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# Roles of *Pax*-Genes in Developing and Adult Brain as Suggested by Expression Patterns

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**We have examined the transcript distribution of six members of the murine paired box–containing gene family (*Pax*-gene family) in midgestation embryo and adult brain using *in situ* hybridization analysis. The expression domains of several *Pax*-genes in the embryo brain were found to correspond with anatomical boundaries that coincide with neuromere landmarks and therefore respect former neuromere territories in the forebrain. The results are consistent with the concept of brain segmentation and suggest a role for *Pax*-genes in the brain regionalization. In the adult brain the expression of *Pax*-genes was observed in discreet areas, with a caudal to rostral restriction in the number of the expressed genes. In general the distribution of transcripts along the anterior–posterior axis was similar to that found in midgestation embryo brain, suggesting a role for *Pax*-genes in the commitment of the precursor cells to different neuronal cell fates and in the maintenance of specific brain cell subtypes. In the cerebellar cortex, the granular cell layer was found to express high levels of the *Pax-6* gene, while putative Bergmann glia and cells surrounding the Purkinje cells contained *Pax-3* transcripts. The main adult brain structures that expressed distinct *Pax*-mRNAs were the periglomerular and granular cell layer of olfactory bulb, nuclei of the septum, amygdala, and isthmus, which suggests a role for the *Pax*-gene family in the specification of the subcortical domains of the evolutionary old limbic system.**

***[Key words:* Pax-*genes, brain development, pattern formation, brain segmentation, adult brain expression]***

Developing and adult multicellular organism depends on a precisely regulated genetic program. Recent evidence has shown that the gene products of several large families may act as transcriptional regulators that contain different evolutionary conserved DNA-binding motifs: the *homeodomain* (reviewed in Scott et al., 1989), the *paired-domain* (Bopp et al., 1986; Dressler et al., 1988; reviewed in Kessel and Gruss, 1990; Deutsch and Gruss, 1991), the *POU domain* (Herr et al., 1988; reviewed in Rosenfeld, 1991), the *helix-loop-helix* (Murre et al., 1989), the *zinc finger domain* (reviewed in Beato, 1989), the *leucine zipper* (Landschulz et al., 1988), and the *fork head domain* (Weigel and Jäckle, 1990). It also appears that members of these multigene families have important roles in the control of development, regional specification, or cellular determination during the complex morphogenesis of the nervous system.

The murine *Pax*-gene family consists of eight members, referred to as *Pax-1* to *Pax-8* (reviewed in Dressler and Douglas, 1988; Walther et al., 1991). All of them encode a conserved sequence, the paired box, initially found in the *Drosophila* segmentation genes *paired* (prd), *gooseberry-distal* (gsb-d), and *gooseberry-proximal* (gsb-p) (Bopp et al., 1986; Baumgartner et al., 1987) as well as in the products of the tissue-specific *Drosophila* paired box–containing genes *Pox meso* and *Pox neuro* (Bopp et al., 1989). The same DNA-binding motif was identified in the genome of a wide variety of organisms as zebrafish (Krauss et al., 1991a,b; Püschel et al., 1992a,b), chicken (Goulding et al., 1992), nematode, frog, turtle, and human (Dressler et al., 1988; Burri et al., 1989). Seven of the isolated murine *Pax*-genes have been characterized in more detail.

The paired domain encompasses 128 amino acids and is located close to the amino terminus of the protein. Three *Pax*-genes, *Pax-3, Pax-6,* and *Pax-7,* contain in addition a second conserved paired type homeodomain of 61 amino acids located toward the carboxy terminus of the protein. *Pax-1, Pax-2*, and *Pax-8* genes have only the first helix of the paired type homeobox, and thus are missing the whole helix-loop-helix part of second DNA-binding motif.

The paired box–containing genes of different species have been subdivided into six classes, based on the paired box and the overall sequence homology (Deutsch and Gruss, 1991; Walther et al., 1991). A particularly high sequence similarity has been found for some genes and their genomic organization within the same species, designated as paralogous genes. The paralogous genes have similar expression patterns as reported for the murine genes *Pax-3/Pax-7* and for *Pax-2/Pax-8* (reviewed in Deutsch and Gruss, 1991; Gruss and Walther, 1992). Recently, evidence was provided that *Pax* proteins may act as transcriptional regulators. Among them are the specific DNA-binding capacity of the *Drosophila paired* gene product (Treisman et al., 1989, 1991) and of the products of the murine *Pax-1* (Chalepakis et al., 1991), *Pax-2* (Dressler et al., 1992), *Pax-3* (Goulding et al., 1991), and *Pax-5* genes (Adams et al., 1992).

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In addition, a transcriptional transactivation has been shown for *Pax-1* (Chalepakis et al., 1991).

At least three murine developmental phenotypes are caused by mutations in *Pax*-genes (reviewed in Chalepakis et al., 1992; Gruss and Walther, 1992): *Pax-1* in *undulated* (Balling et al., 1988), *Pax-3* in *Splotch* (Epstein et al., 1991), and *Pax-6* in *Small eye* (Hill et al., 1991). In addition it appears that the molecular basis of the human Waardenburg syndrome (individuals have defects in the CNS as well as deafness, heterochromia irides, pigmentary deficiency, lateral displacement of the inner canthi of the eye) and aniridia (partial or complete absence of iris, impaired vision, cataracta, optic nerve hypoplasia) can be correlated with genetic lesions in the human *PAX-3* (Baldwin et al., 1992; Tassabehji et al., 1992) or the *PAX-6* gene, respectively (Ton et al., 1991; Jordan et al., 1992). These results indicate that *Pax*-gene products are important regulatory factors during development.

All murine *Pax*-genes except *Pax-1* are expressed in the developing neural tube. In general, *Pax*-genes that encode a paired domain and paired-type homeodomain (*Pax-3, Pax-6, Pax-7*) start to be expressed around embryonic day 8–8.5 post coitum (E8–E8.5 p.c.), thus before the onset of the cellular differentiation. The interesting feature of this group of *Pax*-genes is that they show restricted expression patterns relative to the dorsoventral axis of the developing spinal cord. The paralogous genes *Pax-3* and *Pax-7* are expressed in the mitotic active ventricular zone of the dorsal alar plate along the entire axis including (*Pax-3*) or excluding (*Pax-7*) the roof plate (Goulding et al., 1991; Jostes et al., 1991). In contrast, *Pax-6* has been detected only in the ventricular zone of the basal and medial plate of the developing neural tube (Walther and Gruss, 1991). Recent notochord transplantation studies in chicken have supported the hypothesis that *Pax-3* and *Pax-6* genes may be involved in the dorsoventral polarization of the spinal cord directed by the inductive signals of the underlaying notochord (Goulding et al., 1992). The expression of the group of *Pax-2, Pax-8,* and *Pax-5* genes in the developing neural tube commences later (around E10 p.c.) with an initial distribution of the transcripts in the postmitotic cells on both sides of the sulcus limitans that delineates the alar from the basal plate (Nornes et al., 1990; Plachov et al., 1990; Asano and Gruss, 1992). In later stages their expression domains are confined to differentiating cells in the ventral part of the intermediate zone of the neural tube. Recent data have shown that the *Pax-5* gene encodes a transcriptional factor involved in the regulation of the CD19 gene, which codes for a B-lymphoid–specific transmembrane receptor (Adams et al., 1992; Kozmik et al., 1992).

*Pax*-genes are also active in the developing brain. Transcripts of *Pax-2* and *Pax-8* were detected in the intermediate zone of the myelencephalon and metencephalon until late midgestation stages (Nornes et al., 1990; Plachov et al., 1990). In addition to these expression areas, particularly strong and early accumulation of transcripts has been reported for the *Pax-5* gene at the hindbrain–midbrain boundary and the posterior mesencephalic tegmentum (Asano and Gruss, 1992). The genes from the early-expressed group, *Pax-3, Pax-7,* and *Pax-6*, were found active from E8–E8.5 p.c. through the whole prosencephalon and subsequently in its derivatives, the telencephalon and the diencephalon, in the mesencephalon, and in the hindbrain. In later stages the expression domains of *Pax-3* (Goulding et al., 1991) and of *Pax-7* (Jostes et al., 1991) are retracted to a rostral limit at the level of the diencephalon, while *Pax-6* gene expression is excluded in the roof of the mesencephalon from the very early developmental stages (Walther and Gruss, 1991).

The main purpose of the present study was to gain insight into the expression patterns and the possible roles of the *Pax* multigene family in the adult brain. If *Pax*-genes are involved in the early commitment of the neuroepithelium in different brain areas and have a role for the maintenance of specific subsets of brain cells, one would expect a correlation between the expression patterns found in adult brain and in embryonic brain at a stage when the differentiation processes are active. We therefore investigated using a comparative *in situ* hybridization analysis the transcript distribution of the *Pax*-genes, known to be expressed in the nervous system, in E13 p.c. and in young adult mouse brain (4 weeks). As already shown in our previous publications, this embryonic stage is adequate time to provide representative expression patterns of the *Pax*-genes during development. The data obtained indicate that *Pax*-genes may have roles in fundamental aspects of the developing and mature CNS such as (1) brain pattern formation; (2) differentiation and maintenance of restricted subsets of brain cells that originate from the telencephalon, diencephalon, mesencephalon, isthmus, from the ventricular zone and the external granular layer of the cerebellum; and (3) specification of some brainstem nuclei and of subcortical nuclear domains of the limbic system in the adult brain.

## Materials and Methods

*Mouse embryos and animals.* Embryos and mouse were obtained from cross-matings between (C57Bl6 × SJL)F1 mice, and the midday of the vaginal plug was considered as E0.5 p.c. Immediately after dissection the embryos were fixed overnight at 4°C with 4% paraformaldehyde (PFA) prepared in phosphate-buffered saline (PBS). Adult animals (C57B16 × SJL) were perfused with 4% PFA and their brains were further fixed overnight in the same buffer at 4°C. The next day, embryos and adult brains were dehydrated through ethanol/saline solutions and, after xylene treatment, specimens were embedded in Paraplast (Monoject Scientific).

In situ *hybridization.* A procedure derived from the protocols of Dressler and Gruss (1989) and Graham et al. (1989) as described by Kessel and Gruss (1991) was used for RNA *in situ* analysis. Sections (8 μm) were cut and dried onto chromalum-gelatin slices. The slices were processed through the following steps: dewaxing in xylene, dehydration, washing in PBS, refixing in 4% PFA, washing, protease K treatment (0.02 mg/ml), washing, 4% PFA treatment, washing, 0.1 M triethanolamine treatment, washing, and dehydration. $^{35}$S-labeled RNA probes (specific for different *Pax*-genes) were synthesized using T7- or T3-polymerase, according to the supplier's directions (Promega) from corresponding linearized plasmid templates as described for *Pax-2* in Dressler et al. (1990), *Pax-3* in Goulding et al. (1991), *Pax-5* in Asano and Gruss (1992), *Pax-6* in Walther and Gruss (1991), *Pax-7* in Jostes et al. (1991), and *Pax-8* in Plachov et al. (1990). Probes ($1 \times 10^8$ cpm/ml) were dissolved in hybridization buffer [300 mM NaCl, 10 mM Tris, 10 mM sodium phosphate, 5 mM EDTA, 100 mM dithiothreithol (DTT), 10% dextran sulfate, 50% formamide, 2 mg/ml bovine serum albumin, 2 mg/ml Ficoll, 2 mg/ml polyvinylpyrrolidone]. The hybridization mix was boiled, applied directly onto the sections, and covered with siliconized coverslips. After overnight hybridization at 55°C the following washing procedure was followed: 5× saline–sodium citrate (SSC), 10 mM DTT (30 min, 37°C); 50% formamide, 2× SSC (30 min, 65°C); 0.5 M NaCl, 10 mM Tris (pH 7.4), 5 mM EDTA) (10 min, 37°C); 0.5 M NaCl, 10 mM Tris, 5 mM EDTA, 0.02 mg/ml RNase A (30 min, 37°C); 2× SSC, 10 mM DTT, 50% formamide (15 min, 37°C); 2× SSC (15 min, 37°C); and 0.1× SSC (15 min, 37°C). The sections were then dehydrated in ethanol and air dried.

For autoradiography slices were dipped in Kodak NTB-2 emulsion diluted 1:1 with water. Embryos and adult brain slices were exposed for 10 or 25 d, respectively, and then they were developed in Kodak D-19 solution. For morphological identification the sections were stained with Giemsa (embryo) or toluidine blue (brain) and coverslips were mounted in Eukitt (O. Kindler GmbH, Freiburg, Germany). The ter-

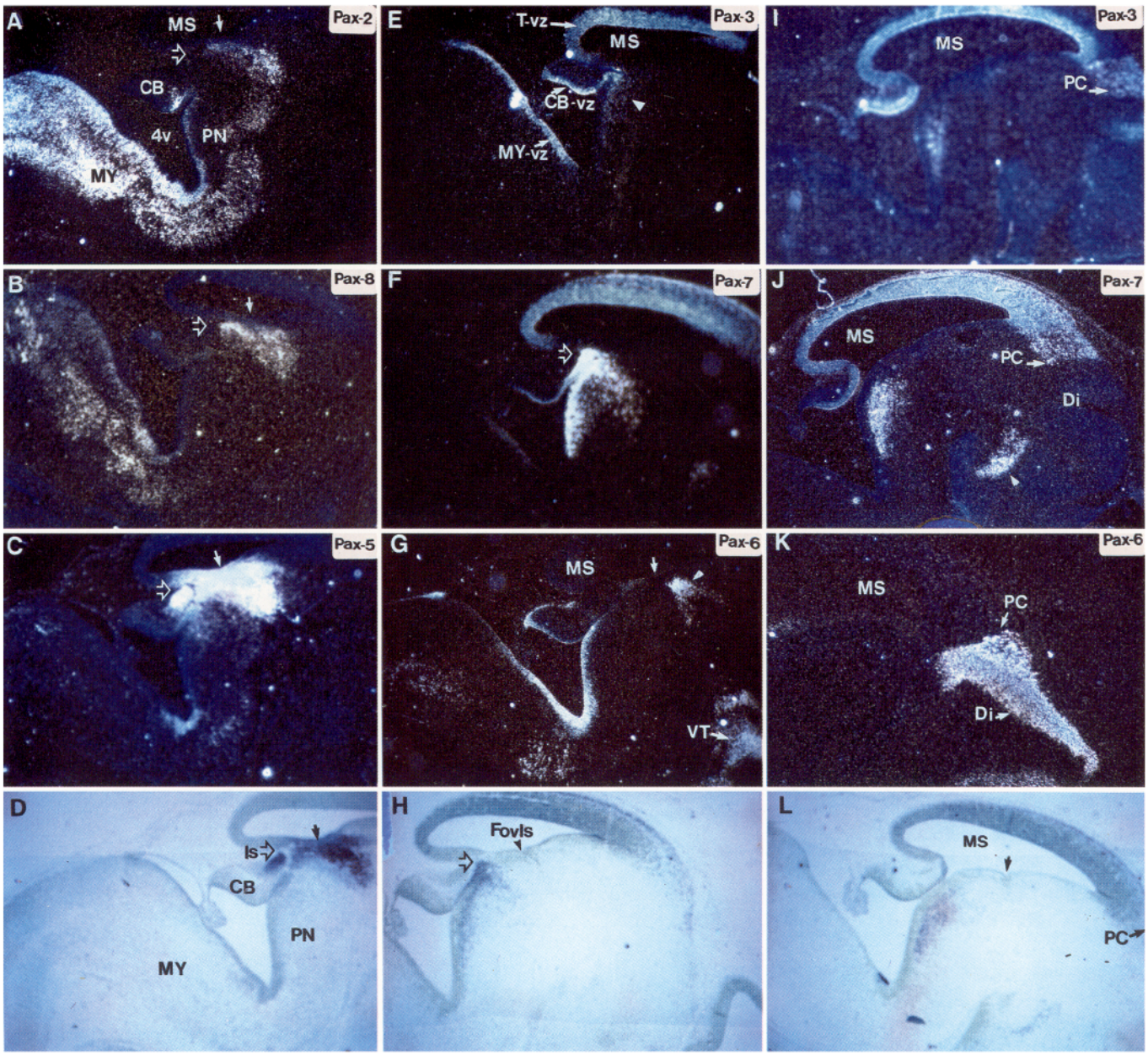


*Figure 1.* Expression of *Pax*-genes at boundaries of midgestation embryo brain. Adjacent almost midsagittal sections (*left* and *middle row*) or parasagittal sections (*right row*) of a 13-d-old embryo were hybridized with antisense RNAs probes for various *Pax*-genes as indicated. *A–H*, Sections showing colocalization of *Pax*-gene transcripts in the hindbrain, including the most rostral domains at the hindbrain–midbrain boundary demarcated by rhombencephalic isthmus (*A–D, F,* and *H, large open arrow labeled Is* in *D*) and fovea isthmi (*H, arrowhead labeled FovIs,* and the *small arrows* in *A–D, G*). *I–L*, At the boundary between the telencephalon and diencephalon, the posterior commissure (*PC*) delineates rostrally the expression domains of the *Pax-3* (*I*) and *Pax-7* (*J*) genes. The same landmark applies for the caudal expression limit of *Pax-6* (*K*) in the forebrain. The views in *D, H,* and *L* are bright-field photomicrographs of the dark-field photos in *C, F,* and *J*, respectively. The *small arrowhead* in *J* points to the expression domain of *Pax-7* in subthalamus. The *arrowheads* in *E* and *G* point to the expression domain at the midbrain–hindbrain boundary for *Pax-3* and *Pax-6*, respectively. *CB*, cerebellum; *Di*, diencephalon; *MS*, mesencephalon; *MY*, myelencephalon; *PC*, posterior commissure; *PN*, pons; *T*, tectum; *vz*, ventricular zone; *4v*, fourth ventricle. Maginification, 40×.

minology used is according to Schambra et al. (1992) for embryo brain and Paxinos and Watson (1986) for adult brain.

## Results

### *Expression of* Pax*-genes in midgestation embryo brain*

To determine the overall *Pax*-gene expression patterns in the midgestation brain, hybridizations were performed on adjacent sagittal and coronal sections of E13 p.c. mouse brain using antisense cRNA probes, specific for *Pax-2, Pax-3, Pax-5, Pax-6, Pax-7,* and *Pax-8* genes (see Materials and Methods). The analysis revealed that the expression domains of different *Pax*-genes correlate with several anatomical landmarks that correspond to former segmental territories.

As depicted on sagittal sections, transcripts of all *Pax*-genes were detected around the hindbrain–midbrain boundary, delineated by the rhombencephalic isthmus and fovea isthmi (Fig. 1). Consistent with our previous observations (Nornes et al., 1990; Plachov et al., 1990; Jostes et al., 1991; Walther and

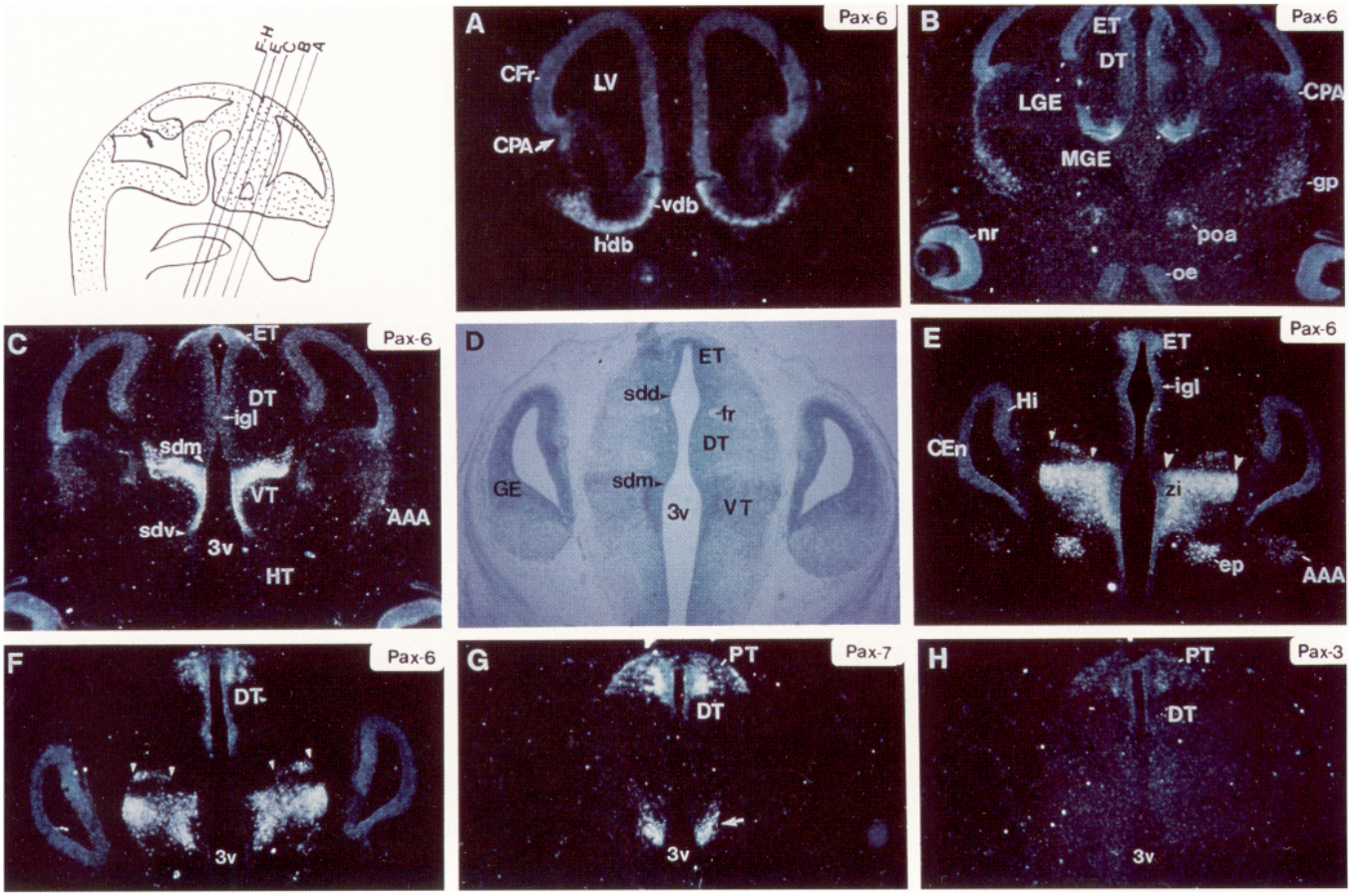


*Figure 2.* Expression of the *Pax*-genes in the forebrain of a 13-d-old embryo. Coronal (slightly transversal) adjacent sections were hybridized with different *Pax*-probes. *A*, A very anterior section showing expression of *Pax-6* in the dorsolateral frontal cortex (*CFr*) and in the region of the horizonal and vertical limb of diagonal band nucleus of Broca (*hdb, vdb*). *B*, Dark-field picture through anterior diencephalon showing localization of *Pax-6* transcripts in the dorsolateral parietal cortex up to the caudatopallial angle (*CPA*), in the preoptic area (*poa*), globus pallidus (*gp*), olfactory epithelium (*oe*), and retina (*nr*). The neuroepithelium of the lateral and medial ganglionic eminence (*LGE, MGE*) lacks a signal. *C–H*, "Segment"-like expression of *Pax*-genes in diencephalon. The subdivision of the diencephalon into the four Herrick's zones [epithalamus (*ET*), dorsal thalamus (*DT*), ventral thalamus (*VT*), and hypothalamus (*HT*)] through the diencephalic sulci [dorsalis, medius, and ventralis (*sdd, sdm, sdv*)] are indicated in *C* and *D*. *D* and *E*, Bright-field (*D*) and dark-field (*E*) views of a section in median plane through the diencephalon show the restricted distribution of *Pax-6* transcripts in the ventral thalamus with a sharp anterior border just below the zona limitans intrathalamica (*large arrowheads*) and in the entopeduncular nucleus (*ep*). *Pax-6* transcripts are also seen in the internal germinal layer (*igl*) of the dorsal thalamus (*DT*), in the epithalamus (*ET*), and in the anterior amygdaloid area (*AAA*); see also *C*, which is a dark-field view of an adjacent anterior to *D* and *E*. *F–H*, More posterior sections illustrating "segment"-like expression, strong for *Pax-7* (*G*) but faint for the *Pax-3* (*H*), confined to the entire pretectum (*PT*). In *E* and *F* the *small arrowheads* point to a thin cell layer in the DT expressing *Pax-6*. In *G* the *arrow* points to the presumptive region of the differentiating periventricular hypothalamic nucleus. *CEn*, entorhinal cortex; *Hi*, hippocampus; *zi*, zona incerta; *LV*, lateral ventricle; *3v*, third ventricle; *fr*, fasciculus retroflexus. Magnification, 40×.

Gruss, 1991; Asano and Gruss, 1992), we find that at this stage some of the *Pax*-genes were expressed with decreasing activity from the spinal cord through the myelencephalon and pons, up to the same boundary, being mainly confined to the intermediate zone (*Pax-2, Pax-8*, Fig. 1*A,B*), to the ventricular zone (*Pax-3*, Fig. 1*E*) or to both the ventricular and intermediate zone (*Pax-7, Pax-6*, Fig. 1*F,G*). The strong hybridization signal detected for *Pax-7* mRNA in the intermediate zone of the pons, in an area slightly posteriorly to the hindbrain–midbrain boundary (Fig. 1*F*), probably represents a group of differentiating cells of the pontine reticular nucleus (see also Fig. 3*E*). The *Pax-6* probe showed a hybridization signal of medium intensity on cells at that boundary (Fig. 1*G*, arrowhead), while for *Pax-5* an extremely high accumulation of transcripts was observed on the both sides of fovea isthmi (Fig. 1*C*). Thus, although not all *Pax*-gene transcripts are strictly colocalized within the hindbrain–midbrain boundary, it is obvious that as upper expression limit in the hindbrain, the *Pax*-genes include the most anterior hindbrain areas and the isthmus. Consistent with the distribution of the *Pax*-gene transcripts in this area, we find overlapping and complementary domains of expression for *Pax-3* and *Pax-7* in derivatives of the first branchial arch (maxilla and mandible) that originate from rhombomere 1 (data not shown).

Our results show that in the midgestation embryonic brain the hindbrain–midbrain boundary delineates the most rostral expression domains of *Pax-2, Pax-8*, and *Pax-5* (see also Fig. 7). Thus, at E13 p.c. in the mesencephalic tectum, only the transcripts of *Pax-3, Pax-7*, and *Pax-6* were detected. The expression of *Pax-3* gene was still found in the ventricular zone of the mesencephalon (Fig. 1*E,I*; see Fig. 3*C*) while the *Pax-7* transcripts were abundantly distributed throughout both the ventricular and the intermediate zone as well as in the most superficial mesencephalic layers close to the pial surface (Fig. 1*F,J*). In more lateral plane of sections both genes include in their expression domains the pretectal areas (the former synencephalon), encompassing the posterior commissure (Fig. 1*I,J*)

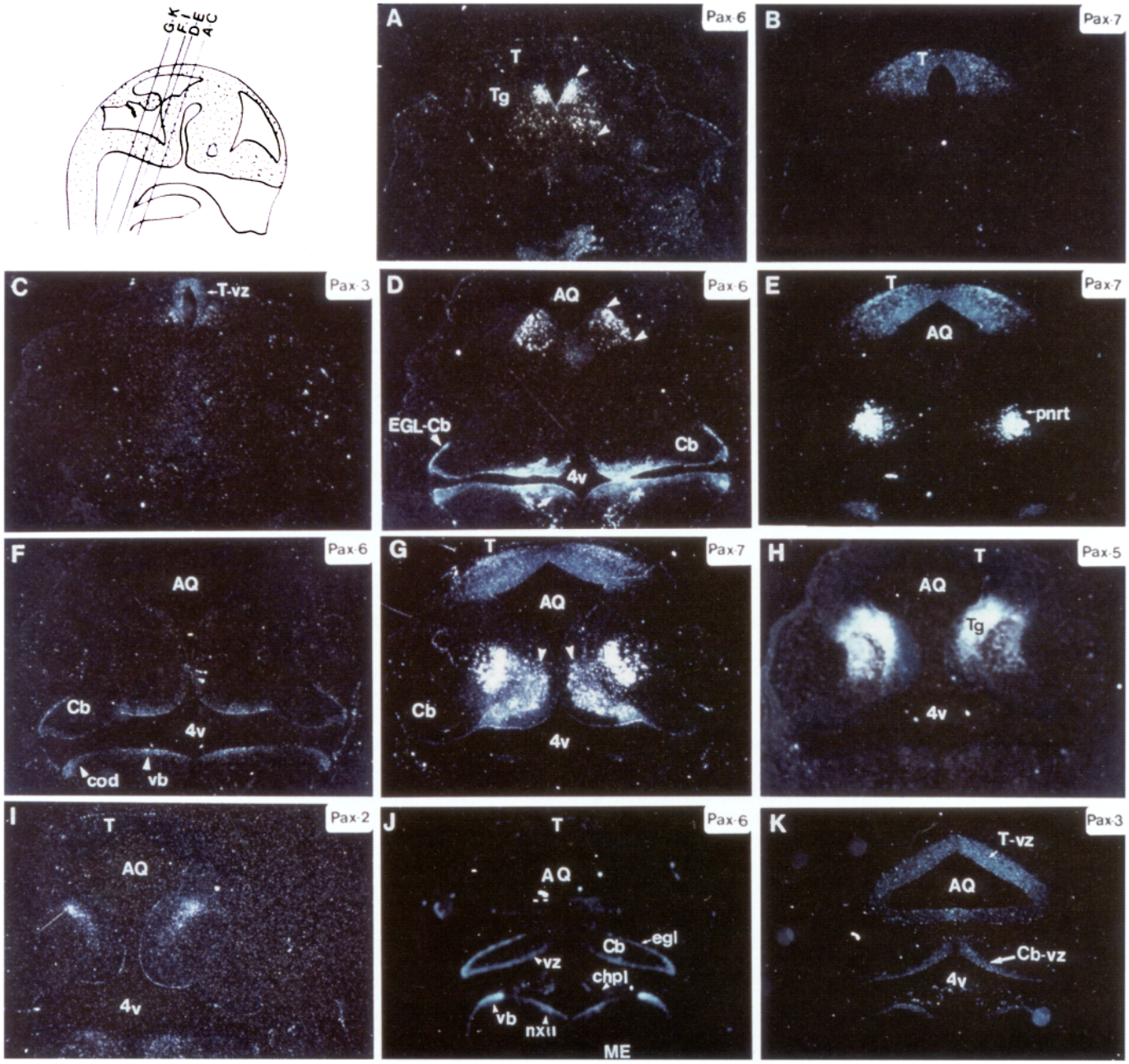


*Figure 3.* Expression of the *Pax*-genes in midbrain and hindbrain of a 13-d-old embryo. Consecutive coronal sections that are progressively more posterior those shown in Figure 2*H* have been hybridized with various *Pax*- gene probes as indicated. *A–C*, Sections through the rostral midbrain showing complimentary expression regions for *Pax-6* (*A*) and *Pax-7* (*B*) in the mesencephalic tegmentum (*Tg*) or tectum (*T*), respectively. The *arrowheads* in *A* point to the presumptive region of the differentiating substantia nigra. *C*, *Pax-3* transcripts are still detectable in the dorsolateral areas of the ventricular zone of the mesencephalon. *D* and *E*, More posterior sections showing localization of *Pax-6* (*D*) and *Pax-7* (*E*) transcripts in the region of the nucleus raphe dorsalis (*arrowheads* in *D*) and in the pontine reticular nucleus (*pnrt*), respectively. *F–I*, Sections through the midbrain–hindbrain region illustrating the most rostral expression limit for *Pax-5* (*H*) as compared with *Pax-6* (*F*), *Pax-7* (*G*), and *Pax-2* (*I*). The *arrowheads* in *G* point to the presumptive area of the differentiating interpeduncular nucleus. In very posterior sections through the hindbrain, *Pax-6* transcripts (*J*) are detected in the region of the vestibular and the hypoglossal nuclei (*vb, nXII*) and in the ventricular zone (*vz*) plus the external granular layer (*egl*) of the cerebellum (*Cb*), while *Pax-3* transcripts (*K*) are detected only in the ventricular zone of the cerebellum (*Cb-vz*). *AQ*, aqueduct of Sylvius; *cod*, dorsal cochlear nucleus; *chpl*, choroid plexus; *ME*, medulla; *vb*, vestibular nucleus; *4v*, fourth ventricle. Magnification, 40×.

and the epithalamic region (Fig. 2*G,H*; see also Fig. 7). The posterior commissure is the anatomical landmark of the border between the last prosencephalic neuromere, the synencephalon, and the mesencephalic rostral neuromere (Puelles et al., 1987). Interestingly, this anatomical boundary is the caudal limit of expression for the *Pax-6* gene at this stage in the dorsal forebrain, extending from the telencephalon and the dorsal diencephalon (Fig. 1*K*; see also Walther and Gruss, 1991) that are derivatives of the forebrain neuromeres, the secondary prosencephalon, and parencephalon posterior, respectively (Puelles et al., 1987). These data indicate that *Pax*-genes have restricted expression domains along longitudinal regions of the developing forebrain.

Analysis of the spatial expression patterns of the *Pax*-genes

in coronal sections of embryonic brain revealed that in addition to longitudinal planes, the expression domains of distinct *Pax*-genes respect neuromere boundaries in transverse planes as well. In very frontal sections, *Pax-6* transcripts were found in the frontodorsal cortex up to the caudatopallial angle and in differentiating cell groups in the septal area (horizontal and vertical limb of the diagonal band nucleus of Broca, Fig. 2*A*) as well as in globus pallidus, preoptic area, olfactory neuroepithelium, retina (Fig. 2*B*), and in the amygdaloid area (Fig. 2*C,E*). In section planes through the diencephalon, three *Pax*-genes showed "segment"-like expression patterns. We used the Herrick's schema for the subdivision of the diencephalon in coronal plane into epithalamus, dorsal thalamus, ventral thalamus, and hypothalamus by the ventricular sulci—sulcus diencephalicus dorsalis, medius, and ventralis (Herrick et al., 1948). It is agreed that almost all vertebrates can be fitted into this scheme (Kuhlenbeck, 1973), and it is widely used in recent molecular studies on expression of various genes during brain development. As shown in Figure 2*C–F*, the *Pax-6* is strongly expressed in the ventral thalamus with a sharp upper limit just bellow the zona limitans intrathalamica (the large arrowheads in Fig. 2*E*). This low-cell-density region [designated also zona limitans interparencephalica (Puelles et al., 1987) or reticular protuberance (Altman and Bayer, 1986)] separates the dorsal from the ventral thalamus. It develops at the furrow between the forebrain neuromeres—the parencephalon anterior and the parencephalon posterior (Keyser, 1972; Puelles et al., 1987; equivalent to neuromere I and II of Coggeshall, 1964). In accordance with the reported caudorostral gradient of the differentiation of the diencephalon (Angevine, 1970), the expression of *Pax-6* in a more anterior section plane was detected in both the ventricular and mantle layer of the ventral thalamus (Fig. 2*C*). In a more posterior section, however, the hybridization grains were distributed only in the mantle layer of the ventral thalamus (Fig. 2*E,F*) where the zona incerta, the reticular nucleus, and entopeduncular nucleus start to differentiate (Altman and Bayer, 1986). In the dorsal thalamus and epithalamus, the *Pax-6* gene transcripts were still detected in the internal germinative layer (Fig. 2*C–F*), consistent with the described sharp ventrodorsal gradient of differentiation of the diencephalon (Angevine, 1970; Keyser, 1972). In addition, a thin layer of *Pax-6*–positive cells close to the zona limitans was detected in the dorsal thalamus (Fig. 2*E,F,* small arrowheads). In more posterior sections, a hybridization signal of moderate intensity was observed for the *Pax-6* gene in the internal germinative layer and the mantle layer of the epithalamus (Fig. 2*F*) while the *Pax-7* (Fig. 2*G*) and *Pax-3* genes (Fig. 2*H*) were abundantly or faintly expressed in the entire diencephalic region above the fasciculus retroflexus including the epithalamus and the pretectum. In addition, a group of strongly hybridizing cells were detected with the *Pax-7* probe in the presumptive region of the hypothalamic periventricular nucleus (the arrow in Fig. 2*G*). Taken together these results show that in E13 p.c. mouse brain, the main expression domain of *Pax-6* in the developing diencephalon is confined to the ventral thalamus (the former parencephalon anterior), while in the dorsal thalamus (the former parencephalon posterior) it delineates the epithalamic region. In the pretectum (the former synencephalon), in its commissural as well as its precommissural part (Keyser, 1972), *Pax-7* and *Pax-3* genes are expressed while *Pax-6* expression is detected in the precommissural part of this region (see also Fig. 7).

The distinct expression patterns of *Pax*-genes in more posterior coronal sections are shown in Figure 3. At the level of superior colliculus, complementary expression with a sharp boundary at the sulcus limitans was detected in the tegmentum or the tectum of the mesencephalon for *Pax-6* (Fig. 3*A*) and *Pax-7* (Fig. 3*B*), respectively. It is of interest that the hybridization signal for *Pax-6* extends also lateral into the tegmentum in the presumptive region of the differentiating substantia nigra (arrowheads in Fig. 3*A*). As already noted, *Pax-3* gene transcripts at this stage were still detectable in the dorsolateral part of the ventricular zone of the mesencephalon (Fig. 3*C*; Goulding et al., 1991). Strongly labeled cells were observed with the *Pax-6* probe in the presumable area of differentiating dorsal raphe (arrowheads in Fig. 3*D*), while *Pax-7* transcripts were abundantly accumulated in the pontine reticular nucleus (Fig. 3*E,G*) and in a possibly interpeduncular areas (arrowheads in Fig. 3*G*). In a more posterior plane, *Pax-6* transcripts were localized in the region of the cochlear, vestibular (Fig. 3*F*) and hypoglossal nuclei (Fig. 3*J*) as well as in the ventricular zone and the external germinative layer of the cerebellum (Fig. 3*J*; see also Fig. 1*G*). In contrast, in the cerebellum *Pax-3* mRNA–positive cells were found only in the ventricular zone (Fig. 3*K*; see also Fig. 1*E*). As reported previously, *Pax-2, Pax-5, Pax-7,* and *Pax-8* transcripts were also present in midgestation cerebellum (Fig. 1*A–C,F,J*).

### *Distribution of the* Pax*-gene transcripts in young adult brain*

The most interesting expression patterns of the *Pax*-gene in 4-week-old mouse brain are shown in Figures 4–6. The results of the study on their transcript localization in brain are summarized and diagrammatically represented in Figure 8. Similar data were obtained when hybridizations were done on brain sections of later stages (6 and 12 weeks old, data not shown).

*Telencephalon and diencephalon.* As shown in Figure 4*A–F*, only one member of the *Pax*-gene family, *Pax-6*, was expressed in discreet areas of the forebrain. In the olfactory bulb, the periglomerular cells of the glomerular layer and the internal granular layer of the main and the accessory olfactory bulb showed high and medium accumulation of the *Pax-6* transcripts, respectively (Figs. 4*A,B*, 8*a*). Within the septal area, a moderate hybridization signal was observed for the *Pax-6* in the lateral septal nucleus (Figs. 4*C*, 8*b,c*) and a strong signal in the medial septal nucleus and in the horizontal and vertical limb of diagonal band nucleus (Broca) (Figs. 4*D*, 8*e*). Moderate expression of *Pax-6* was detected in medium-sized cells, lightly colored with the toluidine blue, in the nuclei of the basolateral complex of the amygdala (Figs. 4*E*, 8*d*), while the other nuclei (except for the basomedial nucleus) had background level of labeling. In the basal ganglia, labeled cells were found in a small region of the ventral pallidum (Figs. 4*D*, 8*c*) and in the entopeduncular nucleus (Figs. 4*E*, 8*d*). A hybridization signal was also detected for *Pax-6* mRNA in zona incerta (Fig. 4*E*) and in its lateral extension into a discreet area of the thalamic reticular nucleus (Fig. 8*d*). The entire cerebral cortex and the hippocampus lacked detectable hybridization for any of the *Pax*-mRNAs.

*Midbrain.* A very high accumulation of *Pax-6* gene transcripts was found in a subset of big and medium sized neuronal cells in the dorsolateral part of substantia nigra reticularis (Figs. 4*F*, 8*e*), which are lightly colored upon staining with toluidine blue. The rostral part of the midbrain central gray also showed expression of *Pax-6* (Figs. 4*F*, 8*e*). With the *Pax-7* probe, a strong hybridization signal was detected in the superior colliculus, especially in the superficial, intermediate, and deep gray layers (Figs. 4*G*, 8*e,f*) and a faint signal in the dorsal part of the midbrain central gray (Fig. 8*e,f*). The *Pax-7* gene was also strongly

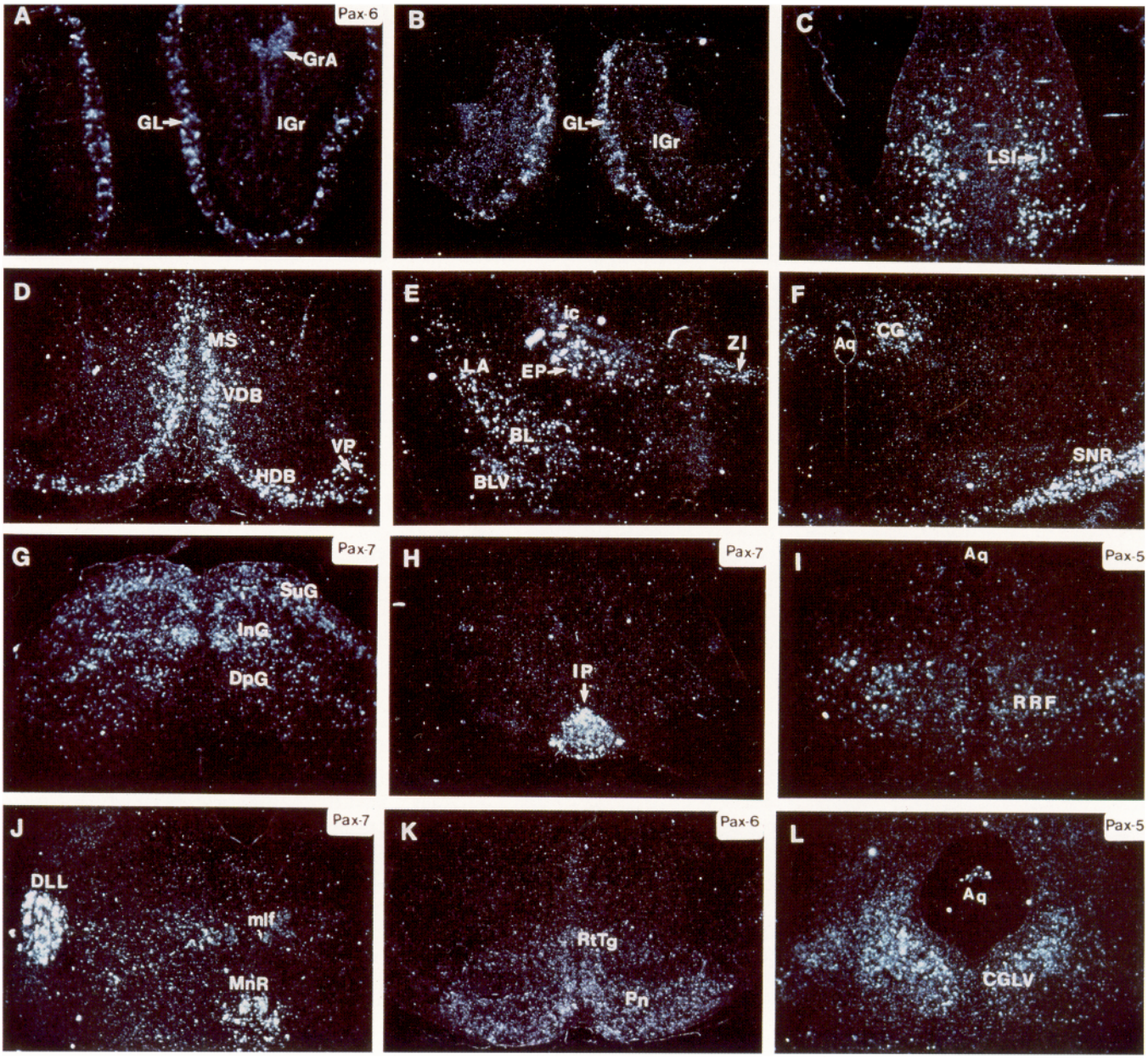


*Figure 4.* Regional distribution of the *Pax*-gene mRNAs in forebrain and midbrain in coronal sections of young adult mouse brain. Consecutive sections were hybridized with different *Pax*-probes as indicated. The pictures of the dark-field photomicrographs are presented in a rostrocaudal direction. *A–F*, Photomicrographs show the expression of *Pax-6* in defined areas of the rostral forebrain. *A* and *B*, In the olfactory bulb, *Pax-6* transcripts are seen in the periglomerular cells of the glomerular layer (*GL*), in the cells of the accessory olfactory bulb (*GrA*) and in the internal granular layer (*IGr*). *C* and *D*, Sections through the septal area showing the presence of *Pax-6* mRNA in the intermediate lateral and medial septal nuclei (*LSI, MS*), in the vertical and horizontal limb of the diagonal band nucleus of Broca (*VDB, HDB*), and in a part of the ventral pallidum (*VP*). *E*, Section through the diencephalon showing localization of *Pax-6* mRNA in the zona incerta (*ZI*), entopeduncular nucleus (*EP*), and the lateral (*LA*), basolateral (*BL*), and basolateral ventral subnucleus (*BLV*) of the amygdala. *F–L*, Rostral to caudal sections through the midbrain. *F*, High accumulation of *Pax-6* mRNA in cells in the dorsolateral part of the zona reticularis of the substantia nigra (*SNR*) and in the central gray (*CG*). *G*, Strong expression of *Pax-7* in optic tectum, especially in the superficial, intermediate, and deep gray layer (*SuG, InG, DpG*) of the superior colliculus. *H*, Strong expression of *Pax-7* in the interpeduncular nucleus, pars compacta (*IP*). *I*, At the same plane *Pax-5* is moderately expressed in dispersed cells in the retrorubral field (*RRF*). *J–L*, Sections through the most caudal plane of the midbrain. *J*, High accumulation of *Pax-7* transcripts in the median raphe nucleus (*MnR*) and in the dorsal nucleus of medial lemniscus (*DLL*). *K*, Strong signal with the *Pax-6* probe in the pontine nuclei (*Pn*) and in the reticulotegmental nucleus of pons (*RtTg*). *L*, Moderate level of *Pax-5* expression in the lateroventral part of the mesencephalic central gray (*CGLV*). *ic*, internal capsule; *mlf*, medial longitudinal fasciculus. Magnification, 40×.

expressed in the interpeduncular nucleus, central (Figs. 4*H*, 8*e*), in some of its subnuclei localized in the more posterior planes—apical and paramedian subdivision of the interpeduncular nucleus (Fig. 8*f*)—in the dorsal nucleus of the lateral lemniscus, and the median raphe nucleus (Figs. 4*J*, 8*g*). It is of note that, while *Pax-6* transcripts were detected in the rostral part of the midbrain central gray (Figs. 4*F*, 8*e*), *Pax-5* mRNA was localized in the more caudal lateral and the ventral parts of the central gray (Figs. 4*L*, 8*g*), including the region of the mesencephalic nucleus of the Vth cranial nerve. In the midbrain tegmentum,

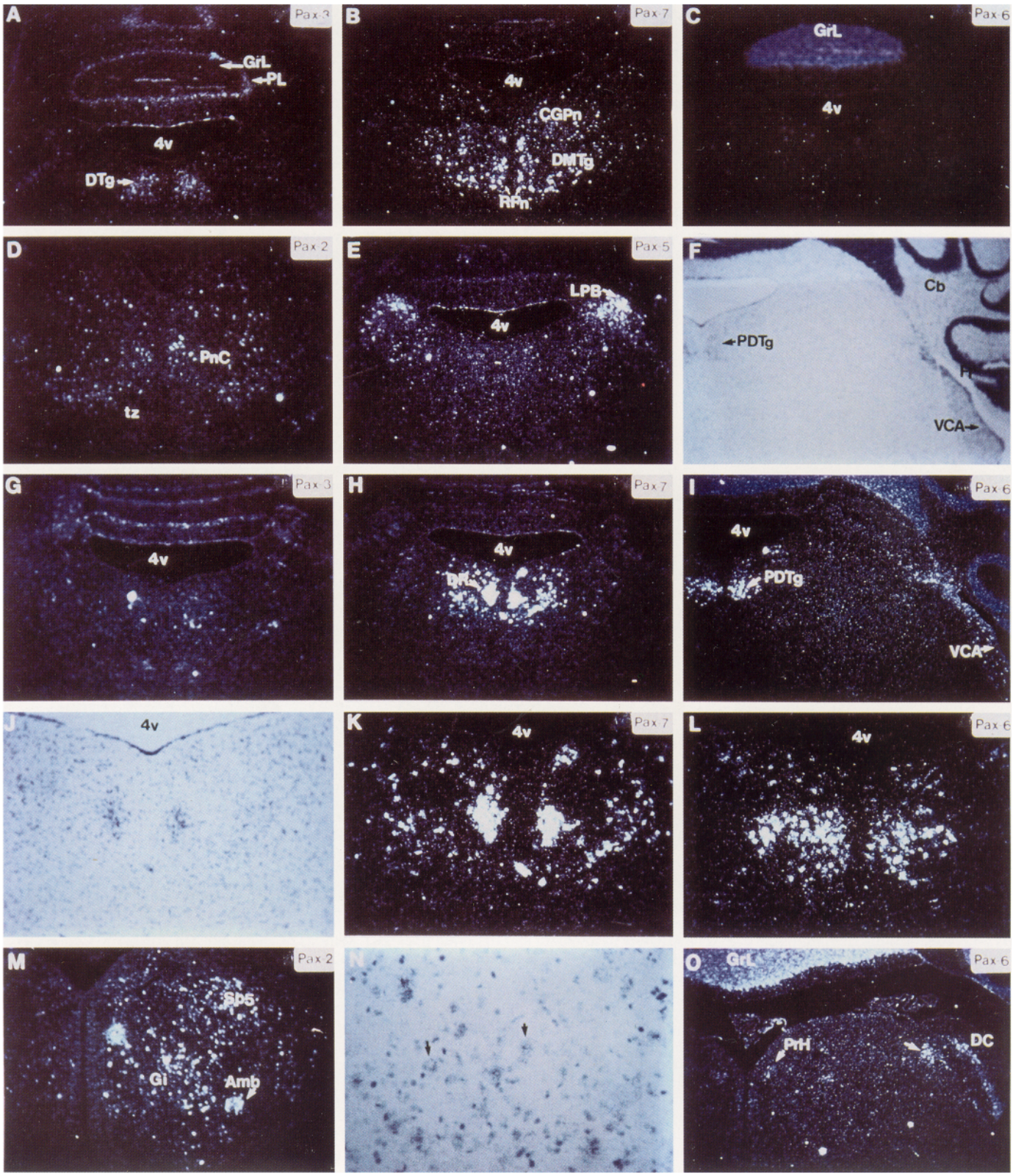


*Figure 5.* Expression of the *Pax*-genes in the hindbrain of young adult mouse brain. Adjacent coronal sections progressively more posterior that in Figure 4*L* were hybridized with various *Pax*-gene probes. The most interesting patterns are presented in a rostrocaudal direction. *A–E*, Sections through a rostral plane of the metencephalon. *A*, Labeled cells in the dorsal tegmental nucleus, central (*DTg*), and in the Purkinje cell layer (*PL*) of the cerebellar cortex with the *Pax-3* probe. *B*, Labeled cells in the pontine central gray (*CGPn*), in the dorsomedial tegmental area (*DMTg*), and in raphe pontis (*RPn*) with the *Pax-7* probe. *C*, Labeling with *Pax-6* probe of the cerebellar granular layer (*GrL*). Note that at this plane of section, *Pax-6* transcripts are not detected in the DTg. *D*, Hybridizing cells in the caudal pontine reticular nucleus (*PnC*) with the *Pax-2* probe. *E*, Strong expression of *Pax-5* in lateral parabrachial nuclei (*LPB*). *F–L*, Sections through the midbrain in a more posterior plane. Bright-field (*F*) and dark-field (*I*) photomicrographs show high accumulation of *Pax-6* transcripts in the posterior dorsal tegmental nucleus (*PDTg*) and labeled cells in the anterior ventral cochlear nucleus (*VCA*). *H*, Section adjacent to the one shown in *I* illustrates a strong expression of *Pax-7* in cells of the caudal

*Pax-5* was also expressed in cells of the retrorubral field (Figs. 4*I*, 8*f*) as well as in few cells distributed in the ventral region of the reticular zone of substantia nigra and the ventral tegmental area (Fig. 8*e*). In the ventral tegmental area, a few scattered cells also contained *Pax-6* transcripts (Fig. 8*e*). Several nuclei of the reticular formation showed moderate to low levels of the hybridization signal for *Pax-6* in the deep mesencephalic nucleus (Fig. 8*f*) and in the reticulotegmental nucleus of the pons (Figs. 4*K*, 8*g*) and for *Pax-2* in the oral and caudal part of the pontine reticular nucleus (Figs. 5*D*, 8*h–j*). It should be noted that the *Pax-8* gene (the paralog of *Pax-2*) showed only occasionally a hybridization signal above the background levels; therefore, these data are not included in the scheme presented in Figure 8.

*Pons and medulla.* Transcripts of *Pax-6* were detected in the pontine nuclei in the trapezoid body and in the reticulotegmental nucleus of pons (Figs. 4*K*, 8*g*), while *Pax-7* transcripts were found in cells of the dorsomedial tegmental area as well as in cells of the oral part of the pontine reticular nucleus (Figs. 5*B*, 8*h*). The main isthmic nuclei showed accumulation of distinct *Pax*-gene transcripts. In the rostral part of the laterodorsal tegmentum, *Pax-3* transcripts were detected at low level in the dorsal tegmental nucleus of Gudden (Figs. 5*A*, 8*h*). In a more caudal section, the posterior dorsal tegmental nucleus showed strong hybridization signal with the *Pax-6* probe (Figs. 5*I,L*; 8*i*). High accumulation of *Pax-7* transcripts was detected in the cells in the most caudal part of the dorsal raphe nucleus and in the cells surrounding the posterior dorsal tegmental nucleus (Figs. 5*H,K*; 8*i*), while the *Pax-2* probe labeled the gigantocellular reticular nucleus in the medulla oblongata (Figs. 5*M,N*; 8*j*). The *Pax-5* gene was found to be strongly expressed in the lateral parabrachial nuclei (Figs. 5*E*, 8*h*). In both the ventral and the dorsal cochlear nuclei (Fig. 5*I,O*) and in the prepositus of the hypoglossal nucleus (Fig. 5*O*), transcripts of the *Pax-6* gene were detected (see also Fig. 8*g–j*). Highly labeled cells with the *Pax-2* probe were observed in the medulla in the gigantocellular reticular nucleus, in the nucleus of the spinal tract of the trigeminus nucleus, and in the ambiguus nucleus (Figs. 5*M,N*; 8*j*), while the nucleus gracilis and the nucleus cuneatus showed a moderate hybridization signal with the same probe (not shown).

*Cerebellar cortex.* Hybridization with *Pax-2, Pax-3*, and *Pax-6* probes showed expression in different cells of the cerebellar cortex. The cells labeled with the *Pax-2* probe had the distribution and appearance of Golgi neurons, scattered through the granular layer (arrows in Fig. 6*F*). The homogeneously distributed grains seen over the molecular layer could indicate unspecific labeling of cells or cell processes since a similar labeling was detected with the sense probe (not shown). The Purkinje cell layer was strongly labeled with the *Pax-3* probe (Fig. 6*A*). The examination of a high-power dark and bright fields of the sections (Fig. 6*B,C*) revealed that the Purkinje cells (Fig. 6*B,C*, large arrows) occasionally had grains above background level; therefore, we considered them to be unlabeled. The clusters of silver grains were also detected on small cells beneath the Purkinje cells, the putative Bergmann glia (Fig. 6*B,C*, small arrows) as well as on small cells surrounding the Purkinje cells laterally, representing probably a subset of basket neurons (Fig. 6*B,C*, arrowheads). In contrast, the *Pax-6* hybridization probe labeled strongly mostly the granule cells in the granular layer of the cerebellar cortex (Fig. 6*D,E*) but labeling of glial cells cannot be excluded.

## Discussion

The present study is the first to investigate the postnatal expression patterns in the CNS of a family of developmental control genes in relation to their embryonic expression profiles. The results from the performed comparative *in situ* hybridization study suggest that *Pax*-genes are involved in the early regionalization of the embryo brain and may have a role in the differentiation, maintenance, and functional assembly of specific subsets of cells in the adult brain.

### Pax-*genes and brain segmentation*

We report here that the expression domains of several *Pax*-genes correspond to morphological landmarks that delineate former neuromeric structures in the developing CNS. Neuromeres, as repetitive transverse bulges of the developing CNS, are segmental entities that are observable during early stages of development in most vertebrates. In the epichordal CNS (spinal cord and hindbrain), inductive signals from the underlaying notochord and floorplate may be involved in the formation of the neuromeres (Lim et al., 1991; Yamada et al., 1991). Recent neuroanatomical and molecular studies confirmed the segmented nature of the hindbrain and have revealed that the hindbrain neuromeres (rhombomeres) are polyclonal lineage restriction units that are specified by the expression of distinct combinations of *Hox*-genes and *Krox-20* (Lumsden and Keynes, 1989; Fraser et al., 1990; Keynes and Lumsden, 1990; Hunt et al., 1991; Wilkinson et al., 1989; reviewed in Lumsden, 1990; McGinnis and Krumlauf, 1992). The most anterior boundary of the expression of the *Hox*-genes (class I) is between rhombomere 1 and rhombomere 2, as shown for *Hox-A2* (reviewed by Krumlauf, 1993). It is of note that rhombomere 1 and the associated structures in the first branchial arch do not express *Hox* genes. In contrast, as shown here, the expression domains of all *Pax*-genes in hindbrain extend farther rostrally including the rhombencephalic isthmus (rhombomere 0, according to Vaage, 1969), thus surpassing the rostral limit of expression of all *Hox*-genes. This suggests an involvement of *Pax*-genes in the regionalization of the most anterior hindbrain domains. As previously reported, in early-stage embryo (E10 p.c.), the upper expression border of *Pax-2* in the developing spinal cord and hindbrain is at the hindbrain–midbrain boundary (Nornes et al., 1990), while for *Pax-5*, an extremely strong expression was detected at the same boundary as early as E9 p.c. (Asano and Gruss, 1992). It may be expected, therefore, that members of the *Pax*-gene family (best candidates being *Pax-2* and *Pax-5*) may have an important role in setting up the midbrain–hindbrain boundary. This assumption is supported by recent experiments showing that injection of an antibody raised against the

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part of the dorsal raphe (*DR*) and in the cells surrounding the PDTg, the latter being negative. *K* and *L* are higher-magnification images, respectively, of the sections shown in *H* and *I* better illustrating the complementary domains of expression for *Pax-7* and *Pax-6* in the dorsal tegmentum. *J* is bright-field view of *K*. *M* and *N*, In the most caudal plane, *Pax-2* mRNA (*M*, *N*) is detected in the gigantocellular reticular nucleus (*Gi*), ambiguus nucleus (*Amb*), and in the spinal trigeminal nucleus (*Sp5*). *O*, *Pax-6* transcripts are seen in the dorsal cochlear nucleus (*DC*); a very faint signal is detected in the prepositus of hypoglossal nucleus (*PrH*) and in the lateral vestibular nucleus (*arrow*). The *arrows* in *N* point to highly labeled cells in the gigantocellular reticular nucleus with *Pax-2* probe. *Cb*, cerebellum; *tz*, nuclei of the trapezoid body; *4v*, fourth ventricle. Magnifications: *A–I*, *M*, and *O*, 40×; *J–L*, 100×; *N*, 250×.

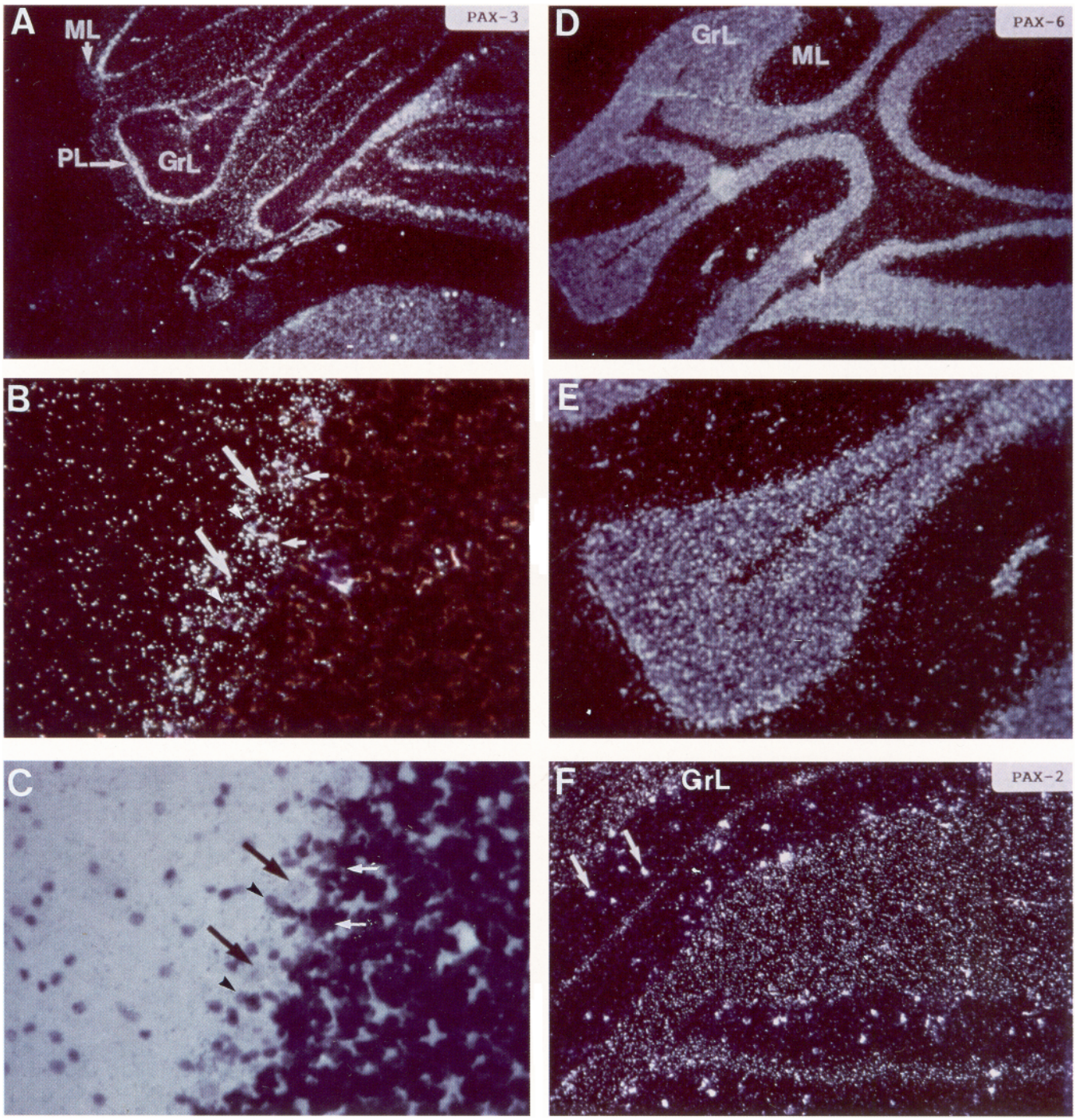


*Figure 6.* Expression of the *Pax*-genes in cerebellar cortex of young adult brain. *A*, Dark-field photomicrograph of a coronal section through posterior cerebellum showing labeling of the Purkinje cell layer with *Pax-3* probe. *B* and *C*, Dark-field (*B*) and bright-field (*C*) views of the same section under higher magnification. The signal seen over the Purkinje cells (*large arrows*) was occasionally above the background level. Labeling is mainly detected on small cells localized beneath the Purkinje cells (putative Bergmann glia, *small arrows*) and on small cells surrounding the Purkinje cells laterally at the depth of the molecular layer (probably a subset of basket cells, *arrowheads*). *D* and *E*, Strong expression of *Pax-6* in the granular cell layer. *F*, Dark-field photomicrograph of a section hybridized with *Pax-2* probe, showing labeling of putative Golgi neurons (*arrows*) spread in the granular layer. The homogeneous signal seen in the molecular layer is considered at present to be unspecific labeling of glial processes or cells since similar labeling was detected with the sense probe. *GrL, ML, PL,* granular, molecular, and Purkinje cell layers of the cerebellar cortex, respectively. Magnifications: *A* and *D*, 100×; *B* and *C*, 400×; *E* and *F*, 250×.

zebrafish *Pax-2* results in a localized malformation at the midbrain–hindbrain border (Krauss et al., 1992). The potential relationship between such members of the *Pax*-gene family and other genes, also known to be expressed around this boundary (*En-1, En-2,* Davidson et al., 1988; Davis and Joyner, 1988; Davis et al., 1988; *Wnt-1,* Wilkinson et al., 1987), is an interesting field for further research.

The prechordal CNS (midbrain and forebrain) develops in-

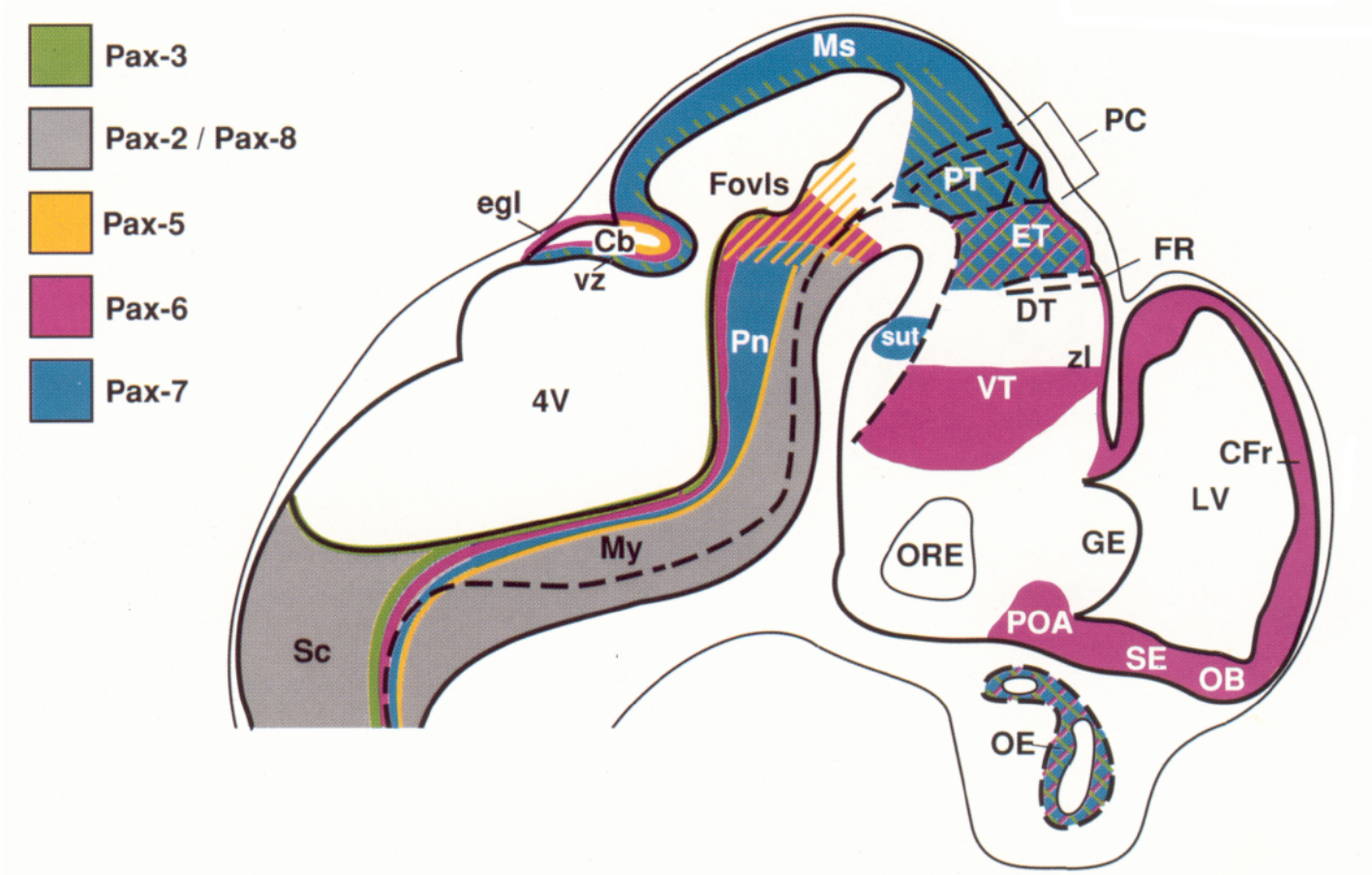


*Figure 7.* Schematic representation of the expression of *Pax*-genes in embryonic brain. The major expression domains of the *Pax*-genes, illustrating the restricted transcripts distribution at brain boundaries in E13 p.c., are indicated by different colors. The lines in hindbrain and spinal cord represent the presence of corresponding *Pax*-gene transcripts and do not reflect their regional distribution along the dorsoventral axis. The computer drawing of the sagittal section of the embryo brain is based on the atlas of Schambra et al. (1992). *Cb*, cerebellum; *CFr*, frontal cortex; *DT*, dorsal thalamus; *egl*, external granular layer of cerebellum; *ET*, epithalamus; *FovIs*, fovea isthmi; *GE*, ganglionic eminence; *LV*, lateral ventricle; *Ms*, mesencephalon; *My*, myelencephalon; *OB*, olfactory bulb; *OE*, olfactory neuroepithelium; *ORE*, optic recess; *PC*, posterior commissure; *Pn*, pons; *PT*, pretectum; *Sc*, spinal cord; *SE*, septum; *sut*, subthalamus; *vz*, ventricular zone; *4V*, fourth ventricle.

dependently from the inductive effects of the notochord and the floorplate. Despite the morphologically well-documented presence of segmental entities in this part of the brain at early embryonal stages in frog (Jacobson, 1983), mouse (Niimi et al., 1962; Jacobson and Tam, 1982; Sakai, 1987), rat (Bergquist and Kallen, 1954; Coggeshall, 1964), chinese hamster (Keyser, 1972), chicken (Puelles et al., 1987), and human (Gilbert, 1935; Dekaban, 1954), data concerning their number, localization, and significance still remain contradictory and the molecular basis of segmental organization of the forebrain is unknown. It is agreed, however, that within the caudal part of the diencephalon, the interneuromeric borders can be traced into the adult stage. Before the interneuromere borders fade, fiber tracts and fiber laminae develop that make it possible to recognize these borders even after the development of the mantle layer and also in the adult brain. At early embryonal stages, the primitive prosencephalon divides into the secondary prosencephalon and the diencephalon (Puelles et al., 1987). The secondary prosencephalon will form later the telencephalon. The diencephalon subsequently gives rise to three neuromeres: parencephalon anterior (ventral thalamus), parencephalon posterior (dorsal thalamus), and synencephalon (pretectum). With the advance of differentiation, the interparencephalic border between the dorsal and ventral thalamus is delineated initially by the tract of zona limitans intrathalamica and later by the external medullary lamina that develops at the same place. Similarly, the syn-parencephalic border is outlined by the fasciculus retroflexus (tractus habenulo-interpeduncularis), while the posterior commissure marks the boundary between the synencephalon and the mesencephalic bulge (mesomere).

Our results demonstrate "segment"-like expression pattern for three of the *Pax*-genes on coronal sections through the developing diencephalon. The distribution of the *Pax-6* transcripts strictly respects the boundaries of the ventral thalamus (the previous parencephalon anterior). The expression domains of the *Pax-7* and *Pax-3* genes are overlapping and confined to the entire diencephalic region dorsal of the fasciculus retroflexus, thus including the epithalamus and the pretectal areas (the former synencephalon), while *Pax-6* transcripts are observed mainly in the epithalamus. The expression pattern of these genes on sagittal sections of embryo brain also support such a conclusion (see Fig. 7). Opinions concerning the neuromere origin of the epithalamus are contradictory. According to Coggeshall (1964) and Niimi et al., (1962) the epithalamus corresponds entirely to the synencephalon, thus encompassing the habenular and also all pretectal nuclei. However, most of the authors consider that

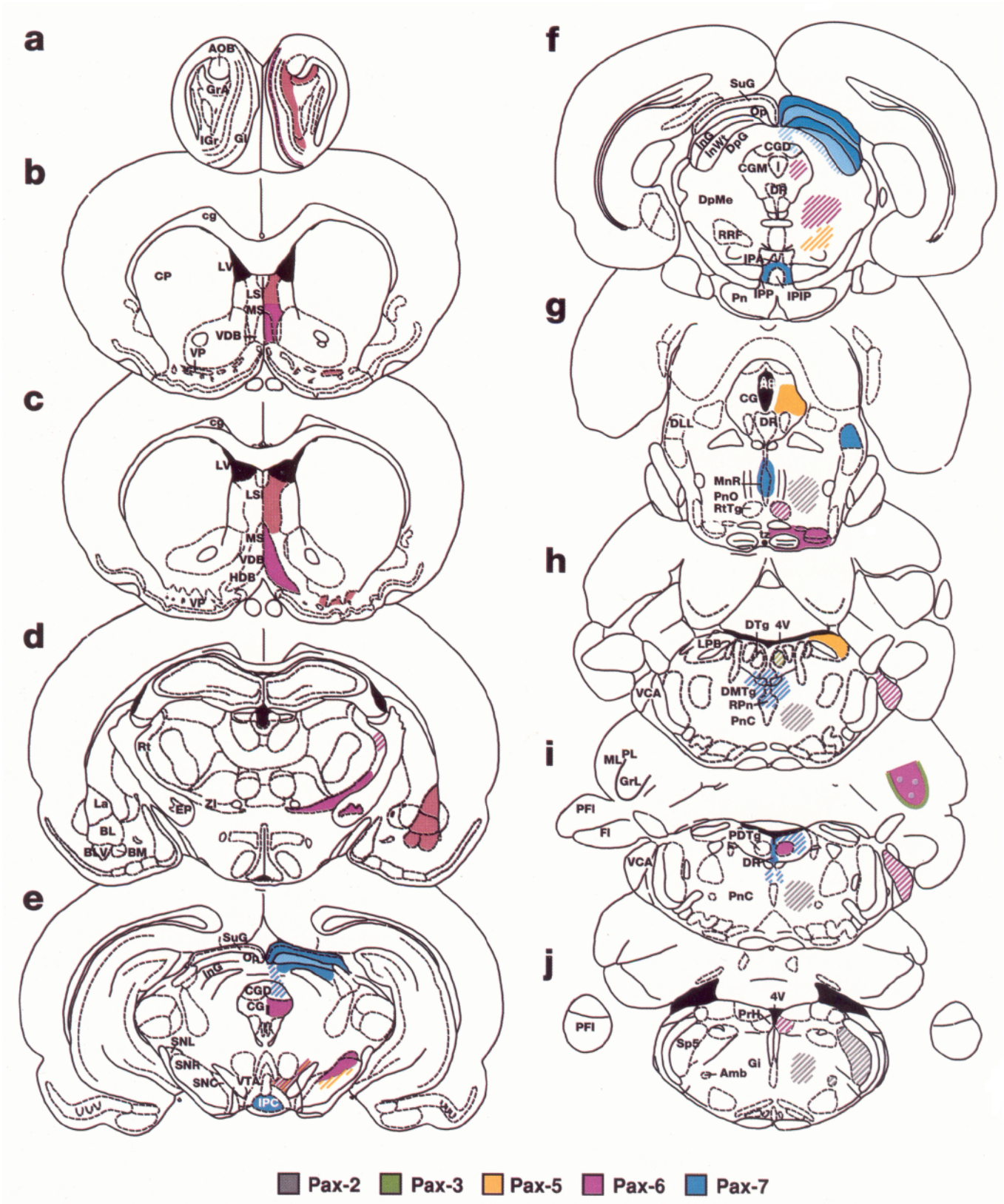


*Figure 8.* Schematic representation of the *Pax*-gene transcript distributions in young adult mouse brain. Regions in which the majority of the cells exhibit strong expression of the respective mRNA are represented by intensive colors as indicated for the various *Pax*-genes. Regions exhibiting moderate expression in most of the cells are indicated by the corresponding light colors and those exhibiting weak expression or contain only scattered positive cells are *hatched*. The computer drawings of the coronal brain sections are based on the atlas of Paxinos and Watson (1982) and arranged from rostral to caudal. For abbreviations, see Appendix.

both the dorsal thalamus and the epithalamus are derivatives of the parencephalon posterior, the epithalamus extending along the entire dorsal border of this neuromere while the pretectum is a derivative of the synencephalon (Keyser, 1972; Puelles et al., 1987). Accordingly, it is reasonable to state that in the caudal diencephalon of E13 p.c. embryo brain, *Pax*-genes have restricted expression domains along longitudinal and transverse regions of former neuromere territories in the developing forebrain and may be useful markers for the segmental organization of the brain. The restricted expression pattern of the *Pax-6* gene to the ventral thalamus is observed as early as E10.5 p.c. (Walther and Gruss, 1991). Around E11 p.c. the expression domains of *Pax-3* and *Pax-7* genes in the forebrain are retracted to the mesencephalon, including the pretectal areas (Fig. 1*G,H*; see also Goulding et al., 1991; Jostes et al., 1991). The first visible signs of diencephalic segmentation appear around E13 p.c. in the rat embryo (Coggeshall, 1964), which is equivalent to the murine E11 p.c. As the patterns of expression of the *Pax-3, Pax-7*, and *Pax-6* genes and their relation to neuromere boundaries are established before the occurrence of any segmentation and axonal outgrowth in the forebrain, it may be that these *Pax*-genes play a role in establishing segment identities and axonal pathways at the borders of their expression domains. This assumption is supported by the reported data showing that the borders of expression of two zebrafish *Pax*- genes coincide with pathways of primary axon tracts generated in the embryonic brain a few hours after the onset of the expression of these genes (Krauss et al., 1991c).

Recent studies have identified a group of genes that are probably involved into the specification of the anterior part of the head. The expression patterns of the homeodomain-encoding protein genes *TTF-1* (Lazzaro et al., 1991); *Nkx-2.1, Nkx-2.2* (Price et al., 1992); *Dlx-1, Dlx-2/Tes-1, Gbx-2* (Porteus et al., 1991; Price et al., 1991; Robinson et al., 1991; Bulfone et al., 1993); *Emx-1, Emx-2* (Simeone et al., 1992a); and *Otx-1, Otx-2* (Simeone et al., 1992b), and the putative secreted factors of the *Wnt*-gene family (*Wnt-3, Wnt-3A, Wnt-5A, Wnt-7B*; Roelink and Nusse, 1991; McMahon et al., 1992; reviewed in Nusse and Varmus, 1992) coincide with anatomical boundaries in the developing prosencephalon. Interestingly, similar to the "segment"-like expression pattern shown here for the *Pax-6* gene, the *Dlx*-transcripts were localized in the ventral thalamus with a sharp anterior boundary at the zona limitans intrathalamica (Price et al., 1991, 1992; Roelink and Nuss, 1991; Salinas and Nusse, 1992). At this border, the transcripts of *Pax-6* and *Dlx*-genes abut upon the expression domains of *Wnt-3*, restricted to the dorsal thalamus as well as to a region of expression of *Nkx-2.2* that is localized in a thin layer of cells in the dorsal thalamus above the boundary expressing also *Pax-6* (Fig. 2*E*; Price et al., 1992). This raises the possibility that the regional specification of the developing diencephalon may be directed by the products of these genes in a chain of events. The earliest time that *Dlx*-transcripts were detected was at E10 p.c., thus being 2 d later than the beginning of the expression at E8–E8.5 p.c. of the *Pax-6* gene (Walther and Gruss, 1991). It seems reasonable to predict, therefore, that *Pax-6* acts before the *Dlx*-genes in a presumed cascade of regulatory processes specifying the ventral thalamus during development. Experiments are in progress to investigate the temporal and spacial expression patterns of *Dlx, Nkx-2.2*, and *Wnt-3* in *Small eye*, the mouse mutant for the *Pax-6* gene. In addition, both *Pax-6* and *Dlx*-genes are also coexpressed in the olfactory pit, the developing olfactory bulb, the anterior hypothalamus (preoptic area, pituitary), and the telencephalon (Price et al., 1991, 1992; Walther and Gruss, 1992). Interestingly, *Pax-6* transcripts are detected in the telencephalic dorsolateral cortex only up to the caudatopallial angle (Fig. 2*B,C*), while high accumulations of transcripts were reported for *Dlx*-genes (Price et al., 1991, 1992) and for *TTF-1* (Lazzaro et al., 1991), respectively, in the entire or only in the medial part of the neuroepithelium of the ganglionic eminence (the future corpus striatum). The mature corpus striatum belongs to the basal ganglia and has an interesting morphology. It consists of caudate-putamen (referred to as neostriatum), which are nuclei with telencephalic origin, and globus pallidus (referred to as paleostriatum), with diencephalic origin. As reported in this article, *Pax-6* transcripts are detected in the region of the differentiating globus pallidus and in the entopeduncular nucleus, which is the rodent equivalent of the mammalian internal pallidal segment (Heimer et al., 1985). Furthermore, in the adult brain, we observed a signal of medium intensity for the *Pax-6* gene in a small area in the ventral pallidum representing the ventral extension of the globus pallidus. Taken together, these facts strongly suggest that the *Pax-6*-gene together with the *Dlx* and *TTF-1* genes might encode important developmental regulators of the processes underlying the formation of the striatum.

### Pax-*genes and cell differentiation*

We made several observations indicating the possibility that *Pax*-genes may participate in cell differentiation in specific regions of the brain. During development, the establishment of spatiotemporal gradients (caudorostral, ventrodorsal, mediolateral) during the differentiation events of the brain has been described (Angevine, 1970; Keyser, 1972). Recent autoradiographic studies on the development of the rat thalamus and hypothalamus (Altman and Bayer, 1986, 1988; reviewed in Altman, 1992) and histochemical studies on forebrain development in chick using AChE as a differentiation marker (Puelles et al., 1987, 1991) demonstrated a mosaic-like pattern of appearance of differentiating cell patches within the matrix territory. According to Altman and Bayer (1986, 1988) the first wave of differentiating cells, designated as "reticular hypothalamus," that leave the neuroepithelium of the wall of the third ventricle between E13 and E15 p.c. in rat (equivalent to E11 p.c. in mouse) consists of the neuronal cells of zona incerta, reticular nucleus, entopeduncular nucleus, and the lateral hypothalamus. By E14 p.c. in rat (equivalent to E12 p.c. in mouse), the number of the differentiating cells in these structures increases rapidly. As shown in this article, the expression domains of *Pax-6* in the E13 p.c. mouse diencephalon apparently match the same differentiating regions in the ventral thalamus. On the other hand, a number of immunological studies have localized by E15 p.c. in rat (equivalent to E13 p.c. in mouse) a large accumulation of tyrosine hydroxylase–immunoreactive neurons in the preoptic area, zona incerta, and periventricular and arcuate nuclei (Sprecht et al., 1981; reviewed in Kalsbeek et al., 1992). The first appearance of tyrosine hydroxylase–immunoreactive neurons was observed in the mouse ventral thalamus around E11 p.c., thus soon after the initial restriction of *Pax-6* expression to the ventral thalamus at E10.5 p.c. (Walther and Gruss, 1991). The dopaminergic neurons of the substantia nigra (which are also tyrosine hydroxylase positive) are one of the earliest to differentiate, arising at E11 p.c. in rat (Lauder and Bloom, 1974). We show in this work that *Pax-6* transcripts are detected in the E13

p.c. mouse mesencephalic tegmentum in the region of the differentiating substantia nigra as well as in neurons of the dorsolateral part of the reticular substantia nigra in the adult brain, known to have a group of the dopaminergic neurons (Fallon and Loughlin, 1985). Several *Pax-6*–positive regions in the adult brain including a part of the periglomerular neurons in the olfactory bulb, retina, preoptic area, zona incerta, reticular substantia nigra, ventral tegmental area, and mesencephalic periaqueductal gray were also shown to contain dopaminergic neurons (Lindvall and Björklund, 1983; Kalsbeeck et al., 1992). Although it is difficult to assign cell fates only upon expression pattern, these results suggest that *Pax-6* may be involved in some aspects of the differentiation of the tyrosine hydroxylase–immunoreactive neurons.

Comparing the expression patterns of the *Pax*-genes in embryonic and adult brain, we find it particularly interesting that distinct *Pax*-genes (*Pax-5, Pax-6, Pax-7*) have a specific expression in different brainstem regions (retrorubral field, ventral tegmental area, substantia nigra, interpeduncular nucleus), known to originate from a common precursor complex in the mesencephalic tegmentum on both side of fovea isthmi (reviewed in Lindwall and Björklund, 1983). As shown in the present work, the three *Pax*-genes are expressed in this particular region of the mesencephalic tegmentum in midgestation embryonic brain. Further immunocytochemical characterization will be required to establish the exact neurochemical nature of the brain cells expressing different *Pax*-genes. Neuronal differentiation is a complex process that involves much more than transformation of mitotic precursor cells into postmitotic cells. Whether the *Pax*-genes may affect the developmental potential of the progenitor cells in the complex series of cellular decisions that determine a given phenotype in the CNS is an interesting area for further research.

Our finding of the differential expression of three *Pax*-genes in different cell types of the cerebellar cortex is also noteworthy. *Pax-3* is expressed only in the ventricular zone of the developing cerebellum of the midgestation embryo brain, while in the adult cerebellar cortex, the *Pax-3* probe labels the Purkinje cell layer, but predominantly the small cells beneath the Purkinje cells (most probably Bergmann glia) and the small cells surrounding the Purkinje cells (probably a subset of basket cells). Interestingly, recent study using quail-chick marker system provided evidence that a population of small cells surrounding the Purkinje cells and located at the border between the molecular layer and the inner granular layer may actually originate not from the external germinative layer, but rather from the ventricular zone, as do the Purkinje cells (Hallonet et al., 1990). In contrast to *Pax-3*, the *Pax-6* probe labels the ventricular and external granular layer of the developing cerebellum, the latter being the source for production of the neurons of the granular layer that is strongly expressing the *Pax-6* gene in the adult cerebellar cortex. Accordingly, *Pax-3* and *Pax-6* appear as useful markers to follow the developmental history of distinct cerebellar populations, from their birth in their respective germinative zone up to their mature state.

### Pax-*genes in adult brain*

We show in this work that most of the members of the *Pax*-gene family are expressed in defined areas in the young adult brain. The *in situ* hybridization study was performed with six different probes on consecutive sections; therefore, some very restricted expression areas for a distinct *Pax*-gene might be missing at present. Despite this limitation, we could clearly show that the regional distribution of the different *Pax*-gene transcripts along the anterior–posterior axis is comparable in both the midgestation embryonic and adult brain. Transcripts of all *Pax*-genes were observed in discrete areas in the most posterior regions of the adult brain up to the meso-metencephalic boundary (see Fig. 8*h–j*), similar to the distribution of the transcripts in the midgestation brain (Fig. 7). In accordance with the finding that in the mesencephalon of midgestation embryo, only *Pax-3, Pax-5, Pax-6,* and *Pax-7* genes were detectable, we found in the adult midbrain that neurons in distinct nuclei express the same *Pax*-genes (Figs. 7, 8*f,g*). Good correspondence exists between the adult and embryonic expression patterns for *Pax-7* whose transcripts are abundantly accumulated in the colliculus superior, the adult counterpart of the anterior embryonal mesencephalic roof (Figs. 7, 8*e*). The only *Pax*-gene to be expressed in the most rostral forebrain areas in the adult CNS was the *Pax-6* gene (Fig. 8*a–d*). This is consistent with the regional expression of *Pax-6* in the telencephalon of midgestation embryo brains (Fig. 7) as well as in later embryonal stages (Walther and Gruss, 1991). It seems that concomitant with the differentiation events that proceed in the developing CNS there is a progressive caudorostral restriction of the number of the expressed *Pax*-genes and of their expression domains. A good correlation also exists between the regional distribution of *Pax-6* transcripts in the midgestation embryo and adult forebrain, encompassing the olfactory pit, the nasal neuroepithelium (Fig. 2*B*), the glomerular and the internal granular layer of the olfactory bulb (Fig. 4*A,B*), differentiating areas in the embryo forebrain and their adult counterparts in the septum (Figs. 2*A*, 4*C,D*), amygdala (Figs. 2*C,E*; 4*E*), zona incerta, entopeduncular and reticular nuclei (Figs. 2*F*, 4*E*), substantia nigra (Figs. 3*A*, 4*F*), and cochlear, vestibular, and hypoglossal nuclei (Figs. 3*F,J*; 5*I,O*). Taken together, these results support the notion that distinct *Pax*-genes are not only involved in the specification of spatial domains in the developing brain but may also have an important role in the differentiation and maintenance of specific neuronal subtypes in the mature CNS.

Our present results provide a good basis for the analysis of the brain organization in the *Splotch* and *Small eye* mutants. The homozygous *Splotch* mutants, in addition to the characteristic neural tube and neural crest defects (spina bifida, exencephaly, reduced or absence of dorsal root ganglia, Schwann cell deficiency), present neural overgrowth of the brain resulting in the partial obliteration of the brain ventricles (Auerbach, 1954). As already discussed, in CNS of early and midgestation embryo, *Pax-3* expression is mainly confined to the ventricular zone of the mesencephalon, hindbrain, and cerebellum, where cells are undergoing mitosis, which suggests a function for *Pax-3* in the early proliferation events. Interestingly, in the adult cerebellar cortex *Pax-3* transcripts are detected in the presumptive Bergmann glia and a subset of basket cells around the Purkinje cells. Further analysis of the brain structures (that normally express *Pax-3*) of *Splotch* mutants might shed some light onto the functional role of *Pax-3* in the organization of the brain. The *Small eye* mutant does not develop eye and nose structures. During development, the expression of *Pax-6* is found in the eye and olfactory pit, telencephalon, diencephalon, and ventricular and external germinative layers of the cerebellum. In the adult, *Pax-6* transcripts are detected in the glomerular and granular layer of the olfactory bulb, in a number of nuclei in the septum, diencephalon, midbrain, and isthmus, as well as in

the granular cell layer of the cerebellum. It will of great interest to analyze how the forebrain and cerebellar structures expressing *Pax-6* are affected in the *Small eye* mutant.

Intriguingly, the main expression areas of several *Pax*-genes in the mature brain belong to or are related with the subcortical domains of the limbic system. The limbic system can be parcelled into three main cortico-subcortical divisions. The three nuclear groups located in the septum, amygdala, and anterior thalamus are the main sources of afferents to the respective parts of the limbic cortex (the cingulate cortex and the hippocampus). In addition, studies with modern neuroanatomical techniques have revealed that the isthmic nuclei (the dorsal and the ventral tegmental nuclei of Gudden; the parabrachial nuclei, locus coeruleus, the nuclei of dorsal raphe and the median raphe), despite their remote position at met- and mesencephalic boundary, also project to the nuclei of septum, amygdala, and anterior thalamus. Most of the isthmic nuclei have a monoamine transmitter phenotype and appear to have a similar organization in reptiles, birds, and mammals (Parent, 1970). Functionally, the isthmus appears to be an important region for the integration and transfer of a somatovisceral information that in lower vertebrates is primarily under the influence of the midbrain and that in the evolution of vertebrates becomes extensively connected with the forebrain (MacLean, 1990). The nuclei of the septum and amygdala serve as centers of convergence of impulses from the olfactory apparatus as well as from the nuclei of the brainstem (substantia nigra, ventral tegmental area, interpeduncular nucleus), the midbrain periaqueductal gray, and the reticular formation. Physiological studies have revealed that the limbic subdivision that includes the corticomedial nuclei of amygdala is primarily concerned with autonomic functions (smacking, salivation, chewing movements) whereas the basolateral nuclear complex of the amygdala are largely involved in conscious processes (attention, fear, rage). Septal nuclei are also related to autonomic functions and behavior reactions mostly implicated in the species procreation (MacLean, 1973). According to the theory of the "triune" brain (MacLean, 1973, 1990), during the evolution the human forebrain expanded along three basic subdivisions that anatomically and biochemically reflect an ancestral relationship to reptiles and early and late mammals. It is thought that in the highest mammals, these three assemblies constitute a hierarchy of three brains in one: reptilian, paleomammalian, and neomammalian brains. The limbic system belongs to the paleomammalian brain. It should be expected that common structures and processes to all vertebrates are controlled by evolutionary highly conserved genes. As already discussed, the paired box has been conserved in a variety of organisms as distinct as nematodes, fly, mouse, and human. In several cases, this conservation has been found throughout the entire coding sequence. For instance, the entire amino acid sequence of the murine and the zebrafish *Pax-6* genes has been reported to have 97% identity and the same tissue specificity of the expression (Püschel et al., 1992a). Similarly, the amino acid sequences encoded by the mouse *Pax-2* gene and its zebrafish counterpart were found to be 87% identical (Krauss et al., 1991b), both genes showing a similar tissue distribution of the protein (Püschel et al., 1992b). In comparison, no more than 70% homology has been reported in the fish and mouse counterparts for other putative developmental control genes: *Hox-2.2, Wnt-1, Eng-1, Eng-2* (Fjose et al., 1988; Molven et al., 1991). These data are consistent with our hypothesis that *Pax*-genes are members of an evolutionary old multigene family and may have a role in the development and /or the function of the main subcortical divisions of the limbic system in the brain of vertebrate, a system that has remained essentially unchanged during the evolution.

Similar to *Pax*-genes, a large number of the *POU* domain genes are widely expressed along the entire neural axis during the development (from the neural tube to the telencephalon) and with subsequently restricted patterns of expression in distinct regions of the differentiating and adult brain (reviewed by Rosenfeld, 1991; Schöler, 1991). Interestingly, in contrast to the *Pax*-genes, which lack expression in the adult brain cortex, the expression of some *POU* genes (*Brn-1, Brn-2, Brn-4, Tes-1/SCIP/Oct-6, Oct-2*) have been correlated with the establishment of the cortical lamination as well as with the development of specific neuronal subtypes in the hypothalamus and hippocampus of adult brain (He et al., 1989; Mathis et al., 1992; Stoykova et al., 1992). The available evidence points to the intriguing fact that in contrast to the *Hox*-genes whose expression domains have their most anterior limits before the hindbrain–midbrain boundary, the members of the *POU* and the *Pax* multigene families may exert developmental functions by specifying evolutionary distinct neuronal subsets in the most rostral domains of the CNS—midbrain and forebrain.

## Appendix

*Abbreviations used for designation of the regional distribution of* Pax-*mRNAs in adult mouse brain (Fig. 8)*

| | |
|---|---|
| Amb | Ambiguus nucleus |
| AOB | Accessory olfactory bulb |
| Aq | Cerebral aqueduct |
| BL | Basolateral nucleus of the amygdala |
| BLV | Basolateral amygdaloid nucleus, ventral subdivision |
| BM | Basomedial nucleus of the amygdala |
| cg | Cingulum |
| CG | Midbrain central gray |
| CGD | Midbrain central gray, dorsal |
| CGM | Midbrain central gray, medial |
| CP | Caudate putamen |
| DLL | Dorsal nucleus of the lateral lemniscus |
| DMTg | Dorsomedial tegmental area |
| DpG | Deep gray layer of the superior colliculus |
| DpMe | Deep mesencephalic nucleus |
| DR | Dorsal raphe nucleus |
| DTg | Dorsal tegmental nucleus |
| EP | Entopeduncular nucleus |
| Fl | Flocculus |
| Gi | Gigantocellular reticular nucleus |
| Gl | Glomerular layer of the olfactory bulb |
| GrA | Granular layer of the accessory olfactory bulb |
| GrL | Granular layer of cerebellar cortex |
| HDB | Horizontal limb of the diagonal band nucleus of Broca |
| InG | Intermediate gray layer of the superior colliculus |
| InWt | Intermediate white layer of the superior colliculus |
| IPA | Interpeduncular nucleus, apical |
| IPC | Interpeduncular nucleus, central |
| IPIP | Interpeduncular nucleus, inner posterior |
| IPP | Interpeduncular nucleus, paramedian |
| La | Lateral amygdaloid nucleus |
| LPB | Lateral parabrachial nucleus |
| LS | Lateral septal nucleus |
| LV | Lateral ventricle |
| Me5 | Mesencephalic trigeminal nucleus |
| ML | Molecular layer of the cerebellar cortex |
| MnR | Median raphe nucleus |
| MS | Medial septal nucleus |
| Op | Optic nerve layer of the superior colliculus |
| PDTg | Posterior dorsal tegmental nucleus |
| PFl | Paraflocculus |

| | |
|---|---|
| PL | Purkinje cell layer of cerebellar cortex |
| Pn | Pontine nuclei |
| PnC | Pontine reticular nucleus, caudal part |
| PnO | Pontine reticular nucleus, oral part |
| PrH | Prepositus hypoglossal nucleus |
| RPn | Raphe pontis |
| RRF | Retrorubral field |
| Rt | Reticular thalamic nucleus |
| RtTg | Reticulotegmental nucleus of the pons |
| SNC | Substantia nigra, zona compacta |
| SNL | Substantia nigra, pars lateralis |
| SNR | Substantia nigra, zona reticularis |
| Sp5 | Spinal trigeminal nucleus |
| SuG | Superficial gray layer of the superior colliculus |
| tz | Nuclei of the trapezoid body |
| VCA | Ventral cochlear nucleus, anterior |
| VDB | Vertical limb of the diagonal band nucleus of Broca |
| VP | Ventral pallidum |
| VTA | Ventral tegmental area |
| ZI | Zona incerta |
| 4V | Fourth ventricle |

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