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Expression of *PAX2* gene during human development

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ABSTRACT The expression of human paired-box-containing *PAX2* gene was examined in 7 human conceptuses 6 to 9 weeks old by *in situ* hybridization. The embryos were collected after legal abortions, embedded in paraffin, serially cut in transversal direction and treated with S35 labeled probe for *PAX2*. In the neural tube of 6-week embryos, *PAX2* was expressed in the outer part of the ventricular zone on both sides of the sulcus limitans. At later stages, it was expressed in the intermediate zone of the spinal cord, both in alar and basal plates except in the region of motor neuroblasts. In the brain, expression of *PAX2* extended from mesencephalic-rhombencephalic border along the entire rhombencephalon in a manner similar to that described for the spinal cord. Expression of *PAX2* gene in the eye was seen in the optic cup and stalk, and later in the optic disc and nerve. In the ear, expression was restricted to the part of the otic vesicle flanking the neural tube and later to the utricle and cochlea. Expression of *PAX2* was observed in developing kidneys as well. During human development *PAX2* has a spatially restricted expression along the compartmental boundaries of the neural tube, and within developing eye, ear and kidneys. Differentiation of those organs seems to be mediated by *PAX2* gene at the defined stages of human development.

KEY WORDS: *human embryo, PAX2 gene, neurogenesis, sense organs*

Introduction

Development, regional specification and morphogenesis of the human brain seem to be controlled by multigene families whose products act as transcriptional regulators that bind to specific DNA sequences. Recent evidence from mouse (Nornes *et al.*, 1990; Stoykova and Gruss, 1994; St-Onge *et al.*, 1995) and human embryos (Gerard *et al.*, 1995) indicate that *Pax* genes play important roles in early embryogenesis. *Pax* genes are characterized by the presence of a highly conserved DNA region referred to as paired box, encoding a DNA-binding protein domain of 128 amino acids, the paired domain (Dressler *et al.*, 1988; Deutsch and Gruss, 1991). Some of the *Pax* genes *(Pax3, Pax4, Pax6, Pax7)* additionally have a second conserved DNA region, a homeobox, encoding domain of 61 amino acids (homeodomain) located towards the carboxyterminus of the corresponding protein (Strachan and Read, 1994). Furthermore, a highly conserved sequence that specifies an octapeptide is found in most *Pax* genes (except for *Pax4* and *Pax6)*. Certain isoforms of Pax proteins seem to be tissue specific and could regulate expression of different genes in different tissues (St-Onge *et al.*, 1995).

Pax genes have a restricted expression pattern along the craniocaudal and dorsoventral axes of the neural tube (McGinnis and Krumlauff, 1992; Chalepakis *et al.*, 1993). The dorsoventral patterning of the neural tube occurs later than the craniocaudal patterning, and requires the notochord (Tessier-Lavigne *et al.*, 1988; van Straaten *et al.*, 1988; Placzek *et al.*, 1990, Monsoro-Burq *et al.*, 1995). Experimentally, the expression of the *Pax3* gene in the neural tube changes by extirpation or grafting of an extra notochord (Goulding *et al.*, 1993). In human embryos notochord abnormalities may be associated with dysraphic axial disorders (Saraga-Babić and Saraga, 1993; Saraga-Babić *et al.*, 1993a) and duplication of the spinal cord (Saraga-Babic *et al.*, 1993b). Disruption of *Pax* genes in animals (Balling *et al.*, 1988; Epstein *et al.*, 1991; Hill *et al.*, 1991, Torres *et al.*, 1995,1996) as well as in humans can lead to developmental abnormalities such as Waardenburg syndrome, spina bifida, aniridia, Peter's anomaly or congenital cataract (Tassabehji *et al.*, 1993; Glaser *et al.*, 1994; Strachan and Read, 1994).

Development of the human neural tube involves several temporally restricted processes: cell proliferation, migration, differentiation and cell death. The most caudal part of the human neural tube develops during the process of secondary neurulation (Saraga-Babić *et al.*, 1994,1995,1996a,b). By the end of the 6th week, three zones differentiate in the lateral walls of the human neural tube: the ventricular, the intermediate and the marginal zone. During the following four weeks mitotic activity gradually ceases and the specific neurons differentiate according to their dorsoventral position, thus forming the definitive spinal cord (Fitzgerald and Fitzgerald, 1994).

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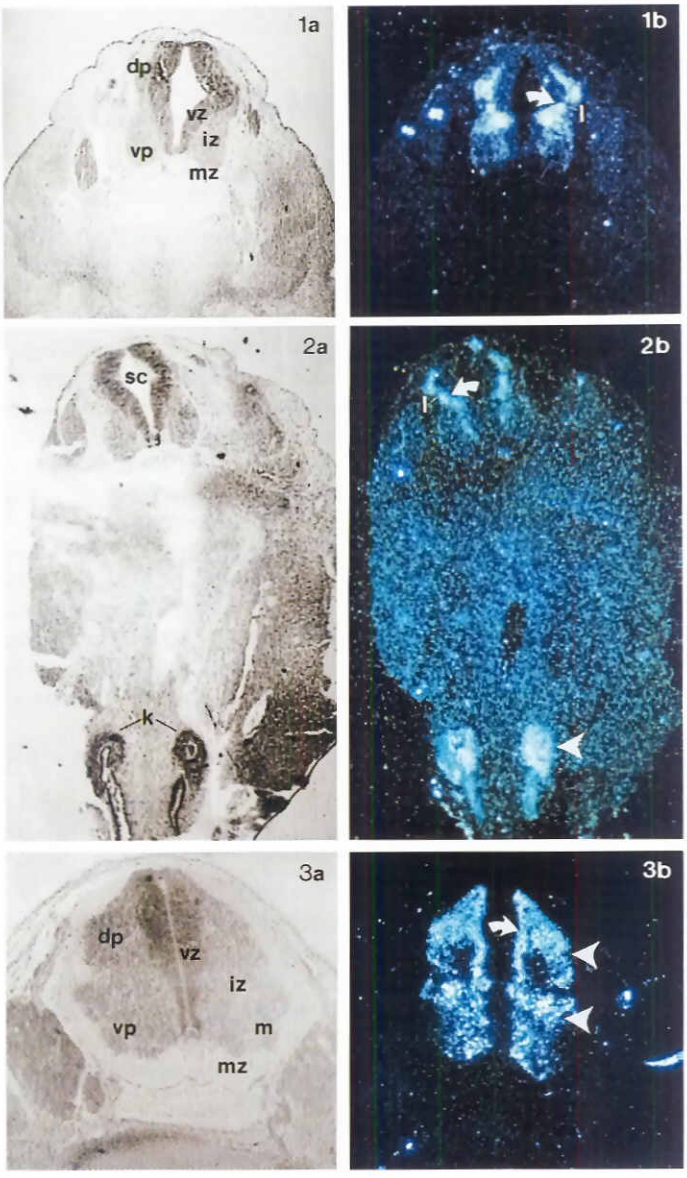

Fig. 1. Transversal section at the cervical level of an early 6-week human embryo. (a) *Under bright-field illumination the spinal cord consists of the ventricular zone (vz), the intermediate zone (iz) and a thin marginal zone (mz). Ventrolateral plates (vp) are more developed than dorsolateral plates (dp).* **(b)** *Same section under dark-field illumination shows intensive PAX2 hybridization in outer part of ventricular zone (arrow) on both sides of sulcus limitans (l). Hybridization is missing in ventral quarter of ventricular zone. Weaker hybridization is noticed in the neighboring intermediate zone (double arrows).*

Fig. 2. Transversal section at the sacral axial level of an early 6-week human embryo (same embryo as in Fig. 1). (a) *Under bright-field illumination the spinal cord (sc) consists of three characteristic zones. Section through developing kidneys (k) is also seen.* **(b)** *Under dark-field illumination PAX2 hybridization is detected as thin and weak strip only in the outer part of ventricular zone (arrow) on both sides of sulcus limitans (l), except in the ventral quarter. Strong hybridization characterizes developing kidneys (arrowhead).*

Fig. 3. Transversal section at the cervical axial level of an 8-week human embryo. (a) *Under bright field illumination the spinal cord has the wider and more developed intermediate zone (iz) and the marginal zone (mz), while the ventricular zone (vz) is much thinner. Both the dorsolateral (dp) and ventrolateral plates(vp) are well developed, with regions od motor neurons (m) positioned ventrolaterally.* **(b)** *Under dark-field illumination PAX2 hybridization is seen in the outer ventricular zone of the dorsolateral plates (arrow). In the intermediate zone, PAX2 hybridization is found in the dorsolateral and ventrolateral plates (arrowheads), except in the area of developing motor neurons.*

In the mouse, the initial distribution of the *Pax2* gene transcripts is in postmitotic cells on both sides of the sulcus limitans of the neural tube, and later in the intermediate zone of the spinal cord, myelencephalon and metencephalon (Nornes *et al.*, 1990; Asano and Gruss, 1992). *Pax2* is also expressed in developing kidneys (Dressler *et al.*, 1990), defined regions of otic vesicle and in the optic cup and stalk (Nornes *et al.*, 1990). An induced deletion of mouse chromosome 19 which contains *Pax2* locus leads to the kidney and retinal defects (Krd mouse) (Keller *et al.*, 1994). Disruption of *Pax2* gene in mouse by homologous recombination displays its role during development of the urogenital system, eye, ear and closure of the neural tube at the midbrain level (Torres *et al.*, 1995,1996; Favor *et al.*, 1996). Spontaneous mutations of *PAX2* gene in humans are associated with optic nerve colobomas, renal anomalies and vesicoureteral reflux, or with optic nerve colobomas and chronic renal failure (Sanyanusin *et al.*, 1995a,b).

Despite numerous investigations on *Pax* genes in animals, only preliminary results exist about developmental role of human *PAX3*, *PAX6* and *PAX5* genes (Gerard *et al.*, 1995), while data about human *PAX2* gene in human development are completely missing. The aim of this study was to determine the spatial and temporal expression of *PAX2* gene during different stages of human development. The data obtained indicate that *PAX2* gene may have an important role in the patterning of the spinal cord at the midbrain-hindbrain boundary, as well as in organogenesis of the human eye, internal ear and kidneys.

Results

The expression of the *PAX2* gene was analyzed in 6-9 weeks old human conceptuses using *in situ* hybridization. Expression of *PAX2* was observed at all developmental stages examined and in four different tissues: neural tube (brain and spinal cord), optic vesicle, otic vesicle and embryonic kidneys. Sense and antisense probes for *PAX6* and for PAX 3 were used as control probes.

Spinal cord

In the 6th week of development, three zones could be distinguished in the wall of the spinal cord: the ventricular zone (future ependymal cells), the intermediate zone (mantle layer, primordium of gray matter) and the marginal zone (primordium of white matter). In the early 6-week embryo, *PAX2* hybridization was detected in the outer parts of the ventricular zone on both sides of the sulcus limitans. Hybridization was more extensive in the dorsolateral plates (alar plates) than in the ventrolateral plates (basal plates). *PAX2* signals were missing in the ventral quarter of the ventricular zone. Weaker hybridization was also present in areas abutting the intermediate zone. (Fig. 1a,b)

In the caudal part of the spinal cord of the same embryo, *PAX2* hybridization was detected as a thin and weak strip in the outer part of the ventricular zone on both sides of the sulcus limitans. The intermediate zone was devoid of *PAX2* expression. Strong hybridization characterized the developing metanephros (both the epithelium of ureteric bud and metanephric mesenchyme) of the same embryo. (Fig. 2a,b)

In the 8-week embryos, the ventricular zone in ventrolateral plates was morphologically much thinner than at earlier stages, and *PAX2* hybridization was missing in these areas. *PAX2* transcripts were still present in the outer ventricular zone of the dorsolateral plates. In the intermediate zone, strong *PAX2* expression was found in the dorsolateral plates and in a part of the ventrolateral plates, except in the area of developing motor neurons (Fig. 3a,b).

In the 9-week fetus the neural canal became the central canal of the spinal cord, the ventricular zone developed into ependymal layer. Ventral and dorsal gray horn (columns), as well as ventral, lateral and dorsal funiculi of the white matter were well defined. Ependymal cells did not express *PAX2*, while its strong expression was present in the intermediate zone of dorsal gray horns and ventral gray horns, except in the area of motor neuroblasts (Fig. 4a,b).

Brain

From the 4th to the 8th week of human development, division and main boundaries between different parts of the brain were established. In the 8-week embryo, expression of *PAX2* gene in medulla oblongata was restricted to the outer part of the ventricular zone and to the intermediate zone within two compartments which correspond to the ventral and dorsal horns of the spinal cord (Fig. 5a,b).

Strong expression of PAX-2 was seen in the outer part of the ventricular zone at the junction of the caudal mesencephalon and rostral rhombencephalon of the 8-week embryo. Weaker hybridization characterized part of the neighboring intermediate zone (Fig. 6a,b).

Otic vesicle

The otic vesicle develops by process of invagination of the otic placode during the 4th week of development. During later embryonic development, each vesicle divides into ventral component (primordium of the sacculae and the cochlear duct) and dorsal component (the primordium of utricle, semicircular canals and endolymphatic duct). In the 6th week, expression of *PAX2* gene was seen in the part of otic vesicle flanking the neural tube (Fig. 7a,b).

In the 8th developmental week, parts of the internal ear were well differentiated. Strong *PAX2* expression was restricted to parts of the utricular wall, while weak expression was present in the cochlea. We observed no expression of *PAX2* in the semicircular canals (Fig. 8a,b).

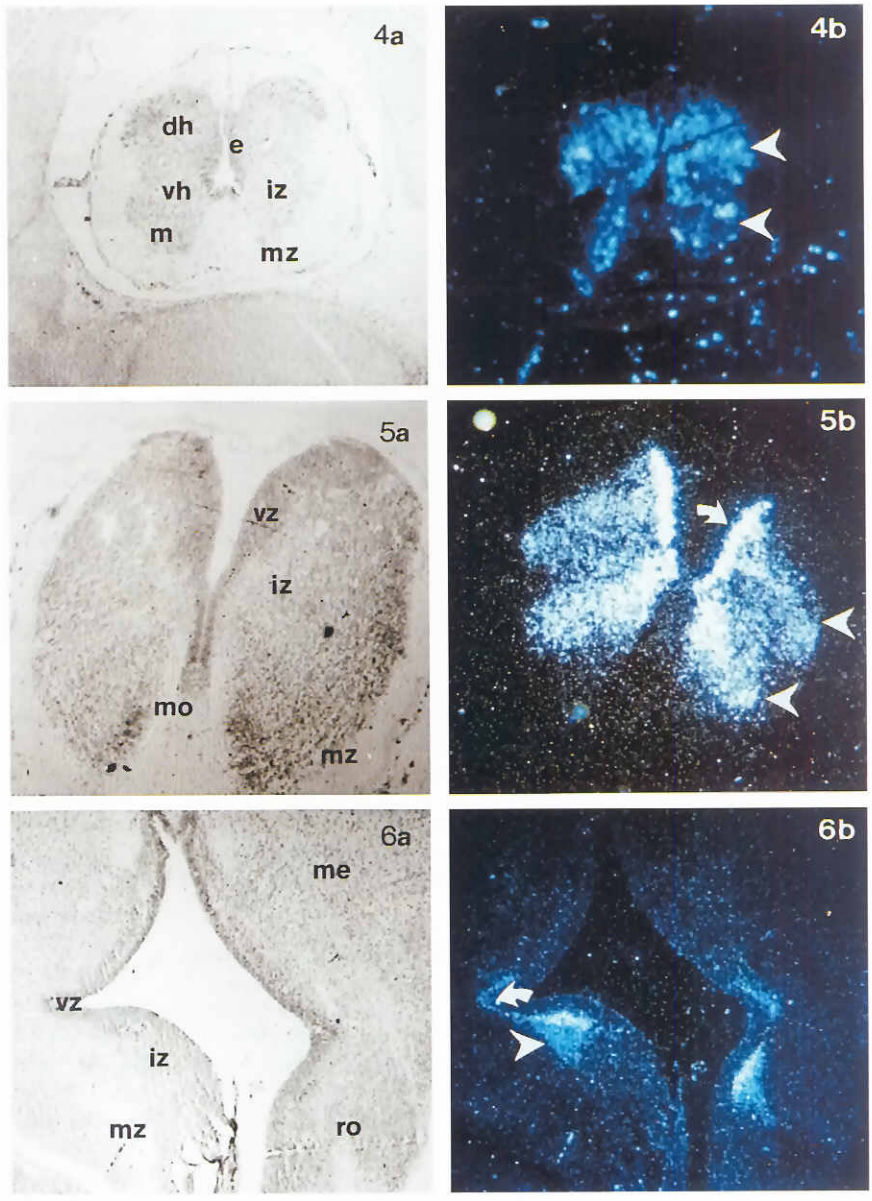

Fig. 4. Transversal section at the cervical axial level of a 9-week human fetus. (a) *Under bright-field illumination well differentiated spinal cord contains ependymal cells (e) surrounding the central canal, the intermediate zone (iz) consisting of dorsal gray horns (dh) and ventral gray horns (vh) with regions of motor neurons (m), and the marginal zone (mz).* **(b)** *Under dark-field illumination PAX2 hybridization is detected exclusively within the intermediate zone: in dorsal gray horns and ventral gray horns (arrowheads), except in the area of motor neurons.*

Fig. 5. Transversal section at the level of medulla oblongata (mo) of a 8-week human embryo. (a) *Under bright-field illumination medulla oblongata is composed of the ventricular zone (vz), the intermediate zone (iz) and the marginal zone (mz).* **(b)** *Under dark-field illumination, strong PAX2 hybridization characterizes outer part of the ventricular zone (arrow). Weaker hybridization is seen in the intermediate zone (arrowheads) in two compartments that correspond to ventral and dorsal horns.*

Fig. 6. Transversal section at the level of junction of mesencephalon (me) and rhombencephalon (ro) in the 8-week human embryo. (a) *Under bright-field illumination the brain tissue still consists of the ventricular zone (vz), the intermediate zone (mz) and the marginal zone (mz).* **(b)** *Under dark-field illumination strong PAX2 hybridization is seen in outer part of the ventricular zone at the mesencephalic-rhombencephalic border (arrows). Weaker hybridization is observed in the neighboring intermediate zone (arrowhead).*

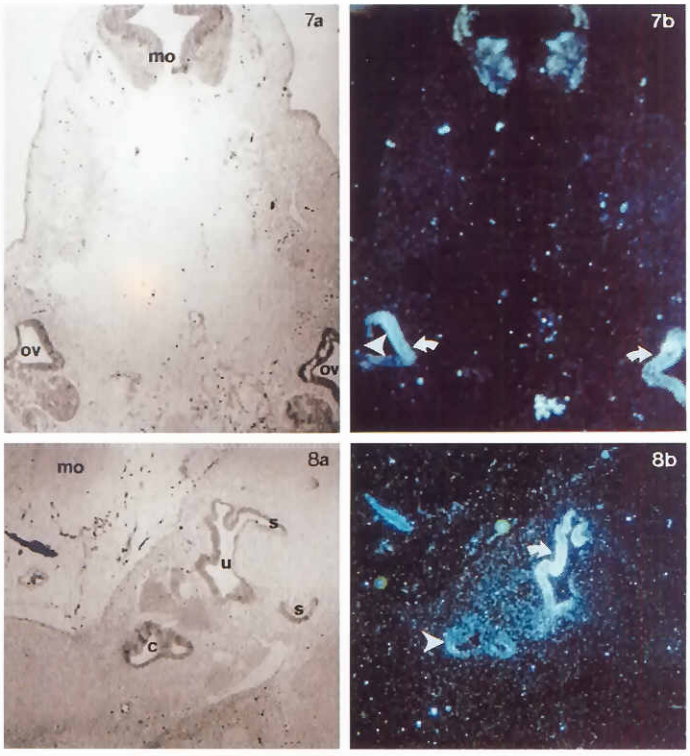

Fig. 7. Transversal section at the level of the otic vesicles (ov) of an early 6-week human embryo. (a) *Medulla oblongata (mo).* **(b)** *Under dark-field illumination strong PAX2 hybridization is seen in medial part of the otic vesicle (arrows), while it is absent in its lateral wall (arrowheads). PAX2 hybridization is characteristically distributed in the medulla oblongata (as shown in Fig. 5).*

Fig. 8. Transversal section at the level of the internal ear and medulla oblongata (mo) of an 8-week embryo. (a) *Under bright field illumination the internal ear consists of utricle (u), cochlea (c) and semicircular canals (s).* **(b)** *Under dark-field illumination strong PAX2 hybridization is seen in the part of utricular wall (arrow), weak hybridization within the cochlear wall (arrowhead), while there is no hybridization in the semicircular canals.*

Optic vesicle

The optic vesicle develops as an outgrowth of the diencephalon during the 4th week of development. Later on, it transforms into the optic cup. The outer layer of the optic cup becomes the pigmented layer of the retina, while the inner layer differentiates into the neural retina. In the early 6th week of development, the *PAX2* gene expression was seen in the dorso- medial part of neural retina (primordium of the optic disc) as well as in the optic stalk (primordium of the optic nerve). Expression in the optic stalk stopped abruptly at the border with the diencephalon (Fig. 9a,b).

In the 8th week of development, *PAX2* hybridization was present in regions of optic disc and optic nerve (Fig. 10a,b).

Hybridization within the pigmented layer of both 6-week and 8-week human eye seems to be an artifact, as in darkfield illumination the pigment appears bright in sections without hybridization probe.

Discussion

In this report we analyzed the expression of the *PAX2* gene during early stages of human development. Similar spatially and temporally restricted pattern of *PAX2* was found in the developing neural tube (brain and spinal cord), eye, ear and kidneys of the mouse (Dressler *et al.*, 1990; Nornes *et al.*, 1990; St-Onge *et al.*, 1995). The *PAX2* gene characteristically defined boundaries within the neural tube, both in cranio-caudal and ventro-dorsal direction.

At the beginning of the 6th week of development, the *PAX2* gene activity in the cranial part of the human spinal cord characterized cells in the outer ventricular zone and cells corresponding to neuroblasts in the intermediate zone. The ventral quarter of the ventricular zone did not show any signal. In the caudal part of the spinal cord, *PAX2* hybridization was restricted only to the outer part of the ventricular zone, while it was missing in the intermediate zone. In humans, like in other species, the most caudal part of the spinal cord derives during secondary neurulation (Muller and O'Rahilly, 1986,1987; Saraga-Babić *et al.*, 1996a,b). Thus, regional differences in expression of *PAX2* gene within the spinal cord could be explained by assuming that the caudal spinal cord differs from its cranial part in mechanism of development and its final destiny. Namely, regression of the caudal spinal cord starts already during the 6th developmental week and therefore never reaches the same maturity of differentiation as the cranial spinal cord (Saraga-Babić *et al.*, 1994). On the other hand, the observed differences for caudal versus sacral neural tube may reflect only temporal differences in neurogenesis. During later developmental stages of postneurogenesis, *PAX2* hybridization was located only in the intermediate zone, both in dorsolateral and ventrolateral plates with exception of areas of motor neurons.

In the brain, expression of the *PAX2* gene was seen in the ventricular zone and in the part of the intermediate zone. From the mesencephalic-rhombencephalic border *PAX2* expression extended along the rhombencephalon and spinal cord, e.g., along the posterior (epichordal) compartment of the neural tube (Nornes *et al.*, 1990). The establishment of compartments in the human neural tube starts with the process of segmentation which seems to occur in all parts of the brain (Muller and O' Rahilly, 1986). The midbrain (mesencephalon) displays two neuromeres, while the hindbrain (rhombencephalon) shows seven to eight rhombomeres (O'Rahilly and Gardner, 1979). During later stages, segmentation is clearly evident only in the peripheral nervous system, while in the central nervous system it gradually disappears (Noback *et al.*, 1991). Similarly to murine development, *PAX2* in human embryos establishes boundaries in the neural tube and seems to be associated with migration and settling of neurons in their final position (Nornes *et al.*, 1990). Thus migration, differentiation and pattern of neuronal distribution are controlled genetically, but can be also influenced by environmental forces operating during development rather than

TABLE 1

AGE AND NUMBER OF HUMAN EMBRYOS USED IN THIS STUDY

Age (weeks)	CRL(mm)	Carnegie stage	No
6	11	17	1
6-7	14	18	1
7	18-22	20	2
8	27	22	1
8-9	32	/	1
9	37	/	1

during postnatal life (Noback *et al.*, 1991). This may explain misexpression of *Pax* genes found in experimentally designed mutants (Balling *et al.*, 1988; Epstein et al., 1991; Hill *et al.*, 1991) and in some human syndromes (Hoth *et al.*, 1993; Tassabehji *et al.*, 1993; Glaser *et al.*, 1994; Strachan and Read, 1994).

The *PAX2* expression was also noted in specific areas of developing sense organs- eye and ear. Similarly to the neural tube, the internal ear is of ectodermal origin as it derives from the thickening of the surface ectoderm on each side of rhombencephalon (Sadler, 1990). At earlier developmental stages, the otic vesicle of human embryos analyzed in our study showed *PAX2* hybridization only in the region nearby the neural tube. Later on, *PAX2* expression was restricted to some derivatives of the otic vesicle such as the utricular and cochlear wall. In the mouse, *Pax2* transcripts were found only in neurogenic regions of otic vesicle (Nornes *et al.*, 1990). Homozygous mouse mutant for the *Pax2* gene displayed absence of the cochlear ganglion and failure of the cochlear duct shaping, while heterozygous mutant showed exencephaly without deafness. Like in homozygous mouse *Pax2* mutants, malfunction of the *PAX2* gene in humans was characterized by hearing defect without exencephaly (Torres *et al.*, 1996). During development, expression of the *PAX2* gene was found in the utricular region of human embryos, while it was seen in the saccular region of mouse embryos (Torres *et al.*, 1996). Although both regions contain maculae which are responsible for the maintenance of equilibrium, malfunction of the *PAX2* gene was not associated with vestibular disfunction neither in mouse mutants (Torres *et al.*, 1996) nor in human syndromes (Sanyanusin *et al.*, 1995a,b). In both species *PAX2* was involved only in the development of the auditory region of the internal ear. Therefore, formation of vestibular region seems to be controled by other genes rather than by the *PAX2* gene.

In the eye, *PAX2* expression characterized dorso-medial parts of the optic cup and stalk at earlier developmental stage, and the optic nerve and optic disc at later stages. The optic vesicle, primordium of the optic cup, develops as an outgrowth of the diencephalon and therefore represents a direct extension of brain tissue. The described pattern of the *PAX2* expression in the eye of human embryos corresponds to the pattern of its expression in the mouse. Mutation of the *PAX2* gene in the adult humans is combined with optic nerve coloboma associated with renal anomalies and vesicoureteral reflux or chronic renal failure (Sanyanusin *et al.*, 1995a,b). The role of *PAX2* gene in formation of those particular organs is confirmed by our study as these organs showed strong expression of the *PAX2* gene already during early development. In human embryogenesis the optic fissure normally closes during the sixth week. The most common anomalies of the human eye are related to the defects in closure of the optic fissure: from simple coloboma affecting the iris to complete cleft extending into the cilliary body, the retina, the choroid and the optic nerve (Sadler, 1990; Moore and Persaud, 1993). Both human and mouse *Pax2* mutants showed abnormal electroretinograms indicating retinal defects (Keller *et al.*, 1994; Sanyanusin *et al.*, 1995a). Non-existence of optic chiasm found in mouse mutants (Torres *et al.*, 1996) can unable transmission of visual messages from both eyes to the primary visual cortex in the occipital lobe (Carola *et al.*, 1992).

Besides the described organs of ectodermal origin, *PAX2* expression was also observed in developing kidneys, which are the mesodermal derivatives. It has been shown in the *Pax2* mouse mutants that this gene has an important role during urogenital development (Torres *et al.*, 1995). In our study the *PAX2* gene was expressed in both the metanephric mesenchyme and the epithelium of the ureteric bud. Deregulation of *PAX2* expression in transgenic mouse resulted in abnormal and disfunctional renal epithelium with properties similar to the congenital nephrotic syndrome (Dressler *et al.*, 1993). It seems likely that transcriptional factors can act as multipotent switches in different parts of the embryo and within organs of different origin and functions (Torres *et al.*, 1996).

In conclusion, expression of the *PAX2* gene in human embryonic and fetal tissues may be associated with the establishment of craniocaudal and ventrodorsal boundaries within developing human neural tube, as well as with migration and settling of cells during neurogenesis, possibly together with *PAX5* and *PAX8*. It also seems to be involved in differentiation of internal ear, eye and developing kidney. Considering the development of neural tube, regional differences in expression of *PAX2* gene found along craniocaudal axis of the spinal cord are in accordance with previous morphological studies on human embryos. Further work focusing on the analysis of different homeobox-containing genes in human development will yield a deeper insight into genetic control of the developing human central nervous system.

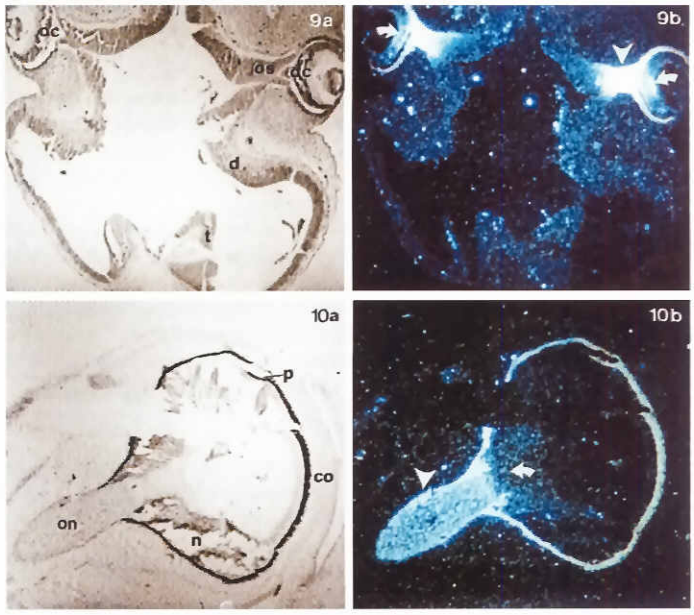

Fig. 9. (a) ***Transversal section at the level of the optic cup (oc), optic stalk (os), diencephalon (d) and telencephalon (t) of an early 6-week embryo.*** **(b)** *Under dark-field illumination PAX2 hybridization is seen in dorso-medial part of neural retina (arrow) and optic stalk (arrowheads), but not in the tissue of the diencephalon and telencephalon.*

Fig. 10. Transversal section through the eye of an 8-week human embryo. (a) *Under bright-field illumination the developing eye consists of pigmented layer (p), neural retina (n), optic nerve (on) and cornea (co).* **(b)** *Under dark-field illumination, strong PAX2 hybridization is seen in the optic nerve (arrowhead), and weaker hybridization in the optic disc (arrow). Hybridization of pigmented epithelium is an artifact.*

Materials and Methods

Human material

Normal human embryos and fetuses between 6-9 developmental weeks were collected after spontaneous or legal abortions from the Department of Gynecology and Obstetrics, Clinical Hospital Split, Croatia. The embryonic tissues were treated as postmortal material with permission of hospital's Drug and Ethical Committee (enclosed).

The postovulatory age was estimated from menstrual data, correlated with the crown-rump length (CRL) and Carnegie stages (O'Rahilly and Gardner, 1971) (Table 1).

Embryos were dissected into two or three pieces, fixed in 4% paraformaldehyde in phosphate buffer for several hours and embedded in paraffin. Tissue blocks were serially cut in transversal direction and mounted on glass-slides coated with Chrome alum (Serva) gelatine.

In situ hybridization

The slices were processed through the following steps: dewaxing in xylene, dehydration, washing in PBS, refixing in 4% PFA, washing, proteinase-K treatment (0.02 mg/ml), washing, 4% PFA, washing, 0.1,M triethanolamine treatment, washing and dehydration.

S ^{35}labeled probe, specific for PAX 2, was synthesized using T3-polymerase, from corresponding linearized plasmid templates as described in Dressler *et al.* (1990). Probe (1x1o 8 cpm/ml) was dissolved in hybridization buffer (300 mM NaCl, 10 mM dithiothretiol-DDT, 10% dextran sulphate, 50% formamide, 2 mg/ml bovine serum albumin, 2 mg/ml Ficoll, 2 mg/ml polyvinylpyrrolidine).

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 used: 2x saline-sodium citrate (SSC), 50% formamide, 10 mM beta-mercaptoethanol (15 min, 37°); 2xSSC, 50% formamide, 10% formamide, 10 mM beta-mercaptoethanol (30 min 65°C); 0.5 M NaCl, 10 mM Tris, 5 mM EDTA (15 min, 37°C); 0.5 M NaCl 10 mM Tris, 5 mM EDTA (15 min, 37°C); 2xSSC, 50% formamide, 10 mM beta-mercaptoethanol (30 min, 37°C); 2xSSC (15 min, room temperature); 0.1xSSC (15 min, room temperature). The sections were dehydrated in ethanol and air dried.

For autoradiography, slices were dipped in Kodak NTB-2 emulsion dilute 1:1 with water. Slices were exposed for up to 21 days and developed in Kodak D-19 solution.

For morphological analysis the sections were stained with Giemsa, coverslips were mounted with Eukitt. Photomicrographs were taken with bright/dark field microscope.

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