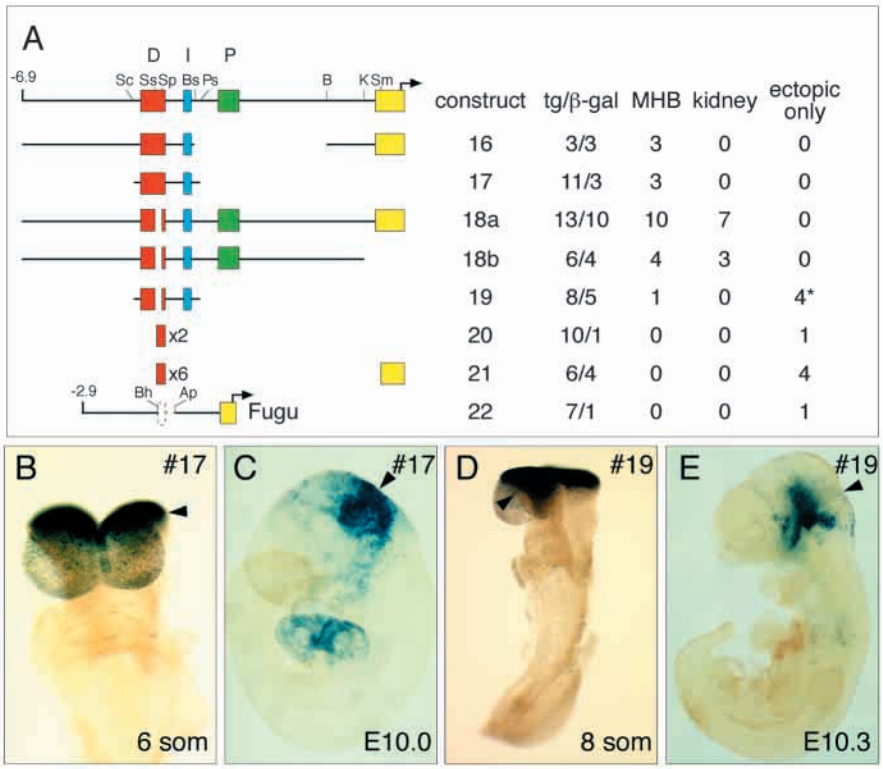

Fig. 5. Presence of a second late MHB enhancer in the *Pax2* upstream region. (A) Transgenic constructs and statistics. Ap, *Apo*I; B, *Bsr*D1; Bh, *Bss*HII; Bs, *Bsm*I; K, *Kpn*I; Sc, *Sca*I; Sp, *Sap*I; Sm, *Sma*I; Ss, *Ssp*I. (B,C) Transgene #17 lacking the proximal enhancer was still expressed in the MHB region (arrowhead). (D,E) Four (* in A) of five embryos expressed transgene #19 only in the ventral MHB region at E10 to E10.5, indicating that the conserved 102 bp sequence in the distal homology region of mammalian and *Fugu Pax2* genes (Fig. 1B) is required for maintaining *lacZ* expression in the dorsal region of the MHB. Embryos are shown in frontal (B), dorsolateral (D) or lateral (C,E) view.

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 (GCAT) 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.

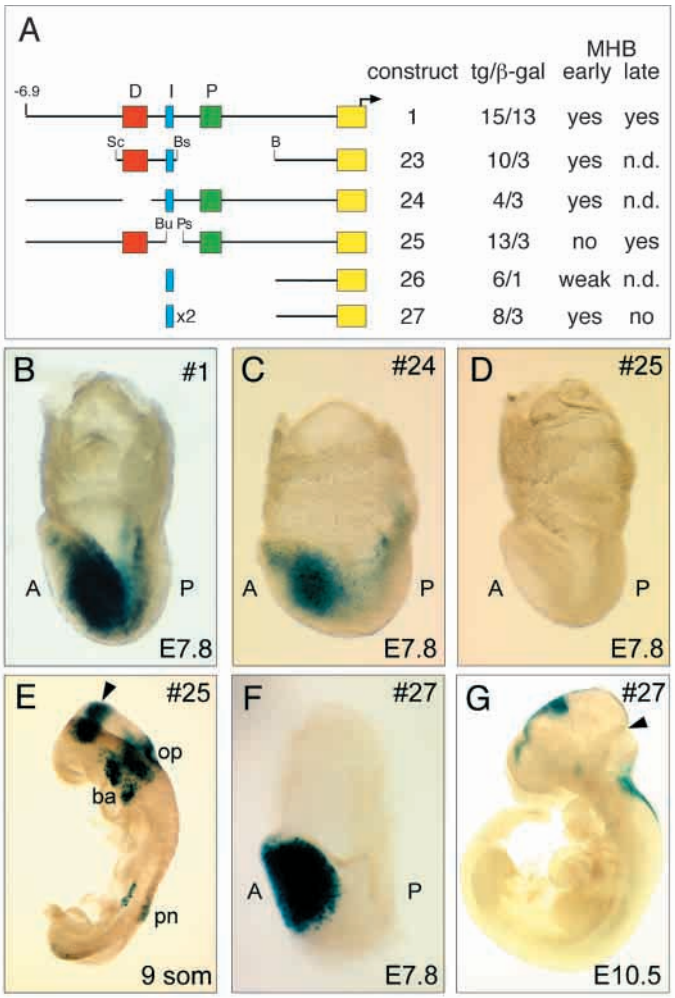

Fig. 6. Identification of the early *Pax2* enhancer. (A) Transgenic constructs and statistics. 'Early' refers to expression in the anterior neural plate of presomitic embryos and 'late' to expression at the MHB beyond the four-somite stage. B, *Bsr*D1; Bu, *Bsu*36I; Bs, *Bsm*I; Ps, *Psh*AI; Sc, *Sca*I. (B,C) Transgenes containing the intermediate homology region of *Pax2* are expressed already in the neural plate of presomitic embryos. (D,E) Loss of the early (D) but not late (E) MHB-specific expression in a permanent transgenic line lacking the intermediate homology region. (F,G) The 120 bp intermediate homology region together with the *Pax2* promoter suffices for anterior neural plate expression (F), but fails to maintain expression in the MHB region (G, arrowhead) at later stages of the same permanent transgenic line. All embryos are shown in lateral view. op, otic placode.

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

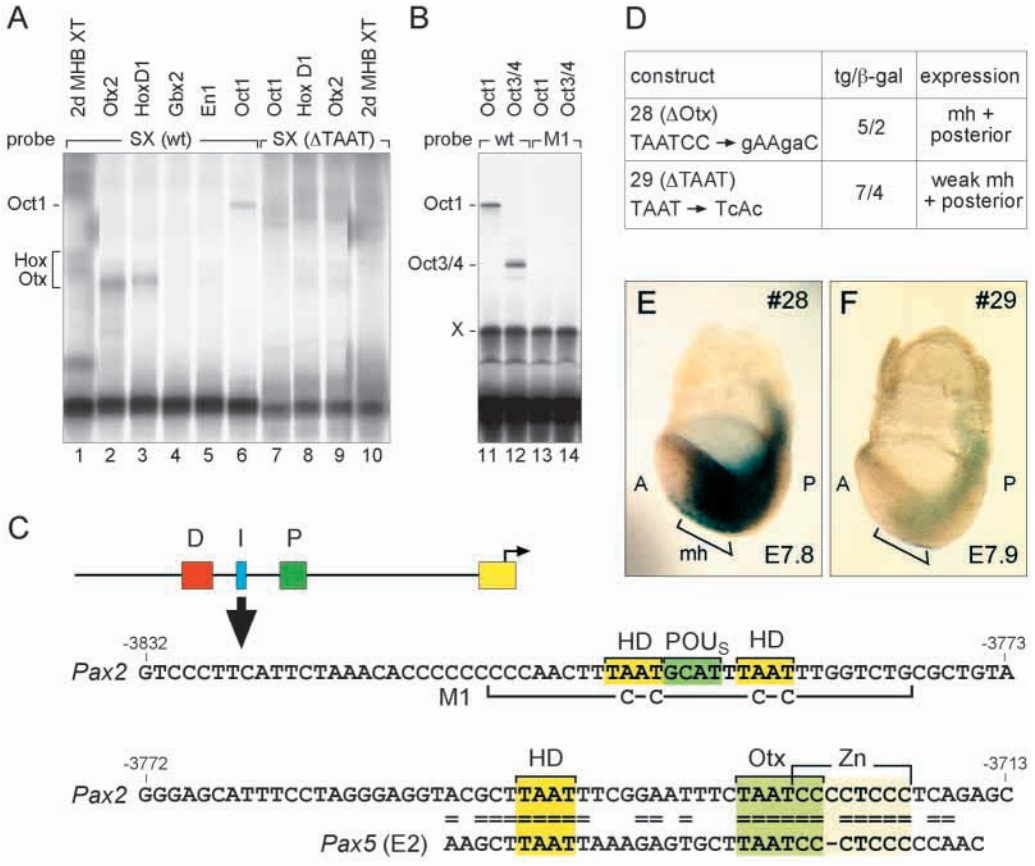

Fig. 7. Mutational analysis of the early *Pax2* enhancer. (A) Binding of homeodomain proteins to the early enhancer. In vitro translated proteins and a MHB extract were analyzed for binding to a 157 bp probe containing the wild-type enhancer (SX[wt], from –3833 to –3677) or the corresponding sequence mutated at all four TAAT sites (SX[ΔTAAT]). Oct1 was analyzed at a 10-fold lower concentration. (B) Interaction of in vitro translated Oct1 and Oct3/4 proteins with a wild-type (wt) or mutated (M1) probe (see C) containing the distal two TAAT motifs. X, nonspecific DNA-binding activity. (C) Sequence of the early enhancer. Recognition sequences for homeo (HD) and POU-specific (POU_S) domains and binding sites for Otx and Sp1-like zinc finger (Zn) proteins are shown together with an alignment of the E2 element present in the MHB-specific enhancer of *Pax5* (Pfeffer et al., 2000). (D-F) Inactivation of the early enhancer by mutation of the TAAT motifs in the context of transgene #1. The introduced mutations are indicated together with the statistical analysis (D) and representative examples of transgenic embryos (E,F). Posterior refers to ectopic expression in the posterior region of late gastrula embryos (D). The mid-hindbrain (mh) territory is indicated by a bracket (E,F).

transgene (#1) 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 (POU_H), 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

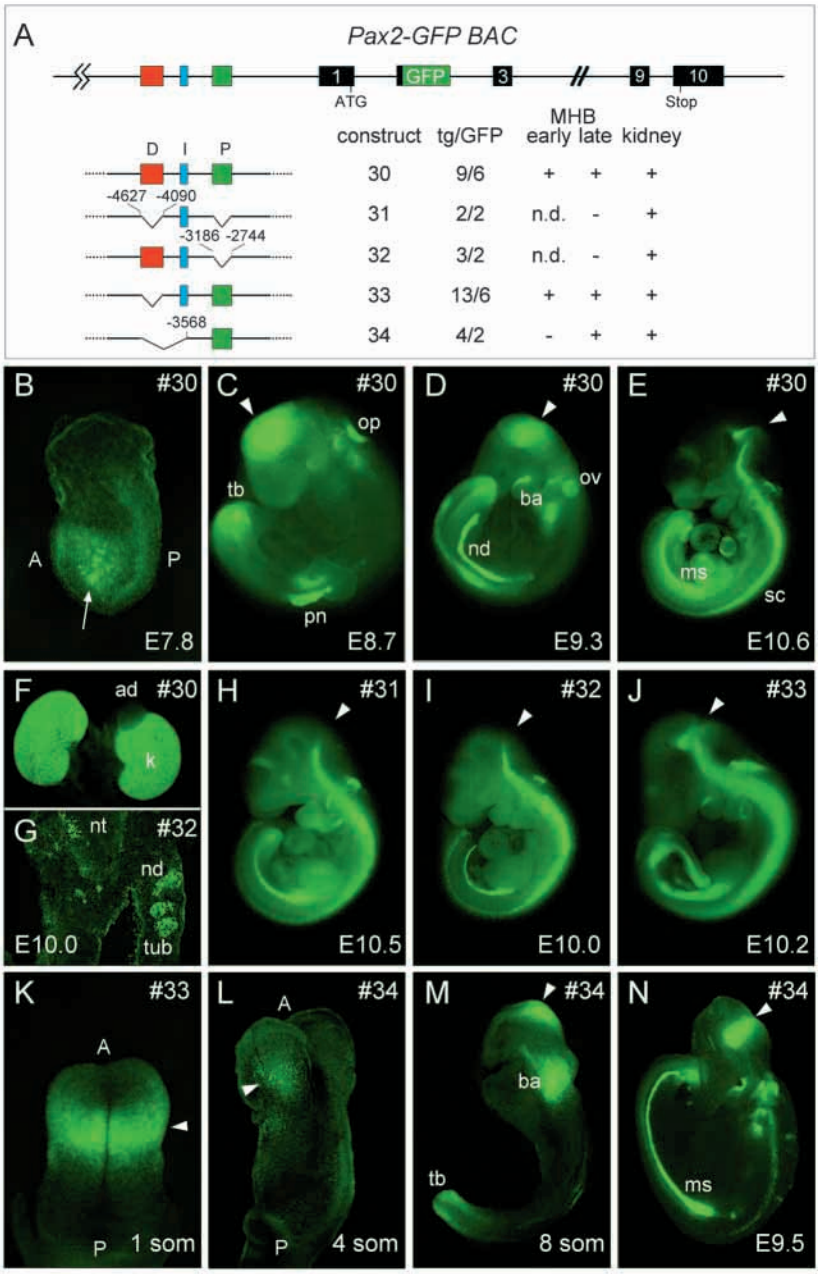

Fig. 8. Functional analyses of the early and late enhancers in the context of the *Pax2* locus. (A) Structure of the *Pax2* BAC #30, which contains an in-frame *GFP* insertion in exon 2 and extends at least 30 kb upstream of *Pax2*. A structurally different *Pax2-GFP* BAC (#76) gave rise to the same 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.

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 *spiel-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

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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