

# Expression pattern of *PAX3* and *PAX6* genes during human embryogenesis

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**ABSTRACT** The expression of human *PAX6* and *PAX3* genes was investigated in 6 human 6-9 week old conceptuses by *in situ* hybridization. During human embryonic development (6-8 developmental weeks), *PAX6* was expressed in the ventricular zone of telencephalon and diencephalon, and in the ventricular and ventral intermediate zones of medulla oblongata and spinal cord. *PAX6* expression was detected in both layers of the optic cup, optic stalk and prospective corneal epithelium. Infundibulum and Rathke's pouch of the pituitary gland showed hybridization signal as well. In the early fetal period (9 developmental weeks) *PAX6* expression increased in the spinal cord. In the eye, hybridization signal characterized the corneal and lens epithelium, pigmented and neural retina, while it was missing in the optic disc and nerve. In a 6-week human embryo, transcripts of *PAX3* gene were observed in the ventricular zone at the mesencephalic-rhombencephalic border, and in the dorsal part of the ventricular zone and the roof plate of the medulla oblongata and the spinal cord. In the 8-9-week fetus, *PAX3* expression increased in dorsal parts of the spinal cord. *PAX3* characterized ectomesenchyme of the upper and lower jaw, and tongue. During early human development, *PAX6* and *PAX3* genes seem to be involved in the brain regionalization and establishment of dorso-ventral polarity of the spinal cord. Additionally, *PAX6* participates in organogenesis of the eye and the pituitary gland, and *PAX3* in the development of face and neck mesenchyme.

**KEY WORDS:** *human embryos, PAX3 gene, PAX6 gene, central nervous system*

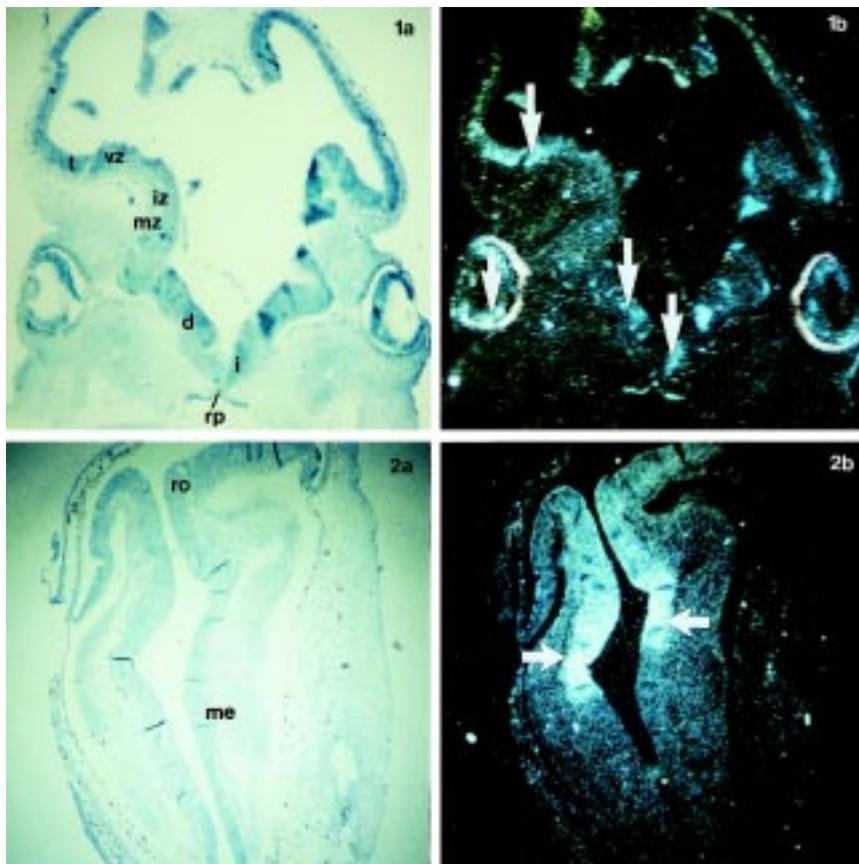
## Introduction

*Pax* genes encode transcription factors which are involved in development and differentiation of the central nervous system both in vertebrates and invertebrates. The murine *Pax* gene family consists of nine members (Walther *et al.*, 1991; Wallin *et al.*, 1993) which are grouped into six different classes (Callaerts *et al.*, 1997). Classification of *Pax* genes is based on the presence of gene products containing the obligatory paired domain, additional content of octapeptide, and complete or partial homeodomain (Callaerts *et al.*, 1997). Except for *Pax1* and *Pax9*, all *Pax* genes are spatially and temporally expressed during development of the central nervous system (Deutsch and Gruss, 1991; Chalepakis *et al.*, 1994; Wallin *et al.*, 1993). At early developmental stages, *Pax3*, *Pax6* and *Pax7* are expressed in the entire developing neural tube, except for the *Pax6* gene which is excluded from the mesencephalic roof (Jostes *et al.*, 1991; Walther and Gruss, 1991; Goulding *et al.*, 1993). At midgestation, the expression of *Pax3/Pax7* is retracted with a rostral limit at the level of posterior commissure, while the *Pax6* expression in the forebrain persists

during embryogenesis and in certain structures in adulthood (Stoykova *et al.*, 1996). The midbrain-hindbrain boundary is the rostral limit of expression of the late *Pax* genes – *Pax2*, *Pax5*, *Pax8* (Nornes *et al.*, 1990; Asano and Gruss, 1992).

Genetic lesions in *Pax3* and *Pax6* genes lead to developmental abnormalities. In *Pax3* mutants, alterations such as deletions and point mutations affect the DNA-binding properties of the product of *Pax3* (Epstain *et al.*, 1991, 1993; Chalepakis *et al.*, 1994). The heterozygous mice *Spotch* mutants have pigmentation disturbances of the abdomen, tail and feet, while homozygous mice mutants die with severe defects of neural tube and neural crest derivatives (Mansouri *et al.*, 1994, 1996). Mutations in the human *PAX3* gene have been described in patients with Waardenburg syndrome (Tassabehji *et al.*, 1993), displaying abnormalities of the eye and nose formation, deafness and pigmentation disturbances. The removal of the notochord or implantation of an additional notochord can alter the dorsoventral patterning of *Pax3* and *Pax6* gene (Goulding *et al.*, 1993). Expression of *Pax3*, *Pax7* and *Pax6* genes can be also regulated by the roof plate and overlying ectoderm possibly via secretion of bone morphoge-

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**Fig. 1. Transversal section through the head region of the 6-week human embryo. (a)** Under bright-field illumination the telencephalon (t), diencephalon (d), and optic cup (c), as well as the developing pituitary gland–infundibulum (i) and Rathke's pouch (rp) close to the junction with pharynx are seen. The brain tissue consists of the ventricular zone (vz), the intermediate zone (iz) and the marginal zone (mz). **(b)** Under dark-field illumination PAX6 hybridization is detected in the ventricular zone of the telencephalon and diencephalon, in the optic cup, infundibulum and Rathke's pouch (arrows).

**Fig. 2. Transversal section through the head region of the 6-week human embryo. (a)** Under bright-field illumination mesencephalon (me) and rhombencephalon (ro) are seen. **(b)** Under dark-field illumination PAX3 hybridization is observed in the ventricular zone at the mesencephalon–rhombencephalon border (arrows).

netic proteins (BMPs) (Liem *et al.*, 1995). In human dysraphic disorders non closure of vertebral arches and neural tube, and defects of surface ectoderm are associated with abnormal notochord (Saraga-Babić and Saraga, 1993; Saraga-Babić *et al.*, 1993; 1996a,b). This suggests a possible role for PAX3 or PAX6 genes in genesis of such disorders. However, until now, in human patients with neural tube defects no significant association with altered PAX3 gene has been found (Hol *et al.*, 1996).

Among *Pax* genes, *Pax6* is the earliest one expressed in the developing central nervous system, eyes and nose. During normal mouse development, early restriction of *Pax6* into the histogenetic fields correlates with formation of distinct forebrain structures and nuclei. In *Small eye* mouse mutants, severe defects of the forebrain are found in regions of *Pax6* expression (Stoykova *et al.*, 1996). Complete loss of the eyes and nasal cavities are seen in homozygous mice mutants, while heterozygous mutants characterize only smaller eyes (Hill *et al.*, 1991). Mutations in the human PAX6 gene

were identified in patients with aniridia syndrome (Glaser *et al.*, 1994) and Peter's anomaly (Hanson *et al.*, 1994).

Preliminary investigations on the early 3-6-week human embryos disclosed importance of several PAX genes in the development of the central nervous system, but in the development of other organs as well. PAX3 is found in the neural groove and later in the closed neural tube in the region of mesencephalon, rhombencephalon and spinal cord (Gerard *et al.*, 1995). PAX6 is expressed very early, just after the closure of the neural tube. Afterwards, it is expressed in the prosencephalon, rhombencephalon, spinal cord, somites and optic vesicle (Gerard *et al.*, 1995).

Recent analyses of congenital human diseases, and transgenic and spontaneous mice mutants revealed the importance of the *Pax* genes in organogenesis of the brain and spinal cord, kidney, pancreas, eye, nose and limb muscles (Dahl *et al.*, 1997). The marked correlation exists between the expression of *Pax* genes in developing organs and their roles in normal and abnormal formation of particular organs in animal mutants and human syndromes. For better understanding of the mechanisms underlying human congenital disorders, a thorough analysis of expression pattern of PAX genes during human development is of essential importance. Preliminary study on early human embryos gave data about organs in which PAX genes appear during development, but detailed description of their distribution and expression pattern is still missing. In this study we investigated the expression pattern of PAX3 and PAX6 genes during embryonic and early fetal period.

## Results

The expression pattern of PAX3 and PAX6 genes was investigated in human embryos between the 6th and 9th developmental week

(during the period of the most intense organogenesis). Both genes were expressed in the developing central nervous system—brain and spinal cord. PAX6 was additionally expressed in the developing eye, while PAX3 in the derivatives of the neural crest—the spinal ganglia and the ectomesenchyme of the face. Sense and antisense probes for PAX3 and PAX6 were used as control probes.

### Brain

In the 6th week of development the human brain already shows signs of division into five main parts: telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon.

Expression of PAX6 gene is seen in the ventricular zone of telencephalon and diencephalon. It is also expressed in the optic cup and stalk. PAX6 hybridization signal characterizes the infundibulum, the evagination of floor of diencephalon (prospective neurohypophysis) and Rathke's pouch, an outpocketing of stomo-

deum (prospective adenohypophysis) (Fig. 1a,b).

Transcripts of *PAX3* gene are observed in the ventricular zone at the mesencephalic-rhombencephalic border in embryo of the same developmental age. (Fig. 2a,b).

At the same developmental age, expression of *PAX6* gene is seen in the middle part of ventricular zone of medulla oblongata and within bilateral clusters of cells in the ventral intermediate zone (Fig. 3a,b). Expression of *PAX3* gene is seen only in the dorsal part of the ventricular zone of medulla oblongata, and not in any part of the intermediate zone (Fig. 4a,b).

### Spinal cord

The spinal cord of a 6-week human embryo shows three characteristic zones: the ventricular zone (prospective ependymal cells), the intermediate zone (prospective gray matter) and the marginal zone (prospective white matter).

*PAX6* gene has a very weak hybridization signal in the middle part of the ventricular zone of a 6-week spinal cord (Fig. 5a,b). Very weak expression of *PAX3* gene is observed in the dorsal part of the ventricular zone in embryo of the same developmental age (not shown). The same embryo displays clear expression of both *PAX6* and *PAX3* genes in specific areas of the brain (see Figs. 1,2,3, and 4).

Subsequent differentiation of the spinal cord is associated with increase of hybridization signal of both *PAX6* and *PAX3* genes. At later developmental stages, in the 8-9-week fetus, strong expression of *PAX6* gene is seen in the ventral part of the ventricular zone, while weaker expression characterizes its middle part. The cluster of cells in the ventral intermediate zone (basal plate) also shows a strong hybridization signal. At the cervico-thoracic region, the hybridization signal in the ventricular zone is thinner (Fig. 6a,b) than at the thoraco-lumbar region (Fig. 7a,b) as differentiation of the spinal cord is more advanced in the cranial part of the embryo. Hybridization signal is excluded from the floor plate and roof plate areas.

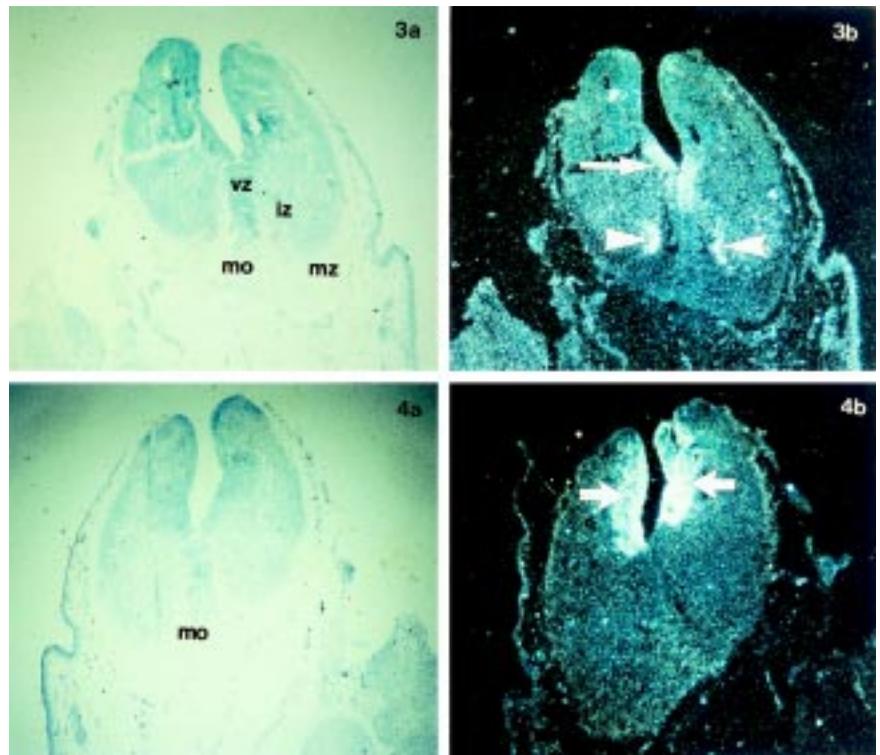
In the 9-week fetus, the strong *PAX3* expression is detected in the dorsal 2/3 of the ventricular zone and in the roof plate, as well as in the spinal ganglia and spinal nerves (Fig. 8a,b).

### Eye

In the 4th week of development, the optic vesicle develops as an outgrowth of the diencephalon, the stem of the vesicle being the optic stalk. Subsequently, under inductive influence of the optic vesicle, the lens placode invaginates to form the lens vesicle. The optic vesicle becomes the double walled optic cup, giving rise to pigmented (outer wall) and neural part (inner wall) of the retina (Sadler, 1995).

In the 6-week embryo, hybridization signal of *PAX6* gene is seen in the surface ectoderm (prospective corneal epithelium) and in the neural and pigmented retinal layers of the optic cup (Fig. 9a,b,c). It is also seen in the optic stalk (prospective optic nerve)(not shown).

Later on, the cells in the posterior wall of the lens vesicle elongate to form the lens fibers. In a 8-9-week fetus, *PAX6* expression characterizes the corneal epithelium, lens epithelium



**Fig. 3. Transversal section at the level of medulla oblongata (mo) of the 6-week human embryo. (a)** Under bright-field illumination the medulla oblongata consists of the ventricular zone (vz), the intermediate zone (iz) and the marginal zone (mz). **(b)** Under dark-field illumination, expression of *PAX6* gene is seen in the middle part of the ventricular zone (arrow) and in part of the ventral intermediate zone (arrowheads).

**Fig. 4. Transversal section at the level of medulla oblongata (mo) of the 6-week human embryo. (a)** Under bright-field illumination the medulla oblongata consists of the three characteristic zones (see Fig. 3). **(b)** Under dark-field illumination expression of *PAX3* gene is seen in the dorsal part of the ventricular zone (arrows).

and the pigmented and neural epithelium of developing retina and iris. Hybridization signal is not present in the optic disc and optic nerve (Fig. 10a,b).

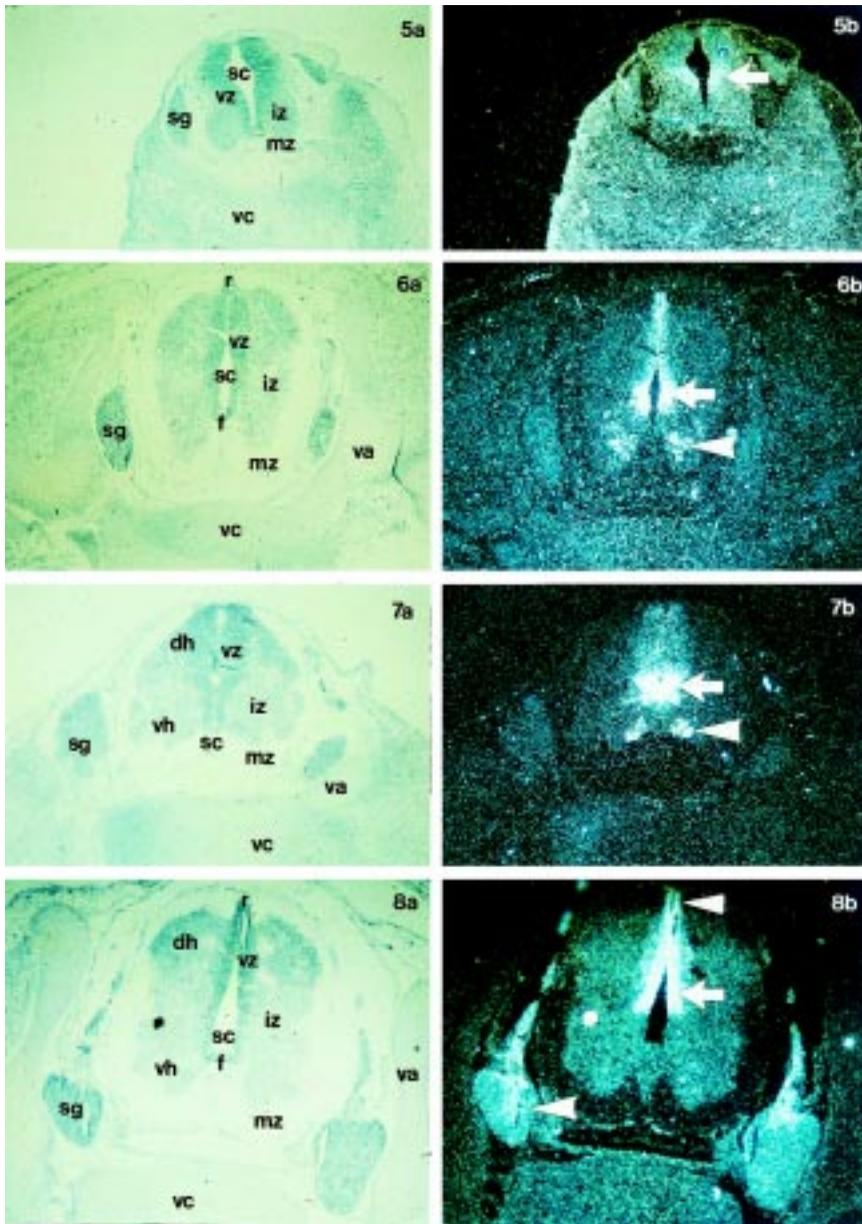
### Derivatives of pharyngeal arches (jaws, tongue)

Mesoderm which forms the embryonic connective tissue of the face is ectomesenchyme derived from the neural crest cells. The first pharyngeal arch is involved in the development of the face (including jaws), while the tongue develops from parts of all four pharyngeal arches (Sadler, 1995).

In the 8-9 week human fetus, *PAX3* hybridization characterizes connective tissue (lamina propria) of the developing upper and lower jaw, and tongue. Expression is particularly strong in the connective tissue immediately below the epithelium and around the excretory ducts of salivary glands. Hybridization signal is not present in the surface epithelium, myoblasts and Meckel's cartilage (Fig. 11a,b).

### Discussion

Investigations on the *Pax* genes during development of different animal species (Bopp *et al.*, 1986; Dressler *et al.*, 1988; Krauss *et al.*, 1991; Goulding *et al.*, 1993) and humans (Gerard *et al.*, 1995; Terzić *et al.*, 1998) disclosed important roles of those genes in body



**Fig. 5. Transversal section through the thoracic part of axial organs in the 6-week human embryo. (a)** Under bright-field illumination, three zones can be distinguished in the spinal cord (sc): the ventricular zone (vz), the intermediate zone (iz) and the marginal zone (mz). Spinal ganglia (sg), vertebral column (vc). **(b)** Under dark-field illumination, a very weak hybridization signal of PAX6 gene is seen in the middle part of the ventricular zone (arrow).

**Fig. 6. Transversal section through the cervico-thoracic level of axial organs in the 8-9 week human fetus. (a)** Under bright-field illumination the spinal cord (sc) consists of a thin ventricular zone (vz) and the well developed intermediate (iz) and marginal zones (mz), roof plate (r) and floor plate (f). Spinal ganglia (sg), vertebral column (vc) and vertebral arches (va) are seen as well. **(b)** Under dark-field illumination PAX6 hybridization signal is seen in the middle ventricular zone (arrow) except the floor plate area, and bilaterally in the ventral intermediate zone (arrowhead).

**Fig. 7. Transversal section through the thoraco-lumbar level of axial organs in the 8-9 week human fetus. (a)** Under bright-field illumination the ventricular zone (vz) of the spinal cord (sc) is thinner ventrally than dorsally. Ventral gray horns (vh) of the intermediate zone (iz) are better developed than dorsal horns (dh). Marginal zone (mz), spinal ganglia (sg), vertebral column (vc), vertebral arches (va). **(b)** Under dark-field illumination PAX6 is expressed in the middle part of the ventricular zone (arrow) and bilaterally in part of the ventral gray horns (arrowhead).

**Fig. 8. Transversal section through the thoracic level of axial organs in the 9-week fetus. (a)** Under bright-field illumination the ventricular zone (vz) of the spinal cord (sc) is thin. The intermediate zone (iz) with well developed ventral (vh) and dorsal gray horns (dh), and the marginal zone (mz) are seen. Floor plate (f), roof plate (r), spinal ganglia (sg), vertebral column (vc), vertebral arches (va). **(b)** Under dark-field illumination PAX3 hybridization characterizes dorsal 2/3 of ventricular zone (arrow) including the roof plate, and spinal ganglia (arrowheads).

patterning. They were found to be involved in organogenesis of the central nervous system, sense organs, kidneys, pancreas, nose and limb muscles (Mansouri *et al.*, 1994; Dahl *et al.*, 1997; Terzić *et al.*, 1998). *Pax* genes were also associated with appearance of different syndromes and oncogenesis in humans and with animal mutants (Wehr and Gruss, 1996). They are evolutionary highly conserved in structure and function as they have similar expression patterns in different species, including man.

Due to numerous investigations in experimental animals, particularly mouse, knowledge on *Pax* genes has significantly improved. During normal mouse development expression of *Pax6* gene is seen in the developing spinal cord and brain. In the spinal cord it is found in the ventricular zone of the intermediate and basal plates, and in the part of ventral intermediate zone. Early *Pax6* expression characterizes prosencephalon, mesencephalon and

rhombencephalon. Later on, it is seen in all parts of the brain except in the roof of mesencephalon (Callaerts *et al.*, 1997). All structures of developing eye and the olfactory epithelium express *Pax6* gene (Walther and Gruss, 1991). Recent analyses disclose the importance of *Pax6* for the formation of the forebrain (Stoykova *et al.*, 1996) and pancreas (St Onge *et al.*, 1997). In the homozygous mouse *Pax6* mutant *Small eye*, the development of the eye and nose are affected. In human mutation aniridia, patients are suffering from iris defects, but other eye structures are affected as well (Hill *et al.*, 1991; Ton *et al.*, 1991; Glaser *et al.*, 1994).

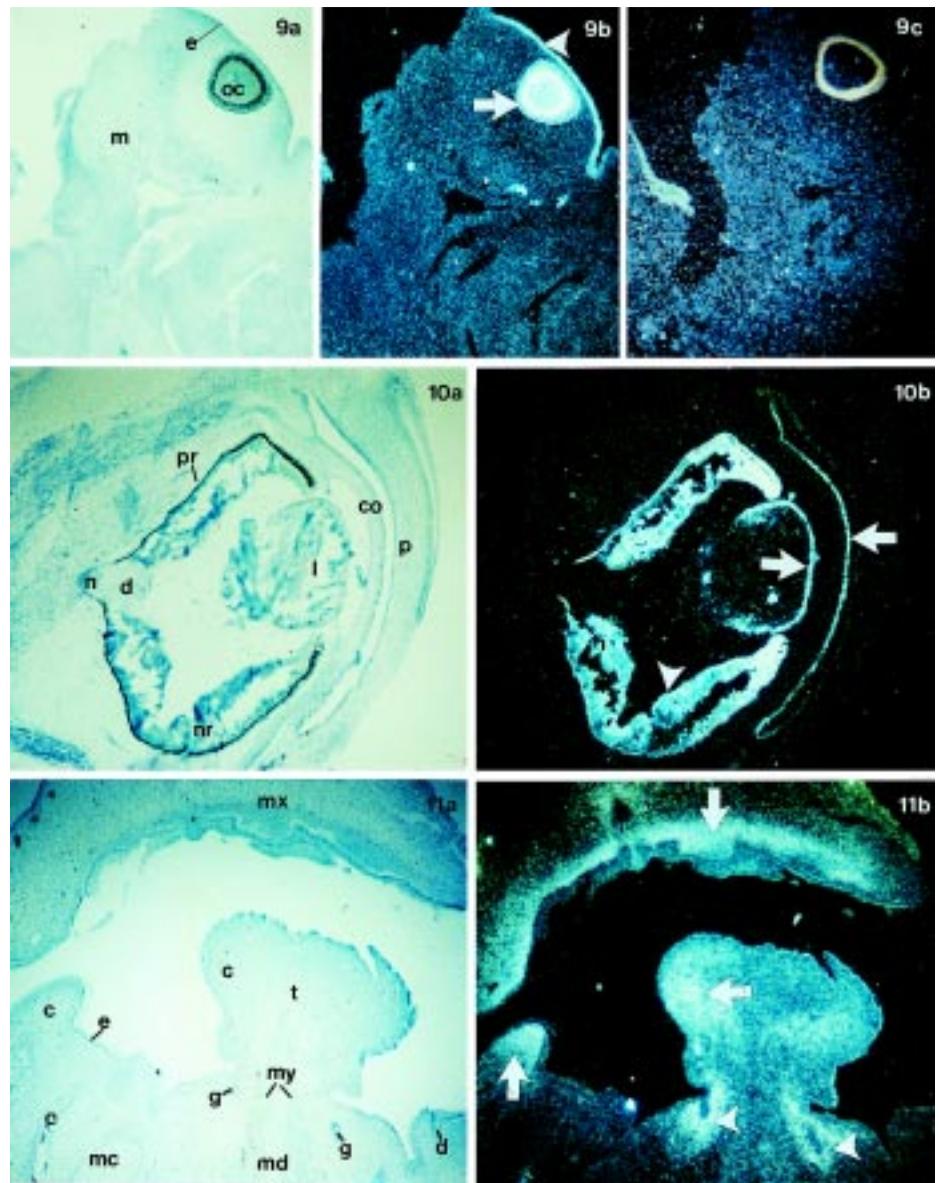
Investigations on 3-6-week human embryos (Gerard *et al.*, 1995) gave only general information on early expression of *PAX* genes in certain organs, but without specific details on their distribution within cell layers. Our study was done on later developmental stages (6-9 postovulatory weeks), describing changes in

expression pattern of *PAX3* and *PAX6* genes during later embryonic and early fetal period. In embryonic stage of human development, *PAX6* was expressed in the mitotically active ventricular zone of telencephalon and diencephalon, as well as in the ventricular zone and part of ventral intermediate zone of myelencephalon and spinal cord. In mouse development, *Pax6* respected anatomical borders of neuromeres, having a segment-like expression pattern in diencephalon (Stoykova and Gruss, 1994; Stoykova *et al.*, 1996). The caudal limit of *Pax6* expression in the human brain was at the diencephalon-mesencephalon border.

Interestingly, in our study the hybridization signal was much stronger in the brain than in the spinal cord of the same young human embryo. At later stages, increase of *PAX6* expression coincided with the differentiation of the human spinal cord. In animals, expression of *Pax6* gene could be influenced by neurotrophin (Kioussi and Gruss, 1994) and by removal or implantation of the notochord. The notochord transplantation dramatically altered the dorsoventral expression pattern of both *Pax6* and *Pax3* genes (Goulding *et al.*, 1993), suggesting that those genes participate in the establishment of dorso-ventral polarity of the spinal cord (Wehr and Gruss, 1996).

In our study, besides its presence in the nervous structures, *PAX6* was also detected in the surface head ectoderm of the prospective corneal and lens epithelium, and in the optic cup and stalk. At the end of the embryonic period, the expression of *PAX6* gene was missing in the optic disc and nerve (derivatives of optic stalk). Investigation on early human embryos (Gerard *et al.*, 1995) could show only expression of *PAX6* gene in the stage of eye vesicle. In animals, a decrease in expression of *Pax6* gene in pigmented retinal epithelium and optic stalk was described at later stages of development (not investigated in our study). It is believed that *Pax6* is not necessary for the formation of the optic cup, but is required at other stages of eye development (Macdonald and Wilson, 1996). Aniridia and Peters' anomaly in humans displayed phenotypic features that correspond to the developmental expression of *PAX6* described in our study (Glaser *et al.*, 1994; Hanson *et al.*, 1994).

Transcripts of *PAX6* gene were found in both Rathke's pouch and infundibulum of the developing human pituitary gland. Until now, *Pax6* was described only in the primordium



**Fig. 9. Transversal section through the head region of the 6-week human embryo, at the level of the eye. (a)** Under bright-field illumination section through the optic cup (oc), head mesenchyme (m) and surface epithelium (e) is seen. **(b)** Under dark-field illumination PAX6 hybridization characterizes optic cup (arrow) and head epithelium overlying the eye –prospective corneal epithelium (arrowhead). **(c)** Under dark-field illumination, the sense control shows no hybridization signal in the optic cup and prospective corneal epithelium. Remaining hybridization of pigmented epithelium is an artifact as it is visible also in every untreated section.

**Fig. 10. Transversal section through the eye of the 8-9-week human fetus. (a)** Under bright-field illumination the developing eye consists of the cornea (co), lens (l), neural (nr) and pigmented retina (pr). Optic disc (d), initial part of the optic nerve (n) and palpebrae (p) are seen. **(b)** Under dark-field illumination PAX6 hybridization is seen in the corneal and lens epithelium (arrows) and in both parts of retina (arrowhead).

**Fig. 11. Transversal section through the maxilla (mx), mandible (md) and tongue (t) of the 8-9-week human fetus. (a)** Under bright-field illumination the components of the developing jaws are seen: surface epithelium (e), connective tissue (c), Meckel's cartilage (mc), initial ossification (o) and dental lamina (d). The tongue (t) is formed of myoblast strands (my), connective tissue and covering epithelium. Ducts of salivary glands (g). **(b)** Under dark-field illumination PAX3 hybridization is seen in the connective tissue (lamina propria) of oral mucosa and tongue (arrowheads) and around ducts of salivary glands (arrowheads).

of adenohypophysis (Rathke's pouch) of mouse embryos (Walther and Gruss, 1991; Callaerts *et al.*, 1997). In our study, at earlier developmental stages expression of *PAX6* gene extended from diencephalon into the infundibulum as it represents the continuation of the brain structure. Interestingly, in *Pax6* mouse mutants all tissues expressing *Pax6* during development were affected except the pituitary gland (Callaerts *et al.*, 1997). Therefore, other genes rather than *Pax6* seem to have a crucial role during pituitary development.

Belonging to the group of early expressed genes in animals, *Pax3* is found to be active in the whole brain at early developmental stages, but later its expression domain is restricted rostrally at the level of the diencephalon (Jostes *et al.*, 1991; Goulding *et al.*, 1993; Stoykova and Gruss, 1994). *Pax3* is found in the ventricular zone, alar plates and the roof plate of the spinal cord, as well as in the neural crest cells and their derivatives. In the mesoderm, *Pax3* is expressed in the primitive streak (Goulding *et al.*, 1993) and in the undifferentiated somites. Later on, it characterizes dermomyotome and limb muscles. The *Spotch* mouse mutants exhibit spina bifida, exencephaly, neural crest and limb-muscle defects (Epstein *et al.*, 1991; Franz *et al.*, 1993; Tassabehji *et al.*, 1993; Mansouri *et al.*, 1996). *Pax3* mutations have been correlated with human Waardenburg syndrome, which displays numerous defects of neural crest derivatives, deafness, pigmentation deficiency and lateral displacement of the inner corner of the eye (Baldwin *et al.*, 1992; Tassabehji *et al.*, 1993). *PAX3* has been found to be mutated in human tumor rhabdomyosarcoma (Barr *et al.*, 1993; Shapiro *et al.*, 1993).

Results of our investigation on human *PAX3* gene accord with the described data in mouse embryos. In six-week human embryo, strong expression of *PAX3* was found at the mesencephalic-rhombencephalic border and in the dorsal part of ventricular zone of medulla oblongata. These data indicate a role for *PAX3* gene, together with other *PAX* genes in establishment of border between those two parts of the human brain. In mouse development, it is believed that *Pax3*, together with *Pax6* participates in establishment of segmental boundaries in the brain and axonal pathways at the borders of their expression (Stoykova and Gruss, 1994). Similarly to *PAX6* gene, hybridization signal of *PAX3* was very weak in the dorsal ventricular zone of the spinal cord in early human embryos. With advancing differentiation of the spinal cord, hybridization signal increased in dorsal part of ventricular zone and roof plate area. It also characterized spinal ganglia and spinal nerves (derivatives of the neural crest cells). Expression of *PAX3* in dorsal parts of the spinal cord suggests its possible role during proper closure of the neural tube. Experimentally, expression of *Pax3* can

be changed by extirpation or implantation of an additional notochord (Goulding *et al.*, 1993). Morphological studies on human embryos with spina bifida or anencephaly showed that anomalies of the central nervous system are always combined with vertebral and notochord abnormalities (positional changes, dispersed or supernumerary notochord) indicating a role for the notochord in genesis of axial disorders (Saraga-Babić and Saraga, 1993; Saraga-Babić *et al.*, 1996a,b). Normal spatial relationship between the axial organs at critical stages of development seems to be of essential importance for expression of genes controlling proper axial development (Saraga-Babić *et al.*, 1993, 1996a,b; Monsoro-Burq *et al.*, 1995). In adult humans with neural tube defects, a *PAX3* gene variation was detected. An increased, but insignificant frequency of the rare allele of a silent polymorphism in exon 2 was found in such patients (Hol *et al.*, 1996).

As previously reported for *Pax3* in mouse development (Stoykova and Gruss, 1994), expression of human *PAX3* gene was detected within the derivatives of branchial arches –maxilla, mandible and tongue (the limb development was not in the focus of this study). Previous reports on human embryos did not describe such expression (Gerard *et al.*, 1995). During normal human development, neural crest cells invade the central part of pharyngeal arches giving rise to skeletal structures, connective tissue of the face, teeth, stromal components of the glands etc. The muscles of the head and neck differentiate from somitomeres in the head region, e.g. from the original mesoderm of pharyngeal arches. However, further formation of myoblasts is modified by the surrounding mesenchyme derived from neural crest cells (Fitzgerald and Fitzgerald, 1994; Sadler, 1995). Contrary to muscles in the head, differentiation of limb muscles is influenced by the parietal (mesodermal) mesenchyme. Different origin of mesenchyme which modifies the formation of limb and face muscles might be one of possible explanations for the absence of face abnormalities in *Spotch Pax3* mutants. Another possibility is specific expression of paralogous *Pax7* gene in this region (Jostes *et al.*, 1991).

Although highly resembling descriptions in experimental animals, human *PAX6* and *PAX3* genes displayed small differences in their expression pattern when compared to other species. It refers to the weak expression of both genes in the spinal cord of early human embryos. Unlike in other species, *PAX6* was found to be active in the infundibulum of the future pituitary gland. In the eye, it disappeared from the optic stalk earlier than in corresponding stage of mouse development. Expression of *PAX3* gene only in the facial connective tissue of pharyngeal arches indicates that other genes rather than *PAX3* have an important role during face and neck development. Further studies focusing on the limb development and analysis of brain structures in humans should give more information on the developmental roles of *PAX6* and *PAX3* genes.

TABLE 1

#### AGE AND NUMBER OF HUMAN EMBRYOS AND FETUSES USED IN THIS STUDY

Age (weeks)	CRL(mm)	Carnegie stage	No
6	11	16- 17	1
6-7	14	17-18	1
7-8	22	20-21	1
8	27	22	1
8-9	32	/	1
9	37	/	1

## 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.

To ensure the use of healthy material in our study, we controlled anamnestic data of women that underwent abortion. In order to eliminate the malformed or poorly preserved embryonic tissue, collected embryos

and fetuses were first examined macroscopically and then microscopically in sections stained with hematoxylin and eosin.

The postovulatory age was estimated from menstrual data, correlated with the crown-rump length (CRL) and Carnegie stages (Moore and Persaud, 1993) (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) gelatin.

#### 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, 0,1M triethanolamine treatment, washing and dehydration.

$^{35}$ S labeled probe, specific for PAX3 and PAX6, was synthesized using T3-polymerase, according to the supplier's direction (Promega) from corresponding linearized plasmid templates as described in Goulding *et al.* (1991) for PAX3 and in Walther and Gruss (1991) for PAX6. Probe ( $1 \times 10^8$  cpm/ml) was dissolved in hybridization buffer (300 mM NaCl, 10 mM dithiothreitol-DDT, 10% dextran sulphate, 50% formamide, 2 mg/ml bovine serum albumine, 2 mg/ml Ficoll, 2 mg/ml polyvinylpyrrolidone).

The hybridization mix was boiled, applied directly onto the section, and covered with siliconized coverslips. After overnight hybridization at 55°C, the following washing procedure was used: 2 x saline-sodium citrate (SSC), 50% formamide, 10 mM beta-mercaptoethanol (15 min. 37°C); 2xSSC, 50% 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 temp.); 0,1xSSC (15 min at 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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