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MOLECULAR AND GENETIC ANALYSIS OF DNA REGULATORY SEQUENCES CONTROLLING MOUSE Engrailed-2 EMBRYONIC BRAIN EXPRESSION

by

Dongli Song

A thesis submitted in conformity with the requirements for the degree of
Doctor of Philosophy
Graduate Department of Molecular and Medical Genetics
University of Toronto

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0-612-41509-0
Molecular and genetic analysis of DNA regulatory sequences controlling mouse Engrailed-2 embryonic brain expression.
by Dongli Song
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ABSTRACT

The vertebrate Engrailed (En) genes, homologues of the Drosophila segment polarity gene engrailed (en), encode a class of highly conserved homeodomain-containing transcription factors. Two murine En genes, En-1 and En-2, have been identified and characterized. The En genes display spatially and temporally restricted expression in the developing midbrain and cerebellum that is essential for development of these brain regions. This thesis has been conducted to elucidate the molecular and genetic mechanisms controlling En-2 embryonic brain expression.

Minimal En-2 DNA regulatory regions were identified by analyzing lacZ reporter genes in transgenic mice. A 1.0 kb enhancer fragment, which contains multiple positive and negative regulatory elements, was shown to be sufficient to direct En-2-like lacZ expression from embryonic day (E) 8.5 to 11.5. Cross species sequence comparison and transgenic analyses revealed that the En-2 mid-hindbrain regulatory sequences have been conserved in mice and humans.

The vertebrate Pax genes, homologues of the Drosophila paired box-containing gene paired (prd), encode a family of transcription factors. Nine murine Pax genes have been identified. Pax-2/5/8 show overlapping expression with En in the developing midbrain and hindbrain. Two DNA-binding sites specific for Pax-2/5/8 proteins were identified in the 1.0 kb En-2 enhancer fragment. Mutation of the two Pax-binding sites in the 1.0 kb-En-2 enhancer-lacZ reporter constructs abolished En-2-like mid-hindbrain
expression in transgenic mice. A targeted deletion of the two Pax-binding sites in the En-2 locus was generated using homologous recombination in mouse embryonic stem cells. Embryos homozygous for this mutation were found to have a significant reduction in En-2 expression in the mid-hindbrain region at E8.5 but not later stages, showing that the two Pax-binding sites are required for proper initiation of the endogenous En-2 brain expression. Results from these studies provide direct molecular and genetic evidence that the Pax genes are upstream regulators of En-2 in a genetic cascade controlling early development of the midbrain and cerebellum.
ACKNOWLEDGMENTS

First, I express my gratitude to my supervisor, Dr. Alexandra Joyner, for her support, understanding and guidance throughout the course of this work. Alex, I appreciate your wise career counsel and numerous efforts to improve my writing and oral presentations.

I express my thanks to the members of my advisory committee, Drs. Jack Greenblatt, Manuel Buchwald and the late Dr. Martin Breitman, for their guidance in the project and their editorial help with this Ph.D. thesis. I would also like to express my thanks to Dr. Janet Rossant for her advice and expertise throughout my studies.

I thank all members of the Joyner lab, both in Toronto and in New York, for their stimulating discussions and technical help. Their friendship and good sense of humor have made those hard-working hours enjoyable. In particular, I would like to thank Cairine Logan and Kathy Millen for their invaluable encouragement and help.

I thank all my family members and my friends for their understanding, encouragement and support. I dedicate this thesis to my father Yating Song and my mother Shuqin Liu. Your love and faith have allowed me to reach my goal.
ATTRIBUTION OF DATA

I am responsible for the work reported in this thesis with the exception of the following: The \textit{in vitro} biochemical analysis of Pax-binding sites was conducted by G. Chalepakis. The chimera production in the gene targeting experiment was done by A. Auerbach, W. Auerbach and K. Losos.
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CHAPTER 1: GENERAL INTRODUCTION
GENERAL INTRODUCTION

Regionalization of the neural tube is a critical step during early development of the central nervous system (CNS) in vertebrates. Understanding the molecular and genetic mechanisms controlling this process presents a major challenge for developmental neural biology. Over the past decade, many developmental control genes have been identified that display temporally and spatially restricted expression that marks different regions of the developing CNS. Recent analyses of the phenotypes of mouse and zebrafish mutants with mutations in such genes, together with classic transplantation experiments in avian embryos, have provided direct evidence that such restricted gene expression is essential for early patterning of the CNS. In addition, a combination of in vivo transgenic mouse studies and in vitro biochemical analyses has been employed to identify DNA regulatory sequences and protein transcription factors that control restricted gene expression patterns. Taken together, these various studies should establish the epistatic interactions between developmental control genes, revealing the genetic pathways governing regionalization of the CNS.

THESIS OUTLINE

The research presented in this thesis focuses on the transcriptional regulation of the mouse Engrailed-2 (En-2) gene during early brain development as an approach to understand the molecular and genetic mechanisms underlying early patterning of the mid-hindbrain junction region (mesencephalon and metencephalon). In the first set of experiments (chapter 2) I combined in vivo transgenic mouse analyses and in vitro biochemical assays to identify DNA sequences directing early En-2 mid-hindbrain expression. In particular, the role of Pax proteins in regulating En-2 expression was examined which led to identification of two Pax-binding sites in the En-2 locus. In the second set of experiments (chapter 3), I analyzed the requirements of the two Pax-binding sites for directing endogenous En-2 expression. A targeted deletion of the two Pax-binding sites in the En-2 locus was generated using homologous recombination in mouse embryonic stem cells (ES cells). Embryos
homozygous for this mutation were found to have a significant reduction in \textit{En-2} expression in the mid-hindbrain region at E8.5 but not later stages, showing that the two Pax-binding sites are required for proper initiation of the endogenous \textit{En-2} brain expression. These studies provided the first molecular evidence that the \textit{Pax} genes are directly upstream of \textit{En-2} in the genetic pathway controlling early mid-hindbrain development. The final chapter (4) summarizes the studies in this thesis and describes possible future research. To provide a general background for these studies, I review in the first chapter: 1) the \textit{En} genes and their roles in the midbrain and cerebellum development, 2) the \textit{Pax} genes and their roles in the midbrain and cerebellum development, 3) early development of the midbrain and cerebellum, and 4) genetic control of early midbrain and cerebellum development.

\textbf{THE \textit{En} GENES AND THEIR ROLES IN MIDBRAIN AND CEREBELLUM DEVELOPMENT}

The \textit{En} genes encode a class of highly conserved homeodomain (HD) containing transcription factors (reviewed in Joyner and Hanks, 1991) related to the \textit{Drosophila} segment polarity gene, \textit{enlarged} (\textit{en}) (Fjose et al., 1985; Poole et al., 1985; Kuner et al., 1985). One or more \textit{En} genes have been isolated in a wide range of species including human, by virtue of their homology to \textit{Drosophila} \textit{en}. Comparative amino acid sequence analysis of the invertebrate and vertebrate \textit{En} proteins has revealed the conservation of five domains, designated EH1-5. EH 4 is a DNA-binding HD (Logan et al., 1992). Outside of the five conserved regions, \textit{En} proteins share little amino acid identity across the phyla. In mice, two \textit{En} genes, \textit{En-1} and \textit{En-2}, have been identified and characterized (Joyner et al., 1985; Logan et al., 1992). Overall, the mouse \textit{En} proteins share 55\% amino acid identity with each other and 35\% identity with the \textit{Drosophila} \textit{En} protein. The mouse \textit{En-1} and \textit{En-2} genes map to chromosomes 1 and 5 respectively, and neither gene was found to be allelic to any pre-existing mouse mutants (Joyner and Martin, 1987).
Biochemical analyses have shown that the *Drosophila* en protein can repress activated, but not basal, transcription in both cultured cells and embryos (Jaynes and O'Farrell, 1991; Smith and Jaynes, 1996). Functional dissection of the *Drosophila* en protein has showed that its N-terminal half can confer repression activity to heterologous DNA-binding domains in cultured cells (Jaynes and O'Farrell, 1991). Three repression domains have been identified, two of which are in the evolutionarily conserved regions EH1 and EH5 (Han and Manley, 1993; Smith and Jaynes, 1996). Unlike the *Drosophila* en protein, both repressor and transactivation activities have been demonstrated for the mouse En proteins in cultured cells (Hanks et al., in preparation).

**Dynamic Expression Patterns of the En Genes During CNS Development**

Comparative expression studies have shown that in vertebrates, from zebrafish to mice, the En genes are expressed in a highly specific and similar pattern, indicating a conservation of function (reviewed in Joyner and Hanks, 1991; Davis et al., 1991). In mice, the two En genes show overlapping expression domains in the developing brain (for En-2 see Fig. 1) and divergent expression patterns in other tissues (Davis et al., 1988; Davis and Joyner, 1988; Davis et al., 1991; Millen et al., 1995). In the CNS, expression of the En genes is initiated in patches of cells within the anterior neural plate in the region of the presumptive mid-hindbrain region. *En-1* expression is first detected at the 1 somite stage (E8.0, the day on which a vaginal plug is observed is designated embryonic day 0.5) whereas *En-2* is activated at the 5 somite stage (E8.5) (Fig. 1A). At E9.0, upon neural tube closure, the En expression domain becomes a broad ring of cells spanning the mid-hindbrain region. Between E9.5 (Fig. 1B) and E10.5 (Fig. 1C), both genes are continuously expressed in this region, but the *En-2* expression domain is broader than that of *En-1* both rostrally and caudally. From at least E15.5 onwards, the expression patterns of the two genes in the cerebellum show some divergence but remain similar in the midbrain (Davis and Joyner, 1988; Millen et al., 1995; Wolf and Joyner, unpublished). In the E15.5-E17.5
Figure 1. *En-2* expression in the developing midbrain and hindbrain. *En-2* initiates at E8.5 in two patches of cells in the anterior neuroepithelium marking the presumptive mid-hindbrain region (A). At E9.5 (B) and E10.5 (C) *En-2* is expressed in a broad band of cells spanning the mid-hindbrain region. Expression continues in the developing midbrain and cerebellum at E15.5 (D) and E17.5 (E). (C is reproduced from Davis and Joyner, 1988; and D from Millen et al., 1995).
cerebellar anlage, En-1 expression is limited to a single mid-sagittal stripe in the medial horizontal plane, while En-2 expression becomes restricted to the same mid-sagittal stripe and four bilateral symmetrical stripes (Fig.1E). From late embryonic to early postnatal development, En expression patterns shift continually producing dynamic changes. En-1 cerebellar expression is gradually downregulated and En-2 expression becomes restricted to a broad mid-sagittal domain. By postnatal day 8 (P8), the adult En cerebellum expression patterns are established; En-1 is no longer expressed and En-2 is expressed in all granule cells and cells in the molecular layer. In addition, expressions of both genes can be detected in a number of motor nuclei in the pons region and in cells within the midbrain. In the developing CNS, En-1 is also expressed in two ventrolateral stripes of interneurons that extend from the myelencephalon down the spinal cord (Davis and Joyner, 1988).

Roles of the En Genes in Midbrain and Cerebellum Development

To gain an understanding of the developmental roles of the En proteins, mouse mutants for En-1 and En-2 were generated by homologous recombination in ES cells. Mice homozygous for a targeted En-1 homeodomain deletion (En-1hd) (Wurst et al., 1994) die at birth and display a large deletion of midbrain and hindbrain structures, including most of the colliculi and cerebellum. A deletion at the mid-hindbrain region is observed as early as E9.0. The En-2 expression domain in these mutant embryos is significantly reduced, with only weak expression in the dorsal part of the isthmus. This indicates that the deletion of the mid- and hindbrain structures results from the failure of early specification and/or expansion of the presumptive mid-hindbrain region. In contrast, a relatively mild phenotype is observed in mice homozygous for two En-2 targeted alleles, a homeodomain deletion (En-2hd) and an N-terminal deletion (En-2ntd) (Joyner et al., 1991; Millen et al., 1994). Both mutants are viable and fertile but show a distinct cerebellar phenotype. The first morphological sign of abnormal brain development is seen at E15.5 as a delay in fusion of the cerebellum at the midline and a smaller tectum. During the first postnatal week, there is also a clear delay in
the foliation process and abnormal folding. Adult En-2 homozygous mutant mice have a 30% reduction in cerebellar size and distinct abnormalities in patterning of the cerebellum folds with the most severe defects being in the posterior region. Consistent with this, these animals show abnormal distribution of spinocerebellar mossy fiber terminals in the posterior cerebellum (Vogel et al., 1996). However, the cerebellar cytoarchitecture in these animals is essentially normal. It remains to be determined whether these animals have any alterations in midbrain cytoarchitecture and axon projections.

Unlike most naturally occurring cerebellar mutants that have severe motor problems, En-2 homozygous mutant mice do not display any obvious behavioral or motor deficits. However, in certain behavioral tests, they are not able to master the task as quickly or to the same degree as wild type mice (Gerlai et al., 1996). Interestingly, the performance of heterozygous mice, although morphologically normal, ranges between that of homozygous mutants and that of wild type mice. The pathophysiology underlying the functional deficit in En-2 mutants remains to be determined.

The contrasting brain phenotypes of En-1 and En-2 mutant alleles have been shown to primarily reflect differences in their temporal expression rather than their biochemical activities. This was demonstrated by an elegant experiment in which En-1 coding sequences were replaced with En-2 sequences (En-1\textsuperscript{2ki}) through a gene targeting knock in approach (Hanks et al., 1995). In the resulting En-1\textsuperscript{2ki} animal, the homozygous En-1 brain phenotype appears to be rescued. This indicates that during normal embryogenesis En-2 may also play a role in early mid-hindbrain specification, and that the relatively late manifestation of an En-2 brain phenotype is probably due to the compensation of En-2 by En-1 during early brain development (E8.5-E15.5) when the two genes have significantly overlapping expression. Further support of this view came from the observation that En-1 and En-2 double homozygous mutant embryos and new borns have a larger mid-hindbrain deletion than En-1 homozygous animals (Wurst et al., in preparation.). The functional redundancy of the two En genes and dynamic changes in En-2 expression have complicated the interpretation for the
En-2 cerebellar mutant phenotype. It is not clear when and how the lack of En-2 causes changes in cerebellar size and foliation pattern, and whether the two aspects of the defect are related to each other.

The functional roles of En proteins in patterning the midbrain have been investigated in chick. Expression of chick En-2 protein shows an increasing rostrocaudal gradient in the dorsal midbrain at the stages when the polarity of the retinotectal projection map is being determined (Gardner et al., 1988). This gradient is the earliest known marker for polarity of the chick optic tectum (Itasaki et al., 1991; Itasaki and Nakamura, 1992) and correlates with the expression of two retinal axon-guidance molecules, RAGS (Drscher et al., 1995) and ELF-1 (Cheng et al., 1995). In heterotopic chick-quail transplantation experiments, En protein expression correlates well with the subsequent change in the anterior-posterior (A-P) gradient of tectal cytoarchitecture as well as the pattern of retinotectal projections (Itasaki et al., 1991; Itasaki and Nakamura, 1992). Perturbing the normal En rostrocaudal gradient in the midbrain by misexpression of En-1 or En-2 throughout the chick tectal primordium abolishes the normal midbrain cytoarchitecture gradient along the A-P axis (Itasaki and Nakamura, 1996; Friedman and O’Leary, 1996; Logan et al., 1996). Ectopic En-1 expression in the rostral optic tectum also induces RAGS and ELF-1 expression and perturbs the normal optic projection order (Logan et al., 1996). These results suggest an important function for En in determining optic tectum polarity and their topographic projection patterns.

A late function for En in axon tract formation has been examined in Xenopus brains at the late (39/40) embryonic stage after the tectum has been specified and properly developed (Retaux et al., 1996). At this stage, reduction of En-1 and En-2 levels in these Xenopus brains, using En-1 and En-2 antisense oligonucleotides, resulted in abnormal intertectal commissure (ITC) projections in the posterior tectum, a region that the axonal bundles normally avoid. The observed effect suggests that the normal high level of En in the posterior tectum regulates a repulsive molecule for ITC projections.
Taken together, the results indicate that the *En* genes play multiple roles during normal development of the midbrain and cerebellum. First they are required for early specification and/or expansion of the mid-hindbrain region and then in establishing midbrain A-P polarity. Late in brain development, the *En* genes likely regulate formation of axon projections and cerebellar patterning. All these functions are crucially dependent on precise regulation of *En* expression.

**Regulation of the Mouse En-2 Gene**

Previous work from this laboratory began a study of the transcriptional regulation of the mouse *En-2* gene using a transgenic mouse approach (Logan et al., 1993). The bacterial *lacZ* gene was used as a reporter gene driven either by the endogenous *En-2* promoter or by a heterologous promoter from the mouse *hsp68* gene. *En-2* genomic fragments were cloned into the *lacZ* reporter constructs to test their ability to direct specific *En-2*-like *lacZ* expression patterns in transgenic mice. A 9.5 kb genomic fragment consisting of both 5' (7.0 kb) and 3' (2.5 kb) sequences was shown to be capable of directing *lacZ* expression in an *En-2*-specific manner both temporally and spatially during embryogenesis as well as in the adult. Further dissection of this fragment led to the identification of two adjacent enhancer regions of 1.5 kb and 1.0 kb located 5.3 kb upstream of the *En-2* start site of transcription. The 1.5 kb and 1.0 kb enhancers were shown to independently direct transgene expression in E10.5 embryos to either the mid-hindbrain region or mandibular myoblasts, respectively. In addition, expression of the 9.5 kb *lacZ* reporter gene was shown not to depend on endogenous *En-2* since its expression was not altered in *En-2* null mutant mice. This transgenic analysis provided important first insight into the molecular mechanism by which *En-2* expression is controlled at the transcriptional level.

**Regulation of the Drosophila en Gene**
Recent genetic studies in vertebrates have demonstrated that developmental control genes involved in fly patterning play important roles in vertebrates. Moreover, the genetic pathways elucidated from *Drosophila* studies appear to be employed in various developmental processes in vertebrates. Extrapolation from knowledge acquired through studies of *Drosophila* pattern formation has greatly facilitated research in vertebrate development.

Early in embryogenesis the *Drosophila en* gene is expressed in the posterior compartment of each parasegment and plays a critical role in a regulatory cascade controlling the process of segmentation. Extensive genetic analyses have demonstrated the complex nature of the regulatory mechanisms underlying *en* expression (reviewed in Hoop and Scott, 1992; Perimon. 1994). Heemskerk et al. (1991) have proposed that *en* expression in the parasegments has at least four distinct phases. Initially, different sets of pair-rule genes including *paired* (*prd*) and *fushi tarazu* (*Ftz*) activate *en* expression in even and odd-numbered parasegments (DiNardo and O'Farrell, 1987; Morrissey et al., 1991). The early maintenance of *en* expression then depends on a *wg-en* interdependent regulatory pathway that involves multiple segment polarity genes (DiNardo et al., 1988; Martinez-Arias et al., 1988). Following this *wg-en* interdependent phase, *en* expression becomes autoregulated (Heemskerk et al., 1991). Finally, the late phase of *en* expression is regulated by a new set of positive and negative regulators (Struhl and Akam, 1985; Wedeen et al., 1986; Moazed and O'Farrell, 1992).

Relatively less is known about DNA regulatory elements controlling *en* expression. Mutations affecting *en* gene functions are located as far as 45 kb upstream and 20 kb downstream of the *en* transcription unit (Kuner et al., 1985; Drees et al., 1987). A cis-acting regulatory region extending 7.5 kb 5' of the *en* transcription unit is only able to direct portions of the endogenous *en* expression in transgenic flies (Hama et al., 1990). Enhancers that mediate *Ftz*-dependent even parasegmental expression of *en* have been identified upstream of the promoter (DiNardo et al., 1988) and in the first intron (Kassis, 1990).
Multiple DNA-binding sites for Ftz and Ftz-F1, a zinc finger nuclear receptor, have been shown to be required for the intron enhancer activity (Florence et al., 1997).

**THE Pax GENES AND THEIR ROLES IN MIDBRAIN AND CEREBELLUM DEVELOPMENT**

The *Pax* genes encode a family of important developmental regulators that are characterized by an evolutionarily conserved DNA binding domain, referred to as the paired-domain (PD) (reviewed in Gruss and Walther, 1992; Noll, 1993; Chalepakis et al., 1993; Wallin et al., 1993). The *Drosophila* pair rule gene, *prd*, was the first *Pax* gene to be identified (Bopp et al., 1986). To date, *Pax* genes have been isolated in a variety of species from nematodes to humans, including at least nine *Pax* genes (*Pax-1 to Pax-9*) in mouse and human (Table I). The *Pax* genes have been classified into four subfamilies based on their genomic organization and the paired box sequences. Genes within the same subfamily have the highest degree of sequence homology within the paired box and share intron/exon boundaries. Members of *Pax-3* and *Pax-6* subfamilies also share sequences coding for another DNA-binding domain, a paired-type HD (PrdHD) (Walther et al., 1991). Importantly, members of the same subfamily display related expression patterns and appear to have overlapping functions during development.

The Paired DNA-Binding Domain (PD) and Its Recognition Sequences

The PD consists of a stretch of 128 amino acids located at the N-terminal of all *Pax* proteins. The basic function of *Pax* proteins as transcription regulators is dependent on an intact PD (Chalepakis et al., 1991; Treisman et al., 1991). Recently, the structure of a complex comprising the PD of the *prd* protein and a 15 bp optimized *Pax* binding site has been solved by X-ray crystallography (Xu et al., 1995). This crystallographic analysis revealed that the PD folds as two structurally independent subdomains, and each subdomain
<table>
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<th>Organisms</th>
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<th>Mutations</th>
<th>Human</th>
<th>Zebrafish</th>
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<th>Drosophila</th>
<th>parad</th>
<th>OP HD</th>
<th>pax-d</th>
<th>pax-4</th>
<th>pax-7</th>
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<th>pax-5</th>
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**Table 1:** Vertebrate and *Drosophila* paired box-containing genes (modified from Gross and Waller, 1992)
contains a helix-turn-helix motif that closely resembles that of a HD. In the crystal structure, the N-terminal subdomain makes extensive DNA contacts. All the hydrophobic contacts and all but one of the DNA contacts are made by residues that are absolutely conserved among all PDs. Furthermore, there is a remarkable correlation between the observed DNA contacts and the locations of Pax missense mutations that result in loss-of-function phenotypes in mice and human. These structural features may explain why the N-terminal subdomain of the PD is the most conserved region among different Pax proteins of different subfamilies and species (Walther et al., 1991).

In the crystal structure, the C-terminal subdomain of the PD in the fly prd protein does not make direct DNA contacts (Xu et al., 1995). Biochemical and genetic studies have also shown that the C-terminal subdomain of PD in the fly prd protein is functionally dispensable (Cai et al., 1994; Jun and Desplan, 1996). This is in contrast to other Pax proteins in which the C-terminal subdomain of the PD contributes significantly to the overall sequence-specific DNA-binding (Chalepakis et al., 1991; Treisman et al., 1991; Czerny et al., 1993; Epstein et al., 1994a; Jun and Desplan, 1996). In fact, an alternatively spliced form of Pax-6 that has a 14-amino-acid insertion near the middle of the PD, is capable of interacting with DNA exclusively through its C-terminal subdomain (Epstein et al., 1994b). Thus, the N- and C-subdomains of PDs appear to function as discrete DNA interacting motifs, thus expanding their recognition specificities.

To date, several in vivo target DNA-binding sites for the Pax-5 subfamily have been identified (summarized in Czerny et al., 1993; Song et al., 1996). DNA-binding sequences for Pax-2, Pax-3, Pax-6 and prd have also been determined through an in vitro random oligos selection method (Epstein et al., 1994a; Jun and Desplan, 1996; Xu et al., 1996, Chalepakis and Gruss, 1995). The Pax-binding sites are unusually long, 13 bp or greater, and seemingly divergent. A Pax recognition consensus sequence has been derived which exhibits a bipartite structure, the half-site being represented by two distinct 5' and 3' core motifs (Czerny et al., 1993). Since the N-terminal subdomain of PD alone was shown to be
able to interact with the more extensively conserved 3' core motif, it was predicted that the N-terminal subdomain of the PD recognizes the 3' consensus motif whereas the C-terminal subdomain interacts with the 5' consensus motif. Remarkably, this bipartite PD-DNA interaction model fits well with the recently solved crystal structure of the PD-DNA complex (Xu et al., 1995).

Information is beginning to accumulate about the DNA binding specificity of different classes of PDs. Mutagenesis of the Pax-6 protein has identified three amino acids in the PD that are responsible for discriminating the DNA-binding sites of Pax-6 and Pax-5 (Czerny and Busslinger, 1995). One of these amino acids, residue 47, was shown to be the only non-conserved residue that contacts DNA (Xu et al., 1995). In Pax-6 this residue is an Asn but it is a His in all other PDs. In the crystal structure of the PD of prd protein, His 47 contacts the last position of the 3' core DNA-binding motif. Notably, this nucleotide position is a T in the Pax-6 binding site but a G in other Pax DNA binding sites (Epstein et al., 1994a; Czerny et al., 1993).

Overlapping Expression Patterns of Pax-2, Pax-5 and Pax-8

The vertebrate Pax genes exhibit spatially and temporally restricted expression patterns during embryogenesis (reviewed in Gruss and Walther, 1992; Chalepakis et al., 1993; Stoykova and Gruss, 1994). In mouse, members of the Pax-5 subfamily, Pax-2, Pax-5 and Pax-8, show overlapping expression in the developing midbrain and hindbrain. Pax-2 expression can be first detected at the pre- to early somite stage (E7.5 - E8) in a broad band of cells across the anterior neural plate, the presumptive mid-hindbrain region (Rowitch and McMahon, 1995). Pax-5 expression is activated in this region at the 3-5 somite stage (E8.5) (Urbanek et al., 1994; Song, et al. 1996). Between E8.5 and E9.0, these two Pax genes have very similar expression domains. From E9.0 to E12.5, Pax-2 expression becomes restricted to a narrow ring of cells posterior to the isthmus, whereas Pax-5 continues to be broadly expressed in the midbrain and anterior hindbrain. Pax-8 expression in this region is
initiated at E9.0. Later in embryogenesis, at least up to E15.5, Pax-5 expression becomes more confined to the midbrain, and Pax-2 and Pax-8 to the rostral hindbrain. The three Pax genes also show overlapping expression in the developing myelencephalon and spinal cord. In addition to their common CNS expression patterns, each of the three Pax-5 subfamily members displays unique expression elsewhere: Pax-2 in the forebrain extending to the developing eye and ear and in the developing kidney, Pax-5 in the B cell lineage and Pax-8 in the thyroid.

**Roles of Pax-2 and Pax-5 in Midbrain and Cerebellum Development**

The Pax genes are frequent targets for pathological mutations in mice and humans (summarized in Strachan and Read, 1994 for Pax-1, Pax-3 and Pax-6; Sanyanusin et al., 1995a and b for PAX-2). Naturally occurring mouse mutant alleles have been described for Pax-1, Pax-3 and Pax-6. Mutations in human PAX-2, PAX-3 and PAX-6 have been reported to be associated with syndromes that are similar to the defects seen in the mouse mutants. The developmental defects caused by the mutations in the Pax genes are closely correlated with their expression patterns. Interestingly, most Pax mutant alleles are semi-dominant in nature, since at least a subset of phenotypes are evident in the heterozygous mutants. This indicates that Pax proteins are required in distinct concentrations, and that the activity produced by a single allele of each Pax gene is below the critical threshold, leading to phenotypic alterations. The defects associated with heterozygous mutants of a particular Pax gene are often manifested in the tissues/organs in which no other subfamily members are expressed. This implies functional redundancy between members within the same subfamily and argues against a dominant-negative effect in causing the heterozygous phenotypes.

Both mouse and human mutant alleles have been reported for Pax-5 subfamily members (Table 1). Three mouse Pax-2 mutant alleles, which contain distinct mutations in the Pax-2 locus, have been characterized. The Krd (Kidney and Retinal Defects) mutant mice carry a transgene-induced 7 cM chromosomal deletion at the distal region of chromosome 19.
that includes the entire Pax-2 locus (Keller et al., 1994). A second Pax-2 mutant allele, Pax-21Neu, was identified as a spontaneous mouse mutant strain (Favor et al., 1996). It has a 1 bp frameshift insertion mutation in the beginning of the PD coding region. This mutation results in a premature stop codon and could result in production of a 51 amino acid mutant peptide. The first 24 amino acids of the mutant peptide are identical to those of the wild type protein with the last 9 residues being part of the PD. In the remaining 27 amino acids, there is only 1 residue matching with the wild type peptide. Thus, the resulting mutant peptide is unlikely to function normally. The third Pax-2 mutant allele, Pax-2ntd, is a presumptive null allele generated by gene targeting (Torres et al., 1995 and 1996). In this allele 4 kb of genomic DNA including the translational start codon and sequences encoding the N-terminal half of the PD is replaced by a neomycin resistance gene (Neo). The homozygous Krd mutation is lethal at the preimplantation stage, likely due to deletion of genes other than Pax-2 that are critical for early gestation. Mice with the other two Pax-2 mutant alleles are able to survive until birth. All three mutations in the Pax-2 gene cause developmental abnormalities in the kidney and the eye in a semi-dominant manner. Inner ear defects are also seen in Pax-2ntd and Pax-21Neu homozygotes. No brain abnormalities have been reported for the Krd heterozygous mice, whereas distinct brain phenotypes have been described for Pax-2ntd and Pax-21Neu mutant mice. Pax-21Neu homozygotes, but not heterozygotes, show a deletion of part of the cerebellum and posterior midbrain. The deletion begins very early in development since the morphological alteration is evident at E9.0. The En-2 expression domain in these embryos is significantly reduced, with only weak expression in the dorsal part of the mid-hindbrain junction region. This indicates that the deletion of the midbrain and hindbrain structures results from the failure of early specification and/or expansion of the presumptive midbrain hindbrain region. Surprisingly, a very different brain phenotype is observed in Pax-2ntd homozygous mice. These animals show exencephaly in the midbrain and hindbrain regions, resulting from a failure of the neural folds to close. The phenotype is manifested in both homozygous and heterozygous embryos with a similar degree of severity.
although the phenotype in heterozygous animals has a lower degree of penetrance and is more sensitive to the genetic background. The morphology of the midbrain and hindbrain in the affected embryos is grossly altered due to exencephaly. However, many En-1, Pax-5 and Wnt-1 expressing cells are still present in this region. Judging from the figures presented in the paper (Torres et al., 1996), the size of the entire mid-hindbrain region as well as the En-1 expression domain appears smaller in the mutant embryos than in wild type embryos, despite the exencephaly.

Although the brain defects of both Pax-2 mutant alleles are manifested in the mid-hindbrain region where Pax-2 is normally expressed, the nature of the two defects is quite different. During early brain development, between the pre-somite and the 3 somite stage, Pax-2 is the only family member being expressed in the presumptive mid-hindbrain region. Therefore, an early deletion phenotype associated with Pax-2\textsuperscript{1Nue} mice seems consistent with an important role for Pax-2 in early mid-hindbrain specification. However, the brain phenotype observed in Pax-2\textsuperscript{ntd} mice apparently argues against such a role for Pax-2 and/or suggests an additional role in neural tube closure. At present, it is difficult to explain why the two Pax-2 alleles have distinctly different brain defects and yet share similar phenotypes in other tissues. Neither mutant allele should give rise to a functional protein, and manifestations of the eye and kidney phenotypes in the two mutant alleles are consistent with this prediction. However, this cannot exclude the possibility that the brain phenotypes in the two mutants are not due to lack of function of Pax-2 protein. A possible explanation for the Pax-2\textsuperscript{ntd} brain phenotype is that the presence of Neo in the Pax-2 locus affects expression of an unknown gene nearby that results in exencephaly (Olson et al., 1996). It is also possible that the mutant peptide produced from the Pax-2\textsuperscript{1Nue} allele has some negative effect on mid-hindbrain development leading to a deletion of this region. In addition, upregulation of Pax-5 and Pax-8 expression could influence the brain phenotypes. Finally, strain differences may be attributable to the discrepancy, even though both studies have analyzed the phenotypes in multiple mouse strains.
Two different human PAX-2 mutations have been identified in two patient families with optic nerve coloboma and renal anomalies (Sanyanusin et al., 1995 a and b). One of the mutations is inherited in an autosomal dominant manner, while it was not possible to determine the inheritance of the other mutation due to limited pedigree information. Both mutations are insertional mutations resulting in frameshifts in the coding region. One of the mutations (Sanyanusin et al., 1995b) is identical to that of the mouse Pax-2\textsuperscript{LNue} allele (Favor et al., 1996). The other mutation truncates the C-terminal half of the protein (Sanyanusin et al., 1995a). The eye and kidney phenotypes caused by the two PAX-2 mutations in heterozygotes are similar to each other and closely resemble the abnormalities observed in the Pax-2 heterozygous mutant mice. No additional abnormalities have been described in these patients that could be attributed to any brain defect. At present, no homozygous patients have been identified for either of the mutations.

A Pax-5 mutant allele was generated by gene targeting (Urbanek et al., 1994). Heterozygous Pax-5 mutants are phenotypically normal, whereas homozygous mice die at three months of age because of a lack of B cells. In the brain, these animals have a small deletion in the inferior colliculus and a slight abnormality in the foliation pattern of the anterior cerebellum. The collicular phenotype can be detected at E16.5 as a clear reduction in the size of the posterior midbrain. This phenotype appears not to fully correlate with the broad Pax-5 expression in this region from E9.0 to E15.5.

Pax-5 and Krd compound mutants were analyzed to address whether the relatively mild brain defects in Pax-5 mutants compared to that of Pax-2 mutants was due to functional redundancy between the two subfamily members (Urbanek et al., submitted). While neither Pax-5 nor Krd heterozygous mutant mice have any brain defects, 20% of the double heterozygotes show a deletion of the inferior colliculus and severe disruption of the vermis of the cerebellum due to a failure of the cerebellar primordium to fuse at the midline. Inactivation of the second copy of Pax-5 in Pax-5(-/-)/Krd(+/-) mice leads to a complete loss of the posterior midbrain and cerebellum. As early as E9.0 the compound mutant embryos
show a deletion of the mid-hindbrain region, which is indicated by the lack of expression of Fgf-8, Wnt-1, En-1 and En-2 in the mid-hindbrain region. These results indicate that early cooperative interaction of Pax-2 and Pax-5 is required for normal midbrain and cerebellum development.

The early developmental role of Pax-2 related genes has also been studied in zebrafish, which appear to have only one Pax-2 related homolog, par-b (Krauss et al., 1991; Puschel et al., 1992). As with its mouse counterparts par-b is activated in the presumptive mid-hindbrain junction region at the 2 somite stage. After neural tube closure, the expression domain extends on both sides of the junction overlapping with the fish eng-2 expression domain. Injection of antibodies against par-b protein into fertilized zebrafish eggs causes an early localized malformation at the mid-hindbrain junction region with significant downregulation of eng-2 and wnt-1 (Krauss et al., 1992). Recently, a par-b zebrafish mutant allele, no isthmus (noi), has been characterized (Brand et al., 1996). In noi mutant embryos, the isthmus and cerebellum are not formed and the tectum is initially present but later degenerates. Expression of eng and wnt-1 was not detected in the midbrain and anterior hindbrain of noi mutants. The absence of eng and wnt-1 expression seems not to be due to the loss of tissue in the brain because no eng and wnt-1 expression was detected even at early somite stages before morphological changes occurred. Moreover, expression of the genes was not detected in the tegmentum that is not deleted in mutant embryos. This result suggests that par-b is required for eng and wnt-1 expression. This study demonstrates a similar role for the Pax genes in fish and mammals, and further emphasizes the importance of the Pax genes in midbrain and cerebellum development.

EARLY DEVELOPMENT OF THE MIDBRAIN AND CEREBELLUM
(MESENCEPHALON AND METENCEPHALON)

The Mesencephalon and Metencephalon (Mes-Met) Territory
Transplantation studies using the chick-quail model system have provided extensive information about early development of the mesencephalon (midbrain) and metencephalon (rostral hindbrain). Fate mapping experiments involving homotopic chick-quail transplantation have revealed that at early somite stages (10-12 somites, chick stage 10HH, (Hamburger and Hamilton, 1951) the caudal mesencephalon cells contribute to the isthmic nuclei and mediorostral cerebellum, despite the morphological appearance suggesting that the cerebellum should be exclusively derived from the rostral metencephalon (Martinez and Alvarado-Mallart, 1989; Hallonet et al., 1990; Alvarado-Mallart et al., 1990; Hallonet and LeDouarin, 1993). Detailed fate mapping has defined a mesencephalic-metencephalic territory (mes-met) that extends from the mesencephalic-prosencephalic constriction to the rhombomere 1/2 border. Notably, the chick En-2 expression domain significantly overlaps with the mes-met territory at early stages (Martinez and Alvarado-Mallart, 1990; Martinez et al., 1991). The neuroepithelium within the alar plate of the mes-met contains the primordia, from anterior to posterior, for the tectum, mesencephalic grisea, isthmic region and cerebellum.

In other regions of the brain, morphologically defined neuromeres have been implicated in regional specification. The rhombomeres in the hindbrain, transiently visible during early neural tube development, are separated by clear morphological boundaries that coincide with the expressions borders of some of the Hox genes (reviewed in Krumlauf et al., 1993; Lumsden and Krumlauf, 1996). Cell lineage studies have demonstrated that at certain stages these morphogenetic units are lineage restricted and each rhombomere develops into a defined section of the hindbrain (reviewed in Lumsden, 1990). The segmental subdivisions in the forebrain, the prosomeres, have also recently been implicated in regional development based on morphological as well as gene expression data (reviewed in Rubenstein et al., 1994; Puelles, 1995)

Neither the mesencephalon nor metencephalon is subdivided into morphologically defined neuromeres. Moreover, the fact that the cerebellar primordium extends on both sides
of the mes-met constriction has raised the question of whether the early morphological constriction represents the true mes-met boundary. A recent transplant study has suggested the presence of a functional interneuromeric boundary at the caudal limit of mesencephalic Otx-2 expression that separates the primordia of mesencephalon and cerebellum (Millet et al., 1996). The Otx genes are homeobox containing genes related to the Drosophila developmental control gene otd (Simeone et al., 1992 and 1993). In mouse and chick, Otx-2 is expressed throughout the anterior neural tube with a sharp caudal mesencephalic border (Simeone et al., 1992 and 1993; Ang et al., 1994; Bally-Cuif et al., 1995). Interestingly, the position of the posterior limit of Otx-2 expression changes at early stages with respect to the mes-met constriction (Millet et al., 1996): it is rostral to the constriction at early somite stages (before 20HH in chick and E10.5 in mouse) with the caudal fifth of the mesencephalic neuroepithelium being Otx-2-negative, and later Otx-2 expression extends to the constriction.

By analyzing the relationships of the Otx-2 caudal expression limit with the fate of cells in the mesencephalon, Millet et al. (1996) have demonstrated that the early Otx-2-positive and -negative mesencephalic neuroepithelium give rise to the mesencephalon and rostral rhombencephalon, respectively. Thus, the caudal limit of Otx-2 expression, rather than the morphological constriction, appears to mark the early mes-met boundary. This view is consistent with a previous mouse study in which perturbation of the Otx-2 caudal expression border by reducing Wnt-1 activity (see below) was shown to correlate with mixing of the mesencephalic and cerebellar cells (Bally-Cuif and Wassef 1994).

The Isthmus Organizing Center

The developmental potential and commitment of the mes-met neuroepithelium have been investigated using heterotopic chick-quail transplantation experiments (Fig. 2) (Nakamura et al., 1986; Alvarado-Mallart et al., 1990; Martinez et al., 1995). At the 10-14 somite stages (HH10-11), the neuroepithelium in the mes-met junction region is already determined as it is capable of maintaining its developmental fate in heterotopic brain.
Figure 2. Chick and quail transplantation showing organizing activity of the isthmus. Dorsal views of chick and quail early somite-stage embryos showing En-2 expression following transplantation of the isthmus tissues into the diencephalon and myelencephalon. The structures that will later develop from the host or donor tissues are indicated to the right. T, telencephalon; D, diencephalon; Mes, mesencephalon; Met, metencephalon; Mey, myelencephalon.
locations. When grafted into the diencephalon or the myelencephalon, the grafted mes-met junction cells form a supernumerary tectum and/or cerebellum in the new host environment, which is/are present in a mirror image orientation relative to the normal mid- and hindbrain structures. Detailed analysis of the chimeric embryos has revealed that the host cells also contribute to the ectopic tectal/cerebellar structures (Alvarado-Mallart et al., 1990). This important finding indicates that the mes-met junction explants have inducing and polarizing activities that can respecify the surrounding host tissue. Interestingly, the outcome of the induction event somewhat depends on the host environment. Diencephalon cells in response to putative signals from mes-met explants form a tectum, whereas rhombencephalic cells give rise to cerebellar structures. Furthermore, the telencephalon, ventral thalamus (ventral diencephalon), and basal plate of the rhombencephalon are not influenced by the explants, suggesting different brain regions, at least at the stages tested, have different levels of competence to respond to the isthmus signal. Such heterotopic transplant experiments have also been performed in combination with gene expression analyses (Gardner and Barald, 1991; Martinez et al., 1991 and 1995; Bally-Cuif and Wassef, 1994; Bloch-Gallego et al., 1996). Significantly, gene expression patterns correlate well with the phenotype of the brain tissues that develop. In the explants, expression of two mes-met marker genes, En-2 and Wnt-1, are maintained, reflecting the determined mes-met fate. In the surrounding transformed host tissues, expression of En-2 and Wnt-1 is induced. In addition, when the diencephalon (P1 or 2) is the host site, Pax-6 expression, which marks diencephalic cells, is downregulated in the induced tissues (Bloch-Gallego et al., 1996). Interestingly, Wnt-1 expressing cells from both explants and host tissues are reorganized into a continuous line, possibly marking a new mes-met border (Bally-Cuif and Wassef, 1994). The regulation of gene expression in host tissues occurs 15-20 hours after grafting, preceding the morphological transformation. These sequential changes in gene expression and then phenotypic transformation indicate that En-2 and Wnt-1 could be involved in respecification of the host tissues. Furthermore, a mouse mes-met graft can also induce En-2 and Wnt-1
ectopic expression in a chick host, indicating that the factors regulating En-2 and Wnt-1 expression and patternning the mes-met are phylogenetically conserved (Martinez et al., 1991). The results obtained from these heterotopic transplantation studies have important implications for normal mes-met development. The studies suggest that the isthmus region functions as an organizing center that specifies and/or patterns the mid- and hindbrain regions along the A-P axis, and that this involves regulating mes-met controlling genes such as En and Wnt-1.

GENETIC CONTROL OF EARLY MIDBRAIN AND CEREBELLUM DEVELOPMENT

Mes-Met Control Genes

Embryological transplant studies indicate that the mes and met are specified as an integrated developmental unit that is patterned along the A-P axis by the isthmus organizing center. Furthermore, pre-somite and early somite stages (E7.5-E9.5 in mouse) represent a critical period when the mes-met is specified and the isthmic organizer is established. In addition to the genetic evidence that the En and Pax genes are essential in regulating these processes, a number of other genes including Otx-2, Gbx-2, Fgf-8 and Wnt-1 have been shown to be required for mes-met development. Gbx-2 is a homeobox containing gene related to the Drosophila developmental control gene unplugged (Bouillet et al., 1995). Wnt-1 is a homolog of the Drosophila segmentation gene wingless (wg) and encodes a secreted signalling molecule (Nusse and Varmus, 1982; Papkoff et al., 1987). Fgf-8 encodes another secreted molecule that belongs to the fibroblast growth factor super-family (Crossley and Martin, 1995; Mahmood et al., 1995).

The earliest indication of patterning of the mes-met is the differential expression of homeodomain transcription factors Otx-2 and Gbx-2. By E7.5 in mouse, Otx-2 is expressed from the anterior limit of the neural plate to a border in the presumptive mid-hindbrain
junction region (Ang, et al., 1994). Gbx-2 is expressed in the posterior of the embryo with an anterior border apparently abutting the Otx-2 caudal border (Wassarman et al., submitted). Thus, the anterior and posterior expression domains of Otx-2 and Gbx-2 appear to define two different populations of neuroectoderm (Fig. 3A). Meinhardt (1983) has proposed that cooperative interactions between juxtaposed cell populations of a different nature stimulate the production of diffusible morphogens, leading to the formation of new positional information organized around the boundary. According to Meinhardt's model, the border between Otx-2 and Gbx-2 expression domains could function as such an organizing boundary. Consistent with this idea, during the pre-somite to early somite stages, transcription of other known mes-met regulatory genes is activated in broad domains surrounding the Otx/Gbx boundary. Pax-2 is the first gene detected at the pre-somite stage (Rowitch and McMahon, 1995). At the 1 somite stage, expression of Wnt-1 and En-1 is initiated in subdomains of the Pax-2 expression domain followed by activation of En-2, Fgf-8, Pax-5 and Pax-8 at the 3-10 somite stage (Fig. 3B,C) (Davis and Joyner, 1988; Song et al., 1996; Rowitch and McMahon, 1995; Crossley and Martin, 1995) By E9.0 when the neural tube closes and the mes-met constriction forms, the gene expression patterns are refined to their final spatial organization (Fig. 3D). The complementary expression relationship of Otx-2 and Gbx-2 is maintained. En-1, En-2, Pax-5 and Pax-8 are continuously expressed in broad domains spanning the mes-met junction, whereas expression of Wnt-1, Pax-2 and Fgf-8 becomes restricted to narrow rings around the isthmus, with Wnt-1 just anterior to the isthmus and sharing a posterior limit with Otx-2, and with Fgf-8 and Pax-2 overlapping just posterior to Wnt-1.

**Roles of Otx-2 and Gbx-2 in Midbrain and Anterior Hindbrain Specification and in Establishment of the Mid-Hindbrain Boundary**

Inactivation of Otx-2 in mouse results in a deletion of the head tissues that includes the neural tube anterior to rhombomere 3 (Acampora et al., 1995; Matsuo et al., 1995; Ang et
Figure 3. Expression patterns for mes-met control genes from E7.5 to E9.5. (A) E7.5, (B and C) E8.0-E8.5 and (D) E9.5. RNA expression patterns are shown in purple. ant, anterior; pos, posterior.
A  Headfold: lateral view

\[ \text{ant} \quad \text{pos} \quad \text{ant} \quad \text{pos} \]

\[ Otx-2 \quad Gbx-2 \]

B  1 somite: dorsal view

\[ \text{ant} \quad \text{pos} \]

\[ Otx-2 \quad Gbx-2 \quad Pax-2 \quad Wnt-1 \quad En-1 \]

C  5 somites: dorsal view

\[ En-2 \quad Pax-5 \quad Fgf-8 \]

D  12-14 somites: lateral view

\[ Otx-2 \quad Gbx-2 \quad En-1/2 \quad Pax-5 \quad Pax-2 \]

\[ Wnt-1 \quad Fgf-8 \]
The primary defect may be due to a function for Otx-2 in the underlying mesoderm and/or endoderm. Nevertheless, the deletion of neural tube structures posterior to the isthmus, which is far beyond its normal posterior expression limit, is consistent with a role for Otx-2 in setting up and/or maintaining the organizer.

Ablation of mouse Gbx-2 (Wassarman et al., submitted) causes a deletion of the structures derived from rhombomeres 1-3 including the cerebellum, suggesting Gbx-2 is required for normal development of these structures. Furthermore, the mes-met boundary of the mutant brain is not formed properly. In Gbx-2 mutant brains, expression of Otx-2, Fgf-8 and Wnt-1 at the mes-met junction is more diffuse and their normal sharp boundaries are disrupted. Patches of Otx-2 and Wnt-1 expressing cells are also found in the region caudal to the Otx-2 domain. In addition, the posterior expression limits of Otx-2 and Wnt-1 shift caudally so that they overlap with that of Fgf-8. By E12.5, it is also clear that the inferior colliculi are anteriorized and expand caudally. These alterations in morphology and gene expression patterns indicate that the mid-hindbrain boundary could be shifted caudally in Gbx-2 mutants, and suggest that Gbx-2 is required for anterior hindbrain specification and development but normally also acts to suppress midbrain development.

As described above, fate mapping studies in avian embryos have shown that the Otx-2 posterior boundary correlates with the junction of the mesencephalic and cerebellar primordia. The Otx-2 and Gbx-2 mouse mutant phenotypes have further demonstrated that Otx-2 and Gbx-2 are essential for specifying the midbrain and anterior hindbrain, respectively. Moreover, it appears that the two genes play key roles in establishing the mes-met boundary and induction of a normal isthmus organizing center.

Roles of En-1, En-2, Pax-2, Pax-5, Wnt-1 and Fgf-8 in Mes-Met Specification and/or Expansion

The common feature of the loss-of-function brain phenotypes for En-1, En-2, Pax-2 (Pax-21Neu), Pax-5, Wnt-1 and Fgf-8 mouse mutants is a deletion of mes-met cells and/or
their derivative structures (Table II). This suggests that these genes represent a gene network required for mes-met specification and/or proliferation. Functional disruption of the early expressing genes, *Wnt-1* (McMahon and Bradley, 1990), *En-1* (Wurst et al., 1994) and *Pax-2* (*Pax-21Neu*, Favor et al., 1996) leads to an extensive deletion of mes-met structures which is evident by E9.0, indicating these genes are essential for early mes-met structures which is evident by E9.0, indicating these genes are essential for early mes-met specification and/or proliferation. Similar, but much milder, brain phenotypes have been observed in mouse mutants for *En-2* (Joyner et al. 1991) and *Pax-5* (Urbanek et al., 1994), the two genes with relatively later onsets of expression. There is no obvious early mes-met deletion in *En-2* and *Pax-5* mutant embryos, but the adult mutant brains show a reduction in the size of the colliculi (*Pax-5*) and cerebellum (*En-2*) as well as cerebellar foliation defects. As discussed above, analyses of *En-1/En-2* or *Pax-2 (Krd)/Pax-5* double mutant mice have revealed that *En-2* and *Pax-5* also function early in specification of the mes-met (Urbanek et al., submitted).

A zebrafish *Fgf-8* hypomorphic mutant, *acerebellar (ace)*, has recently been identified and characterized (Brand et al., 1996; Stainer personal communication). In *ace* mutants, the isthmus and cerebellum are deleted. Analysis of marker gene expression showed that mes-met expression of *eng, wnt-1* and *pax-b* was initiated properly at the end of gastrulation but then progressively lost. The loss of gene expression in the mes-met junction could be due to lack of *Fgf-8* signalling or secondary to deletion of isthmus tissue. This study suggests that *Fgf-8* is critical for maintaining the isthmus and/or the isthmus organizer signalling is required for maintaining *eng, wnt-1* and *pax-b* mes-met expression.

**A Late Function for Wnt-1 in Maintaining the Mes-Met Boundary**

Analysis of a *Wnt-1* hypomorphic mutant allele, *swaying (Wnt-1sw)*, has revealed a function for *Wnt-1* in maintaining the mes-met boundary (Thomas et al., 1991; Bally-Cuif et
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<td>ace(-/-)</td>
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al., 1994). \textit{Wnt-1} is a frameshift mutation that causes premature termination. A large portion of the C-terminal of Wnt-1 is deleted. The \textit{Wnt-1SW} mutant mice have a less severe brain phenotype compared to the targeted \textit{Wnt-1} null mutants, with the midbrain and hindbrain regions partially reduced. Analysis of the relatively late (E9.0 and onward) midbrain and hindbrain phenotype of \textit{Wnt-1SW} mutants showed that formation of the straight \textit{Otx-2} and \textit{Wnt-1} caudal expression limits was perturbed and small ectopic islands of \textit{Otx-2} expressing or not expressing cells were located inappropriately within the hindbrain or midbrain, respectively. Moreover, \textit{Wnt-1} expression was induced at the border of the mes and met islands, suggesting that interactions between the mes (\textit{Otx-2}-positive) and met (\textit{Otx-2}-negative) cells positively regulate \textit{Wnt-1} expression at the boundary.

\textbf{The Isthmus Signalling Molecules Fgf-8 and Wnt-1}

The biochemical nature of the isthmus inducing activities is not yet fully understood. Several studies in chick and mouse have indicated that Fgf-8 represents one of the isthmus signals (Crossley and Martin, 1995; Mahmood et al., 1995; Crossley et al, 1996). During normal development Fgfs and their receptors mediate many signalling pathways that play essential roles in regulating cellular proliferation, differentiation and tissue patterning (reviewed in Yamaguchi and Rossant, 1995). In chick and mouse embryos, \textit{Fgf-8} expression is associated with regions that are known to direct outgrowth and patterning, including the isthmus (Crossley and Martin, 1995; Mahmood et al., 1995; Crossley et al, 1996). Fgf-8 protein alone has been shown to have the same inducing and polarizing effects as isthmic tissue in chick embryos. Implantation of a bead soaked in recombinant Fgf-8 protein (Fgf-8-bead) into the diencephalon (P2) of stage 9-12 embryos induces the surrounding tissues to ectopically express mes-met marker genes such as \textit{En-1}, \textit{En-2} and \textit{Wnt-1} within 48 hours (Crossley et al., 1996). Interestingly, Fgf-8-beads also induce expression of the endogenous \textit{Fgf-8} gene. By stage 25, following implantation of an Fgf-8 bead the caudal diencephalon becomes transformed to form an ectopic midbrain in a mirror
image orientation to the normal midbrain. Similarly, implantation of an Fgf-8-bead into the caudal diencephalon (P1) and mesencephalon induces formation of ectopic cerebellar structures (Martinez and Martin, personal communication). These observations suggest that either a new "isthmus-like" organizing center is formed under the influence of the implanted Fgf-8-bead or that Fgf-8 alone represents the isthmus inducing activity. However, unlike the isthmus explants, an Fgf-8-bead is not sufficient to induce ectopic En expression or mes-met structures in the myelencephalon (Crossley et al., 1996), indicating that other or additional isthmus signals are involved in patterning the hindbrain. An Fgf-8 midbrain inducing effect has also been observed in transgenic mice in which Fgf-8 is ectopically expressed in the midbrain and dorsal forebrain from E8.0 under the control of a Wnt-I DNA regulatory element (Lee et al., 1997). Such transgenic embryos have an expanded tectal structure that expresses En-2. In the mouse transgenic experiment, in contrast to the chick Fgf-8-bead studies, Fgf-8 primarily induces proliferation rather than extensive A-P repatterning.

Wnt-1 is another secreted molecule produced by the isthmus tissue (reviewed in McMahon, 1992; Parr and McMahon, 1994). As described above, a function of Wnt-1 in early specification of the mid-hindbrain region has been demonstrated in Wnt-I null mutant mice (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). The Drosophila wg protein is known to act as a signaling molecule mediating inductive events during development (Zecca et al., 1996). The isthmus expression pattern of Wnt-I and the fact that Wnt-I null mutants have a deletion of the entire midbrain and cerebellum, including cells that do not express Wnt-I, suggest that Wnt-1 may be a component of the isthmic organizer signals. However, in E8.5-E10.5 mouse embryos, ectopic expression of Wnt-I in the ventricular zone of the hindbrain and spinal cord, from the rhombomere 6/7 boundary to the caudal extent of the axis, induces an overgrowth but has no effect on patterning (Dickinson et al., 1994). This negative result must be taken cautiously because the spinal cord may not be competent to respond to a Wnt-1 inductive action in patterning. A cooperative interaction of Wnt-1 and Fgf-8 molecules has been shown to accelerate mammary tumor formation.
(MacArthur et al., 1995), and this could be the case in the isthmus since Fgf-8 and Wnt-1 expression domains abut each other at the mes-met junction. Thus, an alternative explanation consistent with all the data is that Fgf-8 and Wnt-1 act together. In the cases of ectopic induction by Fgf-8, both in chick and mouse, Wnt-1 expressing cells were near or overlapping with the sites of Fgf-8 action. However, when Wnt-1 was expressed ectopically, no Fgf-8 was present.

In summary, the pre-somite and early somite stages represent a critical period when the mes-met is specified and the isthmus organizer is established. A growing number of genes have been shown to be involved in regulating these processes. Otx-2 and Gbx-2 play an important role in the specification of the midbrain and anterior hindbrain, respectively. This likely sets up an initial A-P difference in the mes-met region and establishes the mes-met boundary, an event that could be essential for formation of the isthmus organizing center. En-1, En-2, Pax-2, Pax-5, Wnt-1 and Fgf-8 constitute a gene network and act in concert to specify and/or expand the mes-met region and to establish the isthmus organizing center. Two secreted molecules, Wnt-1 and Fgf-8, have been identified in the organizing center. While an inducing activity of Wnt-1 remains to be demonstrated, Fgf-8 appears to be a component of the isthmic organizer signals (Fig. 4).

**Potential Interactions between the Mes-Met Control Genes**

At present, little is known about the interactions between the various mes-met genes. The expression studies and functional analyses have provided a framework for further studying their complex cross-regulatory and cooperative interactions. Knowledge obtained from genetic studies in *Drosophila* has also contributed to establishing their possible epistatic relationships. The vertebrate Pax, En and Wnt genes are homologs of *Drosophila* segmentation genes. As described above, in fly, initiation of *en* and *wg* transcription
**Figure 4.** Summary of mes-met control gene expression patterns at E9.5. T, telencephalon; D, diencephalon; Mes, mesencephalon; Met, metencephalon; Mey, myelencephalon. (Modified from Joyner 1996).
Mey

organizer region

- Gbx2
- Otx2
- Wnt1 + Otx2
- Fgf8 + Gbx2
- En/Pax5,8

hindbrain
requires the prd gene. Following their activation, en and wg expression undergoes a phase
of interdependence (reviewed in Hooper and Scott, 1992; Perrimon, 1994). Such interactions
may be conserved in the genetic cascade controlling vertebrate mes-met development. The
prd related Pax genes, Pax-2, Pax-5 and Pax-8 have expression domains in the mes-met that
overlap with those of Wnt-1, En-1 and En-2. In particular, Pax-2 expression precedes and
encompasses En-1 and Wnt-1 expression in the presumptive mes-met region (Rowitch and
McMahon, 1995). This raises the possibility that the Pax proteins regulate En and Wnt-1
expression. Consistent with this, the Pax-2/Neu and En-1 and Wnt-1 null mutants have
similar phenotypes (Favor et al., 1996; Wurst et al., 1994; McMahon and Bradley,
1990). Furthermore, studies of zebrafish pax-b mutants have shown that eng and wnt-1 mes-
met expression is dependent on pax-b protein (Krausss et al. 1992; Brand et al., 1996).

Several lines of evidence have indicated that Wnt-1 is required to maintain En
expression. In Wnt-1 null mutant mice, En expression is initiated normally, but it is quickly
lost (McMahon et al., 1992). At about the 5 somite stage, the En expression domain is
reduced in size and completely lost by E9.5. Furthermore, the mes-met structures deleted in
the Wnt-1 mutants, including the cerebellum, are comparable to those seen in En-1 and En-2
double homozygous mutants (McMahon and Bradley, 1990), although Wnt-1 is normally not
expressed in the cerebellar primordium. An elegant transgenic study was recently performed
to test whether the En genes are targets of Wnt-1 signaling (Danielian and McMahon, 1996).
A transgene (Wexp3En-1) that expresses En-1 from a Wnt-1 regulatory element was
introduced into Wnt-1 null mutant mice. In some of the resulting animals, the Wnt-1 brain
mutant phenotype was nearly completely rescued, demonstrating that En-1 can rescue the
effects of loss of Wnt-1 function, strongly suggesting that a key role of Wnt-1 signaling in
mes-met development is to maintain En expression. In the E9.5 rescued embryos, the
endogenous En mes-met expression was also restored in the areas outside of the domain of
transgene expression, indicating that at this later stage En is not dependent on Wnt-1
signalling. This observation together with the fact that En expression in Wnt-1 mutants is not
downregulated until after E8.5, indicates that the E8.5-E9.5 period is the time window when En expression is dependent on Wnt-1 signaling. Moreover, in the E9.5 rescued mes-met of Wexp3En-l transgenic Wnt-1 mutants, Fgf-8 and Pax-5 expression was maintained, indicating that Wnt-1 signalling at this stage may not play a direct role in regulating expression of these mes-met genes.

Ectopic expression of Fgf-8 in chick and mouse was shown to induce En and Wnt-l expression (Crossley et al., 1996). During normal development, it is unlikely that Fgf-8 is involved in initiation of En-1 and Wnt-l because expression of these genes in the presumptive mes-met region precedes Fgf-8 expression. However, Fgf-8 could activate En-2 and Pax-5 expression since they are activated at the same time as Fgf-8. Furthermore, recent data from analysis of an Fgf-8 zebrafish mutant, ace, suggest that Fgf-8 is required for maintaining eng, wnt-l and pax-b mes-met expression (Brand et al., 1996; Stainier personal communication).

Phenotypic analyses of both loss-of-function and gain-of-function mutants in different vertebrates have demonstrated that the mes-met regulatory genes are essential for specification and patterning of this brain region. However, such analyses have only provided limited information about interactions between these mes-met regulatory genes. Moreover, these analyses do not address whether the potential interactions are direct. Identification of DNA regulatory sequences and characterization of their interactions with potential protein regulators represents an important approach for determining direct transcriptional regulation of a locus by transcription factors higher up in the regulatory hierarchy. Such an approach has been employed in this thesis to provide evidence for direct transcriptional regulation of En-2 by Pax proteins. Furthermore, a gene targeting approach was used to address the in vivo function of the Pax DNA-binding sites identified in the initial analyses. These are the first studies to provide molecular evidence that the Pax genes are
direct upstream regulators of En-2 in the genetic cascade controlling midbrain and hindbrain development. Furthermore, this study represents the first step toward dissecting a genetic pathway controlling development of the mid-hindbrain region. The transgenic and biochemical analyses are summarized in chapter 2, and the gene targeting experiment is presented in chapter 3.
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CHAPTER 2: TWO Pax-BINDING SITES ARE REQUIRED FOR EARLY EMBRYONIC BRAIN EXPRESSION OF AN Engrailed-2 TRANSGENE

This chapter is a slightly modified version of the following publication: Song, D.-L., Chalepakis, G., Gruss, P. and Joyner, A. L. (1996). Two Pax-binding sites are required for early embryonic brain expression of an Engrailed-2 transgene. Development 122, 627-635.

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I am responsible for the following work: generation of lacZ reporter constructs (Fig. 1); generation of most of the transgenic mice/embryos (Fig. 2 and 6); En-2 and Pax-2/5/8 RNA in situ hybridization (Fig. 3); sequencing of the mouse (1.0 kb) and human (0.5 kb) En-2 enhancer fragments and sequence comparison analysis (Figure 4).
ABSTRACT

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-2/5/8 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 midbrain and 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.

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 expression that marks 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 *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 homeodomain-containing transcription factors (Joyner et al., 1986; 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 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 region, 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 region diverges after E12.5 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 E9.0 (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). Furthermore, *En-1/En-2* double mutants have a more severe deletion phenotype than that of *En-1* mutants (Wurst et al., in preparation) 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 region 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 which 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-2/5/8 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 phspPTlacZpA 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 Clal-HindIII fragment located 6.3 kb upstream of the translation start site (Fig. 1) (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 Clal-XbaI fragment. Construct XH/lacZ contains a 500 bp Xbal-HindIII fragment. Construct CA/lacZ contains a 750 bp Clal-AccI fragment. Construct (S2X)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 Clal-SstI fragment, respectively. Constructs S/S2/lacZ and (S/S2)2/lacZ contain one or two copies of a 250 bp SstI-SstI fragment, respectively. Construct ΔCX/lacZ has the Clal-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 Clal-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 ΔCX and **CX fragments were introduced by polymerase chain reaction (PCR) (Clackon et al., 1992) using specific primers; ΔCX: 5'-CCCGCATGCACAC-3' and 5'-GAGGCATGCAAGTTGC-3'; **CX: 5'-GCAGACCCGGGCA-3' and 5'-CCACTCTTCAGCTGAG-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
Figure 1. Identification of mouse and human genomic DNA fragments that direct En-2-like brain expression. The top schematic 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 ClaI-HindIII 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, S2X, CS1 and S1S2 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. Δ indicates a 66-bp deletion; *, point mutations; Tg, number of G0 E10.5 transgenic embryos analyzed; lacZ, number of transgenics expressing lacZ; m/h, number of transgenics expressing lacZ in the mid-hindbrain region; a, small dorsal patches of expression in the mid-hindbrain junction. b, variability was observed in the size of the mid-hindbrain expression domains. Restriction sites: A, AccI; C, ClaI; H, HindIII; S1 and S2, SstI; X, XbaI.
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 (G1) males with CD-1 females and subsequently interbreeding animals homozygous for the transgene. Transgene expression patterns were analyzed primarily at E10.5 in generation 0 (G0) embryos or in transgenic lines from E7.5 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**

β-galactosidase (β-gal) activity was detected in whole-mount embryos by using X-gal (5-bromo-4-choro-3-indolyl β-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 BgIII/XbaI 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 pBluescript vector (Strategene, 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 $^{32}\text{P}$-dCTP using the Klenow fragment of DNA 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 binding affinities of BS-I and BS-II to Pax-8 protein were determined by saturation binding experiments. The COS-7 cell extracts containing Pax-8 protein were diluted to 0.1 μg/ul of total cellular protein in a buffer containing 20 mM KCl, 20 mM HEPES (pH 7.9), 20% glycerol, 1mM DTT, 5 μg/ml of BSA, and 0.1 mM PMSF. One microliter of this diluted extract, 0.5 fmole of labeled probe, decreasing amounts of specific competitor oligonucleotides, and 1 μg of nonspecific competitor poly [d(I-C)] were incubated at room temperature for 20 min in 20 ul of a buffer containing 10 mM HEPES (pH 7.9), 100 mM KCl, 1mM EDTA, and 1 mM DTT. Following gel electrophoretic separation, the bands corresponding to protein-bound and free oligonucleotides were quantitated on a PhosphorImager, and the molar concentrations of bound and unbound specific sites were calculated, knowing the total amount of specific sites used in each reaction. The relative binding affinities (Kr), the modified values of Kd, were determined according to a modified Scatchard analysis (Emerson, et al., 1985; Calzone et al. 1988), which takes into consideration protein binding to the nonspecific competitor DNA poly [d(I-C)].

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
E10.5-E12.5 (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 E10.5 transgenic embryos carrying the $CX/lacZ$ construct, nine showed high levels of $\beta$-gal activity after 30 minutes of X-gal staining in a broad ring of cells across the mid-hindbrain 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 region. In contrast, three $lacZ$ expressing transgenic embryos carrying $XH/lacZ$ did not result in any $En-2$-like transgene expression (data not shown). These results localize the $En-2$ regulatory elements to within the 1.0 kb $CX$ fragment. 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.

Two overlapping subfragments of $CX$, $CA$ (750 bp) and $S2X$ (350 bp) were then analyzed (Fig. 1). Two copies of $S2X$ 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 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 region.

To further locate the regulatory sequences, two subfragments, $CS1$ (460 bp) and $S1S2$ (240 bp), were analyzed (Fig. 1). One copy of $CS1$ 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-hindbrain junction (Fig. 2F). Two copies, but not one copy, of $S1S2$ directed
Figure 2. Mid-hindbrain expression patterns of lacZ reporter genes directed by mouse and human En-2 genomic fragments. Sagittal (A, C, D, F, G, I) and dorsal (B, E, H) views of whole-mount E10.5 transgenic embryos stained for β-gal activity. Different mouse and human En-2 genomic fragments used to generate transgenics are indicated (see also Fig. 1.): (A, B) one copy of CX; (C) one copy of CA; (D, E) one copy of CS1; (F) two copies of CS1; (G, H) two copies of S1S2; (I) two copies of hEH.
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 region whereas neither CSI nor SI/S2, 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 E7.5 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. β-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 region (Fig. 3B). The mid-hindbrain expression continued up to E11.5 and then decreased (data not shown). By E15.5, only a few lacZ expressing cells were seen at the junction of the midbrain and cerebellum (data not
Figure 3. Comparison of the temporal and spatial patterns of expression for the *CX/lacZ* transgene and *En-2, Pax-2, Pax-5* and *Pax-8*. (A, B). Lateral views of whole-mount E8.5 (A) and E9.5 (B) *CX/lacZ* transgenic embryos stained for β-gal activity. (C-H) Lateral views of whole-mount E8.5 (C, E, F) and E9.5 (D, F, H) embryos hybridized with RNA probes of *Pax-2* (C, D), *Pax-5* (E, F), *En-2* (G) and *Pax-8* (H).
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 sequence 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 G0 transgenic embryos for lacZ expression at E10.5. 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. 21). 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.
Figure 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% similarity 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).
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 these 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 E9.5 the *CX/lacZ* and *Pax-5* expression domains were extensively overlapping in the mid-hindbrain 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 E9.0 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 Krs 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),
Figure 5A. Pax DNA-binding sites BS-I and BS-II. Top schematic 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.
Pax-2 Consensus  
TCACGCGTGAC

Pax-5 Consensus  
A
G..CA.TG..GCGTGACCA

Mouse BS-I Point Mutations  
GGAAGCAGCAGACCCGTCGGCACCACCACGGAGG

Mouse BS-II Point Mutations  
GCCACTCTTTTCACTGAGCCCATGACAAAGAGG

CG G

CAG
Figure 5B. 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.

Figure 5C. Point mutations in BS-I and BS-II abolish their binding to Pax-8 in EMSA.
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 E10.5 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 (ΔCX, Fig. 1) led to a complete loss of En-2-like lacZ expression in the mid-hindbrain region at E10.5 in all lacZ expressing G0 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, 3/8 from mutating BS-I and 1/5 from mutating BS-II) to normal expression (5/13 transgenics) (data not shown). These results suggest that both Pax-binding sites are critical for directing En-2-like transgene expression. Four transgenic lines carrying the ΔCX/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 E8.5 (Fig. 6B) to E12.5 (data not shown). These results suggest that the Pax-binding sites are important 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 in order to identify genetic events which establish regional diversity in the developing brain. Our
The Pax-binding sites, BS-I and BS-II, are required for transgene expression in the mid-hindbrain region. (A-E) Dorsal views of whole-mount transgenic embryos stained for β-gal activity. (A,C) Transgenic embryos carrying reporter gene CX/lacZ showing En-2-like mid-hindbrain expression pattern at E8.5 (A, arrow) and E 10.5 (C, arrow). (B,D) Transgenic embryos caring reporter gene ΔCX/lacZ which contains a deletion of BS-I and BS-II showing no En-2-like lacZ expression in the anterior neurepithilum at E8.5 (B, arrow) nor in the mid-hindbrain region at E10.5 (D, arrow). (E) A transgenic embryo carrying reporter gene **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 (arrow head).
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 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-2/5/8. 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 E8.5 to E11.5. This minimum control region consists of two enhancer fragments, *CSI* and *S1S2*, and one repressor fragment, *AX*. The two positive regulatory fragments appear to act cooperatively to specify an integrated transgene expression domain. Each enhancer fragment, *CSI* in one copy, or *S1S2* 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 *CSI* in two copies, act in a synergistic manner to produce a broad ring of expression across the mid-hindbrain region. 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 studies identified two Pax-binding sites in the *CSI* fragment that are required for *En-2*-like transgene expression, whereas no Pax-
binding sites were found in the $S_1S_2$ 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 were 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 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 E11.5. 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 specificities 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.,
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-2/5/8 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 E8.5 to E12.5, indicating that the Pax-binding sites are essential both for initiation and maintenance of the early phase of En-2-like expression in transgenic 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. Furthermore, it remains to determine whether these sites are essential for normal En-2 expression within the endogenous En-2 locus.

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 which are only expressed in the presumptive mid-hindbrain 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. 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; Hoop 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 inter- and intra-cellular 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.
EFERENCES


CHAPTER 3: TWO Pax2/5/8-BINDING SITES ARE REQUIRED FOR PROPER INITIATION OF ENDOGENOUS Engrailed-2 EXPRESSION IN THE MOUSE BRAIN
ABSTRACT

During early brain development En-2 exhibits spatially restricted expression in the mid-hindbrain region. Evidence from gene expression data, promoter analysis in transgenic mice and mutant phenotypic analysis has suggested a role for Pax-2, Pax-5 and Pax-8 in regulating En-2 mid-hindbrain expression. Previously, we identified two Pax-2/5/8-binding sites in a 1.0 kb En-2 early mid-hindbrain enhancer that directed β-galactosidase (β-gal) activity similar to En-2 from E8.5 to E11.5 and showed that the two Pax-2/5/8-binding sites are essential for directing mid-hindbrain expression in transgenic mice. We have now examined the functional requirements of these two binding sites in directing endogenous En-2 expression using a gene targeting approach. The two Pax-2/5/8-binding sites were deleted from the En-2 locus by homologous recombination in mouse stem cells. Embryos homozygous for this mutation had a significant reduction in En-2 expression in the presumptive mid-hindbrain region at the 5-7 somite stage. However, from E9.0 onwards, the mutant embryos showed an En-2 expression pattern comparable to that seen in wild type embryos. This result demonstrates that the two Pax-2/5/8-binding sites are required for proper initiation of En-2 expression but are not critical for En-2 expression at later stages. We also compared mid-hindbrain expression patterns of lacZ RNA and protein directed by the 1.0 kb enhancer. This comparison revealed that the lacZ protein expression pattern, assayed by β-gal activity, gave a broader expression domain than the actual RNA pattern. This suggests that lacZ protein serves as a lineage marker in the mid-hindbrain region in the sense that it persists long after its mRNA has disappeared.

INTRODUCTION

Early patterning of the vertebrate central nervous system (CNS) results in regional specialization along the anterior-posterior (A-P) axis. Genetic and experimental evidence has indicated that the midbrain and anterior hindbrain are specified as an integrated
morphogenetic field that gives rise to the midbrain structures and cerebellum. A growing number of molecules, including the transcription factors En, Pax-2, Pax-5, Pax-8, Otx-2 and Gbx-2, as well as the secreted factors Fgf-8 and Wnt-1, have been found to be involved in midbrain and cerebellum specification (reviewed in Wassef and Joyner, 1997). However, little is known about the epistatic interactions between these developmental regulators and the genetic pathways that control midbrain and cerebellum development.

The homeobox-containing mouse En genes, En-1 and En-2, were identified on the basis of their sequence homology to the Drosophila segmentation gene engrailed (en) (Joyner et al., 1985; Joyner and Martin, 1987). During brain development the two En genes are expressed in the midbrain and cerebellum in a spatially and temporally restricted manner (Davis and Joyner, 1988; Davis et al., 1988 and 1991). En-1 expression is first detected at the 1 somite stage (E8.0) in the anterior neuroepithelium, and En-2 is expressed in the same region at the 5 somite stage (E8.5). The early En expression domain marks the presumptive mid-hindbrain region. After neural tube closure, both En genes are expressed in a broad band of cells surrounding the mid-hindbrain region, and expression continues in the developing midbrain and cerebellum throughout embryogenesis. Studies of mouse En-1 and En-2 mutants have shown that both genes are required for normal development of these brain regions. Mice homozygous for an En-1 targeted mutant allele die at birth and have an early embryonic deletion that results in loss of most midbrain and cerebellar structures (Wurst et al., 1994). Mice homozygous for two En-2 targeted alleles are viable but show a 30% reduction in the size of the cerebellum and a specific cerebellar foliation defect (Joyner et al., 1991; Millen et al., 1994). The two En proteins have been shown to have similar biochemical activities in the brain, since the En-1 mutant brain phenotype can be rescued by replacing En-1 with En-2 coding sequences (Hanks et al., 1995). Furthermore, En-1/En-2 compound homozygous mutant mice have a deletion of the entire midbrain and cerebellum that is significantly more extensive than the deletion seen in En-1
homozygotes (Wurst and Joyner, unpublished), indicating that the two En genes normally have overlapping functions in the early specification of this brain region.

The vertebrate Pax genes, homologues of the Drosophila paired box-containing gene paired (prd), encode a family of transcription factors (reviewed in Gruss and Walther, 1992). Members of the Pax-5 subfamily, including Pax-2, Pax-5 and Pax-8 in mouse as well as pax-b in zebrafish, show overlapping expression with En in the mid-hindbrain region. In mouse, Pax-2 expression is detected in the presumptive mid-hindbrain region at the pre-somite stage, preceding En-1 expression (Rowitch and MacMahon, 1995). Pax-5 expression (Adams et al., 1992; Asano et al., 1992) initiates at about the same time as En-2 followed by Pax-8 activation at about the 12 somite stage (Stoykova and Gruss, 1994, Song et al., 1996). From E9.0 to E12.5, the three Pax genes are expressed in domains that overlap with those of the En genes in the developing midbrain and anterior hindbrain. Functional disruption of Pax-2, Pax-5 and pax-b causes deletion and/or malformation of the mid-hindbrain region and its derivative structures. Two different brain phenotypes have been reported for two Pax-2 mouse mutant alleles. Homozygotes for a point mutation allele, Pax-2^{INue}, have an early deletion of the mid-hindbrain region (Favor et al., 1996), which is very similar to the En-1 mutant brain phenotype. Mice mutant for a targeted N-terminal deletion Pax-2 allele have an open neural tube and show exencephaly in the midbrain (Torres et al., 1996). Pax-5 homozygous mutant mice, like En-2 mutants, have a relatively mild brain phenotype showing a partial deletion of the inferior colliculus (Urbanek et al., 1994). The pax-b gene, the zebrafish homolog of the mouse Pax-5 subfamily, shows restricted expression in the mid-hindbrain region in zebrafish embryos (Krauss et al., 1991), and has been shown to play an important role in development of this brain region. Zebrafish embryos homozygous for a pax-b mutant allele, no isthmus (noi), fail to form the mid-hindbrain junction region and have a deletion of dorsal midbrain structures and the cerebellum (Brand et al., 1996). A similar phenotype was elicited by
injection of pax-b antibodies into zebrafish embryos at the two cell stage (Krauss et al., 1992).

The overlapping expression patterns of the En and Pax genes and the similarities of their mutant brain phenotypes suggest that these genes belong to the same genetic pathway controlling early development of the mid-hindbrain region. Furthermore, genetic analyses in Drosophila have indicated that prd is an important upstream activator of en (reviewed in Hooper and Scott, 1992). Our previous biochemical and transgenic analysis demonstrated that proteins of the Pax-5 subfamily are involved in regulating early En-2 mid-hindbrain expression (Song et al., 1996). A 1.0 kb Clal-Xhol En-2 mid-hindbrain enhancer fragment (En-2CX) was shown to be sufficient to produce lacZ expression in a similar domain to endogenous En-2 in transgenic embryos between E8.5 and E12.5. Two DNA sites that specifically bind to Pax-2, Pax-5 and Pax-8 proteins were identified within the En-2CX enhancer fragment. Deletion of these two Pax-2/5/8-binding sites from the En-2 enhancer abolished the En-2-like transgene expression in the mid-hindbrain region in E8.5 to E12.5 transgenic embryos. These results provided strong molecular and genetic evidence that the Pax genes are immediately upstream of En-2 in the genetic cascade controlling early regional development of the mouse midbrain and anterior hindbrain. The noi mutant provided further genetic evidence that Pax-5 family proteins regulate En and Wnt genes in other vertebrates, since in these mutants En and Wnt expression is not initiated (Brand et al., 1996).

In the present study, we further tested the functional requirement of these two Pax-2/5/8-binding sites in regulating endogenous En-2 expression through a gene targeting approach. A targeted deletion of the two Pax-2/5/8-binding sites in the En-2 locus was generated by homologous recombination in mouse embryonic stem (ES) cells. In situ hybridization analysis of En-2 RNA from the mutant allele showed that deletion of the two DNA-binding sites greatly reduced the number of cells expressing En-2 in the presumptive mid-hindbrain region at the 5-7 somite stage, demonstrating that these two Pax-2/5/8-
binding sites are required for proper initiation of En-2 expression. However, this deletion mutation had no obvious effect on En-2 expression in the midbrain and cerebellum at later stages (E9.0 onwards). These results suggest that the Pax-2/5/8-binding sites are not critically required for maintaining En-2 expression, and that other regulatory sequences, possibly including additional Pax-binding sites, are required to fully activate and then maintain En-2 expression. Consistent with these results, re-evaluation of lacZ RNA expression, rather than β-galactosidase (β-gal) protein activity, from the En-2CX/lacZ transgene showed that the 1.0 kb En-2CX enhancer fragment can drive En-2-like transcription most accurately only at E8.5 but not later, stages.

MATERIALS AND METHODS

Construction of targeting vector

A 9.6 kb EcoRI-XhoI En-2 5' genomic fragment was isolated from a 129/VJ mouse genomic library and was used for constructing the targeting vector. The genomic fragment was first cut into three subfragments (Fig. 1A, top line), EcoRI-XhoI (1.6 kb), XhoI-AccI (640 bp) and AccI-XhoI (7.4 kb), and subcloned into pBluescript II KS+ (Stratagene). A 66 bp region, which contains the two adjacent Pax-2/5/8-binding sites, was deleted from the XhoI-AccI fragment using the polymerase chain reaction (PCR) (Song et al., 1996) with two pairs of En-2 specific primers: sense strand 5'-CCCGCATGCACAC and antisense strand 5'-GAGGCATGCAAGTTGC; and sense strand 5'-GCAGACCCGGGCA and antisense strand 5'-CCACTCTTCAGCTGAG. A SphI site was introduced at the site of the deletion. The XhoI-SphI and SphI-AccI fragments were ligated to the EcoRI-XhoI fragment and AccI-HindIII fragment (6.8 kb), respectively. The resulting 1.8 kb EcoRI-SphI and 7.2 kb SphI-HindIII fragments were subcloned into pBluescript II KS+ (Stratagene) plasmids and they were used as the 5' and
**Figure 1.** Targeted deletion of the Pax-2/5/8-binding sites in the En-2 regulatory region by homologous recombination using the cre-\(loxP\) system. (A) Top line, schematic of En-2 genomic locus. The En-2 exons are represented as boxes with coding regions filled. Ovals represent the approximate positions of the two Pax-binding sites. Second line, linearized \(En-2^{pbdneo}\) targeting vector. The selectable neomycin resistance (PGKNeo) and thymidine kinase (PGKT) cassettes are shown in open boxes. The locations of the \(loxP\) sites flanking the PGKNeo cassette are depicted by arrow heads. Dashed lines indicate the regions of homology between the locus and the targeting vector. Third lines, schematic of the expected gene replacement at En-2 regulatory region. Bottom line, schematic of the expected excision of PGKNeo by cre. After excision of PGKNeo, a single \(loxP\) site was left in the locus and an additional HindIII site (H) was introduced, which was used for genotyping. Approximate positions of the 5' and 3' probes used to identify the targeting event are shown on the top. Approximate positions of the PCR primers used for genotyping are shown (a and b). (B,C) Southern blot analysis of genomic DNA from control (W4) and targeted (W4-20) ES cells. (B) DNA was digested with KpnI and detected by the 5' probe, giving a 4.5 kb wild type fragment and a 6.5 kb targeted fragment. (C) DNA was digested with BamHI and detected by the 3' probe, giving a 8.5 kb wild type fragment and a 10.5 kb targeted fragment. (D) PCR genotyping of yolk sacs of embryos from heterozygous intercrosses of \(En-2^{pbdneo}\) heterozygous mice. PCR amplified a 382 bp wild type fragment and a 230 bp mutant fragment. (E) PCR genotyping of yolk sacs of embryos from heterozygous intercrosses of \(En-2^{pbd}\) heterozygous mice. PCR amplified a 382 bp wild type fragment and a 436 bp mutant fragment which contains the additional HindIII site. Half of the PCR products were cut with HindIII, and the mutant fragment was cut into two subfragments of 224 bp and 212 bp, respectively. A, AccI; B, BamHI; C, ClaI; E, EcoRI; H, HindIII; K, KpnI; X, XbaI; Xh, XhoI; uc, uncut; ct, cut.
3' arms, respectively, to construct the targeting vector. The loxP/PGKNeo/PGKTK vector, in which the PGKNeo cassette is flanked by two loxP sites (Hanks et al., 1996), was used to build the targeting vector. The two arms were cloned on either side of the loxP/PGKNeo cassette, with the neo gene in the same transcriptional orientation as En-2 (Fig. 1A, second line). After correct targeting, the 66 bp region, which contains the two Pax-2/5/8-binding sites, should be replaced by loxP/PGKNeo (Fig. 1A, third line). A targeted allele with such sequences is referred to as En-2pbd (En-2 Pax-2/5/8-binding sites deletion). Following cre mediated excision of the PGKNeo cassette from the targeted locus, a single loxP site (34 bp) and a 86 bp polylinker sequence containing an extra HindIII site should be left in the targeted locus (Fig. 1A, bottom line). The resulting allele is referred to as En-2pbdneo (En-2 Pax-2/5/8-binding sites deletion with neo).

**Generation of En-2pbdneo and En-2pbd mice**

Targeted ES cell lines were produced by electroporating NotI linearized targeting vector into R1 (Nagy et al., 1993) ES cells and a new line of 129/SvEv-derived ES cells, W4, established in the Joyner laboratory (W. Auerbach and Joyner, unpublished). The ES cell culture, selection and electroporation were carried out as described (Wurst and Joyner, 1993). ES cell colonies that were resistant to G418 and Gancyclovir were analyzed by Southern blot analysis for homologous recombination events (Fig 1B and C).

Chimeras were generated by aggregating ES cells, obtained from 3 R1-derived and 3 W4-derived independently targeted cell lines, with diploid CD1 morulae (Wood et al., 1993). R1-derived and W4-derived chimeric males, which had 50-100% ES cell contribution to their coat color, were bred to 129/SvEv females to establish F0 heterozygotes. Homozygotes were generated by interbreeding heterozygotes and then breeding homozygotes to either homozygous or heterozygous animals. Mouse lines carrying the En-2pbdneo allele were maintained on a CD1 outbred background.
The PGKneo cassette in the targeted En-2 locus was deleted in vivo by crossing En-2pb\textit{dneo} mice with transgenic mice that express the bacterial \textit{cre} gene under the control of a cytomegalovirus (CMV) promoter (W. Auerbach and Joyner, unpublished). The En-2\textit{pad} mice produced from such crosses also carried a CMV\textit{cre} transgene. The double transgenic mice were bred to wild-type CD1 mice, and the offspring that carried only En-2\textit{pb}\textit{d} but not \textit{cre} were selected and used to establish En-2\textit{pb}\textit{d} homozygous mouse lines by interbreeding heterozygotes.

\textbf{Genotyping of wild-type, En-2pb\textit{dneo} and En-2pb\textit{d} alleles}

Genotyping of wild-type and mutant alleles was performed by Southern blot analysis using genomic DNA samples prepared from ES cells or tail tissue as described (Wurst and Joyner, 1993). Both the 5' and 3' sides of the recombinant En-2pb\textit{dneo} allele were analyzed using restriction enzyme cutting sites that flanked the predicted homologous recombination event (Fig. 1A). The 5' side was examined with KpnI digestion and probed with a 0.6 kb HindIII-XhoI internal probe, which detected a 4.5 kb wild-type fragment and a 6.5 kb mutant fragment (Fig. 1B). The 3' side was analyzed by BamHI digestion and probed with a 0.7 kb HindIII-XhoI external probe, which detected a 8.5 kb wild-type fragment and a 10.5 kb mutant fragment (Fig. 1C).

The \textit{cre}-mediated excision of the PGKNeo cassette from the targeted locus was confirmed by hybridization of BamHI-digested DNA with a neo probe (data not shown). The resulting En-2\textit{pb}\textit{d} allele was genotyped using Southern blot analysis based on the presence of an extra HindIII polylinker site that was introduced into the targeted locus (Fig. 1A). Southern blot analysis was performed on DNA digested with HindIII, using the 5' HindIII-XhoI fragment as a probe. The wild-type allele produced a 2.2 kb band, and the targeted allele generated a 0.7 kb fragment (data not shown).

Embryos from heterozygote intercrosses or homozygote-heterozygote crosses were genotyped by PCR using yolk sac DNA. Tissue samples were digested at 60°C for 6-8
hours in a non-ionic detergent proteinase K buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 2.0 mM MgCl₂, 0.1 mg/ml gelatin (Sigma), 0.45% Nonidet P-40 (Sigma), 0.45% Tween-20 (Sigma) and 0.005% proteinase K (Sigma). The digested samples were denatured at 94°C for 10 minutes and then amplified by PCR for 30 cycles (94°C, 1 minute; 55°C, 1 minute; 72°C, 1 minute). PCR amplified fragments were visualized by 2.7% agarose gel electrophoresis and ethidium bromide staining. The wild type allele was detected as a 382 bp fragment (Fig. 1D) using a pair of En-2 specific primers: sense strand 5'-CTTTGTGATGAGGCGATGTACTG-3' and antisense strand 5'-CCAATGAGCGGTGCTATAAAT-3'. In the En-2pbdneo allele, PGKNeo was inserted into the amplified region and therefore was not amplified with the PCR conditions used. The En-2pbdneo mutant allele was detected as a 230 bp fragment (Fig. 1D) using a set of neo specific primers: sense strand 5'-GAACAAGATGGATTGCACGCAG, and antisense strand 5'-TTCAGTGACAAGTCGAGCACA. The En-2pbd allele was amplified as a 436 bp fragment (Fig. 1E) using the En-2 specific primers. Furthermore, the 436 bp mutant fragment could be cut with HindIII to generate two subfragments of 212 and 224 bp (Fig. 1E).

**Whole-mount RNA in situ hybridization analysis and lacZ staining for β-gal activity**

Mid-day of the day on which the vaginal plug was detected was considered as day 0.5 post coitum in the staging of embryos. For whole mount RNA in situ hybridization, whole embryos, or dissected brains from embryos older than E10.5, were fixed overnight in 4% paraformaldehyde at 4°C. Whole-mount RNA in situ hybridization of embryos was performed as described (Parr et al., 1993), with modifications (Knecht et al., 1995). The staining patterns were observed after 1 hour of staining, and the color reaction was stopped after 6-8 hours of staining. Pictures of whole-mount RNA in situ hybridized embryos were taken after color staining was completed. Single-stranded RNA probes labeled with
digoxigenin-UTP were synthesized from linearized template DNA as directed by manufacturer (Boehringer Mannhein Biochemicals). Two En-2 probes containing a 800 bp BglII/XbaI fragment within the 3' untranslated region (Millen et al., 1994) and a 600 bp XhoI-ClaI fragment within the coding region were used for RNA in situ hybridization analyses. The two probes produced comparable En-2 RNA expression patterns in wild type embryos (Wolf and Joyner, unpublished results). The BglII/XbaI fragment was employed in experiments using the En-2<sup>pb<sub>d</sub>neo</sup> and En-2<sup>pb<sub>d</sub></sup> alleles. The XhoI-ClaI fragment was employed in experiments using the En-2<sup>pb<sub>d</sub>/ntd</sup> allele since this probe is derived from sequences deleted from the En-2<sup>ntd</sup> allele (Millen et al, 1994). The <i>lacZ</i> probe contained a 800 bp EcoRV/SacI fragment (Kalnins et al., 1983). β-gal activity was assayed by whole-mount X-gal (5-bromo-4-choro-3-indolyl β-D-galactopyranoside) staining as described (Logan et al., 1993).

RESULTS

Generation of mice with a targeted deletion of two Pax-2/5/8-binding sites in the <i>En-2</i> locus

In order to determine whether the two essential Pax-2/5/8-binding sites identified in a 1.0 kb enhancer fragment in our transgenic experiments were also necessary for expression of the endogenous <i>En-2</i> gene in the normal genomic environment, we generated mice carrying a targeted deletion of the two Pax-binding sites by homologous recombination in ES cells (Mansour et al., 1988). A positive/negative targeting vector containing 1.8 kb of 5' and 7.2 kb of 3' <i>En-2</i> genomic sequences was constructed to replace the two adjacent DNA-binding sites (66 bp) with a loxP/PGKneo cassette (Fig. 1A).

In this study we used two ES cell lines, R1 (Nagy et al., 1993) and W4. Since this was the first time that the 129 SvEv(Taconic)-derived W4 cell line was used to generate a
Table I. Generation of targeted ES cells, chimeras and germline transmission of \textit{En-2^{pbdneo}} using R1 and W4 ES cells

<table>
<thead>
<tr>
<th>ES cell line</th>
<th># of cells electroporated</th>
<th># of Neo/Neo+GC resistant colonies</th>
<th># of targeted colonies</th>
<th>ES cell lines aggregated</th>
<th># of chimeras generated</th>
<th># of germline transmitters</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>2X10^7</td>
<td>602/102</td>
<td>8</td>
<td>1-4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14-3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11-4</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>W4</td>
<td>2X10^7</td>
<td>130/142</td>
<td>9</td>
<td>5-4</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17-7</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20-5</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

Neo: Neomycin; GC: gancyclovir
targeted allele, we compared the W4 cell line with the well-established R1 ES cell line in its frequency of targeting events, chimera generation and germline contribution (Table I). Approximately 2X10^7 cells from each ES cell line were electroporated with 400 ug of vector DNA followed by G418 (positive) and Gancyclovir (negative) selection (Wurst and Joyner, 1993). One hundred and two R1 and 142 W4 double-resistant colonies were generated and examined by Southern blot analysis (see Materials and Methods). Eight R1 and 9 W4 double-resistant colonies were identified that had undergone the predicted homologous recombination replacement event to produce the mutant allele, En-2pbdneo. Three R1 and 3 W4 targeted cell lines were used to generate chimeras. Twenty eight chimeric mice, 14 R1-derived and 14 W4-derived, with 50-90% ES cell contribution to the coat color were produced. All these chimeras were tested for germline transmission, and 4 R1-derived and 5 W4-derived chimeras transmitted the En-2pbdneo allele to their offspring. These results demonstrated that the two ES cell lines had comparable overall efficiencies in generating the En-2pbdneo targeted mouse allele. Two R1-derived and two W4-derived chimeric males were bred to 129/SvEv females to establish F0 heterozygotes. Subsequently, the 4 targeted mouse lines were kept on a CD1 outbred background. Intercrossing of heterozygotes produced En-2pbdneo homozygous mice that were viable and fertile. This was expected since En-2 null homozygotes are viable and fertile (Joyner et al, 1991). The analysis reported here is primarily from one W4 mouse line (W20-5).

**En-2 expression in En-2pbdneo mutant embryos**

To determine whether the two Pax-2/5/8-binding sites were required for expression of the endogenous En-2 gene, we examined En-2 expression in E8.5-E17.5 En-2pbdneo homozygous mutant embryos by RNA in situ hybridization analysis. In wild type embryos, the En-2 gene is first detected in two triangular patches of cells within the anterior neuroepithelium at the 5-7 somite stage (E8.5; Fig. 2A). After the neural tube closes (E9.5-E10.5) En-2 is expressed in a broad band of cells spanning the mid-hindbrain region.
Figure 2. Whole-mount in situ analysis of $En$-2 expression patterns in wild type embryos (A,C,E) and $En$-2$^{pbdneo/bdneo}$ mutant embryos (B,D,F) at E8.5 (A,B, dorsal view), E9.5 (B,C, lateral view) and E10.5 (E,F, lateral view).
In E8.5 En-2pdneo homozygous mutant embryos (n=5), En-2 RNA expression (Fig. 2B) was significantly weaker than that seen in wild type embryos. In E9.5 (n=6) and E10.5 (n=6) homozygous mutant embryos, the En-2 expression was essentially normal in pattern, but a reduction in strength of expression was consistently observed (Fig. 2D,F). No change in En-2 expression was observed in E12.5 (n=3), E15.5 (n=3) and E17.5 (n=3) homozygous mutant embryos as compared to wild type (data not shown).

**En-2 expression in En-2pd mutant embryos**

The reduction in En-2 expression observed in En-2pdneo mutants could result from the deletion of the two Pax-2/5/8-binding sites. Alternatively, or in addition, it could be due to the insertion of the PGKneo cassette into the En-2 regulatory region. Interference by an inserted neo promoter on an adjacent gene's promoter has been reported in other loci (reviewed in Olson et al., 1996). To address this issue, the En-2pdneo mice were crossed with transgenic mice carrying a CMV/cre transgene (see Materials and Methods) to obtain in vivo cre-loxP-mediated excision of the PGKneo cassette. In the double transgenic offspring from such crosses, the targeted En-2 allele, En-2pd, should contain a single 34 bp loxP site plus 86 bp of polylinker sequences in place of the 66 bp region containing the two Pax-2/5/8-binding sites (Fig. 1A). En-2pd heterozygotes were intercrossed to produce mice homozygous for the En-2pd allele that were viable and fertile. En-2 expression in En-2pd homozygous mutants was analyzed from E8.5 to E17.5 by RNA in situ hybridization analysis. In wild type embryos En-2 expression is activated in the anterior neuroepithelium at the 5-7 somite stage (E8.5, Fig. 3A). In contrast, in the En-2pd homozygous embryos of the same stage (n=6) only weak En-2 RNA signals were detected in the most anterior part of the normal En-2 expression domain (Fig. 3B). As development proceeded, at the 11-14 somite stage (E9.0), En-2 expression in mutant embryos (n=3, Fig. 2D) appeared similar to that seen in the wild type embryos (Fig. 3C).
Figure 3. *En-2* expression pattern in homozygous *En-2*mutant embryos. Whole-mount in situ analysis of *En-2* expression patterns in wild type embryos (A,C,E) and *En-2*mutant embryos (B,D,F) at E8.5 (A,B, dorsal view), E9.0 (C, D, lateral view) and E9.5 (E,F, lateral view).
Later in development, at E9.5 (n=4, Fig. 3E,F), E10.5, E12.5 and E17.5 (n=3 for each stage, data not shown) no detectable differences in En-2 expression patterns were observed between mutant and wild type embryos. These results indicate that deletion of the Pax-2/5/8-binding sites affected initiation of En-2 expression but not later expression, and that the reduction of En-2 expression observed in E9.5-E10.5 En-2pbdneo homozygous embryos was due to the presence of PGKNeo in the En-2 regulatory region.

**Brain morphologies of En-2pbdneo/pbdneo, En-2pbd/pbd and En-2pbd/ntd mutants**

Ablation of En-2 expression specifically affects cerebellum development (Joyner et al., 1991; Millen et al., 1994). In homozygous En-2 mutant mice (En-2ntd), the size of the cerebellum is reduced and abnormal foliation is seen in the posterior cerebellum. Heterozygous En-2 animals have a morphologically normal brain. To test whether the early reduction in En-2 expression levels in the presumptive mid-hindbrain region of En-2pbd/pbd and En-2pbdneo/pbdneo mutant causes any adult brain phenotype, we analyzed the brain morphologies of animals homozygous for either mutation. No significant brain morphological changes were observed in these animals (En-2pbd/pbd, n=4; En-2pbdneo/pbdneo, n=6; data not shown). Moreover, to further decrease the En-2 expression level, we crossed En-2pbdneo mice with En-2ntd mice. The brain morphologies of the resulting transheterozygous mutant mice En-2pbdneo/ntd also appeared normal (n=4, data not shown). These results suggest that a more than 50% reduction in the En-2 expression levels between E8.5 and E10.5 does not affect normal cerebellar development.

**Expression patterns of endogenous En-2 RNA, En-2CX/lacZ RNA and En-2CX/lacZ β-gal activity**

Our En-2pbd gene targeting experiments showed that deletion of the Pax-2/5/8-binding sites from the endogenous En-2CX enhancer region only transiently affected
initiation of \textit{En-2} expression but had no detectable effect on \textit{En-2} expression at later stages. This is apparently a different result from what we obtained in our previous transgenic analysis in which deletion of the two Pax-2/5/8-binding sites in the context of the 1.0 kb \textit{En-2CX} enhancer completely abolished the enhancer activity in directing \textit{En-2}-like early embryonic \textit{lacZ} expression from E8.5 to E12.5 (Song et al., 1996). There are several possible explanations for this difference (see Discussion), one of them being that the \textit{En-2CX} enhancer does not actually act in an \textit{En-2}-like manner as shown by \textit{β-gal} activity.

A recent transgenic experiment in our laboratory suggested that the transcriptional control directed by the mid-hindbrain brain enhancer fragments did not mimic endogenous early embryonic \textit{En-2} expression. A 2.5 kb \textit{ClaI-EcoRI (CE)} \textit{En-2} enhancer fragment, which contains the 1.0 kb \textit{CX} fragment, was used to direct \textit{En-2} cDNA expression (\textit{En-2CE/En2}) in an attempt to rescue the \textit{En-2} null (\textit{En-2\textsuperscript{ntd}}) mutant phenotype (Wolf and Joyner, unpublished). In the \textit{En-2\textsuperscript{ntd/ntd}} mutants carrying the \textit{En-2CE/En2} transgene, the domain of transgene expression at E9.5 and E10.5, as detected by \textit{En-2} RNA \textit{in situ} hybridization analysis, was found to be significantly smaller than the X-gal staining pattern seen in \textit{En-2CE/lacZ} transgenic embryos. Furthermore, in another transgenic experiment the \textit{En-2CE} enhancer was found to direct \textit{cre} RNA expression in a similar narrow domain in the mid-hindbrain junction region (Zinyk et al, 1998). Two factors could account for this discrepancy: RNA \textit{in situ} hybridization analysis could be much less sensitive than \textit{β-gal} activity in detecting transgene expression; alternatively, \textit{lacZ} RNA and/or \textit{lacZ} protein are more stable than \textit{En-2} or \textit{cre} RNA. If the latter is the case, it is possible that \textit{β-gal} activity would be detected in cells that express the transgene at the stage of analysis as well as in cells that have expressed the transgene many hours before. This raised an important question as to whether the \textit{lacZ} staining pattern previously observed in the \textit{En-2CX/lacZ} transgenic embryos only reflected \textit{de novo} transgene expression or was also acting as a short term lineage marker for cells that had expressed \textit{lacZ} RNA/protein.
To address this issue, we compared the patterns of expression of lacZ RNA and lacZ protein directed by the same transgene. Transgenic embryos derived from mice homozygous for one of our En-2CX/lacZ transgenic lines were obtained and half of the embryos were assayed by RNA in situ hybridization and the other half by X-gal staining. In order to compare the sensitivities of the RNA in situ hybridization and X-gal staining methods, we examined the expression patterns of lacZ RNA and X-gal staining at E8.5, since at this stage the transgene expression is first initiated and thus there should be no issue of RNA and protein stability. At this stage the staining patterns of lacZ RNA (n=4, Fig. 4B) and X-gal (n=4, Fig. 4C) were quite similar to each other, indicating that these two methods have comparable sensitivities. However, at E9.0 (11-13 somites) the expression domain of En-2CX/lacZ RNA (n=4, Fig. 4E) was significantly smaller than the En-2CX/lacZ X-gal staining pattern (n=5, Fig. 4F), and the difference became even more pronounced at E9.5 (n=4 for each group, Fig. 4H,I). At E10.5 and later, only a small number of transgene expressing cells could be detected at the dorsal mid-hindbrain junction region by RNA in situ analysis (n=3, Fig. 4K). However, a broad band of cells spanning the mid-hindbrain region were still detected by X-gal staining (n=2, Fig. 4L). These results demonstrate that lacZ protein has a significantly longer half life than lacZ RNA.

Similar results were also obtained in experiments performed with embryos from a different transgenic line (En-2CE/lacZ) in which the lacZ reporter gene is directed by the 2.5 kb En-2CE enhancer fragment, the enhancer used to direct En-2 and cre expression described above. It is worth noting that the mRNA expression patterns of lacZ, En-2 and cre directed by the 2.5 kb En-2CE enhancer were very similar to each other at E9.5 and E10.5, indicating that the lacZ mRNA has an half life similar to those of other mRNAs.

Taken together, data from the comparative analyses of RNA and protein expression indicate that β-gal activity may not accurately reflect transcription from the transgene at any given stage, and that lacZ RNA transcripts appear to be a better indicator of a lacZ transgene expression pattern. We therefore re-evaluated the regulatory activity of the
En-2CX enhancer by comparing the expression of En-2CX/lacZ RNA and endogenous En-2 RNA. At E8.5, En-2CX/lacZ RNA (n=4, Fig. 4B) and En-2 RNA (Fig. 4A) showed very similar expression patterns. At E9.0 (n=4) and E9.5 (n=3), the En-2lacZ RNA expression domains (Fig. 4E,H) were significantly smaller than the endogenous En-2 expression pattern (Fig. 4D,G). At E10.5 (n=3, Fig. 4K) and E12.5 (data not shown) the transgene expression was restricted to a small dorsal patch at the mid-hindbrain junction region, but at these stages endogenous En-2 expresses broadly in the mid-hindbrain region (E10.5, n=4, Fig. 4J). These results indicate that the regulatory elements contained in the 1.0 kb En-2 enhancer are sufficient to initiate transgene expression in an En-2-like manner. The regulatory sequences are also capable of directing transgene expression to the mid-hindbrain junction region up to E12.5. However, they are not sufficient to direct expressions in a pattern that closely mimics the endogenous En-2 expression pattern from E9.0 onwards. This conclusion is consistent with our gene targeting experiment in that the main regulatory requirement of the 1.0 kb enhancer, which is dependent on the two Pax2/5/8-binding sites, is to initiate En-2 mid-hindbrain expression.

**DISCUSSION**

In this study we tested the functional requirement of the two Pax-2/5/8 DNA-binding sites in a 5' En-2 enhancer region for regulating endogenous En-2 expression using a Cre-loxP gene targeting strategy. Deletion of the two binding sites from the En-2 locus disturbed initiation of En-2 expression in the presumptive mid-hindbrain junction region at the 5-7 somite stage. This demonstrates a role for the two DNA-binding sites in activating En-2 transcription. This mutation, however, did not completely abolish En-2 activation at E8.5, nor did it affect expression at later stages, demonstrating that these binding sites alone are not critically required for En-2 expression. Since this result appears different from the one obtained in our previous transgenic study, we re-evaluated the expression pattern of a lacZ reporter gene directed by the enhancer fragment containing the
Pax-2/5/8 binding sites using RNA *in situ* hybridization analysis. The lacZ RNA expression was found to be similar to that of endogenous *En-2* RNA only at E8.5. In contrast, unlike β-gal activity, lacZ RNA was only detected in a much more restricted domain than normal *En-2* RNA at later stages. This comparative expression analysis indicates that the stable lacZ protein was acting as a transient lineage marker, and as such led to an over-representation of the cell population transcribing the lacZ reporter gene. Thus, the *in vivo* gene targeting results of deletion of Pax-2/5/8-binding sites are consistent with the actual regulatory function of the *En-2* enhancer fragment that requires the Pax-2/5/8-binding sites for activity.

**Pax-2/5/8-binding sites are involved in initiation of *En-2* expression but are not essential for early embryonic *En-2* expression**

During development, *En-2* shows spatially and temporally regulated expression in the presumptive midbrain and cerebellum. *En-2* expression can be divided into at least three phases: early embryonic regionally restricted expression in the mid-hindbrain region (E8.5-E12.5); late embryonic expression in specific cell-types in the midbrain and domain-restricted cerebellar expression (E12.5-first week postnatal); and adult cell-type-specific expression in the midbrain structures and the cerebellum. Our previous transgenic analyses have shown that the different phases of *En-2* expression involve different sets of cis-acting DNA regulatory elements. A 9.5 kb genomic fragment is sufficient for conferring an *En-2*-like expression pattern throughout development (Logan et al., 1993). A 1.0 kb subfragment from the 9.5 kb region, located 5.8 kb 5' of the start of *En-2* transcription, seemed initially to be capable of initiating and maintaining the early transgene expression in the mid-hindbrain region (up to E11.5), but not for later phases of expression (Song et al., 1996). A systematic functional analysis of the 1.0 kb enhancer in transgenic mice revealed that the 1.0 kb enhancer contains multiple positive and negative DNA regulatory elements: a 5' 460 bp *En-2* specific mid-hindbrain enhancer element, a middle 240 bp general enhancer
element and a 3' 300 bp repressor element, that all function in concert to establish the early mid-hindbrain expression. *In vitro* biochemical and sequence analyses of the 1.0 kb enhancer identified two Pax-2/5/8-binding sites located in the 5' *En-2* mid-hindbrain specific enhancer element. Mutagenesis and transgenic assays showed that the Pax-2/5/8-binding sites are essential for the regulatory activity of the 1.0 kb enhancer (Song et al., 1996).

In the present study we further addressed the functional role of the Pax-2/5/8-binding sites in directing endogenous *En-2* expression. In *En-2phd* homozygous mutant embryos that lack these sites, *En-2* expression was activated at the right time, the 5-7 somite stage (E8.5), but its expression domain in the presumptive mid-hindbrain region was greatly reduced as compared to wild type embryos. This effect was transient, since by the 11-14 somite stage (E9.0) onwards the mutant and wild type embryos showed comparable *En-2* expression patterns. These results demonstrate that these Pax-2/5/8-binding sites are involved in initiation of *En-2* expression but are not critically required for maintaining early *En-2* expression.

During early mouse brain development, expression of *Pax-2*, *Pax-5* and *Pax-8* significantly overlaps with *En* expression in the mid-hindbrain junction region from E8.5 to E12.5. Moreover, in zebrafish *eng-2* expression is completely lost in *noi* mutant embryos that contain a mutation in *pax-b* (Brand et al., 1996), demonstrating that the *Pax* genes play an important role in *En* expression in the mid-hindbrain region of another vertebrate. Since deletion of the two Pax-2/5/8-binding sites from the mouse *En-2* locus only has an effect on initiation of *En-2* expression, it seems likely that there are other Pax-2/5/8-binding sites in the *En-2* locus located outside the 1.0 kb enhancer region that play a role in *En-2* transcription. Consistent with this, our previous transgenic study (Song et al., 1996) showed that a single copy of the 460 bp 5' *En-2* specific enhancer element, which contains the two Pax-2/5/8-binding sites, could only direct a low level of β-gal activity in a very restricted domain in the dorsal mid-hindbrain junction. However, a high level of β-gal
activity, which gave rise to a broad mid-hindbrain domain of expression, was conferred by two copies of the enhancer element or by the combination of the 5' En-2 specific enhancer element with the middle 240 bp general transcriptional enhancer element. These transgenic results indicate that during normal development the endogenous En-2 mid-hindbrain expression is established through the cooperative actions of multiple enhancer elements that likely include many Pax-binding sites.

**Insertion of PGKNeo in the En-2 regulatory region interferes with the early embryonic En-2 expression in the mid-hindbrain region**

In the homozygous En-2\textsuperscript{pbdneo} mutant embryos a decrease in the level of En-2 expression was observed from E8.5 to E10.5, indicating that both initiation and early maintenance of En-2 expression were affected. The later effect appears to be mainly a result of the insertion of PGKNeo, rather than the deletion of the of the Pax-2/5/8-binding sites, because such an effect on En-2 expression was not seen in the En-2\textsuperscript{pbd} mutant allele in which PGKNeo was excised from the targeted locus. There is increasing evidence from other experiments that insertion of PGKNeo into a locus can perturb expression of neighboring genes (reviewed in Oselin et al., 1996). It has been suggested that PGKNeo may disrupt chromatin configuration, interfere with the function of cis-regulatory elements and/or compete with the promoters of neighboring genes for basic transcription factors. For example, in a study of β-globin regulation (Fiering et al., 1995), a targeted deletion of the 5' DNase hypersensitive site 2 was generated in the locus control region of the β-globin locus and a significant change in β-globin gene expression was observed when the PGKNeo selection cassette was present in the targeted locus. However, after removal of PGKNeo, β-globin expression became essentially normal. Our study again emphasizes that in order to obtain an accurate analysis for the effect of a deletion of regulatory sequences, it is important to remove any selection cassette from the targeted locus, even if it is located some distance from the start of transcription.
The *En-2CX/lacZ* transgene expression domain detected by X-gal staining was significantly broader than that detected by RNA *in situ* hybridization analysis.

In this study we compared the developmental expression profiles of lacZ RNA and protein directed by the 1.0 and 2.5 kb *En-2* enhancer fragments. At E8.5, when the transgene was first expressed, the patterns of RNA expression and X-gal staining were quite similar. However, from E9.0 onwards, the transgene expression domain detected by X-gal staining was significantly broader than that detected by RNA *in situ* analysis. The difference became more pronounced as development proceeded. At E10.5, a broad band of X-gal staining cells was still readily seen in the mid-hindbrain region, whereas only a very narrow band of cells were positive for *lacZ* RNA. The difference in expression patterns is not likely to be primarily due to different sensitivities of the two detection methods, because both methods detected similar transgene expression patterns at E8.5 when transgene expression is initiated. In addition, the 2.5 kb *En-2* enhancer has been used in other transgenic studies for directing expression of *En-2* and *cre*, respectively (Wolf and Joyner, unpublished results; Zinyk et al., submitted). The expression patterns of these transgenes, analyzed by RNA *in situ* hybridization, were very similar to the *lacZ* RNA pattern in the *En-2CX/lacZ* transgenic embryos, indicating that the half life of *lacZ* mRNA is comparable to those of *En-2* and *cre* mRNAs. Thus, the most likely explanation for the difference in *lacZ* RNA and protein is that *lacZ* protein has a longer half life than *lacZ* RNA.

From E8.5 to E10.5, the mid-hindbrain region, like other brain regions, undergoes rapid cell proliferation. It has been shown in the chick that there are morphogenetic movements of cells in the isthmus region that translocate dorsal cells anteriorly and posteriorly for long distances (Millet et al., 1996). The broad X-gal staining pattern observed with the *En-2CX/lacZ* transgene likely reflects similar morphogenetic movements in the mouse with stable *lacZ* protein acting as a transient lineage marker. These results are consistent with fate mapping studies from our laboratory in which the fates of cells
expressing cre from the En-2 mid-hindbrain enhancer were analyzed using a site specific recombination approach (Zinyk, et al, submitted). Consequently, X-gal staining marks two different cell populations, the cells that express the transgene at the stage analyzed and the cells that have expressed the transgene previously. This leads to X-gal staining giving an apparently broader transgene expression domain and longer expression time in the mid-hindbrain region than actual transcription.

Our comparative expression analysis raises several important issues regarding the use of X-gal staining, a very commonly used method, to assay lacZ reporter transgene expression in vivo. First, it is not suitable for determining when transcription of a transgene is turned off due to the long half life of lacZ protein. Second, caution should be taken in interpreting a pattern of transgene expression in a region that has rapid cell proliferation and/or substantial cell movement because in these situations X-gal staining acts as a lineage marker rather than a reflection of de novo reporter gene expression. A possible example of this situation comes from a study of Wnt-1 regulatory sequences in which a Wnt-1/lacZ transgene was found to be expressed in migrating neural crest cells whereas Wnt-1 RNA was only detected in the dorsal neural tube (Echelard et al., 1994). In this case, lacZ RNA in situ hybridization analysis could be employed to determine whether the X-gal expression pattern came from de novo activation of the transgene in the migrating cell population or resulted from a persistence of lacZ protein in neural crest cells after they migrated out from the dorsal midline.

Due to our finding that lacZ protein is not a reliable marker for transcription, it is necessary to re-evaluate the regulatory activity of the 1.0 kb En-2 mid-hindbrain enhancer using RNA in situ hybridization analysis. According to the lacZ RNA expression pattern, the 1.0 kb enhancer contains sufficient regulatory sequences to initiate En-2-like transgene expression in the presumptive mid-hindbrain region at E8.5 and to direct transgene expression in the correct spatial domain surrounding the mid-hindbrain junction up to E12.5. However, from E9.0 to E12.5 the transgene expression domain is not as broad as
the endogenous *En-2* expression domain, indicating that the 1.0 kb enhancer only contains a subset of the regulatory elements that are responsible for early embryonic *En-2* expression in the brain. This regulatory activity profile is in agreement with the result obtained from our gene targeting experiment in that only the initiation of *En-2* expression and not later expression is affected by deletion of the Pax-2/5/8-binding sites.
REFERENCES


CHAPTER 4: GENERAL DISCUSSION
GENERAL DISCUSSION

Summary
A combination of molecular genetic, biochemical, and embryological assays has begun to reveal the identity and function of molecules that control development of the mesencephalon and metencephalon, the primordia of the adult midbrain and cerebellum, respectively. The mouse En and Pax genes, which encode transcription factors, display spatially and temporally restricted expression in the mid-hindbrain region which is essential for development of this region. In this thesis I have investigated how En-2 mid-hindbrain expression is established and the role of Pax proteins in this process. This work represents one of the first studies dissecting aspects of the genetic pathways that control early midbrain and cerebellum development.

In chapter 2, I analyzed transcriptional regulation of En-2 expression using in vitro protein-DNA binding assays and in vivo reporter gene expression in transgenic mice. A 1.0 kb minimal En-2 mid-hindbrain enhancer fragment was defined and the DNA regulatory sequences within the enhancer fragment were systematically characterized. I identified multiple positive and negative regulatory elements within the 1.0 kb enhancer fragment, and showed that these elements function cooperatively to establish an early embryonic En-2-like mid-hindbrain expression pattern. I also found that the mid-hindbrain regulatory sequences are conserved in humans. Importantly, I demonstrated that two Pax-2/5/8-binding sites in the 1.0 kb enhancer fragment are essential for the enhancer activity in transgenic mice. These results provided the first biochemical evidence that the Pax genes are directly upstream of En-2 in a genetic pathway controlling mid-hindbrain development.

In chapter 3, I studied the functional requirement of the two Pax-2/5/8-binding sites in directing endogenous En-2 expression. A mouse mutant allele, En-2pbd, carrying a deletion mutation of the two Pax-2/5/8-binding sites in the En-2 locus was generated. Examination of En-2 expression in En-2pbd homozygous mutant embryos revealed that the
two Pax-2/5/8-binding sites are required for proper activation of endogenous *En-2* expression in the presumptive mid-hindbrain region, but are not essential for further expression in this region. Morphological analysis of *En-2*<sub>pbd</sub> homozygous mutant adult brains showed no significant morphological changes as compared to wild type adult brains, indicating that the transient effect on early *En-2* mid-hindbrain expression does not perturb normal development of the mid-hindbrain region. Additionally, I re-evaluated the regulatory activity of the 1.0 kb enhancer by RNA *in situ* hybridization analysis. This analysis showed that the 1.0 kb enhancer directs an *En-2*-like transcription most accurately only during the initial phase of expression. This is in agreement with the regulatory function of the two Pax-2/5/8-binding sites within the endogenous *En-2* locus demonstrated in the gene targeting experiment.

The research presented in this thesis provided evidence for some of the molecular genetic mechanisms that regulate *En-2* mid-hindbrain expression. However, many aspects of this process remain unknown, and several new questions have been raised from this study. In this chapter, I define these issues and discuss possible future research directions.

**Multiple temporal and spatial DNA regulatory elements are involved in *En-2* expression**

During development, *En-2* exhibits a dynamic expression profile that can be divided into three phases: *early embryonic* regionally restricted expression in the mid-hindbrain region (E8.5-E12.5); *late embryonic* expression in specific cell types in the midbrain and domain-restricted expression in the cerebellum (E12.5-first week postnatal), and *adult* cell-type-specific expression in the midbrain and cerebellum. These different phases of expression correlate with different stages of midbrain and cerebellum development. Hence the regulatory program underlying *En-2* brain expression is predicted to be complex, involving multiple modes of control. Indeed, data from our transgenic analysis have shown that different *En-2* regulatory elements function to direct *En-2* expression at
different developmental stages and/or in subsets of the En-2 expression domain (Fig. 1). Previously, a 9.5 kb En-2 genomic DNA fragment, which consists of both a 5' 7.0 kb and a 3' 2.5 kb En-2 genomic fragments, was shown to be capable of conferring an En-2-like expression pattern throughout development, although the late embryonic and adult expression was variable (Logan et al., 1993). Work from this thesis has demonstrated that regulatory sequences sufficient for initiating embryonic mid-hindbrain expression are located in a 1.0 kb enhancer region within the 5' end of the 9.5 kb fragment (Song et al., 1996 and chapter 3). However, additional En-2 DNA regulatory sequences must be responsible for continuing the early embryonic expression in a broad mes-met domain as well as for the late embryonic and adult expression.

Identification of additional DNA sequences regulating early embryonic En-2 brain expression

My initial transgenic analysis showed that the En-2 1.0 kb enhancer fragment is capable of initiating and maintaining En-2-like β-gal activity during early mid-hindbrain development (E8.5-E12.5, Chapter 2). However, more recent experiments in the laboratory (Zinyk et al., 1998) indicated that this En-2 early mid-hindbrain enhancer is not sufficient to direct En-2-like transgene expression from E9.0 onwards, although it can direct expression to the mid-hindbrain junction where En-2 expression is highest. I addressed this apparent discrepancy by comparing the expression patterns of lacZ RNA and lacZ protein directed by the 5' 1.0 kb and 2.5 kb En-2 enhancer fragments (Fig. 1) in the transgenic mice (Chapter 3). The results of this expression comparison analysis demonstrated that the lacZ RNA staining pattern more precisely reflects transcription of the transgene than the β-gal activity. According to the lacZ RNA expression pattern, the regulatory sequences contained in the 1.0 kb and 2.5 kb enhancer fragments contain only
**Figure 1.** The top schematic shows the mouse *En-2* locus in which exons are represented as boxes with coding regions filled. The lower panel shows the mouse *En-2* genomic DNA fragments tested. Tg: transgene; hsp: mouse heat shock promoter; PA: SV40 poly A sequences. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hind*III; P, *Pvu*II; X, *Xba*I.
EN-2-like 7 expression pattern

Mouse EN-2

Fragment

1.0 Kb
2.5 Kb
5.5 Kb
7.0 Kb
7.0 + 2.5 Kb
110a

X E

EN-2 RNA: narrow
X-Ea: broad

EN-2 RNA: broad
X-Ea: narrow

not tested
X-Ea: narrow

not tested
X-Ea: narrow

not tested
X-Ea: broad

not tested
X-Ea: broad

neat cleave
not tested

Gale Cradwell

Mid-hindbrain
Early embryonic
Adult

late embryonic

neat cleave
a subset of the regulatory elements needed to direct the complete early (E8.5-E12.5) *En-2* expression pattern and which are mainly involved in the initiation of *En-2* mid-hindbrain expression and directing expression to the dorsal mes-met junction. Additional regulatory sequences are required for maintaining and/or expanding the early mid-hindbrain expression domain to cover much of the mes-met and not just the mes-met junction.

The earlier studies in our laboratory in which the 9.5 kb *En-2* enhancer fragment was shown to direct *En-2*-like expression throughout development and in the adult was done by assaying β-gal activity. Since RNA expression analysis is a better reflection of transcription from a transgene, it is not clear whether these sequences can direct broad mid-hindbrain expression. However, few additional *En-2* regulatory sequences are likely to be present within the 7.0 kb fragment (Fig. 1) since the RNA expression pattern of an *En-2* cDNA directed by this fragment (Wolf and Joyner, unpublished) was comparable to the *lacZ* RNA expression pattern directed by the 1.0 kb enhancer fragment. Furthermore, another *lacZ* reporter construct containing the *En-2* promoter (2.5 kb) and 3' (2.5kb) sequences did not show any specific transgene expression (Logan et al., 1993; Fig. 1). Whether the 5' end 1.0 kb and the 3' end 2.5 kb fragments could function together to direct a broader expression pattern remains to be tested. Clearly, additional *En-2* genomic DNA fragments need to be isolated and tested in transgenic animals to identify the complete *En-2* mid-hindbrain regulatory region. It is difficult to predict whether these additional mid-hindbrain regulatory sequences could function independent of the 1.0 kb enhancer and, therefore, new DNA fragments should be tested both alone and together with the 1.0 kb fragment. It is important that RNA *in situ* hybridization analysis, rather than X-gal staining, is used to analyze *lacZ* transgene expression pattern.

Once new *En-2* enhancer fragments are identified, DNase footprinting and/or band shift experiments using protein extracts isolated from E9.0-E12.5 brain tissue could be employed to identify DNA binding site(s) for potential regulatory protein(s). In addition, the DNA sequence of these fragments should be determined to search for potential binding
sites for known transcription factors, such as Pax proteins, which are normally expressed in the mid-hindbrain region. The in vivo requirements of these binding sites, either for known or unknown transcription factors, should be verified by mutation analyses in transgenic mice similar to that described in Chapter 2. The important binding sites should then be tested for their requirements in directing endogenous En-2 expression in their native chromosomal positions by a gene targeting approach, as described in Chapter 3.

**Identification of DNA regulatory sequences for late embryonic and adult En-2 cerebellum expression**

From E12.5 onwards, En-2 expression in the developing cerebellum undergoes dynamic changes (Davis and Joyner, 1988; Davis et al., 1988 and 1991). At E15.5-E17.5, En-2 shows transient expression in at least 7 sagittal stripes (Millen et al., 1994). Interestingly, a number of developmental control genes, including En-1, Pax-2, Pax-6 and Wnt-7b, are also expressed in sagittal stripes which are overlapping with or complementary to the stripes of high En-2 expression. Ongoing research in this laboratory has begun to address the functional significance of such striking spatial gene expression patterns (Campbell and Joyner, unpublished). Analysis of the underlying transcriptional regulatory mechanisms controlling these gene expression patterns should bring new insight into the molecular genetic control of cerebellum patterning. In addition, any stripe-specific enhancers that are identified should provide useful tools for directing ectopic gene expression in these cerebellar domains.

Although the 9.5 kb fragment has been shown to be able to direct the late embryonic En-2 cerebellum expression, several aspects of the transgene expression remain unclear. First, the previous transgene expression was analyzed by X-gal staining. Given the dynamic nature of En-2 expression in the developing cerebellum, it is necessary to re-evaluate the transgene expression by lacZ RNA in situ hybridization analysis. Moreover, since the preliminary cerebellum expression analysis of the transgene was performed on
sagittal sections (Logan and Joyner unpublished), it is unclear whether the lacZ transgene is in fact expressed in sagittal stripes mimicking the endogenous En-2 expression in E15-E17.5 cerebellum. Thus, to gain a more detailed three-dimensional understanding of the transgene expression pattern, the transgene expression should be re-examined in both sagittal and coronal sections, as well as in whole mount brains to compare with En-2 expression. Once a more precise transgene expression pattern is determined, further deletion mapping of the 9.5 kb fragment in transgenic animals should be carried out to delineate the enhancer elements required to achieve En-2-like cerebellum expression. Subsequently, these regulatory elements should be analyzed by DNase footprinting and/or band shift experiments using protein extracts isolated from cerebellar and non-cerebellar brain tissues at appropriate developmental stages to identify binding site(s) for potential regulatory protein(s). Since Pax-2, Pax-3 and Pax-6 are expressed in patterns that overlap with, or are complementary to, the sagittal stripes of En-2 expression (Millen et al., 1995), they may regulate En-2 expression at this stage. DNA binding consensus sequences for these Pax proteins have been characterized (Czerny et al., 1993; Epstein et al., 1994; Chalepakis and Gruss, 1995, Song et al., 1996), making it possible to identify DNA binding sites for these Pax proteins and test their binding capacity.

Although the 9.5 kb fragment contains regulatory sequences for adult En-2 cerebellum and midbrain expression, the transgene expression pattern was not entirely reproduced consistently in the different transgenic lines that were analyzed (Logan et al., 1993), indicating there are additional En-2 regulatory sequences outside the 9.5 kb fragment for adult expression. Therefore, more En-2 genomic DNA fragments need to be isolated and tested for their possible regulatory activities in adult transgenic animals.

Identification of additional Pax-2/5/8/-binding sites involved in early embryonic En-2 mid-hindbrain expression
Evidence from gene expression studies and mutant analysis in mice and zebrafish has suggested a role for Pax-2/5/8 proteins in regulating En early mid-hindbrain expression. I have identified two Pax-2/5/8-binding sites in an En-2 mid-hindbrain enhancer. Targeted deletion of the two Pax2/5/8-binding sites decreased initiation of endogenous En-2 expression in the anterior neuroepithelium at E8.5. However, this effect was only transient, as En-2 expression in E9.0 and later mutant embryos appeared quite normal. Since Pax-2/5/8 expression in the mid-hindbrain region overlaps with En-2 expression from E8.5 to E12.5, the Pax genes could have roles both in activating as well as continuing En-2 expression in this region. One possible explanation for the transient disruption of En-2 expression observed following deletion of the two Pax-2/5/8-binding sites is that there are other Pax-binding sites in the En-2 locus. Another interpretation for this result is that En-2 expression does not completely depend on the Pax proteins. To address this issue, mouse En-2 expression in Pax-2/5/8 mutant animals needs to be analyzed. In mouse, the role of Pax genes in En regulation is complicated by the possible functional redundancy between the three Pax genes. However, in zebrafish, only one Pax-2/5/8 related Pax gene, pax-b, has been identified (Krauss et al., 1991; Gruss and Walther, 1992) and eng-2 expression was completely lost in a zebrafish pax-b mutant allele, noi (Brand et al., 1996). This result is consistent with the prediction that Pax-2/5/8 are required for both activation and continuation of mouse En-2 mid-hindbrain expression. It is possible that there are multiple Pax-binding sites functioning within En-2 enhancers that confer different temporal and spatial aspects of En-2 expression. The main regulatory role of the 1.0 kb En-2 enhancer appears to be to activate En-2 expression and this activity is dependent on the two Pax-2/5/8-binding sites. Once additional En-2 mid-hindbrain enhancer elements are identified, they should be analyzed as described above by footprint experiments using Pax-2/5/8 protein. Any protected regions should then be sequenced to determine whether they contain potential Pax-binding site(s) based on the known Pax-2/5/8
binding consensus sequences (Czerny et al., 1993). The in vivo function of any identified Pax-binding site(s) should then be tested by mutation analysis in transgenic mice.

**Roles of Pax-2/5/8 proteins in activating En-1 and En-2 mid-hindbrain expression**

Gene expression and mutant phenotype studies have suggested a role for Pax-2/5/8 in regulating En-1 and En-2 mid-hindbrain expression. Our direct biochemical evidence that Pax-2/5/8 proteins interact with regulatory sequences in En-2 DNA further supports the possibility of En-1 being regulated by the Pax proteins given the fact that the two En genes have very similar expression patterns in the mid-hindbrain region. Once regulatory sequences in En-1 DNA that can direct En-1-like embryonic expression are identified, they should be analyzed for the presence of potential Pax-2/5/8-binding sites. Knowledge of the Pax protein-DNA interaction obtained in the research presented in this thesis and other biochemical analyses (Czerny et al., 1993; Epstein et al., 1994; Song et al., 1996) should facilitate identifying Pax-binding sites in the En-1 locus.

Although the two En genes have very similar expression patterns in the mid-hindbrain neuroepithelium, a major difference is that En-1 is expressed a half day earlier than En-2. If Pax-2/5/8 proteins are involved in regulating both En genes, an interesting question is why En-2 is not activated by Pax-2 before E8.5. Expression of each of the three Pax genes is initiated in the mes-met region in a specific temporal order: Pax-2 turns on at the pre-somite stage, prior to En-1; Pax-5 is activated at the same stage as En-2 at the 5 somite stage followed by Pax-8 at the 13-14 somite stage. This sequential activation of the three Pax genes leads to differences in the concentration of total Pax-2/5/8 proteins along the A-P axis and at different somite stages. A recent study of Pax-2 (Krd) and Pax-5 double mutant mice has suggested that the gene dosage of the two Pax genes affects mid-hindbrain development (Urbanek et al., 1997). One interesting hypothesis derived from these results is that the concentration of Pax-2 alone is sufficient for activating En-1,
whereas En-2 activation requires both Pax-2 and Pax-5. If this is the case, activation of En-2, but not En-1, should be affected in a Pax-5 mutant. I have analyzed expression of both En genes in Pax-5 mutant mice at E8.5-E9.5 and found no significant change of expression for either En gene (Song and Joyner, unpublished), indicating that the differential activation of En-1 and En-2 is not a result of the different levels of Pax proteins. Other possible explanations for the different expression patterns of the two En genes are that En-2 activation requires other protein factor(s) that are not present prior to E8.5, and/or that En-2 transcription is inhibited prior to E8.5. Future studies of En-1 and En-2 regulatory sequences should provide an answer to this question.

**Identification of Fgf-8 response element(s) in the En loci**

Fgf-8 has recently been identified as a likely candidate for one of the signaling molecules in the isthmus organizer center (Crossley and Martin, 1995; Crossley et al., 1996). Fgf-8 has been shown to induce ectopic En-2 and Wnt-1 expression in the diencephalon and mesencephalon and subsequently transform these tissues into mes-met tissues. En-1 was also shown to be induced by Fgf-8 in chick (Martin, personal communication) and in mice (Liu and Joyner, unpublished). It is of great interest to understand how the isthmus organizer center patterns the mid-hindbrain region. Elucidation of the Fgf-8 signaling pathway that can induce En-2 expression ectopically is likely to provide some important molecular genetic information about the normal process of mid-hindbrain patterning by the isthmus. Although the Fgf signaling pathway has been studied extensively (reviewed in Van der Geer et al., 1994), its nuclear components are largely unknown. One starting point to further dissect this pathway is to identify Fgf-8 response elements within En-2 DNA regulatory sequences. To this end, I have tested whether the En-2 9.5 kb enhancer fragment contains any Fgf-8 response element(s) (Song, Losos and Joyner, unpublished). Fgf-8 was ectopically expressed in the E8.5-E9 mesencephalon and later mid-hindbrain junction and dorsal midline of the brain (Echelard et
al., 1994) in transgenic mice carrying a lacZ reporter gene directed by the 9.5 kb En-2 enhancer fragment (Logan et al., 1993). The Fgf-8 transgenic mice were previously showed to have enlarged midbrains in response to the ectopic Fgf-8 expression, and En-2 expression was expanded in the enlarged tectum at E10-E12 (Lee et al., 1997). However, in our double Fgf-8/En-2 lacZ transgenic mice, the lacZ transgene was not induced in the expanded tectum, indicating that Fgf-8 response elements are not present in the 9.5 kb fragment. Similar studies should be performed once additional En-2 enhancer regions are identified.

Identification of other protein factors that interact with En-2 regulatory sequences

The spatially and temporally restricted En-2 expression pattern requires cooperative interactions between multiple DNA regulatory elements and transcription factors (Chapter 2 and 3). The candidate gene approach used in this thesis has established a direct interaction between the Pax-2/5/8 proteins and DNA regulatory sequences important for a normal early En-2 mid-hindbrain expression pattern. Such an approach was first successfully applied in demonstrating that Krox-20 acts as an upstream regulator of Hox-2B expression in rhombomeres 1 and 3 of the hindbrain (Sham et al., 1993). As more mid-hindbrain regulatory genes are isolated from different organisms, this approach will continue to be an effective means to study the regulation of En-2 expression.

We have studied En-2 DNA regulatory elements with an ultimate goal of identifying novel transcription factors that are involved in En-2 expression. To date, identification of transcription factors has mainly relied on their known DNA binding properties. DNA-affinity chromatography, combined with conventional protein purification techniques, has been successfully used in identify many eukaryotic transcription factors (Briggs et al. 1986; Hai et al. 1988). However, this method requires a large amount of tissue as the starting material for purification. Expression screening has also been employed to clone genes that
encode sequence-specific DNA-binding proteins (Singh et al. 1988; Staudt et al., 1988; Drolet et al. 1991). This approach, by virtue of the specific binding between a protein factor and its cognate DNA sequence, allows one to isolate DNA-binding proteins from more limited tissue sources. Thus, this direct cloning method is likely to be a better choice for identifying En-2 regulatory factors. Once En-2 DNA regulatory sequences are identified by footprinting and bandshift assays, oligonucleotides containing protein binding sequences can be used as probes to screen expression libraries constructed from mouse embryos obtained at different stages.

Due to non-specific DNA-protein interactions, some of the isolated cDNA clones may not be involved in En-2 transcription. Therefore, the expression patterns of the identified genes should be examined by RNA in situ analysis to determine if they are expressed in a temporal and spatial manner consistent with a role in regulating En-2 expression. The genes that show overlapping or complementary expression patterns with En-2 are good candidates to act as En-2 activators or inhibitors, respectively. These genes can then be further characterized by in vitro biochemical assays to characterize their interactions with En-2 DNA regulatory sequences. Ultimately, their developmental function can be determined by gene targeting studies.

Our transgenic analysis has identified three DNA regulatory elements that are involved in En-2 activation in the presumptive mid-hindbrain region. Further biochemical analysis of DNA binding sites present in these elements and molecular cloning of their interacting transcription factors should provide important new insights into the genetic control of early mid-hindbrain development.
REFERENCES


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