

## Two Pax-binding sites are required for early embryonic brain expression of an *Engrailed-2* transgene

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### SUMMARY

The temporally and spatially restricted expression of the mouse *Engrailed (En)* genes is essential for development of the midbrain and cerebellum. The regulation of *En-2* expression was studied using *in vitro* protein-DNA binding assays and *in vivo* expression analysis in transgenic mice to gain insight into the genetic events that lead to regionalization of the developing brain. A minimum *En-2* 1.0 kb enhancer fragment was defined and found to contain multiple positive and negative regulatory elements that function in concert to establish the early embryonic mid-hindbrain expression. Furthermore, the mid-hindbrain regulatory sequences were shown to be structurally and functionally conserved in humans.

The mouse paired-box-containing genes *Pax-2*, *Pax-5* and *Pax-8* show overlapping expression with the *En* genes

in the developing brain. Significantly, two DNA-binding sites for Pax-2, Pax-5 and Pax-8 proteins were identified in the 1.0 kb *En-2* regulatory sequences, and mutation of the binding sites disrupted initiation and maintenance of expression in transgenic mice. These results present strong molecular evidence that the Pax genes are direct upstream regulators of *En-2* in the genetic cascade controlling mid-hindbrain development. These mouse studies, taken together with others in *Drosophila* and zebrafish on the role of Pax genes in controlling expression of *En* family members, indicate that a Pax-En genetic pathway has been conserved during evolution.

Key words: *En*, *Pax*, mid-hindbrain, development, regionalization, evolution, enhancer, transgenic mice, *lacZ*, mouse

### INTRODUCTION

Regionalization of the neural tube is an early critical event during patterning of the central nervous system (CNS) in vertebrates. Over the past decade, a large effort has been directed toward identifying genes involved in regulating this process. Many candidate genes display temporally and spatially restricted expressions that mark different regions of the neural tube (reviewed in McGinnis and Krumlauf, 1992; Puelles and Rubenstein, 1993). Recent analysis of mutant phenotypes in mice has provided direct evidence that such restricted gene expression is in fact critical for early patterning of the CNS (reviewed in Joyner and Guillemot, 1994). An important question that remains from these studies is the nature of the genetic pathways that regulate early spatially restricted gene expression and govern regionalization.

In the developing brain, a number of genes, including members of the *Engrailed (En)*, *Pax* and *Wnt* gene families, have been shown to be critical for patterning the midbrain and rostral hindbrain (mesencephalon and metencephalon), which give rise to the colliculi, tegmentum, isthmus region and cerebellum. The *En-1* and *En-2* genes, homologues of the *Drosophila* segmentation gene *engrailed (en)*, encode homeo-

domain-containing transcription factors (Joyner et al., 1985; reviewed in Joyner, 1996). The Pax gene family consists of nine members that encode paired domain-containing transcription factors related to *Drosophila* paired and gooseberry (reviewed in Gruss and Walther, 1992; Noll, 1993). The *Wnt* genes, homologues of the *Drosophila* segmentation gene *wingless (wg)*, encode short-range signaling molecules (reviewed in McMahon, 1992; Nusse and Varmus, 1992). In mouse embryos, *En-1*, *En-2*, *Wnt-1*, *Pax-2*, *Pax-5* and *Pax-8* show spatially and temporally overlapping expression domains that encompass the developing mid-hindbrain junction region (Davis et al., 1988; Davis and Joyner, 1988; Adams et al., 1992; Asano and Gruss, 1992; Püschel et al., 1992; Rowitch and McMahon, 1995). Expression of all but *Pax-8* is initiated in similar domains shortly following formation of the neural plate. After neural tube closure, *En-1*, *En-2*, *Pax-5* and *Pax-8* are expressed in broad rings spanning the mid-hindbrain junction, whereas expression of *Wnt-1* and *Pax-2* becomes restricted to a narrower ring near the mid-hindbrain constriction. Expression of these genes in the mid-hindbrain junction region diverges after 12.5 days post coitum (d.p.c.) as neurogenesis proceeds. Similar early overlapping expression patterns of the *En*, *Pax* and *Wnt* genes in the developing brain

have also been observed in chick, *Xenopus* and zebrafish (Davis et al., 1991; Krauss et al., 1991).

Loss-of-function mutants for *En-1*, *En-2*, *Wnt-1* and *Pax-5* have been generated by gene targeting. *En-1* and *Wnt-1* homozygous mutants show deletions of the midbrain and cerebellum that can be detected as early as 9.0 d.p.c. (Wurst et al., 1994; McMahon and Bradley, 1990; Thomas and Capecchi, 1990). *En-2* and *Pax-5* homozygous mutants have milder phenotypes; *En-2* mutants show reduction in the size of the cerebellum and an abnormal foliation pattern (Joyner et al., 1991; Millen et al., 1994), and *Pax-5* mutants primarily show a partial deletion of the inferior colliculus (Urbánek et al., 1994). The more severe and earlier phenotype of *En-1* mutants compared to that of *En-2* mutants was shown to be due to the earlier expression of *En-1* (Hanks et al., 1995). Consistent with this, *En-1/En-2* double mutants have a more severe deletion phenotype than that of *En-1* mutants (W. Wurst and A. L. Joyner, unpublished data) suggesting that both genes are required for proper development of this region. By analogy, the mild *Pax-5* mutant phenotype may be due to compensation by the related *Pax-2* and *Pax-8* genes. Consistent with this idea, zebrafish embryos injected with an antibody against pax[zf-b], the only Pax-5-related protein identified in zebrafish, show early malformations in the mid-hindbrain junction region (Krauss et al., 1992).

Taken together, the early overlapping expression of the *En*, *Pax* and *Wnt* genes in the mid-hindbrain and the similarity of mutant brain phenotypes suggests that these genes are part of a genetic network that controls the development of this region. Significantly, in *Drosophila*, a genetic network utilizing homologues of these genes is employed to regulate segmentation, suggesting that the genetic pathway may have been conserved through evolution (reviewed in Ingham and Martinez-Arias, 1992; Hooper and Scott, 1992).

At present, little is known about interactions between these regulatory genes in the mouse. A study of *En* expression in *Wnt-1* targeted mutants found that the expression of *En* genes was initiated normally but quickly lost (McMahon et al., 1992). This suggests that *Wnt-1* is not required to initiate *En* expression but could be involved in the maintenance of *En* expression. Alternatively, the loss of *En* expression in *Wnt-1* mutants could reflect the loss of *En*-expressing cells due to the absence of a *Wnt-1* signal. Due to the early deletion phenotypes and/or functional overlaps between paralogous genes, mutant mice have provided limited information regarding potential gene interactions. Identification of DNA regulatory sequences and characterization of their interactions with potential protein regulators are required to directly address this issue.

To study the potential interactions between the *En*, *Wnt* and *Pax* genes in mice, we have analyzed the regulation of *En-2* using *lacZ* reporter constructs in transgenic mice. Previously, we identified a 9.5 kb *En-2* genomic fragment that contains sufficient regulatory information to direct *En-2*-like expression during embryogenesis and in the adult (Logan et al., 1993). A 1.5 kb enhancer fragment was located that is sufficient for at least the embryonic mid-hindbrain expression. In the present study, we have further dissected this 1.5 kb enhancer fragment and found that it contains multiple positive and negative regulatory elements that function together to establish the early embryonic mid-hindbrain expression. A minimum enhancer

fragment was used to test whether Pax proteins regulate *En-2* expression. Using in vitro electrophoretic mobility shift assays (EMSA), we identified two DNA-binding sites for Pax-2, Pax-5 and Pax-8 proteins. Significantly, the Pax-binding sites are required for initiation and maintenance of the *En-2*-like early embryonic expression in transgenic mice. These results present strong molecular evidence that the *Pax* genes are the direct upstream regulators of *En-2* in a genetic cascade controlling mid-hindbrain development.

## MATERIALS AND METHODS

### DNA constructs

All transgenic constructs were made by inserting mouse and human *En-2* genomic fragments into the *SmaI* site upstream of *phspPT-lacZpA* in a modified pBluescript vector (Kothary et al., 1989; Logan et al., 1993). The *phspPTlacZpA* contains promoter sequences (−664 to +224 relative to the start of transcription) from the mouse *hsp68* gene including the translation start site, fused in frame to the *lacZ* gene from pMC1871, followed by a 240 bp SV40 polyadenylation signal. Construct *CH/lacZ* contains a 1.5 kb *ClaI-HindIII* fragment located 6.3 kb upstream of the translation start site (Logan et al. 1993). The other mouse *En-2* genomic fragments cloned in the *lacZ* reporter constructs were derived from the *CH* fragment. Construct *CX/lacZ* contains a 1.0 kb *ClaI-XbaI* fragment. Construct *XH/lacZ* contains a 500 bp *XbaI-HindIII* fragment. Construct *CA/lacZ* contains a 750 bp *ClaI-AccI* fragment. Construct  $(S_2X)_2/lacZ$  contains two copies of a 350 bp *SstI-XbaI* fragment. Constructs *CS/lacZ* and  $(CS)_2/lacZ$  contain one or two copies of a 460 bp *ClaI-SstI* fragment, respectively. Constructs  $S_1S_2/lacZ$  and  $(S_1S_2)_2/lacZ$  contain one or two copies of a 250 bp *SstI-SstI* fragment, respectively. Construct  $\Delta CX/lacZ$  has the *ClaI-XbaI* fragment that contains a 66 bp internal deletion (bp 221–286 relative to the 5′ end of the *CH* fragment). The deleted sequences include the two Pax-binding sites, BS-I (bp 228–256) and BS-II (bp 257–286). Construct  $**CX/lacZ$  has the *ClaI-XbaI* fragment that contains point mutations in both Pax-binding sites. Constructs *hEH/lacZ* and  $(hEH)_2/lacZ$  contain one or two copies of a 550 bp *EcoRI-HindIII* fragment from the human *EN2* locus, which is located 6.8 kb upstream of the translation start site.

The deletion and site-directed mutagenesis of point mutations in the  $\Delta CX$  and  $**CX$  fragments were introduced by polymerase chain reaction (PCR) (Clackon et al., 1992) using specific primers;  $\Delta CX$ : 5′-CCCAGTGCACAC-3′ and 5′-GAGGCATGCAAGTTGC-3′;  $**CX$ : 5′-GCAGACCCGGGCA-3′ and 5′-CCACTCTTCAGTGTAG-3′. The DNA sequences of the PCR amplified regions were confirmed by sequencing.

### Production of transgenic mice and analysis of transgene expression

Outbred CD-1 mice were used to produce transgenic embryos and mouse lines as described (Hogan et al., 1986). Transgenic embryos and mice were identified by Southern blot analysis of DNA extracted from yolk sacs or tail biopsies, using *lacZ*- or *En-2*-specific DNA probes. Transgenic lines were established by breeding founder and/or generation 1 (G<sub>1</sub>) males with CD-1 females and subsequently interbreeding animals homozygous for the transgene. Transgene expression patterns were analyzed primarily at 10.5 d.p.c. in generation 0 (G<sub>0</sub>) embryos or in transgenic lines from 7.5 d.p.c. to the adult. The day on which a vaginal plug was observed was designated day 0.5 of gestation.

### Whole-mount *lacZ* staining and in situ hybridization

$\beta$ -galactosidase ( $\beta$ -gal) activity was detected in whole-mount embryos by using X-gal (5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside) as described (Logan et al., 1993). The embryos were

stained for periods ranging from 30 minutes to overnight according to the strength of transgene expression. Whole-mount RNA in situ hybridization of embryos was performed essentially as described (Conlon and Rossant, 1992). Single-stranded RNA probes labeled with digoxigenin-UTP were synthesized from linearized template DNA as directed by the manufacturer (Boehringer Mannheim Biochemicals). The *En-2* probe contained a 800 bp *Bgl*III-*Xba*I fragment within the 3' untranslated region; *Pax-2*, *Pax-5* and *Pax-8* probes were as described (Asano and Gruss, 1992).

### Sequence analysis

The mouse (1.0 kb) and human (550 bp) enhancer fragments were restricted into 250-350 bp subfragments and subcloned into the pBlue-script vector (Stratagene, LaJolla, CA). These subclones were sequenced by the dideoxy chain termination method using the Sequenase DNA Sequencing Kit (United States Biochemical, Cleveland, OH). All sequences were analyzed using the University of Wisconsin GCG sequence analysis program package (Devereux et al., 1984).

### DNA-binding assays

EMSA were performed essentially as described previously (Chalepakis et al., 1991). DNA fragments, synthetic oligonucleotides of BS-I and BS-II or mutant variants were 3' end-labeled with [<sup>32</sup>P]dCTP using Klenow polymerase. The different Pax proteins were expressed under the control of *hCMV* promoter/enhancer in transient transfected COS-7 cells as described (Maulbecker and Gruss, 1993) and whole cell extracts were used. The relative binding affinity (K<sub>r</sub>) values were determined by saturation binding experiments as described previously (Emerson et al., 1985).

## RESULTS

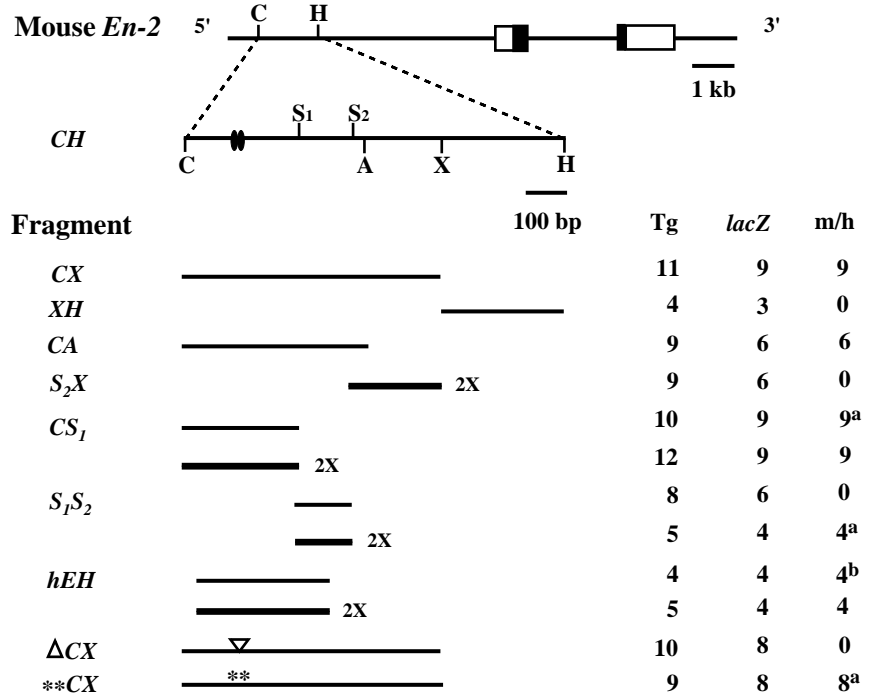
### Multiple regulatory elements are required for early embryonic *En-2*-like mid-hindbrain expression

To localize the *En-2* regulatory elements, we further dissected the 1.5 kb *En-2* genomic fragment (*CH*, Fig. 1) that is sufficient to drive *En-2*-like brain expression at 10.5-12.5 d.p.c. (Logan et al., 1993) using a transient transgenic assay (see Materials and Methods). Initially, two subfragments of *CH*, *CX* (1.0 kb) and *XH* (500 bp), were tested (Fig. 1). Of eleven 10.5 d.p.c. transgenic embryos carrying the *CX/lacZ* construct, nine showed high levels of β-gal activity after 30 minutes of X-gal staining in a broad ring of cells across the mid-hindbrain junction region (Fig. 2A,B). This expression pattern is comparable to that directed by the 1.5 kb *CH* enhancer fragment and to the endogenous expression pattern of *En-2* in the mid-hindbrain. In contrast, three *lacZ*-expressing transgenic embryos carrying *XH/lacZ* did not result in any *En-2*-like transgene expression (data not shown). As observed previously, all *lacZ*-expressing transgenic embryos showed consistent *lacZ* expression in the spinal cord attributable to the mouse *hsp68* promoter (Logan et al., 1993). In addition, variable ectopic expression outside the mid-hindbrain junction and spinal cord was seen in many embryos. These results

localize the *En-2* regulatory elements to within the 1.0 kb *CX* fragment.

Two overlapping subfragments of *CX*, *CA* (750 bp) and *S<sub>2</sub>X* (350 bp) were then analyzed (Fig. 1). Two copies of *S<sub>2</sub>X* failed to direct any *En-2*-like expression (data not shown). One copy of *CA* retained enhancer activity for directing strong *lacZ* expression to the mid-hindbrain junction region (Fig. 2C). Thus, the enhancer elements were further localized to the *CA* region. Interestingly, the *CA* fragment consistently gave additional low level *lacZ* expression throughout the CNS with variable higher levels in regions of the forebrain and spinal cord. This observation suggests that the 3' region of the *CX* fragment, not present in the *CA* fragment, contains elements that repress expression outside the mid-hindbrain junction region.

To further locate the regulatory sequences, two subfragments, *CS<sub>1</sub>* (460 bp) and *S<sub>1</sub>S<sub>2</sub>* (240 bp), were analyzed (Fig. 1). One copy of *CS<sub>1</sub>* gave strong *lacZ* expression which was restricted to only the dorsal part of the mid-hindbrain junction (Fig. 2D,E). Two copies of this subfragment, moreover, conferred strong and broad expression across the mid-



**Fig. 1.** Identification of mouse and human genomic DNA fragments that direct *En-2*-like brain expression. The top diagram shows the mouse *En-2* locus in which exons are represented as boxes with coding regions shaded. The enlarged region below represents the previously identified 1.5 kb *Cla*I-*Hind*III enhancer fragment (*CH*) (Logan et al., 1993). Ovals represent the approximate positions of the Pax-binding sites. The lower panel shows the mouse and human *En-2* genomic DNA fragments present in the *lacZ* reporter constructs tested. *CX*, *XH*, *CA*, *S<sub>2</sub>X*, *CS<sub>1</sub>* and *S<sub>1</sub>S<sub>2</sub>* are subfragments derived from the *CH* fragment. *hEH* is a human genomic fragment derived from the human *EN-2* locus (Logan et al., 1992). *hEH* is aligned relative to the *CH* mouse genomic fragment based on sequence homology. 2X represents two copies of the DNA fragments (indicated as thicker lines). All fragments were attached to the *hsp68* promoter for analysis of *lacZ* expression.  $\Delta$  indicates a 66 bp deletion; \*, point mutations; Tg, number of G<sub>0</sub> 10.5 d.p.c. transgenic embryos analyzed; lacZ, number of transgenics expressing *lacZ*; m/h, number of transgenics expressing *lacZ* in the mid-hindbrain junction region; a, small dorsal patches of expression in the mid-hindbrain junction. b, variability was observed in the size of the mid-hindbrain junction expression domains. Restriction sites: A, *Acc*I; C, *Cla*I; H, *Hind*III; S<sub>1</sub> and S<sub>2</sub>, *Sst*I; X, *Xba*I.

hindbrain junction (Fig. 2F). Two copies, but not one copy, of *S<sub>1</sub>S<sub>2</sub>* directed very weak *lacZ* expression restricted to the dorsal mid-hindbrain junction, which was detected only after 4 hours of X-gal staining (Fig. 2G,H). Since *CA* in one copy gives high level expression across the mid-hindbrain junction whereas neither *CS<sub>1</sub>* nor *S<sub>1</sub>S<sub>2</sub>*, in one copy, confers such an expression pattern, this suggests that enhancer elements located in both subfragments act cooperatively. Furthermore, the broad expression domain appears to reflect a higher level of expression since increasing the copy number of enhancer elements either by oligomerizing the same element or by combining different elements resulted in broader expression.

In summary, the 1.0 kb *CX* subfragment seems to represent a minimal control region that, in one copy, is capable of reconstructing *En-2*-like mid-hindbrain expression. This fragment appears to contain multiple positive and negative regulatory elements that act cooperatively to establish a strong and spatially restricted mid-hindbrain expression pattern in transgenic embryos.

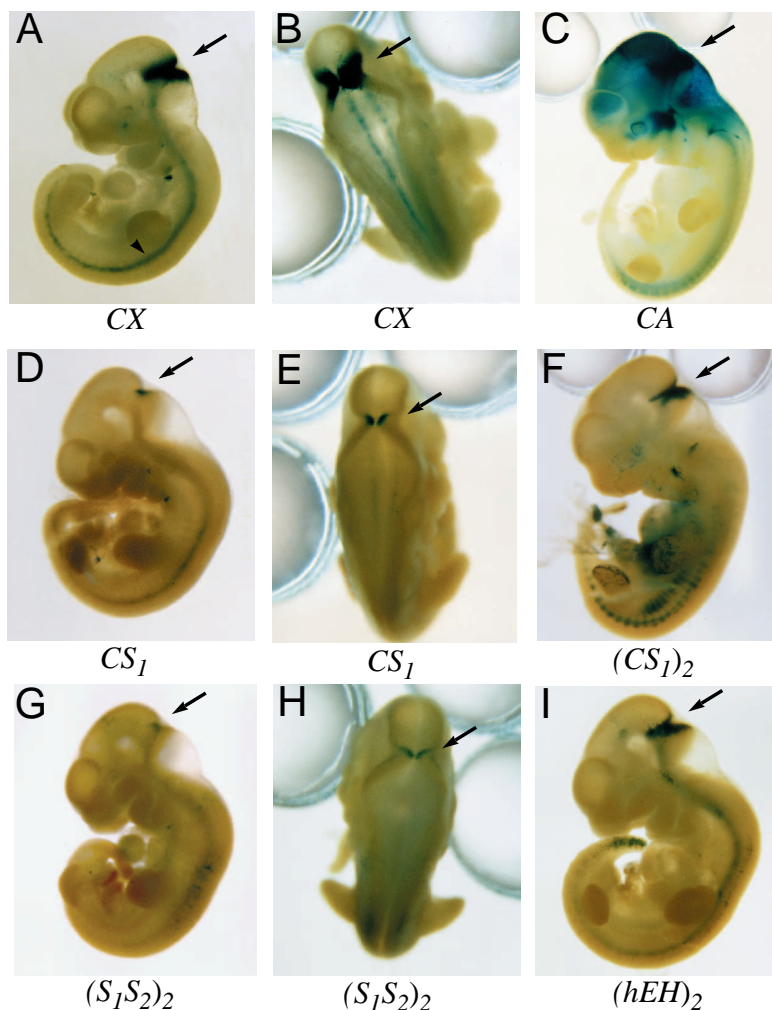
#### The 1.0 kb *CX* fragment functions only as an early embryonic *En-2* mid-hindbrain enhancer

During embryogenesis, the *En-2* gene first shows spatially defined mid-hindbrain expression and then gradually becomes restricted to specific groups of neurons in the midbrain and cerebellum. This expression pattern was replicated by a *lacZ* reporter gene containing 9.5 kb of *En-2* genomic DNA (Logan et al., 1993). We therefore examined the temporal transgene expression profile directed by the 1.0 kb *CX* enhancer fragment at stages from 7.5 d.p.c. to adult in six transgenic lines carrying *CH/lacZ* ( $n=2$ ) and *CX/lacZ* ( $n=4$ ). All the transgenic lines analyzed showed a similar *lacZ* expression profile in the developing brain.  $\beta$ -gal activity was first detected at the 5-somite stage in two lateral patches of the anterior neural plate (Fig. 3A). Expression in this region then expanded to form a ring surrounding the mid-hindbrain junction (Fig. 3B). The mid-hindbrain junction expression continued up to 11.5 d.p.c. and then decreased (data not shown). By 15.5 d.p.c., only a few *lacZ*-expressing cells were seen at the junction of the midbrain and cerebellum (data not shown). No appreciable *lacZ* expression was detected in the adult brain (data not shown). These results demonstrate that the 1.0 kb *CX* region contains sufficient regulatory information for initiating and maintaining early embryonic *En-2* brain expression, but not for later cell-type-specific expression.

#### The early embryonic *En-2* mid-hindbrain regulatory sequences are conserved in the human *EN2* locus

Cross-species homology was used to determine whether the mid-hindbrain regulatory sequences were evolutionarily conserved. A human *EN2* genomic clone containing 7.4 kb of sequences upstream of the coding region was examined by Southern blot analysis using the mouse 1.0 kb *CX* fragment as a probe. A 550 bp human *EN2* fragment located 6.8 kb upstream of

the *EN2* coding region was found to hybridize to the mouse 1.0 kb *CX* fragment (data not shown). Sequence analysis revealed that the human *EN2* fragment had 74% nucleotide identity to the 5' end of the mouse 1.0 kb *CX* fragment over a 364 bp region (Fig. 4). We tested whether the human fragment had enhancer activity in vivo by cloning it into the *lacZ* reporter construct and analyzing *G<sub>0</sub>* transgenic embryos for *lacZ* expression at 10.5 d.p.c. One copy of the human fragment directed strong *lacZ* expression to the mid-hindbrain region, although the size of the expression domain varied from a narrow band to dorsal patches (data not shown). Moreover, two copies of this human fragment gave reproducible broad *En-2*-like expression (Fig. 2I). These results indicate that the essential *En-2* regulatory sequences required for embryonic *En-2* expression have been functionally conserved in mice and humans, and support the localization of these sequences to the 5' region of the mouse 1.0 kb *CX* fragment.



**Fig. 2.** Mid-hindbrain expression patterns of *lacZ* reporter genes directed by mouse and human *En-2* genomic fragments. Lateral (A,C,D,F,G,I) and dorsal (B,E,H) views of whole-mount 10.5 d.p.c. transgenic embryos stained for  $\beta$ -galactosidase activity show *lacZ* expression in the mid-hindbrain junction region (arrows). The different mouse and human *En-2* genomic fragments used to generate the transgenics are indicated below each photograph (see Fig. 1 for descriptions). The *lacZ* expression in the spinal cord (arrow head) is due to the *hsp68* promoter (Logan et al., 1993).

### Pax proteins bind to *En-2* mid-hindbrain regulatory elements

The early mid-hindbrain expression patterns of *En-2* and the *CX/lacZ* transgene were compared to those of *Pax-2*, *Pax-5* and *Pax-8* to determine whether it is feasible that the *Pax* genes regulate *En-2* expression. At 8.5 d.p.c. *CX/lacZ*, *En-2*, *Pax-2* and *Pax-5* shared very similar, if not identical, expression domains in the anterior neural folds (Fig. 3A,C,E,G). At 9.5 d.p.c. the *CX/lacZ* and *Pax-5* expression domains were extensively overlapping in the mid-hindbrain junction region (Fig. 3B,D), whereas the *Pax-2* domain in this region became restricted to a narrow ring within the *En-2* expression domain (Fig. 3F). *Pax-8* expression was first detected at 9.0 d.p.c. at the mid-hindbrain junction region (data not shown) and soon expanded caudally to encompass the *En-2* hindbrain expression domain (Fig. 3H).

To directly address whether Pax proteins regulate *En-2* expression, we examined representative members of each of the four Pax protein subclasses, Pax-1, Pax-3, Pax-5 and Pax-6 (Walther et al., 1991), by EMSA for their ability to bind DNA subfragments derived from the 1.0 kb *CX* fragment. Pax-1 and Pax-5 were found to form specific protein-DNA complexes with sequences located on a 102 bp *AluI-StuI* subfragment. Further deletion analysis showed that two independent Pax-binding sites were present in this region (data not shown).

Two putative Pax-binding sequences, BS-I and BS-II, were defined (Fig. 5A) by comparing the DNA sequence of the *AluI-StuI* fragment with reported Pax-binding sites and their degenerate consensus sequences (Zannini et al., 1992; Czerny et al., 1993; Epstein et al., 1994). Each binding site was shown to bind, with high affinity, to Pax-1 and members of the Pax-5 subclass (Fig. 5B). The  $K_{rs}$  of BS-I and BS-II to Pax-8 were  $3.33 \times 10^5$  and  $0.32 \times 10^5$ , respectively, which are comparable to the values of other Pax-binding sites (Czerny et al., 1993). Furthermore, point mutations (Fig. 5A) in each binding site abolished binding activity to Pax-8 in EMSA (Fig. 5C). The 550 bp human fragment was also found to bind to the proteins of the Pax-5 subfamily (data not shown), and one putative Pax-binding sequence similar to BS-I was identified based on sequence analysis.

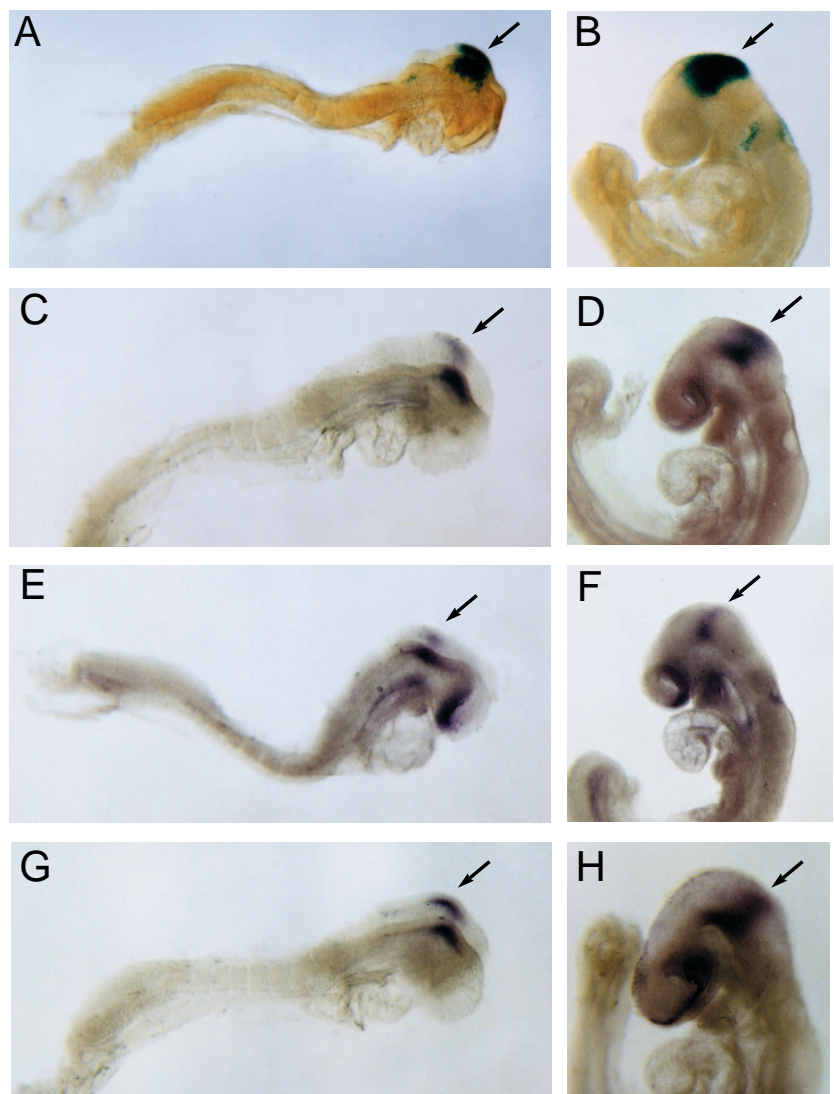
### BS-I and BS-II are required for early embryonic *En-2* mid-hindbrain expression

The *in vivo* role of the Pax-binding sites was tested in 10.5 d.p.c. transgenic embryos by mutagenesis of these sites within the *CX/lacZ* construct. A 66 bp internal deletion that removed both BS-I and BS-II from the *CX* fragment ( $\Delta CX$ , Fig. 1) led to a complete loss of *En-2*-like *lacZ* expression in the mid-hindbrain junction region at 10.5 d.p.c. in all *lacZ*-expressing  $G_0$  transgenic embryos analyzed (Fig. 6D). Point mutations in both BS-I and BS-II (Fig. 5A) together within *CX* (\*\**CX*; Fig. 1) consistently resulted in expression of only weak small dorsal patches (8/8) (Fig. 6E). In contrast, point mutations in either binding site

alone resulted in variable mid-hindbrain expression ranging from only small patches of dorsal expression (4/13 transgenics) to normal expression (5/13 transgenics) (data not shown). These results suggest that both Pax-binding sites are critical for *En-2* expression. Four transgenic lines carrying the  $\Delta CX/lacZ$  construct were analyzed to examine the temporal aspect of the requirement for these Pax-binding sites. No *En-2*-like expression was detected in any of the lines from 8.5 (Fig. 6B) to 12.5 d.p.c. (data not shown). These results demonstrate that the Pax-binding sites are required for establishing and maintaining the *En-2*-like transgene expression in the early embryonic mouse brain.

### DISCUSSION

We have studied regulation of *En-2* expression in transgenic mice



**Fig. 3.** Comparison of the temporal and spatial patterns of expression for the *CX/lacZ* transgene and the *En-2*, *Pax-2*, *Pax-5* and *Pax-8* genes. (A,B) Lateral views of whole-mount 8.5 d.p.c. (A) and 9.5 d.p.c. (B) *CX/lacZ* transgenic embryos stained for  $\beta$ -galactosidase activity. (C-H) Lateral views of whole-mount 8.5 d.p.c. (C,E,G) and 9.5 d.p.c. (D,F,H) embryos hybridized to *Pax-2* (E,F), *Pax-5* (C,D), *En-2* (G) and *Pax-8* (H) RNA probes. Arrows indicate mid-hind brain expression.

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244 ACCGTGCCACCC...CGGAGGGCCACTTCTTCACTGAGCCATGACAAGAGGC 292
159 ACCGTGGGGCCCGGGGAGAGCCGCCACCCATTTGCTGAGCCCGAC...ACAGGC 207
293 TTTGAAGTTCGCTGCTCTCTGCTAGCCCTCCCTCCGCAATCCCGCTCTGGCGT 342
208 TTTGAAGTTCGCTGAGCTGGCTCAGCC...CTTCCCGCTCCGSCCTTGCGCA 256
343 GCACAGCAAGCACCCGAGTCCGCTCAGTGTCCCAACTTTTGTGGTAA 392
257 GCGC...GGTCCCGCAGAGTCCAGGCCGT.GCCTCCGTTTCGACGCTCA 301
393 GCACCTTATAGCAGCCGCTCATTTGACCCACGTCCTTGGCCGAGCACAGCC 442
302 GACCTTATTTGCGGGGCTCATTTGACCCACGTCCTTGGGGGATTCGACGCC 351
443 CTCGTGATCTGTGAGCTGGGAGAGGCTCAG...CGTTCATTCACAAATC...CG 489
352 C...CTGATCTGTGAGCTGCAAAAGAGCTCCGAGTTTCATTCACAAATTCGG 400
490 CGCCTACCAGTCCCTCCATG...GGTGGGGCAGGGGAG... 525
401 CCCCCAGCCGTCCTCCACGCCACCCGAGCCACCTCCCGACCCGAGAGCC 450
526 ...CGGATCTGGG...GAGGGGAGGTCATTTCTTGTGCGACCTTCGT 570
451 CGGGGCTTTGGGCGTGCGGAGGAGGCCCGCTTCTGCTCCCTTCAC 500
571 TTCACGACGGCCTTCAGGGCAGAGATTTGTAGCGAGCG 610
501 CTC...CAGGCC...GGGAGGACTTTGGGGGAGGGG 532

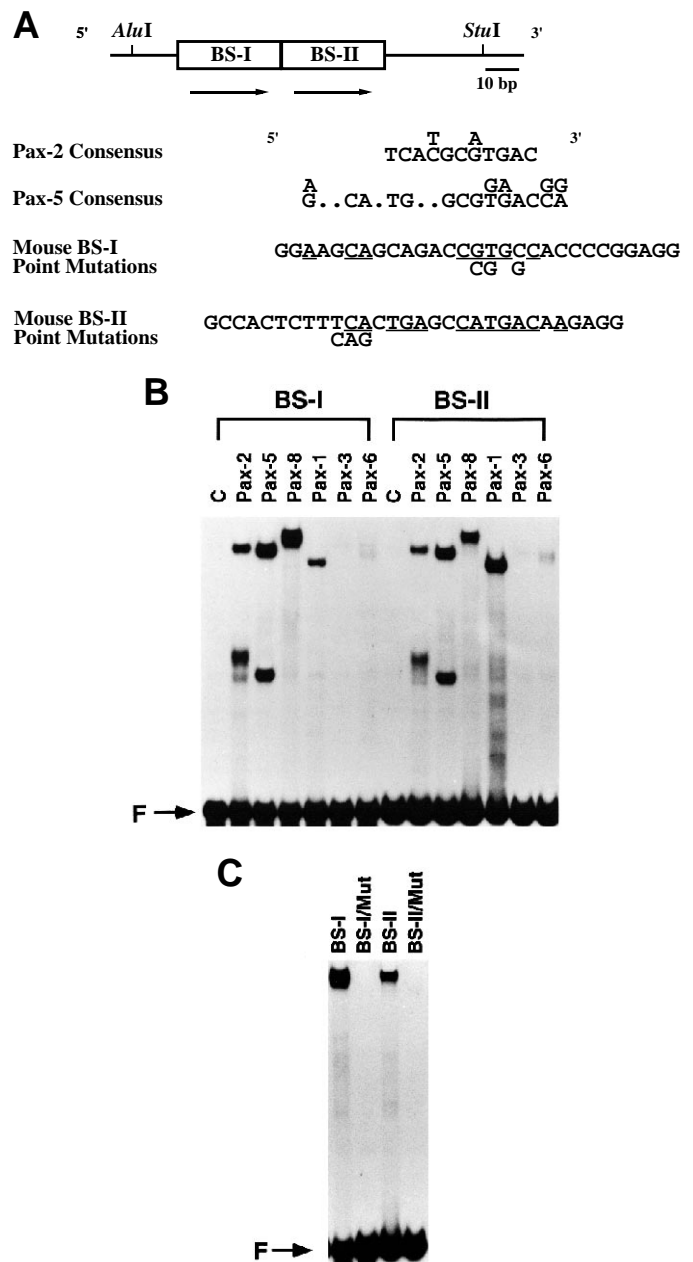
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**Fig. 4.** Sequence conservation of the mouse and human *En-2* DNA enhancer fragments. Sequence comparison of the mouse (upper line, *CX*, bp 244–610) and human enhancer fragments (lower line, *hEH*, bp 159–532) show 74% identity between the two sequences. Alignment was performed using BestFit from the GCG package (Devereux et al., 1984, gap weight: 5.0, length weight: 0.3, average match: 1.0, average mismatch: –0.9). GenBank accession no. U41757.

in order to identify genetic events that establish regional diversity in the developing brain. Our results demonstrate that the dynamic *En-2* expression pattern involves at least two phases of regulation. The initiation and maintenance of the early embryonic expression in the mid-hindbrain junction region depends on *cis*-acting DNA regulatory elements that are located within a minimum region of 1.0 kb, which have both cooperative positive and negative effects on transcription. These DNA sequences have been conserved in humans. Additional and/or different regulatory elements must be required to direct later *En-2* brain expression. This suggests that multiple *trans*-acting protein factors participate in the regulation of *En-2* expression throughout development. By characterizing the 1.0 kb regulatory sequences both *in vitro* and in transgenic mice, we present strong molecular evidence that one set of critical factors are the Pax proteins, Pax-2, Pax-5 and Pax-8. These proteins appear to be direct upstream activators of early *En-2* mid-hindbrain expression.

### Multiple DNA regulatory elements are required to specify *En-2* expression in the developing brain

Our transgenic analysis has defined a 1.0 kb *CX* fragment as a minimum control region that is capable of reconstructing *En-2*-like transgene expression in the mid-hindbrain junction region from 8.5 to 11.5 d.p.c. This minimum control region consists of two enhancer fragments, *CS*<sub>1</sub> and *S*<sub>1</sub>*S*<sub>2</sub>, and one repressor fragment, *AX*. The two positive regulatory fragments appear to act cooperatively to specify an integrated transgene expression domain. Each enhancer fragment, *CS*<sub>1</sub> in one copy, or *S*<sub>1</sub>*S*<sub>2</sub> in two copies, is capable of directing specific mid-hindbrain expression but only to small dorsal patches of cells at the mid-hindbrain junction. However, the two enhancer fragments together, or *CS*<sub>1</sub> in two copies, act in a synergistic manner to produce a broad ring of expression across the mid-hindbrain junction. This suggests that *En-2* mid-hindbrain expression in normal embryos depends on cooperative actions between multiple protein factors interacting with at least two DNA regulatory elements. Biochemical



**Fig. 5.** Binding of Pax proteins to BS-I and BS-II. (A) Top diagram shows the positions and orientation (arrows) of BS-I and BS-II in the *En-2* locus. Lower panel shows DNA sequence alignments of BS-I and BS-II with known Pax-2 and Pax-5 recognition consensus sequences (Czerny et al., 1993; Epstein et al., 1994). The base matches of both BS-I and BS-II with Pax-2 and Pax-5 recognition consensus sequences are underlined. Point mutations introduced in BS-I and BS-II are shown below the wild type sequences. (B) Binding of Pax proteins to BS-I and BS-II in EMSA. The Pax proteins analyzed are indicated above each lane. *C*, refers to control extracts from cells transfected with the expression vector alone. The migrating positions for full-length protein-DNA complexes are indicated (arrow). The faster migrating protein-DNA complexes in the Pax-2 and Pax-5 lanes likely contain degradation products of the Pax proteins. *F* refers to free DNA. (C) Point mutations in BS-I and BS-II abolish their binding to Pax-8 in EMSA.

studies identified two Pax-binding sites in the *CS*<sub>1</sub> fragment that are required for *En-2*-like transgene expression, whereas no Pax-

binding sites were found in the *S<sub>1</sub>S<sub>2</sub>* enhancer fragment. Furthermore, no apparent DNA sequence similarity was found between the two enhancer fragments, indicating that they interact with different trans-acting protein factors. It will be interesting to further locate other DNA regulatory sequences and to identify the protein factors that interact with them.

Sequences located within the 3' half of the 1.0 kb enhancer appear to contain DNA-binding sites for a repressor(s), since deletion of a 3' 350 bp AX fragment resulted in a low level of transgene expression throughout the CNS. Variable higher levels of expression was also observed in some regions in the CNS. In normal embryos, *En-2* expression is not uniform; it is strongest at the mid-hindbrain junction and gradually decreases rostrally and caudally. Chick/quail transplantation experiments have suggested that the rostral gradient of expression is mediated by an inhibitory activity emanating from the mesencephalic-diencephalic constriction (reviewed in Alvarado-Mallart, 1993). Since the additional transgene expression observed here was throughout the CNS, it is unlikely that the repressor elements located within the AX fragment are responsible for setting up the normal rostrocaudal expression gradients. Rather, they may play a role in down regulating *En-2* expression outside the mid-hindbrain junction region.

Analysis of the developmental expression profile of the 1.0 kb enhancer demonstrated that the transgene contained sufficient regulatory sequences for initiating *En-2*-like expression, however, the expression was not maintained beyond 11.5 d.p.c. Our previous transgenic analysis showed that a 9.5 kb genomic fragment is capable of conferring *En-2*-like expression throughout development and in the adult (Logan et al., 1993). Based on these two results, regulation of *En-2* brain expression can be divided into at least two phases: the initiation and early regionally restricted expression and later cell-type-specific expression, which require different *cis*-acting DNA regulatory elements. The two phases of *En-2* regulation may reflect distinct genetic programs that control different stages of development of this region; the early phase corresponding to regional specification and the late phase to neural differentiation and maturation.

### Role of Pax-2, Pax-5 and Pax-8 in regulating early *En-2* mid-hindbrain expression

The *Pax* genes can be grouped into four subfamilies (Gruss and Walther 1992; Walther et al., 1991). The paralogues of each subfamily share similar genomic organization and protein structure, and a high degree of sequence identity in the paired domain. All but *Pax-1* exhibit temporally and spatially restricted expression patterns in the developing CNS, consistent with roles in regional specification. The developmental importance of *Pax* genes in the CNS, as well as in other systems, has been emphasized by recent studies of mouse mutants and inherited human diseases (reviewed in Gruss and Walther, 1992; Chalepakis et al., 1993; Keller et al., 1994; Sanyanusin et al., 1995).

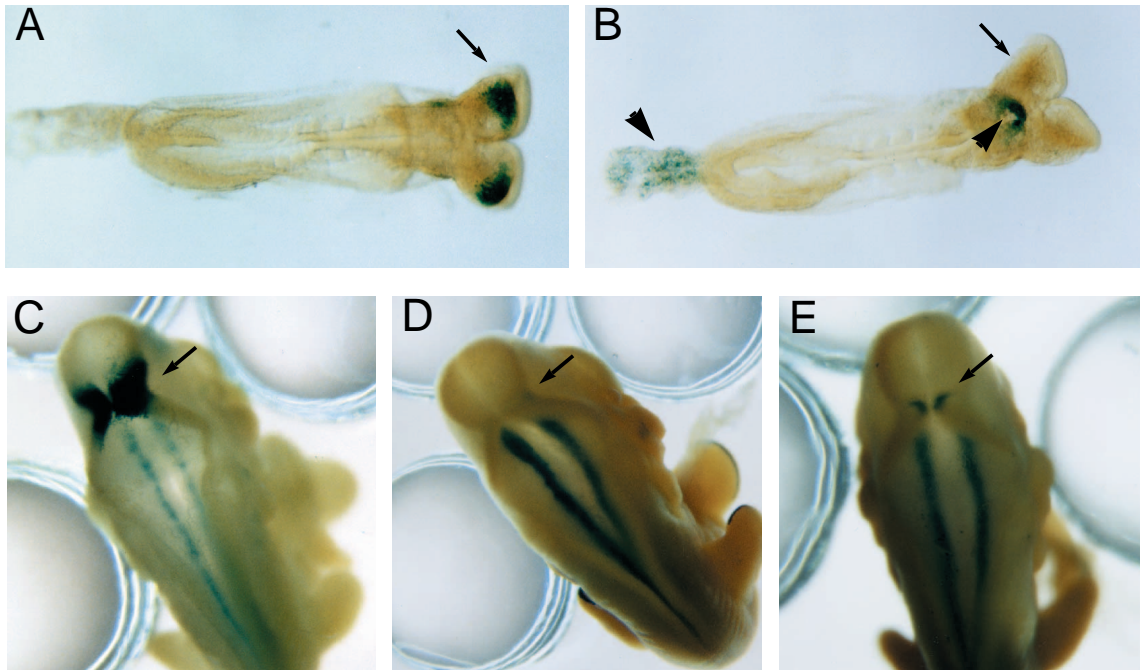
Pax proteins act as transcriptional regulators and their activity has been shown to depend on a specific DNA-binding activity of the paired domain (Treisman et al., 1991). All of the previously identified paired domain recognition sequences, unlike other types of DNA-binding sites, are unusually long (over 20 bp) and seemingly divergent (Czerny et al., 1993). Pax-binding sites exhibit a bipartite structure, with each half-site being represented by a 5' and 3' consensus motif. Using EMSA, we identified two DNA-binding sites, BS-I and BS-II,

within the 1.0 kb *En-2* enhancer fragment for the Pax-5 subfamily proteins and Pax-1. Although BS-I and BS-II share limited sequence similarity to each other, they both show significant base matches with the deduced Pax-binding consensus sequences in both the 5' and 3' half-sites.

Members of the Pax-5 subfamily share over 90% amino acid identity in their paired domains (Walther et al., 1991). Consistent with this, all three Pax proteins had similar affinities for BS-I and BS-II. At present, little is known about the molecular basis of the DNA-binding specificity for different Pax subfamilies. A recent study of the Pax-6 protein has identified three amino acids in the paired domain that are responsible for discriminating the DNA-binding sites of Pax-6 versus Pax-5 (Czerny and Busslinger, 1995). Interestingly, Pax-1 and Pax-5 are identical at these three amino acids (Chalepakis et al., 1991). This may explain why most Pax-5 target sequences, including BS-I and BS-II, are also recognized by Pax-1.

The binding specificity of Pax-2, Pax-5 and Pax-8 for BS-I and BS-II strikingly correlates with the Pax subfamily gene expression patterns, since only the *Pax-5* subfamily shows early coexpression with *En* in the developing mid-hindbrain. Introducing point mutations in the conserved 5' or 3' core motifs of BS-I and BS-II abolished the binding to these Pax proteins *in vitro*. We demonstrated the functional significance of BS-I and BS-II *in vivo* using transgenic mice. Deletion of both DNA-binding sites from the 1.0 kb enhancer completely abolished transgene expression in the mid-hindbrain from 8.5 to 12.5 d.p.c., indicating that the Pax-binding sites are essential both for initiation and maintenance of the early phase of *En-2* expression in normal embryos. Introducing point mutations in both DNA-binding sites significantly reduced transcription of the transgene; only weak *lacZ*-expressing cells were detected in small dorsal patches at the mid-hindbrain junction. The apparent difference between the *in vitro* and transgenic results with point mutations may suggest that *in vivo* the Pax protein-DNA complexes are stabilized by other protein-DNA interactions.

Comparison of the gene expression pattern of *En-2* to that of *Pax-2*, *Pax-5* and *Pax-8* raises questions about the complexity of regulation of *En-2* in the brain if these Pax paralogues have similar functions, as has been shown for the *En* (Hanks et al., 1995) and *Hox* (Condie and Capecchi, 1994) genes. For example, *Pax-2* expression in the mid-hindbrain is initiated at least half a day earlier than that of *En-2*, and *Pax-2* is also expressed in the developing optic cup and otic vesicles where no *En-2* expression has been detected (Davis et al., 1988; Püschel et al., 1992; Rowitch and McMahon, 1995). This suggests that activation of *En-2* by the Pax proteins may require co-factors that are only expressed in the mid-hindbrain junction region from the 5-somite stage, or alternatively that there are inhibitors that repress *En-2* expression at inappropriate times or places during development. Our transgenic analysis is consistent with both possibilities since it indicates that the broad domain of *En-2*-like expression relies on synergistic activation utilizing the Pax-binding site-containing enhancer fragment and adjacent regulatory sequences as well as repression of expression outside this region. Finally, since *Pax-2* is expressed prior to and overlapping with *En-1* and *Wnt-1*, *Pax-2* may have a role in initiating *En-1* and *Wnt-1* expression in the developing brain (Rowitch and McMahon, 1995). Thus, Pax-2, Pax-5 and Pax-8 proteins may have unique as well as overlapping regulatory roles in brain development.



**Fig. 6.** The Pax-binding sites, BS-I and BS-II, are required for transgene expression in the mid-hindbrain junction region. (A-E) Dorsal views of whole-mount transgenic embryos stained for  $\beta$ -galactosidase activity. (A,C) Transgenic embryos carrying reporter construct *CX/lacZ* show an *En-2*-like mid-hindbrain expression pattern at 8.5 d.p.c. (A) and 10.5 d.p.c. (C) (arrows). (B,D) Transgenic embryos carrying reporter construct  $\Delta$ *CX/lacZ* which contains a deletion of BS-I and BS-II show no *En-2*-like *lacZ* expression in the anterior neural folds at 8.5 d.p.c. (B, arrow) nor in the mid-hindbrain junction region at 10.5 d.p.c. (D, arrow). (E) A 10.5 d.p.c. transgenic embryo carrying reporter construct *\*\*CX/lacZ* which contains point mutations in both BS-I and BS-II. The normal mid-hindbrain *lacZ* expression pattern is lost, *lacZ* staining is only detected in a very small dorsal patch in the mid-hindbrain junction region (arrow). The *lacZ* staining seen outside the mid-hindbrain junction region and spinal cord represents ectopic expression (arrowheads).

The target-specificity could be accomplished by cooperative interactions of the Pax proteins with different co-factors.

### Evolutionary conservation of a *Pax-En* genetic pathway

Early in *Drosophila* development, *en* and *wg* are expressed in adjacent stripes that mark the borders between parasegments. This expression is required for establishment and maintenance of segmentation of the body (reviewed in Ingham and Martinez-Arias, 1992; Hooper and Scott, 1992). The initial *en* and *wg* stripes are set up by overlapping expression of an array of pair-rule genes, both activators and repressors (DiNardo and O'Farrell, 1987; DiNardo et al., 1988; Heemskerk et al., 1991; Morrissey et al., 1991). The paired box-containing gene, *paired*, is a pair-rule gene that acts as a positive regulator of *en* and *wg*. Following activation, the expression of *en* and *wg* expression in adjacent cells becomes mutually dependent, mediated by intercellular and intracellular signaling pathways (reviewed in Perimon, 1994). This *en-wg* interdependent regulation is only transient as *en* soon becomes autoregulated (Heemskerk et al., 1991).

Based on expression patterns, some of the genetic pathways involving conserved segmentation genes may have evolved to control divergent developmental processes in vertebrates. In this study, using *in vitro* protein-DNA binding assays and *in vivo* expression analysis in transgenic mice, we have provided strong molecular evidence that Pax-2, Pax-5 and Pax-8 proteins are directly involved in initiation and maintenance of early *En-2* brain expression. We also showed that the *En-2* mid-hindbrain regulatory sequences are structurally and functionally

conserved in humans. A previous study in zebrafish has suggested that *pax[zf-b]*, a member of the Pax-5 subclass, was necessary for normal *eng-2* brain expression in fish (Krauss et al., 1992). Taken together, the studies in *Drosophila*, zebrafish and mouse indicate that the *Pax-En* genetic pathway has been conserved during evolution. Our identification of *cis*-regulating Pax-binding sites for *En-2* expression demonstrates the biochemical nature of this interaction in mammals.

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