Identification of Pax2-regulated genes by expression profiling of the mid-hindbrain organizer region

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Summary

The paired domain transcription factor Pax2 is required for the formation of the isthmic organizer (IsO) at the midbrain-hindbrain boundary, where it initiates expression of the IsO signal Fgf8. To gain further insight into the role of Pax2 in mid-hindbrain patterning, we searched for novel Pax2-regulated genes by cDNA microarray analysis of FACS-sorted GFP$^+$ mid-hindbrain cells from wild-type and *Pax2*$^{-/-}$ embryos carrying a *Pax2*^{GFP} BAC transgene. Here, we report the identification of five genes that depend on Pax2 function for their expression in the mid-hindbrain boundary region. These genes code for the transcription factors En2 and Brn1 (Pou3f3), the intracellular signaling modifiers Sef and Tapp1, and the non-coding RNA *Ncrms*. The *Brn1* gene was further identified as a direct target of Pax2, as two functional Pax2-binding sites in the promoter and in an upstream regulatory element of *Brn1* were essential for *lacZ* transgene expression at the mid-hindbrain boundary. Moreover, ectopic expression of a dominant-negative Brn1 protein in chick embryos implicated Brn1 in *Fgf8* gene regulation. Together, these data defined novel functions of Pax2 in the establishment of distinct transcriptional programs and in the control of intracellular signaling during mid-hindbrain development.

Key words: Mid-hindbrain development, Pax2-regulated genes, *Sef*, *Tapp1*, *Ncrms*, *En2*, *Brn1, Fgf8* regulation, Mouse

Introduction

The midbrain and cerebellum develop from an organizing center that is formed at the junction between the embryonic midbrain and hindbrain, known as the isthmus. This isthmic organizer (IsO) was discovered because of its property of inducing an ectopic midbrain or cerebellum, when transplanted into the chick diencephalon or hindbrain, respectively (reviewed by Liu and Joyner, 2001a; Wurst and Bally-Cuif, 2001). The IsO activity recruits the surrounding tissue into either a midbrain or cerebellum fate by controlling cell survival, proliferation and differentiation along the anteroposterior axis of the mid-hindbrain region. The formation of the IsO is the result of complex cross-regulatory interactions between transcription factors (Otx, Gbx, Pax and En) and secreted proteins (Wnts and Fgfs), culminating in the expression of the signaling molecule Fgf8 at the mid-hindbrain boundary (Liu and Joyner, 2001a; Wurst and Bally-Cuif, 2001; Ye et al., 2001). Fgf8 is the central mediator of IsO activity, as it is both necessary and sufficient for inducing midbrain and cerebellum development (Crossley et al., 1996; Chi et al., 2003). Once formed, the IsO is maintained by a positive feedback loop involving multiple mid-hindbrain-specific regulators. Consequently, the IsO is lost upon individual mutation of these regulators, whereas ectopic expression of a single factor activates most other components of the regulatory cascade (Nakamura, 2001). Owing to this interdependence, the hierarchical relationship among the different regulators remains largely elusive during the maintenance phase of IsO activity (Liu and Joyner, 2001a; Wurst and Bally-Cuif, 2001).

The initiation of IsO development crucially depends on the transcription factor Pax2 (Favor et al., 1996; Brand et al., 1996), which shares similar DNA-binding and transactivation functions with Pax5 and Pax8 of the same paired domain protein subfamily (Kozmik et al., 1993; Dörfler and Busslinger, 1996). *Pax2* is the earliest known gene to be expressed throughout the prospective mid-hindbrain region in late gastrula embryos (Rowitch and McMahon, 1995). The initially broad expression pattern of *Pax2* is progressively refined to a narrow ring centered at the mid-hindbrain boundary by embryonic day 9.5, while the related *Pax5* and *Pax8* genes are activated in the same region at 3-4 and 6-7 somites, respectively (Urbánek et al., 1994; Rowitch and McMahon, 1995; Pfeffer et al., 1998). Consistent with this sequential gene induction, mutation of the *Pax2* gene leads to the loss of the midbrain and cerebellum in mouse and zebrafish embryos (Favor et al., 1996; Brand et al., 1996; Bouchard et al., 2000), whereas the inactivation of *Pax5* or *Pax8* results in a mild

cerebellar midline defect or no brain phenotype at all (Urbánek et al., 1994; Mansouri et al., 1998). The severe mid-hindbrain deletion is, however, only observed in *Pax2*$^{-/-}$ mouse embryos on the C3H/He genetic background (Bouchard et al., 2000), where the compensating *Pax5* and *Pax8* genes fail to be activated at the mid-hindbrain boundary (Pfeffer et al., 2000; Ye et al., 2001) similar to the *Pax2.1* (*noi*) mutant embryos of the zebrafish (Pfeffer et al., 1998). In the absence of Pax2, *Otx2*, *Gbx2* and *Wnt1* are normally transcribed at early somite stages, while the expression of *En1* is reduced in the developing mid-hindbrain region (Ye et al., 2001). Importantly, *Fgf8* expression is never initiated at the mid-hindbrain boundary of *Pax2*$^{-/-}$ C3H/He embryos (Ye et al., 2001), resulting in the complete absence of IsO activity and subsequent apoptotic loss of the mid-hindbrain tissue starting at the 12-somite stage (Pfeffer et al., 2000; Chi et al., 2003).

To further investigate the role of Pax2 at the onset of mid-hindbrain development, we searched for novel Pax2-regulated genes by gene expression profiling of mid-hindbrain cells isolated by FACS sorting from wild-type and *Pax2*$^{-/-}$ E8.5 embryos. This unbiased approach identified the *En2*, *Brn1* (*Pou3f3* – Mouse Genome Informatics), *Sef* (*Il17rd* – Mouse Genome Informatics), *Tapp1* (*Plekha1* – Mouse Genome Informatics) and non-coding *Ncrms* genes as genetic Pax2 targets that are totally dependent on Pax2 function for their expression in the mid-hindbrain region. The transcription factors En2 and Brn1, as well as the signaling modifiers Sef and Tapp1, implicate Pax2 in the establishment of distinct transcriptional programs and the control of intracellular signaling during mid-hindbrain development. Biochemical and transgenic analyses demonstrated that Pax2 directly activates the mid-hindbrain-specific expression of *Brn1* by interacting with two functional Pax2/5/8-binding sites in the promoter and an upstream regulatory element of the *Brn1* gene. Moreover, ectopic expression of a dominant-negative Brn1 protein in chick embryos implicated Brn1 as a novel regulator of *Fgf8* expression. The identification of new Pax2-regulated genes has thus provided important insight into the role of Pax2 in mid-hindbrain development.

Materials and methods

Mice

Pax2$^{+/-}$ and *Pax5*$^{+/-}$ mice as well as the *Pax2*^{GFP} mice carrying the BAC transgene 30 were maintained on the C3H/He background and genotyped as described (Urbánek et al., 1994; Bouchard et al., 2000; Pfeffer et al., 2002).

FACS sorting and linear RNA amplification

The mid-hindbrain region of GFP^{+} E8.5 embryos from *Pax2*$^{+/-}$ *Pax2*^{GFP} intercrosses was dissected with 26-gauge needles and dissociated into single cells at 37°C for 15 minutes in 24-well plates containing 500 μl of 1% trypsin in PBS. The reaction was stopped by transferring the single-cell suspension into 4 ml of cold DMEM containing 10% fetal calf serum followed by centrifugation and resuspension in phenol red-free DMEM containing 10% fetal calf serum and 1 μg/ml propidium iodide (PI). Live PI^{-} GFP^{+} cells of individual embryos were sorted with a FACSVantage TSO flow-cytometer (Becton-Dickinson) directly into the Trizol Reagent (Gibco-BRL), vortexed for 1 minute and then stored in liquid nitrogen. This sorting protocol yielded 5000-10,000 GFP^{+} cells per embryo. Total RNA from selected samples was submitted to linear amplification as described (Hoffmann et al., 2003) with some modifications. Briefly, the total RNA from a minimum of 5000 cells was reverse-transcribed with an oligonucleotide consisting of $d(T)_{15}$ linked to a T7 RNA polymerase recognition site. Following second-strand synthesis, the samples were amplified by T7 polymerase-mediated in vitro transcription. The resulting aRNA was reverse-transcribed using random nonamer oligonucleotides [$pd(N)_9$] and used for a second round of cDNA synthesis and in vitro transcription. Two rounds of amplification from 5000 cells typically yielded 30 to 80 μg of aRNA.

cDNA microarray hybridization

The cDNA microarray screening was essentially performed as described (Cheung et al., 1999). A detailed description of the method used can be found as supplementary information. Briefly, aRNA was reverse-transcribed into cDNA in the presence of Cy3-dUTP or Cy5-dUTP using the Gibco-RT kit. The Cy3- and Cy5-labeled cDNA probes were pooled and ethanol-precipitated together with poly-dA, tRNA and mouse Cot.1 DNA. The precipitated cDNA probes were washed, prehybridized at 50°C for 1 hour in a solution containing 35% formamide, 4×SSPE, 0.5% SDS, 5× Denhardt's solution and 10 μg/ml denatured salmon sperm DNA and then added to microarray slides for overnight hybridization at 50°C. Post-hybridization washes were carried out for 10 minutes in 0.2×SSC, 0.1% SDS and 10 min in 0.2×SSC. The slides were dried and scanned using an Axon GenePix 4000 scanner. The hybridization results were normalized using the marray-package of Bioconductor (http://www.bioconductor.org) and the 'Print Tip Loess' algorithm (Yang et al., 2002). The cDNA microarrays contained 26,000 spotted EST clones (11,000 BMAP clones from Research Genetics and 15,000 NIA clones from the National Institute of Aging), which corresponded to 17,000 UniGene clusters.

In situ hybridization

Embryos were dissected and processed for in situ hybridization with digoxigenin-UTP-labeled RNA probes as described (Henrique et al., 1995). The *En2* probe was previously described (Davis et al., 1988). The *Sef* probe contained a 750 bp cDNA sequence extending from the PCR oligonucleotide 5′-GGAGCCTGACTGGTTTGAGAA-3′ to the *Nde*I site. The *Tapp1* and *Ncrms* probes were derived from the identified ESTs (*Tapp1*, BC020017; *Ncrms*, BE655589). The mouse *Brn1* probe (643 bp) was cloned into the pGEM-Teasy plasmid (Promega) following RT-PCR from E10.5 head cDNA using the primers 5′-GGGCAGAAGTCAAGGGAAGTG-3′ and 5′-TGGCGT-CGTCGGTGGAGAACA-3′ and the chick *Brn1* probe (429 bp) following RT-PCR amplification from chick embryo RNA with the primers 5′-ATGGT(G/C)CAGAG(C/T)GACTTCATGCAGGG-3′ and 5′-GCT(C/T/G)AGCAT(G/A/T)CCGTT(C/T)AC(C/A)GTGAA-3′.
The partial chick *Brn1* cDNA sequence was submitted to GenBank (Accession Number DQ002393).

5′-RACE

The transcriptional start sites of the *Brn1* gene were identified by 5′-RACE, using the SMART RACE cDNA amplification kit (BD Bioscience) according to the manufacturer's instructions. RNA isolated from the head of E10.5 embryos was reverse-transcribed into cDNA with the *Brn1*-specific primer 5′-GCTTCCACGGCAGCGGC-GGCGGCAGCAG-3′ followed by PCR amplification with the oligonucleotides 5′-ACGGGAGACAACAAAGGACGAAGCGGTT-CC-3′ (outer) and 5′-GGAAGAAGAGTGCATTGGTGGAGGTG-GAGA-3′ (inner) in combination with the primers provided with the RACE kit.

Electrophoretic mobility shift assay

The Pax2 protein was synthesized by coupled in vitro transcription/translation and used for EMSA analysis with published *CD19* and *Blnk* oligonucleotide probes as described (Kozmik et al.,

1992; Schebesta et al., 2002). The competitor fragments C, D and P were cloned by PCR with the following primers:

C, 5′-CAGACAAAACAATCACACTCC-3′ and 5′-GGGGAGGA-TAGGAACAGAGCC-3′;

D, 5′-GGCTGCGAGGCTGCTGCTGAG-3′ and 5′-GTTTTGCG-AGGTGGCTGTGAC-3′;

P, 5′-ACAACAGATTTCCAGCTTCTA-3′ and 5′-CTCTCCCTC-TCTCCTTCTCTC-3′.

The following double-stranded *Brn1* oligonucleotides were used as competitor DNA:

Da, 5′-tcagATTCGGAGCACACCGACCGCCGGGTTACGTTCT-CGGCTGCTGCTT-3′ and 5′-tcagAAGCAGCAGCCGAGAACGTA-ACCCGGCGGTCGGTGTGCTCCGAAT-3′;

Db, 5′-tcagTGCTTGGACTAGAAACTGCAGATTGCGGTCCG-GTGCCCTGCAGCT-3′ and 5′-tcagAGCTGCAGGGCACCGGACC-GCAATCTGCAGTTTCTAGTCCAAGCA-3′;

Dc, 5′-tcagTTCTCTTTTTTCTTGGTTCGCTGAGGTTCCTCTG-TatcGCGTTTC-3′ and 5′-tcagGAAACGCgatACAGAGGAACCTC-AGCGAACCAAGAAAAAAGAGAA-3′;

Dd, 5′-tcagTCCTCTGTatcGCGTTTCCGCTTGGCCGCGTCGT-CCCCCCCCCCC-3′ and 5′-tcagGGGGGGGGGGGACGACGCG-GCCAAGCGGAAACGCgatACAGAGGA-3′;

Ddm, 5′-tcagTCCTCTGTatcGCGTTTCCatTTGGCCGCGTCGT-CCCCCCCCCCC-3′ and 5′-tcagGGGGGGGGGGGACGACGCG-GCCAAatGGAAACGCgatACAGAGGA-3′;

Pa, 5′-tcagGGGGGACAACAGATTTCCAGCTTCTACGACGCT-CTGtCaAAATTA-3′ and 5′-tcagTAATTTtGaCAGAGCGTCGTA-GAAGCTGGAAATCTGTTGTCCCCC-3′;

Pb, 5′-tcagGATTTCCAGtcTCTACGACGCTCTGCCTAAATTA-AAAAGCAACCA-3′ and 5′-tcagTGGTTGCTTTTTAATTTAGGC-AGAGCGTCGTAGAgaCTGGAAATC-3′;

Pc, 5′-tcagCTGtCaAAATTAAAAAGCAACCAATCGGAACGG-CCGGAAGGGGGG-3′ and 5′-tcagCCCCCCTTCCGGCCGTTCC-GATTGGTTGCTTTTTAATTTtGaCAG-3′;

Pcm, 5′-tcagCTGtCaAAATTAAAAAGCAACCAATCaGtACGG-CCGGAAGGGGGG-3′ and 5′-tcagCCCCCCTTCCGGCCGTaCtG-ATTGGTTGCTTTTTAATTTtGaCAG-3′.

Brn1 transgenes

lacZ transgenes were generated by insertion of a 3.2-kb *Bgl*II-*Not*I fragment or a 6.2-kb *Afl*II-*Not*I fragment from the 5′ flanking region of *Brn1* into the *Bgl*II-*Not*I sites of pTRAP-PL, which is a modified version of pTRAP (Pfeffer et al., 2000) lacking the minimal promoter. The mutant *lacZ* transgenes were obtained by site-directed mutagenesis, using the QuikChange kit (Stratagene) together with the following oligonucleotides:

Dd, 5′-CTCTGTGCGGCGTTTCCatTTGGCCGCGTCGTCCCC-CCCC-3′ and 5′-GGGGGGGGACGACGCGGCCAAatGGAAACG-CCGCACAGAG-3′;

Pc, 5′-CCTAAATTAAAAAGCAACCAATCaGtACGGCCGGAA-GGG-3′ and 5′-CCCTTCCGGCCGTaCtGATTGGTTGCTTTTTA-ATTTAGGC-3′.

Plasmid-free DNA of the transgene was injected into pronuclei followed by the transfer of zygotes into pseudopregnant females. Transgenic embryos were stained for β-galactosidase activity as described (Pfeffer et al., 2000).

In ovo electroporation

cDNAs for electroporation were cloned into the expression vector pCIE containing a chick β-actin promoter, a polylinker and an internal ribosomal entry sequence (IRES) linked to a *GFP* gene. Full-length mouse Otx2, chick Gbx2 and chick Pax2 constructs have been described (Ye et al., 2001). VP16-Brn1 and EnR-Brn1 constructs were generated by fusing the POU homeodomain of rat Brn1 (amino acids 301-497) in-frame C-terminal to the transcriptional activator domain of VP16 or the repressor domain of *Drosophila* Engrailed (amino acids 1-298), respectively. These constructs were unilaterally electroporated into chick embryos at HH stage 8-10 as described (Hynes et al., 2000). Briefly, DNA at 3-6 mg/ml was microinjected into the central canal of the neural tube, and platinum electrodes flanking the neural tube delivered six square pulses of 28 V with a duration of 40 mseconds and an interpulse interval of 45 mseconds. Two days later, chick embryos were analyzed by in situ hybridization.

Results

FACS sorting and microarray screening of mid-hindbrain cells from wild-type and *Pax2* mutant embryos

As Pax2 is an essential regulator of midbrain and cerebellum development (Favor et al., 1996; Bouchard et al., 2000; Ye et al., 2001), we wanted to further investigate the function of Pax2 in mid-hindbrain formation by identifying novel target genes by cDNA microarray screening. This approach relies on the detection of gene expression difference in the mid-hindbrain region of wild-type and $Pax2^{-/-}$ embryos, but required three technical difficulties to be addressed. First, the prospective mid-hindbrain tissue, though initially formed in $Pax2^{-/-}$ embryos on the C3H/He strain background, rapidly degenerates starting at the 12-somite stage (Pfeffer et al., 2000) owing to failed formation of the IsO (Ye et al., 2001). We therefore dissected the mid-hindbrain region at the four- and six-somite stage before the onset of tissue degeneration in $Pax2^{-/-}$ embryos. Second, to enrich for Pax2-expressing cells in the microdissected mid-hindbrain tissue, we took advantage of the $Pax2^{GFP}$ BAC transgene (Pfeffer et al., 2002), which expresses a *GFP* gene under the control of the *Pax2* locus equally well in $Pax2^{-/-}$ and wild-type embryos (Fig. 1A-C; data not shown). Fluorescence-activated cell sorting (FACS) was thus used to isolate 5000-10,000 live GFP^+ cells from the mid-hindbrain region of individual $Pax2^{GFP}$ embryos (Fig. 1C). Last, because the total RNA isolated from the sorted GFP^+ cells of an individual embryo was very low (5-10 ng), we linearly amplified the poly(A)$^+$ RNA by two consecutive cycles of cDNA synthesis and in vitro transcription (Wang et al., 2000), which resulted in 30-80 μg of aRNA corresponding to an estimated 10^6-fold mRNA amplification.

As the expression of mid-hindbrain-specific genes is upregulated during somitogenesis (Wurst and Bally-Cuif, 2001), we determined the gene expression increase during mid-hindbrain development and used this information, in addition to the genotype comparison, as a second criterion for the identification of Pax2-regulated genes. To this end, we prepared aRNA from $Pax2^{GFP}$-expressing mid-hindbrain cells of control (wild-type or $Pax2^{+/-}$) embryos at the 0-2 somite and 8-9 somite stages. The different aRNAs were reverse-transcribed in the presence of Cy3-dUTP or Cy5-dUTP, and the labeled cDNA probes were hybridized to microarrays, which contain 26,000 cDNA clones corresponding to 17,000 UniGene clusters. Only genes with expression levels that were more than fourfold above background were chosen for further analysis. Among the selected 13,200 ESTs, putative Pax2-regulated genes were identified (1) by an expression ratio of more than 1.7 in at least one genotype comparison of six-somite-stage embryos (103 ESTs) and (2) by an expression difference of more than 2.0 between the 0-2 and 8-9 somite stages (168 ESTs). Selection according to both criteria resulted in 12 candidate genes, including the known Pax2 target gene

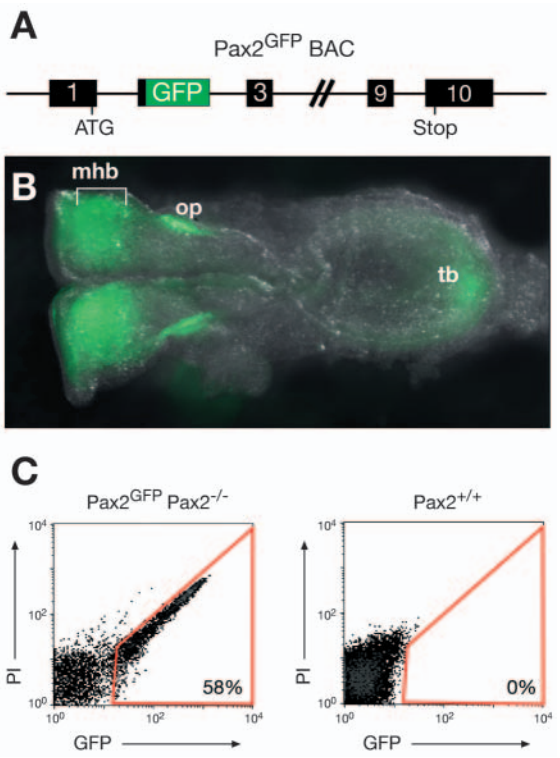

Fig. 1. Isolation of *Pax2*-expressing mid-hindbrain cells from mouse embryos. (A) Structure of the $Pax2^{GFP}$ BAC transgene, which was previously described as transgene 30 containing an in-frame *GFP* insertion in exon 2 of *Pax2* (Pfeffer et al., 2002). (B) GFP expression of the $Pax2^{GFP}$ transgene in the mid-hindbrain boundary (mhb) region, otic placode (op) and tail bud (tb) of E8.5 embryos. (C) Representative FACS sorting of *Pax2*-expressing mid-hindbrain cells. The mid-hindbrain domain (indicated by a bracket in B) was manually dissected from a control wild-type and transgenic $Pax2^{GFP}$ $Pax2^{-/-}$ embryo at E8.5 (seven somites) and dissociated into single cells by trypsin digestion. Live PI^- GFP^+ cells were isolated from individual embryos by fluorescence-activated cell sorting (FACS). The percentage of GFP^+ cells located within the sorting gate is shown for control and transgenic embryos. PI, propidium iodide.

Pax5 (Pfeffer et al., 2000). Six of these genes (shown in Table 1) could subsequently be validated as Pax2-activated genes by in situ hybridization analysis (see below).

Genetic identification of novel Pax2-regulated genes in vivo

As an initial step of validating the microarray results, we investigated whether the candidate genes are expressed in a similar pattern as *Pax2* in the developing mid-hindbrain region at embryonic (E) day 8.5 and 9.5. In situ hybridization analysis of the 11 new candidate genes revealed that the expression of six of them could either not be detected or was not specifically localized to the developing mid-hindbrain region (data not shown). By contrast, the remaining five genes were expressed in the mid-hindbrain domain of wild-type embryos at E8.5 and E9.5 (Fig. 2). Two of these genes, *En2* and *Brn1* (*Pou3f3*), code for homeodomain and POU domain transcription factors, respectively (Joyner and Martin, 1987; Hara et al., 1992). *Sef* and *Tapp1* code for intracellular modulators of Fgf and phosphatidylinositol signaling, respectively (Dowler et al., 2000; Fürthauer et al., 2002; Tsang et al., 2002), and *Ncrms* corresponds to a non-coding RNA gene that is highly expressed in alveolar rhabdomyosarcoma (Chan et al., 2002).

The five genes *En2*, *Brn1*, *Sef*, *Tapp1* and *Ncrms* are expressed at E8.5 in a broad domain corresponding to the prospective midbrain and anterior hindbrain (Fig. 2C,E,G,I,K), which reflects *Pax2* expression at this developmental stage (Fig. 2A). At E9.5, the expression patterns of the putative Pax2-regulated genes have started to diverge in the mid-hindbrain region, while novel expression domains have emerged in other parts of the embryo. At this stage, only *Ncrms* is expressed like *Pax2* in a narrow stripe centered at the mid-hindbrain boundary (Fig. 2B,L). *En2* and *Sef* are broadly expressed in the posterior midbrain and anterior hindbrain (Fig. 2D,H), as previously published (Davis et al., 1988; Fürthauer et al., 2002). *Tapp1* expression is observed in the anterior midbrain (Fig. 2J), whereas *Brn1* is strongly expressed from the forebrain throughout the entire midbrain to the hindbrain (Fig. 2F). In summary, these expression data suggest that Pax2 may control the initiation, but not the maintenance of expression of the candidate genes in the mid-hindbrain boundary region.

We directly tested this hypothesis by comparing the expression pattern of the five putative Pax2 target genes in wild-type and $Pax2^{-/-}$ embryo at the five- to eight-somite stage (E8.5). All five genes failed to be expressed in the developing

Table 1. Identification of Pax2-dependent genes expressed in the mid-hindbrain region

			Genotype comparison				Time course
Gene	EST	Function	4s +/+ vs –/–	6s +/– vs –/–	6s +/+ vs –/–	6s +/+ vs –/–	0-2s vs 8-9s +/+
Pax5	BB219629	TF	2.0	3.4	2.6	3.2	4.9
En2	AI844870	TF	2.3	4.3	8.3	8.8	9.3
Brn1	AI853528	TF	1.0	1.2	1.8	1.8	3.2
Sef	AI428510	Signaling	1.2	2.1	2.4	2.0	5.5
Tapp1	AI849556	Signaling	1.2	1.3	2.2	2.0	2.1
Ncrms	AI853140	Non-coding	0.9	0.9	1.3	3.0	4.0

The Pax2-regulated genes identified by microarray screening are shown together with their EST Accession Number, known function and expression ratio as determined by microarray hybridization. Control and *Pax2* mutant genotypes were compared with each other in independent microarray experiments with Cy3- and Cy5-labeled cDNA probes that were prepared from sorted GFP^+ mid-hindbrain cells of individual four-somite (4s)- or six-somite (6s) stage embryos. Pooled RNA of two or three control ($Pax2^{+/+}$ or $Pax2^{+/-}$) embryos was used to analyze the increase of gene expression between the 0- to two-somite (0-2s) and eight- to nine-somite (8-9s) stage. All expression data are presented as control/mutant or late/early ratios of the normalized fluorescence values determined by microarray hybridization. *Fgf8* could not be identified as a Pax2-regulated gene in these screens because of the absence of *Fgf8* cDNA on the microarrays used. TF, transcription factor.

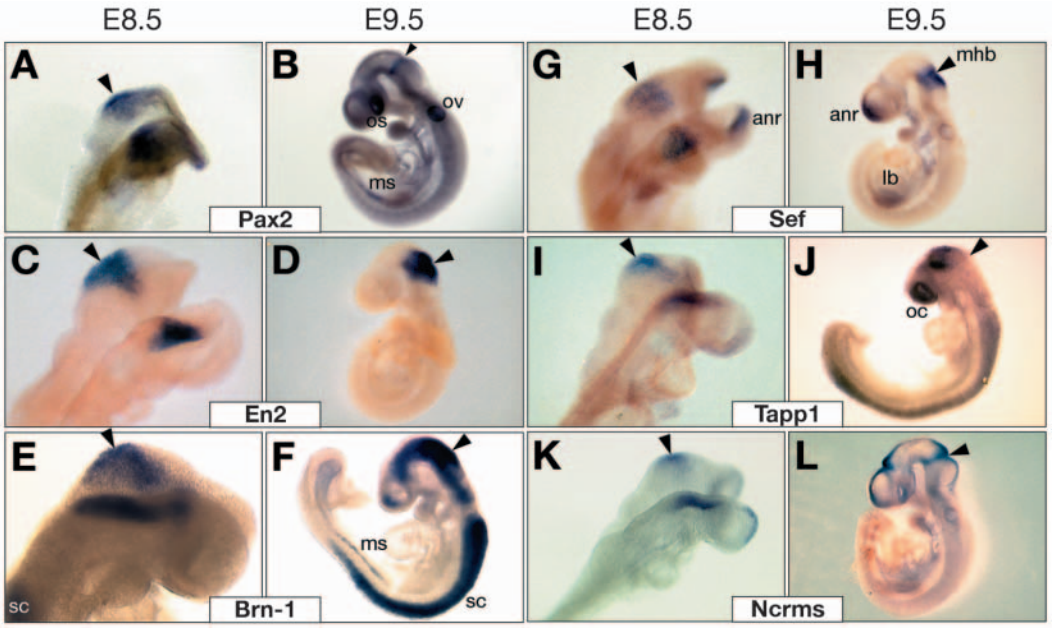

Fig. 2. Expression pattern of the putative Pax2-regulated genes in wild-type embryos. Probes of the indicated genes were used for whole-mount in situ hybridization of embryos at E8.5 (8-10 somites; A,C,E,G,I,K; dorsolateral view) and E9.5 (B,D,F,H,J,L; lateral view). Arrowhead indicates the position of the mid-hindbrain boundary (mhb). anr, anterior neural ridge; lb, limb bud; ms, mesonephros; oc, optic cup; os, optic stalk: ov, otic vesicle; sc, spinal cord.

mid-hindbrain region of *Pax2*$^{-/-}$ embryos (Fig. 3B,D,F,H,J), in contrast to stage-matched control embryos (Fig. 3A,C,E,G,I), while other expression domains remained unaffected by the *Pax2* mutation. These results unequivocally demonstrate that Pax2 controls the mid-hindbrain-specific expression of *En2*, *Brn1*, *Sef*, *Tapp1* and *Ncrms* during early somitogenesis.

Identification of conserved upstream sequences of the *Brn1* gene

The transcription factor Brn1 is a member of the class III POU protein family (Ryan and Rosenfeld, 1997) and has been implicated in patterning of the cerebellum (Sugitani et al., 2002). As the cerebellum develops from the Pax2-dependent expression domain of *Brn1*, we selected this gene for biochemical and transgenic analysis of its regulatory elements to determine whether *Brn1* is a direct transcriptional target of Pax2. We first searched for conserved sequences within the human and mouse genomic sequences that extend from –20 kb upstream to +15 kb downstream of the *Brn1* translation start site. The highest sequence conservation outside of the coding region was found in a 6 kb sequence located immediately upstream of the *Brn1* start codon. This region contains five elements (A, B, C, D and P) with an evolutionary conservation of more than 93% (Fig. 4A; see Fig. S1 in the supplementary material). Four of these elements (B, C, D and P) are even conserved between the *Brn1* genes of mammals and the pufferfish *Fugu rubripes* (Fig. 4A).

Gene promoters are often located within CpG islands (Antequera and Bird, 1999), which are also present in the first

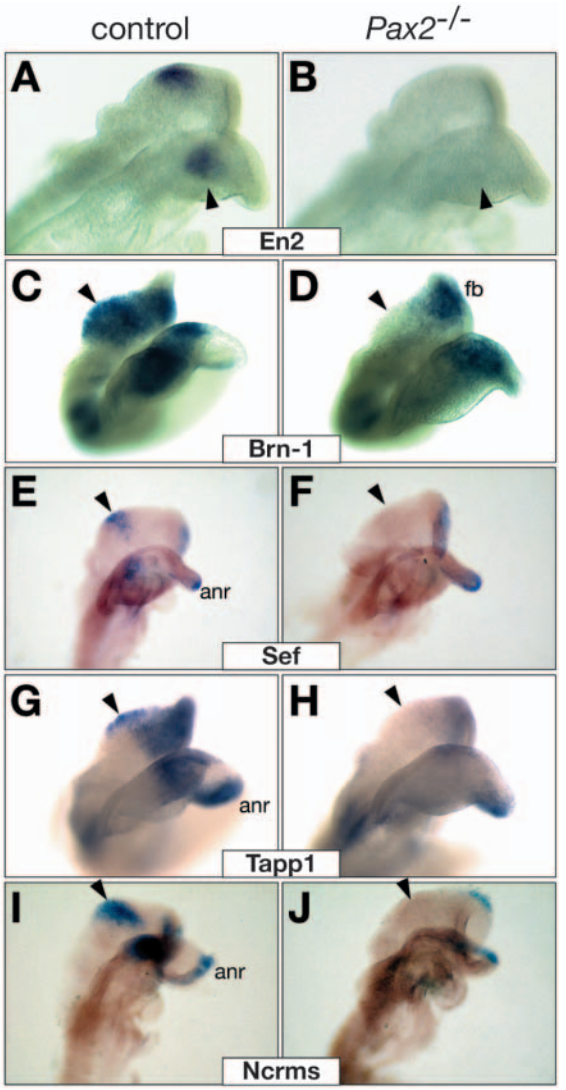

Fig. 3. Pax2-dependent gene expression in the developing mid-hindbrain region. *Pax2*$^{+/+}$ or *Pax2*$^{+/-}$ control embryos (A,C,E,G,I) and *Pax2*$^{-/-}$ embryos (B,D,F,H,J) were analyzed by for expression of the indicated genes by in situ hybridization at the five to six somite (A,B,I,J) or seven to eight somite (C-H) stage. Arrowheads indicate the position of the mid-hindbrain boundary. anr, anterior neural ridge; fb, forebrain.

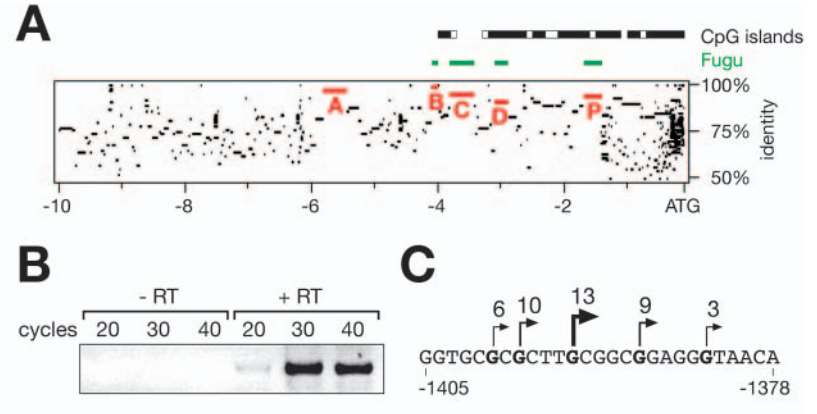

Fig. 4. Sequence conservation and heterogeneous transcription initiation of the *Brn1* gene. (A) Conservation of the 5′ flanking sequences of mammalian and fish *Brn1* genes. The PipMaker program (Schwartz et al., 2000) was used to compare the 10 kb upstream regions of the human and mouse *Brn1* genes. Sequence block with 50% to 100% identical sequence (*y*-axis) are indicated together with their position (*x*-axis) relative to the translation start codon of *Brn1*. The conserved elements A,B,C,D and P share 93.2% (D) to 96.5% (B) sequence identity in the two mammalian species (Fig. 6D; see Fig. S1 in the supplementary material). Four of these regions (B, C, D and P) are even conserved between mammals and the pufferfish *Fugu rubripes* (shown in green). CpG islands with an average GC content of ~60% are present in the first 3.8 kb upstream of the *Brn1* start codon. (B) Mapping of the transcription initiation region. 5′-RACE amplified PCR fragments of ~325 bp from E10.5 head RNA with a primer located downstream of element P (–1098/–1069 relative to start codon). The presence or absence of reverse transcriptase (RT) and the PCR cycles are indicated. (C) Identification of transcription start sites. Sequencing of the 5′-RACE products identified heterogeneous transcriptional start sites within a 17 bp sequence of element P. The number of PCR clones with an identical 5′ end is indicated together with the nucleotide positions relative to the start codon.

4 kb upstream of the *Brn1* start codon (Fig. 4A). The PromoterInspector program (Scherf et al., 2000) identified elements D and P as potential promoter regions. To test this possibility, we determined the start sites of *Brn1* transcription by using primers located in the two putative 5′ untranslated regions for 5′-RACE analysis of E10.5 head RNA. PCR fragments could readily be amplified with an element P primer (Fig. 4B) in contrast to element D sequences (data not shown). Cloning and sequence of 63 RACE products demonstrated that the 5′ ends of 41 clones clustered at five sites spanning a 17 bp region within element P (Fig. 4C). As expected for heterogeneous transcription initiation, no TATA box could be found upstream of these start sites, which are located between positions –1400 and –1383 relative to the initiation codon (Fig. 6D). Taken together, these results identified a single *Brn1* promoter, giving rise to heterogeneous transcription initiation in embryonic brain cells.

High-affinity Pax2-binding sites in the promoter and upstream element D of *Brn1*

The binding of Pax2 to the different conserved regions of *Brn1* was next assessed by electrophoretic mobility shift assay (EMSA). To this end, we cloned the conserved *Brn1* sequences and used them as competitor DNA to prevent the binding of in vitro translated Pax2 protein to a labeled oligonucleotide containing the high-affinity Pax2/5/8-binding site of the *CD19* promoter (Kozmik et al., 1992). In addition, we used the high-affinity Pax2/5/8-binding site of the *Blnk* promoter (Schebesta et al., 2002) as a reference sequence for comparing the competition strength of the different conserved *Brn1* elements. This competition assay revealed the presence of Pax2-binding sites in elements P and D, but not in the conserved regions A, B or C (Fig. 5A; data not shown). Several potential Pax2-binding sites were identified within elements P and D (Fig. 5B,C) by comparison with the consensus Pax2/5/8 recognition sequences (Fig. 5D) (Czerny and Busslinger, 1995). Competition analysis with oligonucleotides of individual candidate sites revealed a single Pax2-binding sequence (Dd) within element D (Fig. 5B). The interaction of Pax2 with promoter element P was further investigated by comparing three overlapping subfragments (P1-P3) in the competition assay, which mapped the Pax2-binding activity to the 5′ region of fragment P1 (Fig. 5C). This region contains three candidate Pax2-binding sequence, only one of which (site Pc) was able to interact with Pax2 (Fig. 5C). Importantly, the recognition sequences Dd and Pc bound Pax2 with similar efficiency as the high-affinity binding site of *Blnk* (Fig. 5B,C) consistent with the fact that the Dd and Pc sites match the consensus recognition sequence at 13 out of 15 positions (Fig. 5D). Moreover, Pax2 binding to these two sites was completely abolished by mutating two consensus nucleotides in each binding site (Ddm, Pcm; Fig. 5B,C). In summary, these experiments identified two high-affinity Pax2-binding sites, which are located 134 (Pc) and 1413 (Dd) bp upstream of the major transcription initiation site of *Brn1*.

Both Pax2-binding sites are essential for mid-hindbrain-specific expression of *Brn1*

We next investigated by transgenic analysis whether the Pax2-binding sites Dd and Pc are important for *Brn1* expression in the developing mid-hindbrain region. For this, we generated the transgenes *3.2wt-lacZ* and *6.2wt-lacZ* by inserting 3.2 kb and 6.2 kb 5′ flanking sequences of *Brn1* (starting at position –59 relative to the ATG codon) upstream of a *lacZ* reporter gene. Transgenic embryos were generated by pronuclear DNA injection and analyzed by X-gal staining for *lacZ* expression at E9.5. The shorter *3.2wt-lacZ* transgene was unable to drive reporter gene expression, indicating that the promoter (P) and upstream element D with their Pax2-binding sites are not sufficient for activating *Brn1* expression in the embryo (not shown). By contrast, the *6.2wt-lacZ* transgene containing the promoter (P) and all four conserved upstream elements (A-D) gave rise to localized *lacZ* expression in the posterior forebrain (diencephalon), mid-hindbrain boundary region and spinal cord (Fig. 6A). This expression pattern differs from that of the endogenous *Brn1* gene at E9.5, as the *6.2wt-lacZ* transgene failed to be expressed in the mesonephros and throughout the entire forebrain-hindbrain region (compare Fig. 2F with Fig. 6A). Hence, the *6.2wt-lacZ* transgene contains the control elements for initiating *Brn1* expression in the mid-hindbrain

boundary region, while lacking regulatory sequences for maintaining *Brn1* expression throughout the entire midbrain and hindbrain. To further study the role of the Pax2-binding sites Dd and Pc in the initiation of mid-hindbrain-specific *Brn1* expression, we mutated each Pax2 recognition sequence individually in the *6.2wt-lacZ* transgene by introducing the two-nucleotide substitutions that abrogate Pax2 binding (Fig. 5B-D). Both mutant transgenes, *6.2Ddm-lacZ* and *6.2Pcm-lacZ*, failed to be expressed above the detection limit in the mid-hindbrain boundary region, although they gave rise to strong β-galactosidase staining in the diencephalon of the forebrain and along the entire spinal cord. The two high-affinity Pax2-binding sites in the promoter and upstream element D are therefore essential for initiating the mid-hindbrain-specific expression of *Brn1* during early somitogenesis. These data unequivocally identify the *Brn1* gene as a direct target of Pax2 during mid-hindbrain development.

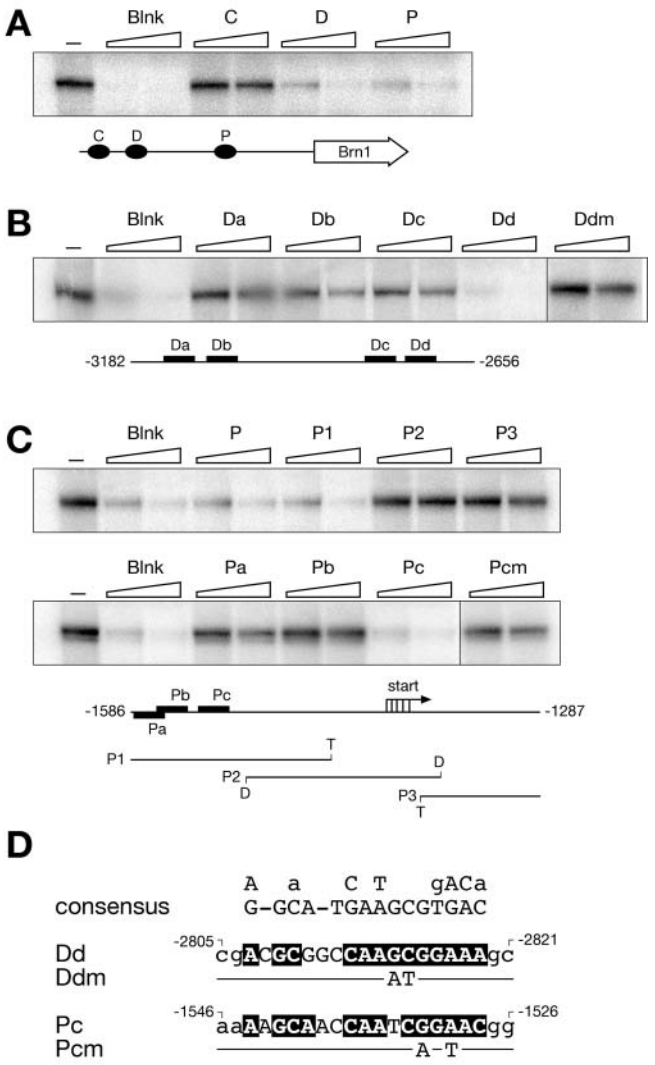

Fig. 5. Mapping of Pax2-binding sites in the conserved 5′ regions of *Brn1*. (A) Presence of high-affinity Pax2-binding sites in elements D and P. The binding of in vitro translated Pax2 protein to a labeled oligonucleotide containing the Pax2/5/8-binding site 1 of the *CD19* promoter (Kozmik et al., 1992) was measured by EMSA in the absence (–) or presence of a 10- or 50-fold molar excess of the indicated competitor DNA. The competition strength of PCR fragments comprising elements C, D and P was compared with that of the high-affinity site 1 of the *Blnk* promoter (Schebesta et al., 2002). The protein-DNA complexes are shown together with a map, indicating the positions of the conserved elements relative to the Brn1-coding sequence. (B,C) Identification of the sites Dd (B) and Pc (C) as high-affinity Pax2-binding sequences. The same competition assay was used to evaluate the interaction of Pax2 with the indicated restriction fragments (P1-P3) or oligonucleotides containing putative Pax2/5/8-binding sites in element D (Da-Dd) and P (Pa-Pc). Substitution of two base pairs prevents binding of Pax2 to the mutant (m) sites Ddm (B) and Pcm (C). D, *Dde*I; T, *Taq*I. (D) Sequence alignment of sites Dd and Pc with the consensus Pax2/5/8 recognition sequence (Czerny and Busslinger, 1995). The positions relative to the *Brn1* start codon and the nucleotide substitutions of the mutant sites are indicated.

EnR-Brn1 induces ectopic *Fgf8* expression in the hindbrain of chick embryos

In addition to Brn1 (Pou3f3), the class III POU protein family consists of Brn2 (Pou3f2 – Mouse Genome Informatics), Brn4 (Pou3f4 – Mouse Genome Informatics) and Tst1 (Pou3f1 – Mouse Genome Informatics). In situ hybridization analyses revealed strong *Brn2* and weak *Brn4* expression in the mid-hindbrain domain of E8.5 embryos (see Fig. S2 in the supplementary material), whereas *Tst1* was not expressed in this CNS region (data not shown). Moreover, *Brn2* expression was downregulated but not lost in the mid-hindbrain domain of *Pax2*$^{-/-}$ embryos (see Fig. S2 in the supplementary material). Consistent with these overlapping expression patterns, brain development is largely normal in *Brn1* or *Brn2* single-mutant mice (Schonemann et al., 1995; Nakai et al., 1995; Nakai et al., 2003), whereas double-mutant mice show a severe defect in cerebellum patterning (Sugitani et al., 2002). To specifically analyze the role of Brn1 in mid-hindbrain development, we next performed ectopic expression experiments in chick embryos by in ovo electroporation. To investigate the expression pattern of the endogenous *Brn1* gene, we cloned a partial chick *Brn1* cDNA (Fig. 7A) and used it as a probe for in situ hybridization of chick embryos at HH stages 14/15 and 19/20 (Fig. 7B,C). These embryos expressed *Brn1* throughout the midbrain, hindbrain and spinal cord as well as in the mesonephros (Fig. 7B,C), which resembles the corresponding expression pattern in the mouse embryo (Fig. 2F). As Fgf8 is the central mediator of IsO activity (Crossley et al., 1996), we used *Fgf8* expression as a read-out for the chick embryo electroporation experiments. Previous studies have demonstrated that ectopic Pax2 expression could efficiently induce endogenous *Fgf8* transcription only in Gbx2^{+} hindbrain cells adjacent to ectopic Otx2-expressing cells (Ye et al., 2001) (Fig. 7F). However, combined expression of rat Brn1 and mouse Otx2 failed to induced *Fgf8* transcription (Fig. 7D), possibly because endogenous Brn1 was already expressed in the hindbrain of electroporated chick embryos (Fig. 7B,C). Next, we fused the VP16 transactivation or Engrailed repression (EnR) sequences to the DNA-binding POU domain of rat Brn1 in an attempt to generate dominant-active or -negative Brn1 proteins, respectively. Whereas electroporation of VP16-Brn1 together with Otx2 had no effect on *Fgf8* expression (Fig. 7E), ectopic expression of EnR-Brn1 and Otx2 strongly induced *Fgf8* transcription in the chick hindbrain.

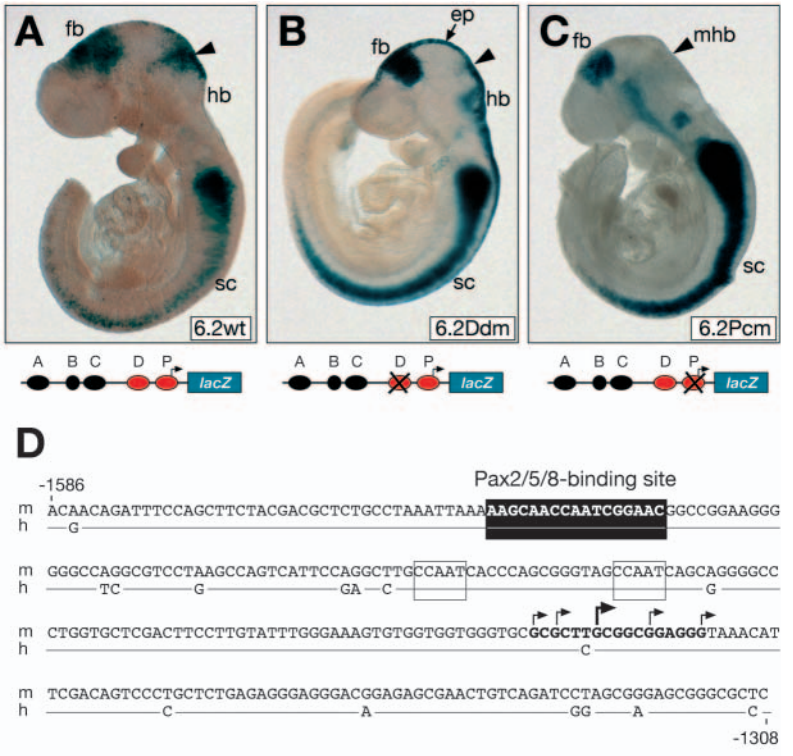

Fig. 6. Both high-affinity Pax2-binding sites are essential for the initiation of mid-hindbrain-specific *Brn1* expression. (A) Expression of the *6.2wt-lacZ* transgene at E9.5. A 6.2 kb *Afl*II-*Not*I fragment from the 5′ region of *Brn1* (–6267/–59 relative to the start codon) directs expression of a *lacZ* reporter gene in the posterior forebrain (fb), mid-hindbrain boundary (arrowhead) region and spinal cord (sc), which correspond to a subset of the endogenous *Brn1* expression domains at E9.5 (see Fig. 2F). (B,C) Inactivation of the Pax2-binding site Dd or Pc prevents mid-hindbrain-specific expression of the *6.2Ddm-lacZ* or *6.2Pcm-lacZ* transgene, respectively, while leaving the forebrain and spinal cord expression unaffected. The expression of all transgenes was analyzed by X-gal staining of injected founder (G_0) embryos at E9.5. Each transgenic construct gave rise to three *lacZ*-expressing embryos with a similar β-galactosidase staining pattern. The embryo shown in B revealed ectopic β-galactosidase expression in the epidermis (ep) from the forebrain to the hindbrain (hb), which was not seen with other embryos carrying the same *6.2Ddm-lacZ* gene. Arrowheads in B,C indicate the midbrain-hindbrain boundary (mhb). (D) *Brn1* promoter sequence. The mouse (m) DNA sequence of promoter element P is shown together with the transcription initiation sites, two conserved CCAAT boxes and the functional Pax2/5/8-binding site Pc. Only the divergent nucleotides of the corresponding human (h) *Brn1* sequence are indicated.

Hence, these data implicate Brn1 in the regulation of *Fgf8* expression during mid-hindbrain development.

Discussion

Pax2 plays a key role in the formation of the isthmic organizer (IsO) that controls midbrain and cerebellum development (Favor et al., 1996; Bouchard et al., 2000; Ye et al., 2001). Despite the importance of Pax2 for mid-hindbrain patterning, relatively little is known about target genes that are activated by Pax2 at the onset of mid-hindbrain development. We have previously shown that Pax2 is essential for initiating expression of the closely related *Pax5* and *Pax8* genes, indicating that the inactivation of *Pax2* is equivalent to mutation of all three *Pax2/5/8* family members at the mid-hindbrain boundary (Pfeffer et al., 1998; Pfeffer et al., 2000; Ye et al., 2001). Pax2 is furthermore necessary and sufficient for inducing the expression of the IsO signal Fgf8 (Ye et al., 2001). Here, we have used gene expression profiling of mid-hindbrain cells from wild-type and *Pax2*$^{-/-}$ embryos as a more systematic strategy for identifying Pax2-regulated genes. This approach relies on transgenic GFP labeling and FACS sorting of Pax2-expressing cells followed by linear RNA amplification, probe preparation and cDNA microarray screening. In this way, we identified five genes, *En2*, *Brn1*, *Sef*, *Tapp1* and *Ncrms*, which are expressed in the developing mid-hindbrain region under the control of Pax2. The molecular nature of these new target genes implicates Pax2 in the control of intracellular signaling and the establishment of transcription factor networks in the mid-hindbrain region.

Control of intracellular signaling by Pax2

Fgf receptor stimulation by the IsO signal Fgf8 activates the Ras/mitogen-activated protein kinase (MAPK) pathway (Kouhara et al., 1997; Corson et al., 2003), which controls cell proliferation and differentiation (Marshall, 1995). Fgf8 signaling also activates the expression of the transmembrane protein Sef (similar expression to Fgf genes), which acts as a negative feedback regulator to limit the duration of Ras-MAPK signaling by inhibiting the tyrosine phosphorylation of Fgf receptors (Fürthauer et al., 2002; Tsang et al., 2002; Kovalenko et al., 2003). Here, we have demonstrated that the expression of *Sef* fails to be induced in the mid-hindbrain region of *Pax2*$^{-/-}$ mouse embryos, similar to *Pax2.1* (*noi*) mutant embryos of the zebrafish (Tsang et al., 2002). As *Sef* expression is also not activated at the mid-hindbrain boundary of *Fgf8* (*ace*) mutant embryos (Fürthauer et al., 2002), it is possible that the absence of *Sef* transcripts in *Pax2*$^{-/-}$ embryos is an indirect consequence of the failed induction of *Fgf8* expression (Ye et al., 2001). However, it is equally likely that Pax2 directly activates the *Sef* gene in cooperation with Fgf signaling.

Pax2 also regulates the midbrain-specific expression of the tandem PH-domain-containing protein 1 (*Tapp1*) gene. Tapp1 was identified as an adaptor molecule that specifically binds to the lipid phosphatidylinositol 3,4-bisphosphate [PI(3,4)P_2] via its C-terminal pleckstrin homology (PH) domain (Dowler et al., 2000). PI(3,4)P_2 is generated by the inositol 5′-phosphatase SHIP from PI(3,4,5)P_3, which in turn is produced through phosphorylation of PI(4,5)P_2 by the phosphatidylinositol 3′-kinase (PI3K) (Rohrschneider et al., 2000). Both PI3K and SHIP are activated by stimulatory and inhibitory tyrosine kinase receptors, respectively, in agreement with the role of their second messengers PI(3,4,5)P_3 and PI(3,4)P_2 in recruiting different PH domain-containing effector proteins to the plasma membrane (Rohrschneider et al., 2000). The PI(3,4,5)P_3-dependent recruitment and activation of the Akt/PKB, PDK1 and Btk kinases promotes cell survival and proliferation

(Rohrschneider et al., 2000). SHIP antagonizes these pathways by metabolizing the lipid ligand PI(3,4,5)P_3 of these kinases to PI(3,4)P_2, which functions as a membrane docking site for adaptors such as Tapp1 (Dowler et al., 2000; Kimber et al., 2002; Marshall et al., 2002). Tapp1 is constitutively associated though its PDZ domain-binding motif with the protein tyrosine phosphatase-like protein 1 (PTPL1/FAP1), which dephosphorylates receptors and adaptor proteins at the plasma membrane, thus further inactivating PI3K signaling (Kimber et al., 2003). Hence, the Pax2-dependent expression of Tapp1 may contribute to feedback inhibition of PI3K signaling during midbrain development.

The non-coding RNA Ncrms

The Pax2-regulated gene *Ncrms* is transcribed into a non-coding RNA that was initially identified because of its higher abundance in alveolar rhabdomyosarcoma compared with the embryonic subtype of this pediatric muscle tumor (Chan et al., 2002). Recently, it has been shown that non-coding RNA genes are almost as prevalent as protein-coding genes in the mammalian genome (Cawley et al., 2004). Among these RNAs, the *Ncrms* transcript belongs with its size of 1.25 kb to the family of long non-coding RNAs, which include the *H19*, *Air* and *Xist* transcripts (Reik and Walter, 2001; Sleutels et al., 2002; Wutz et al., 2002). Analogous to the regulatory functions of these known non-coding RNAs, it is conceivable that the *Ncrms* transcript is involved in the control of mid-hindbrain-specific gene expression.

Pax2-dependent regulation of the *En2* transcription factor gene

Pax2 also controls the expression of the transcriptional regulators En2 and Brn1 in addition to the transcription factors Pax5 and Pax8 in the developing mid-hindbrain region. These data indicate a key role for Pax2 in the activation of distinct transcriptional programs at the onset of mid-hindbrain development. The homeodomain protein En2 is required for normal development of the cerebellum (Joyner et al., 1991; Millen et al., 1994). Interestingly, a 1.0 kb enhancer of the *En2* gene contains two Pax2/5/8-binding sites that are essential for directing *lacZ* transgene expression at the mid-hindbrain boundary (Song et al., 1996). However, mutation of these two sites in the *En2* locus only minimally affects the initiation of endogenous *En2* transcription (Song and Joyner, 2000). Our observation, that the mid-hindbrain-specific expression of *En2* completely depends on Pax2 function, points to the presence of yet unidentified functional Pax2/5/8-binding sites that must lie outside of the 1.0 kb enhancer in the *En2* locus. In contrast to *En2*, *En1* expression is reduced but not absent at the mid-hindbrain boundary of *Pax2*$^{-/-}$ embryos (Ye et al., 2001). Hence, Pax2 acts upstream of En genes in the genetic cascade of mid-hindbrain development, consistent with the fact that both En1 and En2 are not required for the initiation, but for the maintenance, of mid-hindbrain-specific gene expression (Liu and Joyner, 2001b) in marked contrast to Pax2 (Pfeffer et al., 2000; Ye et al., 2001) (this study). Moreover, the combined inactivation of *En1* and *En2* results in a similar mid-hindbrain phenotype (Liu and Joyner, 2001b) as mutation of *Pax2* (Favor et al., 1996; Bouchard et al., 2000) in agreement with the regulation of both En genes by Pax2.

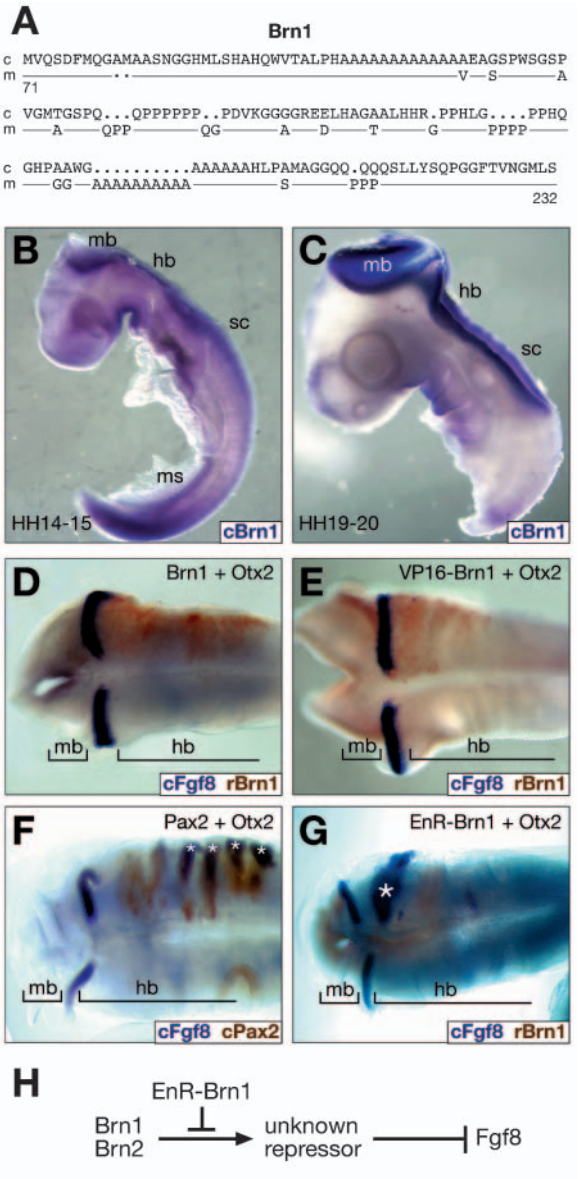

Fig. 7. Induction of *Fgf8* in the hindbrain by ectopic expression of EnR-Brn1. (A) Comparison of chick (c) and mouse (m) Brn1 protein sequences. A partial chick *Brn1* cDNA was PCR-cloned from embryo RNA. Numbers refer to the corresponding amino acids of mouse Brn1, for which only the divergent amino acids are shown. Dots indicate five gaps introduced for optimal sequence alignment. (B,C) *Brn1* expression in chick embryos at HH stages 14-15 and 19-20. (D-G) Ectopic expression of rat (r) Brn1 proteins together with mouse Otx2 in chick embryos. The expression of endogenous chick (c) *Fgf8* (blue) and electroporated rat *Brn1* (D,E,G) or chick *Pax2* (F) genes (brown) was detected by in situ hybridization. Asterisks indicate ectopic *Fgf8* expression in the hindbrain. In G, the strong *Fgf8* signal (blue) covered the *Brn1* signal (brown) because of colocalization of ectopic *EnR-Brn1* and *Fgf8* expression. The upper side of the embryo is electroporated, whereas the lower side serves as a control. (H) Hypothetical interactions that may explain *Fgf8* induction by the ectopically expressed EnR-Brn1. For explanations, see Discussion.

Brn1 function in mid-hindbrain development

The gene coding for the POU domain transcription factor Brn1 was identified as a direct target of Pax2 by two criteria. First,

Brn1 expression fails to initiate in the mid-hindbrain region of *Pax2*$^{-/-}$ embryos, in contrast to wild-type or *Pax5*$^{-/-}$ embryos (see Fig. S3 in the supplementary material). Second, the 5′ region of *Brn1* contains two high-affinity Pax2-binding sites, which are essential for initiating transgene expression in the mid-hindbrain region. Additional regulatory sequence located outside of the 6.2 kb 5′ region analyzed are, however, required for the subsequent maintenance of *Brn1* expression throughout the entire midbrain and hindbrain, which may involve auto- and cross-regulatory interactions of POU proteins with a consensus octamer sequence in the upstream element B (see Fig. S1 in the supplementary material). Of the four members of the class III Pou gene family, only the closely related *Brn1* and *Brn2* genes (Bürglin and Ruvkun, 2001) are abundantly expressed in the mid-hindbrain region of E8.5 embryos. Interestingly, Pax2 also regulates the *Brn2* gene in this brain region, although less stringently than *Brn1* (see Fig. S2 in the supplementary material). Owing to the overlapping expression patterns, midbrain and cerebellum development is largely normal in mice that lack either *Brn1* or *Brn2* (Schonemann et al., 1995; Nakai et al., 1995; Nakai et al., 2003), whereas cerebellum patterning is severely affected in double-mutant mice (Sugitani et al., 2002). Here, we have complemented these loss-of-function analyses by ectopic expression experiments in chick embryos to further study the role of Brn1 and Brn2 in mid-hindbrain development. Ectopic expression of the transcriptional repressor EnR-Brn1 in combination with Otx2 strongly induced *Fgf8* expression in the hindbrain of chick embryos in marked contrast to full-length Brn1 or the transcriptional activator VP16-Brn1. These data and the fact, that endogenous Brn1 is expressed throughout the mid-hindbrain region, suggest that Brn1 functions as transcriptional activator in mid-hindbrain development. In agreement with this conclusion, Brn1 and Brn2 were shown to be important activators of the *protease nexin-1* gene at the mid-hindbrain boundary in transgenic analysis as well as in transient transfection experiments (Mihailescu et al., 1999). According to this hypothesis, the EnR-Brn1 fusion protein functions as a dominant-negative regulator to block the transcriptional activity of full-length Brn1 (Fig. 7H). The EnR-Brn1 protein may thus lead to ectopic *Fgf8* expression by preventing the Brn1/2-dependent expression of a so far unknown repressor of the *Fgf8* gene (Fig. 7H). This hypothesis implicates Brn1 and Brn2 in restricting *Fgf8* expression to the posterior domain of the mid-hindbrain boundary, but fails to explain why Pax2 is able to activate *Fgf8* in this location despite the presence of Brn1/2 proteins. Other regulators that are induced at the Otx2/Gbx2 boundary may antagonize the function of Brn1/2 or the postulated repressor to facilitate *Fgf8* expression in Gbx2^{+} cells of the mid-hindbrain boundary. Although further experiments including *Fgf8* promoter analyses are required to elucidate the precise molecular nature of the Pax2-Brn1/2-Fgf8 regulatory interactions, our experiments have identified the Brn1/2 proteins as novel regulators controlling the expression of the IsO signal Fgf8.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/11/2633/DC1

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