Pax genes and their roles in cell differentiation and development
Ahmed Mansouri*, Marc Hallonet† and Peter Gruss‡

Members of the Pax gene family are expressed in various tissues during ontogenesis. Evidence for their crucial role in morphogenesis, organogenesis, cell differentiation and oncogenesis is provided by rodent mutants and human diseases. Additionally, recent experimental in vivo and in vitro approaches have led to the identification of molecules that interact with Pax proteins.

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Abbreviations
BMP  bone morphogenetic protein
En  Engrailed
FGF  fibroblast growth factor
sey  small eye
Shh  Sonic hedgehog

Introduction
The murine Pax gene family was identified on the basis of sequence homology with Drosophila segmentation genes [1,2] and now consists of nine members. All Pax proteins contain a paired-box, DNA-binding domain of 128 amino acids located at the amino-terminal end. This domain has been highly conserved during evolution and is found in Drosophila and in human, mouse, rat, chicken, quail and zebrafish genes. Distinct classes or subgroups of Pax genes are defined by the presence or absence of a paired-type homeobox and of an octapeptide coding region, in addition to the paired box (see Fig. 1) [3,4]. Paired box containing proteins are transcription factors, as they display sequence-specific DNA-binding activity and can regulate transcription [5,6].

Pax genes display dynamic expression patterns during ontogenesis in a large variety of tissues, and mutant phenotypes correlate very well with the expression patterns (see Fig. 2). For example, Pax1 is expressed in the sclerotome and is mutated in undulated mice suffering from skeletal abnormalities [7]. Pax3 is expressed in the limb muscle, neural tube and neural crest, and is mutated in Splotch mice and human Waardenburg syndrome in which malformations of all these structures occur [8,9]. Pax6 is expressed during eye formation and is mutated in small eye (sey) mice and rats [10-12], and in human aniridia [13]; in all of these, eye defects are displayed. Pax2 is expressed during eye and kidney development [14] and is mutated in a human family with kidney and eye abnormalities [15]. Pax genes clearly play important roles during the formation of many structures. Furthermore, deregulated expression of Pax genes may lead to oncogenesis [16-18].

In this review, we focus on recent data documenting the role of Pax genes, and the interaction of the Pax proteins with other transcription factors, in the development of the nervous system, in organogenesis and in cell proliferation and differentiation.

Pax genes in the central nervous system
The roles of Pax genes are particularly documented at the level of the nervous system, which consequently may represent a model for the study of the function of the Pax genes. All Pax genes, except Pax1 and Pax9, are expressed in various restricted territories in the neural tube. Unlike Hox genes, Pax genes are found in the more rostral domains of the brain: Pax6 is found in the telencephalon [10,19], Pax3 and Pax7 in the mesencephalon [19-21], and Pax2, Pax5 and Pax8 at the midbrain–hindbrain boundary [19,22-24] (see Fig. 2). In the spinal cord, Pax genes display restricted expression patterns along the dorsoventral axis.

In the brain, comparison of the expression domains of forkhead, Wnt, Engrailed (En), and Pax genes with sites of neuronal differentiation suggest that some Pax proteins are morphoregulators of development of the brain [19,25,26]. Several Pax6 semidominant mutations are documented in the mouse, rat and human, with various phenotypic severities. At the cellular level, Pax6 mutations cause a delay in neuronal migrations in a gene-dose-dependent manner, and impair axonal growth and differentiation [27]. In mouse sey mutants, Dlx1 gene expression, which is normally specific for the ganglionic eminence, ectopically extends into the cortex, where Pax6 is normally expressed. Pax6 function is thus necessary for establishing and/or maintaining the frontier between two brain territories [28**]. Overexpression of the chicken En1 gene in medulloblastoma cell lines results in the downregulation of Pax6 expression, thus suggesting that these genes are inversely regulated [29]. In addition, grafting experiments of an Engrailed-expressing midbrain–hindbrain piece of neuroepithelium in which fibroblast growth factor (FGF8) is expressed onto diencephalic structures can induce the expression of En2 in the host adjacent tissues [30]. Moreover, FGF8 has been recently proposed as an organizer of this brain region and could be a major...
Cell differentiation

<table>
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<tr>
<th>Paired domain</th>
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<th>Chromosomal location</th>
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<td>Mouse: Man</td>
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<td>Pax1</td>
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<td>Pax9</td>
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H Helix structure

The Pax proteins. All Pax proteins contain a paired-box, DNA-binding domain (paired domain) of 128 amino acids, located at amino-terminal ends. Distinct subgroups of proteins are defined by the presence or absence of a paired-type homeobox (HD) and of an OP domain. Related genes share the same patterning on the figure. In addition to the paired domain, Pax3, Pax4, Pax6 and Pax7 have a second DNA binding domain, the paired-type homeobox (HD), at their carboxy-terminal ends. This homeobox is truncated and carries only the first helix in Pax2, Pax5 and Pax8, and is totally absent in Pax1 and Pax9. In addition, all Pax proteins, except Pax4 and Pax6, carry an OP domain of unknown function between the paired domain and the homeobox. Pax-related genes share overlapping expression patterns with one another. Although some Pax genes may be present on the same chromosome, they are not clustered, as compared with the Hox genes which are clustered.

The Pax proteins are involved in the establishment of the midbrain-hindbrain boundary, which regionalizes embryonic neuroepithelium [31, 32].

Pax5 and Pax8 are expressed at the midbrain-hindbrain boundary level, as are members of the Wnt, Engrailed, and FGF gene families [33]. Injection of antibodies against the zebrafish Pax[b] protein, a homologue of Pax2, Pax5 and Pax8, causes specific and localized formations at this boundary that are associated with a decrease of Wnt1 and En2 RNA in this area [34]. Similarly, Pax5-knockout mice exhibit defects in tissues arising from the posterior midbrain region and the hindbrain; the inferior colliculus is partially deleted and the foliation of the cerebellum is perturbed in these mice [35]. These results, and the phenotypes observed in Wnt1-/- and En1-/- mice, in which the caudal midbrain and cerebellum are severely affected [33], clearly indicate that Pax, Wnt and En act in concert during the development of the midbrain-hindbrain boundary region.

In the developing eye, Pax2 is expressed exclusively in the optic stalk, and Pax6 in the eye cup [36]. That these two genes are inversely regulated is confirmed by ectopic overexpression of the ventralizing factor Sonic hedgehog (Shh) which leads to overexpression of Pax2...
Figure 2

### Expression patterns

<table>
<thead>
<tr>
<th>Pax genes</th>
<th>Expression pattern</th>
<th>Central nervous system</th>
<th>Mesoderm</th>
<th>Endod</th>
<th>Mutants: affected structure</th>
<th>Regulators</th>
<th>Regulated</th>
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<tr>
<td>Pax 1</td>
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<td>Pharyngeal pouches, thymus</td>
<td>un: thymus, intervertebral disc, shoulder girdles</td>
<td>Shh</td>
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<td>Pax 2</td>
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<td>Pax 5</td>
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<td>Latero-medial</td>
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<td>Pax 8</td>
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<td>Latero-medial</td>
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<td>Thyroid</td>
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<td>Pax 3</td>
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<td>Pax 7</td>
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<td>Latero-medial</td>
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<td>Pax 4</td>
<td>Eye, nasal placodes</td>
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<td>Pax 6</td>
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Schematic summary of the expression patterns of Pax genes, the structures affected by mutations in the Pax genes, and the molecules regulating or being regulated by the Pax proteins. The patterns of expression indicated in this table are a simplification of the complex expression patterns of the Pax genes. The thick black horizontal lines represent expression patterns of the Pax genes. The expression of Pax2, Pax5 and Pax8 is more expanded at the midbrain-hindbrain boundary. Der, dermomyotome; DIE, diencephalon; Endod, endoderm; Mid, midbrain; Hemato, haematopoietic system; Hind, hindbrain; KO, knockout; Krd, kidney and retinal defects; ProsE, prosencephalon; Scler, sclerotome; SC, spinal cord; TelE, telencephalon; thyroglobulin; ThyroPerox, thyroperoxidase; un, undulated mouse mutant; WS, human Waardenburg syndrome; ?, putative interacting candidates; direct binding, target DNA is directly bound by Pax proteins (see text for details). *In this column, human syndromes or diseases are indicated in bold type before the colon, whereas mouse mutants are indicated in normal type before the colon. Affected structures are indicated after the colon.

and downregulation of Pax6 and provokes hypertrophia of the optic stalk and reduction of pigment epithelium and neural retina [37**,38]. Pax2 and Pax6 are thus crucial morphoregulators of the eye and are both under the regulation of ventralizing agents.

In the spinal cord, transplantation experiments in the chick indicate that the expression of Pax3, Pax7 and Pax6 is regulated by the notochord via a secretion of Shh and probably also by the roof plate and overlying ectoderm, possibly via a secretion of bone morphogenetic proteins (BMPs) 4 and 7 [39,40]. Pax3, Pax6 and Pax7 are expressed prior to neural differentiation in mitotically active cells, as opposed to Pax2, Pax5 and Pax8 which are not expressed at this time. It has been reported that ectopic expression of Pax3 in the entire spinal cord of the mouse, using the Hoxb4 enhancer, does not confer dorsal identities as expected but instead leads to lack of the floor plate in the affected areas of the spinal cord of the transgenic animals [41]. Pax genes may thus be important regulators of the dorsoventral patterning of the spinal cord.

The Pax3 gene is inactivated by deletions or point mutations in various murine splotch or human Waardenburg syndrome semidominant alleles. Splotch mutants exhibit spina bifida, exencephaly, and neural-crest and limb-muscle defects [8,9,42]. Recent studies of splotch delayed, which produces a milder phenotype than other splotch alleles, revealed normal neuronal differentiation in dorsal and ventral regions of the affected neural tube
Similarly, Pax7−/− mice do not show any obvious neural tube defect [44*]. Functional redundancy between Pax3 and Pax7 could thus be responsible for the lack of abnormal neuronal differentiation phenotype in these mutants.

The Drosophila gooseberry (gsb) locus codes for two linked and structurally related genes, gsb-proximal and gsb-distal, which are homologues of the Pax3 and Pax7 genes (for review, see [45]). Experiments in which Drosophila embryos carried a deletion removing both gsb transcripts or in which embryos ectopically expressed only one of gsb-distal and gsb-proximal clearly indicate that Gsb specifies row five neuroblasts [46*,47]. Pax3 and Pax7 may also specify neuronal identity in the spinal cord, and only double mutations of both Pax genes may give us a definitive answer about their respective functions.

**Pax genes in organogenesis**

Pax genes are also expressed outside the central nervous system during organogenesis: Pax1 is expressed in thymus, Pax2 and Pax8 in kidney, Pax6 in thyroid, Pax6 in pancreas and eye, and Pax4 in pancreas ([14,24,48,49]; B Sosa-Pineda, P Gruss, unpublished data). Mutation of the Pax1 gene in undulated mice leads to reduced thymus size and affects the maturation of the thymocytes [49]. Accordingly, Pax1 seems to be required in thymus epithelium differentiation and hence influences T-cell maturation [49].

Krd (kidney and retinal defects) mice with a deletion of a chromosomal segment that includes the Pax2 locus have kidney defects [50]. Kidney hypoplasia has been associated with heterozygosity of a human PAX2 point mutation [15]. Experiments using antisense oligonucleotides to Pax2 in kidney organ culture, and inactivation of Pax2 by homologous recombination, demonstrate that this gene is essential for the development of the kidney epithelial components that are derived from the intermediate mesoderm [51,52*].

Pax8−/− mice suffer from a thyroid defect (A Mansouri, P Gruss, unpublished data). Pax4−/− mice suffer from a pancreas defect (B Sosa-Pineda, P Gruss, unpublished data).

Targeted ectopic expression of cDNA encoding Drosophila eyeless or its murine homologue, Pax6, induces morphologically normal eyes in the wings, legs and antennae, demonstrating that eyeless is the master control gene for eye morphogenesis in Drosophila [53*,54]. Analysis of homozygous ey− mice, and studies of chimeric mouse embryos composed of wild-type and ey−mutant cells, indicates that Pax6 acts directly and in a cell-autonomous manner in the optic cup and lens, and is essential for lens and nasal-placode specification from surface ectoderm [55,56*].

Pax proteins are thus crucial regulators of organogenesis in thymus, kidney, thyroid, pancreas and eye.

**Pax genes in cell differentiation**

Analysis of undulated mice demonstrates that Pax1 is essential for the condensation of the mesenchymal sclerotome cells and for the initiation of chondrogenesis [57]. The effect of Pax1 on sclerotome differentiation is regulated by the notochord via secretion of Shh [58,59*]. Inactivation by gene targeting demonstrates that Pax5 is essential for early B-cell differentiation [35]. The limb muscles, whose precursors express Pax3, are specifically disturbed in splotch mice [42]. Additionally, neural-crest derivatives, such as Schwann cells or melanocytes, are also affected in splotch mice [60–62]. In vitro experiments indicate that Pax3 is exclusively expressed in differentiating and nonmyelinating Schwann cells (see [63]). Accordingly, Pax3 involved in the migration, differentiation and possibly the proliferation of the neural-crest cells and myoblasts. Also, Pax7 has been shown to play a role in cephalic neural crest specification, differentiation and/or proliferation [44*].

**Pax genes, cell proliferation and oncogenesis**

Overexpression of Pax genes can transform fibroblasts into tumours in nude mice [18]. Similarly, the expression of PAX2 and the paralogous gene PAX8 are abnormally upregulated in Wilms tumour, a paediatric renal carcinoma of mesenchymal origin [64,65].

Translocations of human PAX3 or PAX7 result in the expression of a PAX–forkhead fusion protein carrying the intact DNA-binding domains of the PAX3 or PAX7 molecules that are probably responsible for the generation of rhabdomyosarcoma [17,66]. In the case of the PAX3–forkhead fusion, the molecule produced is a more efficient transcription factor than PAX3 itself and would be expressed under the normal PAX3 promoter [67]. It has not, however, been directly demonstrated that the fusion protein is driven by the PAX promoter.

Deregulation of PAX5 expression has been reported in humans in malignant astrocytomas [68] and medulloblastomas [69]. In addition, the Pax5 promoter is modified in large-cell lymphomas by insertion of the potent Eμ enhancer of the IgH gene [70]. It is not clear how Pax5 is acting in these tumours but it may be involved in regulating cell proliferation, as in vitro experiments indicate that Pax5 may regulate proliferation in B cells [71].

Thus, the overexpression of Pax genes in tissues in which they are normally expressed may lead to tumorigenesis, suggesting that doses of Pax proteins are critical for their normal function.


**Pax protein target genes**

Little is known about Pax target genes. However, *in vitro* protein–DNA binding assays and *in vivo* expression analysis indicate that a 1.0 kilobase fragment of the En2 enhancer contains binding sites for Pax2, Pax5 and Pax8 [72*]. Mutation of these binding sites disrupts initiation and maintenance of expression of the β-galactosidase reporter gene in the midbrain–hindbrain boundary of transgenic mice [72*].

Pax3 and the tyrosine kinase receptor for hepatocyte growth factor/scatter factor that is encoded by the c-met proto-oncogene are expressed in the lateral dermomyotome, that is, the progenitors of limb muscle [73]. Splotch mice display defects in neural-crest derivatives, failure of neural-tube closure and lack of limb muscles [8,9,42]. Splotch and c-met/+ mice exhibit the same phenotype in the limb. Furthermore, c-met expression is greatly reduced in the dermomyotome of splotch embryos. In addition, Pax3 directly activates c-met expression by binding to the c-met promoter, thus demonstrating that c-met is a target gene of Pax3 [74*,75].

Similarly, Pax5 was identified as a B cell specific transcription factor that regulates the expression of the CD19 gene which encodes a surface protein [76]. In addition, Pax8, which is expressed in the thyroid, binds to the promoter, and regulates the expression, of the thyroperoxidase and thyroglobuline genes [77].

*In vitro* experiments also show that Pax5 can directly inhibit transcription of the p53 gene by binding to the untranslated first exon, and could thereby participate in tumorigenesis [78].

**Conclusions**

Pax gene mutants are powerful tools for the study of Pax gene function. Nevertheless, functional redundancy between related members, alternative splicing and complex promoters complicate the analysis. Consequently, knockout mutants are greatly needed to study the effect of defined mutations. New techniques using conditional knockouts and knockins may be used to specifically inactivate the Pax gene of interest in a selected tissue at a defined time.

Pax protein doses play a crucial role in proper function of the proteins. The ectopic expression of Pax genes in transgenic mice may therefore give new insights into the mechanism of action of Pax proteins.

The possible regulation of cell adhesion by Pax proteins could explain some of the mutant phenotypes. In this context, *in vitro* approaches will most probably contribute to the functional analysis of the Pax genes.

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**References and recommended reading**

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


31. Crossley PH, Martinez S, Martin GR: Midbrain development induced by FGFB in the chick embryo. Nature 1996, 380:56-66. Implantation of FGFB-coated beads into the forebrain mimics the effects of midbrain-hindbrain tissue grafts in inducing the expression of En2 and Wnt1 and the differentiation of mesencephalic-like structures. Accordingly, FGFB is a major regulator of the formation of this brain territory, which is where it is normally expressed.


34. Ruchoux MM, Saule S: Differential expression between Pax6 and En2 and Enl genes. Development 1995, 122:2555-2567. This study demonstrates that lack of gooseberry-distal transforms row five neuroblasts, where it is normally expressed, into row three neuroblasts, one of the other four rows that are normally present in each Drosophila segment. Inversely, ubiquitous expression of the same gene generates the reciprocal transformation, thus demonstrating that gooseberry-distal is one of the prime candidates for controlling specific neuroblast identity.


40. Torres M, Gomez-Pardo E, Dresseler GR, Gruss P: Pax-2 controls multiple steps of urogenital development. Development 1996, 122:4057-4065. Pax2 knock-out mice lack kidneys, ureters and genital tracts. This study demonstrates that Pax2 is essential for the formation of kidney epithelium from the mesenchyme of the intermediate mesoderm [see also (61)].

Targeted ectopic expression of the Drosophila eyeless gene or of the mouse Pax6 gene in various imaginal disc primordia of the Drosophila embryo leads to induction of ectopic eye structures in the wings, legs and antennae. These results support the conclusion that eyeless and the related Pax6 are master control genes for eye morphogenesis.


Using chimeras of eye mutant and wild-type cells, the authors observed that eye cells were excluded from lens and nasal epithelium in these chimeric animals, thus indicating that Pax6 is required in a cell-autonomous manner for the formation of these structures. The authors also confirm that Pax6 is also necessary for the proper formation of the optic cup (see also [55]).


Culture systems of presomitic mesoderm explants were used to demonstrate that Shh induces the expression of Pax1. In addition, different levels of Pax1 expression were induced by different concentrations of Shh. Inversely, the authors also report a repression of the expression of Pax1 in the Shh-treated explants.


83. The En2 enhancer, which can direct β-galactosidase expression in the midbrain–hindbrain boundary of transgenic mice, has two binding sites for the Pax2, Pax5 and Pax6 proteins. Mutation or deletion of the two Pax5-binding sites abolishes the expression of lacZ in this brain area of transgenic animals.


Binding assay experiments identified the Pax3-binding site on the human c-met promoter. Furthermore, future experiments demonstrated that Pax3 can stimulate c-met expression. These results are consistent with the observation that c-met is downregulated in splotch mice (see also [76]).


