Dysgenesis of cephalic neural crest derivatives in \( \text{Pax7}^{-/-} \) mutant mice

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SUMMARY

\( \text{Pax7} \) is a member of the paired box containing gene family. Its expression pattern suggests a function in cephalic neural crest derivatives, skeletal muscle and central nervous system development. To understand the role of \( \text{Pax7} \) during mouse embryogenesis, we used the homologous recombination technique in embryonic stem cells and generated \( \text{Pax7}^{-/-} \) mice. Homozygous animals are born but die shortly after weaning. They exhibit malformations in facial structures involving the maxilla and nose. Our analysis suggests that the observed phenotype is due to a cephalic neural crest defect. No obvious phenotype could be detected in the central nervous system and skeletal muscle. Functional redundancy between \( \text{Pax7} \) and \( \text{Pax3} \) is discussed.

Key words: \( \text{Pax7} \), homologous recombination, mouse, neural crest, ES cells

INTRODUCTION

The mouse \( \text{Pax} \) gene family consists of nine members (see Gruss and Walther, 1992 for review) which have been isolated on the basis of sequence homology to \( \text{Drosophila} \) segmentation genes (Bopp et al., 1986; Coté et al., 1987; Baumgartner et al., 1987). They all contain the paired box, a DNA binding domain of 128 amino acids, which is located close to the amino terminus. The paired box has been highly conserved during evolution and paired box containing genes have been cloned from different organisms such as mouse (Gruss and Walther, 1992), zebrafish (Krauss et al., 1991), chick (Goulding et al., 1993a) and man (Buri et al., 1989). In the mouse, \( \text{Pax} \) genes are expressed in very restricted domains (Deutsch et al., 1988; Dressler et al., 1990; Plachov et al., 1990; Jostes et al., 1991; Walther and Gruss, 1991) and with the exception of \( \text{Pax1} \) and \( \text{Pax9} \) (Deutsch et al., 1988, Neubüser et al., 1995; Wallin et al., 1993; Stapleton et al., 1993), they all exhibit discrete expression patterns in the developing and adult nervous system (Stoykova and Gruss, 1994).

The crucial role that \( \text{Pax} \) genes play in embryonic development, is documented by three mouse mutants with mutations in three corresponding \( \text{Pax} \) genes (for review see Hastie, 1991; Gruss and Walther, 1992; Chalepakis et al., 1993; Mansouri et al., 1994). Furthermore, \( \text{Pax} \) genes are also mutated in three human syndromes (Hill and Van Heyningen, 1992; Strachan and Reed, 1994; Sanyanusin et al., 1995) and \( \text{Pax6} \) is mutated in \( \text{Drosophila} \) \text{eyeless} mutants (Quiring et al., 1994). These developmental defects described for the mouse mutants and human syndromes (for review see Mansouri et al., 1994; Strachan and Reed, 1994) correlate very well with the expression domains described for the respective \( \text{Pax} \) genes.

The mutation of \( \text{Pax3} \) in \( \text{Splotch} \) mice (Epstein et al., 1991) leads to defects in neural crest derivatives and in limb muscle (Auerbach, 1954; Moase and Trasler, 1989; Franz, 1990). In man, \( \text{Pax3} \) mutation leads to Waardenburg syndrome (Tassabehji et al., 1992, 1993; Baldwin et al., 1992; Morell et al., 1992). According to the genomic organisation and sequence similarities in the paired domain, \( \text{Pax} \) genes can be subdivided into subgroups which share common expression domains. \( \text{Pax3} \) and \( \text{Pax7} \) form such a paralogous group. Although the \( \text{Pax3} \) function is revealed by the \( \text{Splotch} \) mutation, no spontaneous mutant is available which indicates the role of \( \text{Pax7} \). Therefore, we wanted to mutate the \( \text{Pax7} \) gene in mice. Using the approach of homologous recombination in embryonic stem cells, we generated mice with a null allele for \( \text{Pax7} \). The heterozygous animals are normal and fertile, while homozygous offspring develop to term but die within three weeks after birth. The analysis of the \( \text{Pax7} \) mutant mice revealed that in all mutant animals mainly facial skeletal structures are affected which could be related to a neural crest cell defect.

MATERIALS AND METHODS

Targeting vectors

For the first targeting vector (Fig. 2B) an EcoRI fragment of 5.4 kb derived from a λ genomic clone obtained from a genomic library (Balb/c) was used. The PGKNeo plasmid kindly provided by P. Soriano (Soriano et al., 1991) was digested with XhoI and the 1.7 kb neo insert containing the PGK promoter and bovine growth hormone Poly(A) was ligated into the unique SalI site in the first exon of the paired box. The PGKneo and the \( \text{Pax7} \) gene have the same transcriptional orientation. At the 5’ end of the construct, 1 kb upstream of the neomycin insertion the HSV-Tk (Mansour et al., 1988) was inserted into a blunt-ended KpnI site. The construct contains 1 kb on the 5’ end and 3.5 kb of homologous sequences on the 3’ end (Fig. 2B).

The second construct is identical to the one described for Balb/c DNA except that the DNA is of isogenic origin isolated from a λ clone.
obtained from a 129Sv genomic library (kindly provided by T. Doetschman). The third construct (Fig. 2C) was also derived from 129Sv origin. In this construct the amount of homology was extended to 15.3 kb on the 5' end while at the 3' end the homology is reduced to 1.1 kb. In the third construct no HSV-Tk gene was included. All cloning procedures were done in bluescript KSII+ using standard protocols.

The construct containing β-galactosidase (data not shown) is identical to the construct in Fig. 2C with the exception of the insertion of β-galactosidase in front of the neomycin gene and in frame with Pax7 sequences.

**Tissue culture and screening**

ES cell line, D3, was kindly provided by R. Kemler and A. Gossler and R1 cells by A. Nagy. ES cells were cultured as described by Robertson (1987). 10⁷ ES cells were mixed with 25 µg of linearized targeting construct in a volume of 800µl PBS and electroporated using the Bio-Rad Gene Pulser (250 Volt, 500 µF, electrode distance 0.4 cm). ES cells were seeded 5 minutes later on 6×10 cm dishes containing embryonic fibroblasts which had been freshly treated with MitomycinC. Drug selection (G418: 300 µg/ml) or (G418 + ganciclovir 2 µM) was started 24 hours later, and after 8 days resistant colonies were picked and grown on 24-well plates. After a few days confluent colonies were trypsinized and three-quarters of each colony was frozen in 24-well plates at −70°C and the remaining quarter was grown on gelatinized 24-well plates until confluent. Genomic DNA was made from cells in the 24 wells and used for further analysis by genomic Southern blot. For analysis, genomic DNA was digested by EcoRI and after agarose gel electrophoresis and blotting, hybridization was performed using an external probe (Fig. 2E). Positive clones were confirmed by using internal (Fig. 2F) and neo probes (data not shown).

**Histological, whole-mount and radioactive in situ hybridization analysis**

Embryos for histological analysis were fixed in Bouin’s solution. Embryos for in situ hybridization were fixed o.n. at 4°C in 4% paraformaldehyde in PBS. Bouin-fixed embryos were washed several times with PBS and then embedded in OCT medium. Serial sections were cut and mounted on gelatin-coated slides. Sections were stained with hematoxylin and eosin. Some sections were hybridized with radioactive probes using the methods described above.
times in 70% ethanol, dehydrated through graded alcohols, cleared in toluene, embedded in paraffin, sectioned at 12 μm and stained with hematoxylin-eosin. Embryos for whole-mount in situ hybridization were washed after fixation twice in PBT (PBS, 1% Tween 20) dehydrated through 25%, 50%, 75% and 100% methanol and kept at −20°C. Once enough homozygous embryos have been collected, embryos were hydrated through graded methanol/PBT and processed for whole-mount in situ hybridization as described by Wilkinson (1992). Radioactive in situ hybridization was performed as described previously (Stoykova and Gruss, 1994). Whole-mount β-galactosidase staining were performed as described by Allen et al. (1988).

**Skeletal analysis**

Whole foetal skeletons were dissected and stained with alizarin red and alcian blue as described previously (Kessel and Gruss, 1991).

**RESULTS**

**Expression of Pax7 during embryonic development**

*Pax7* is detected at 8.5 days post coitum (d.p.c.) in all brain vesicles and this expression is later (11.5 d.p.c.) retracted to the mesencephalon with an anterior boundary at the posterior commissure (Jostes et al., 1991; Stoykova and Gruss, 1994). In the neural tube, *Pax7* is confined to the alar plate and *Pax7* mRNA is first detected after the closure of the neural epithelium (Jostes et al., 1991). The onset of *Pax3* expression in the neural tube is earlier than that of *Pax7* and starts before closure of the neuroepithelium (Goulding et al., 1991).

In the somites, *Pax7* is first detected in the dermomyotome and later in development is confined to the intercostal muscle (Jostes et al., 1991; Mansouri and Gruss, unpublished data) whereas *Pax3* is already down regulated upon activation of the myogenic markers (Goulding et al., 1991, 1994; Williams and Ordahl, 1994).

*Pax7* has been previously detected in the nasal pit and nasal neuroepithelium (Jostes et al., 1991). We performed comparative in situ analysis of the expression of the paralogous *Pax7* and *Pax3* genes on adjacent sagittal and transverse sections of the head of 13.5 d.p.c. embryos. The results are shown in Fig. 1. The expression of *Pax7* is confined to the medial region of the frontonasal mass (Fig. 1E,H, white arrow) including the nasal capsule, while *Pax7* is detected in both the medial and lateral parts of this region (Fig. 1F,I, white arrowhead). Furthermore, *Pax7* is expressed in the mandible (Fig. 1C,I).

We wanted to follow the expression of *Pax7* in early developmental stages in the head region in order to clarify its presumptive involvement in the patterning of the head neural crest. This was done by inserting the gene for β-galactosidase in front of the neomycin gene (shown in the third construct in Fig. 2C, see legend for details). The β-galactosidase gene (*lacZ*) is inserted in-frame to the *Pax7* sequences so that a fusion to the first exon of the paired box was generated (data not shown). Upon electroporation in ES cells, a targeted clone was obtained and the new *Pax7* mutation containing *lacZ* was introduced into the mouse germline.

In animals heterozygous for this mutation, using whole-mount staining for β-galactosidase, we could detect expression domains of *Pax7* during embryogenesis which have not been described previously (Jostes et al., 1991). *Pax7* is expressed in discrete domains in the rhombencephalon. As shown in Fig. 3, in 8.5 and 9.5 d.p.c. embryos, *Pax7* is detected in presumptive
Fig. 3. Expression of Pax7 in cephalic neural crest. Whole-mount staining for β-galactosidase of 8.5 d.p.c. (A,B) and 9.5 d.p.c. (C,D) heterozygous (Pax7+/−) embryos. (C) Lateral view; (D) dorsal view; (A) The arrow indicates cephalic neural crest around the optic vesicle; the arrowhead indicates neural crest cells at the level of the mesencephalon. (B) The arrow indicates neural crest cells migrating at the hindbrain level; (D) arrow indicates neural crest cells migrating from rhombomere 5. R rhombomere. Bar, 200 μm in A and C; 264 μm in B; 160 μm in D.

Fig. 4. Phenotype of Pax7−/− mice in the maxilla. Whole-mount alizarin red/alcan blue stained skeletal preparations (head) of new born Pax7+/+ (A) and Pax7−/− mice. (A,B) Dorsal view and (C,D) lateral view of the wild-type and homozygous animals. The arrowhead indicates the reduced size of maxilla in Pax7−/− animals (B) as compared to wild type (B). In the lateral view, the maxilla is indicated by white dashes and the anterior-posterior shortening is indicated by the distance between the white and the black arrows. Notice also that the lacrimal bone is underdeveloped. N, nasal cartilage; Pm, premaxilla; Mx, maxilla; Zy, zygomatic; L, lacrimal bone; Bar 1 cm in A and B and 2.8 cm in C and D.
**Fig. 5.** Reduced number of tubules of serous glands in the nose of Pax7−/− mice. Transverse sections through the head of new born animals at the level of the nasal cavity. (A) wild-type; (B) homozygous animal. SG, serous gland; S, nasal septum; arrowhead indicates serous glands in the lateral part; arrow indicates serous glands in the inferior part. Bar, 200 μm.

**Fig. 6.** In Pax7−/+ mice the inferior lateral nasal capsule is missing. Transverse sections through the head of new born animals at the level of the nasal cavity. (A) Wild-type; (B) homozygous animal. S, nasal septum; the inferior lateral bone of the nasal capsule, which is missing in Pax7−/+ mice (B) is indicated by arrow in the wild type (A). Bar, 200 μm.

**Fig. 7.** Expression of Pax3 at 9-9.5 d.p.c. Whole-mount in situ hybridization of Pax3 expression using a riboprobe. The rostral part of the hindbrain, with lower expression, is indicated by a black arrowhead and the other domain of lower expression, in rhombomere 5 is indicated by a black arrow. Bar, 200 μm.
rhombomere 1, 3 and 5. This expression pattern is first detected at 8.5 d.p.c. (Fig. 3B) and persists through 9.5 d.p.c. (Fig. 3C,D) until 10 d.p.c. (data not shown) and was confirmed by whole-mount in situ hybridization (data not shown). Furthermore Pax7 has been found to be expressed in streams of cells at the level of the forebrain around the optic vesicle (Fig. 3A, see small arrow), at the level of the midbrain (Fig. 3A, see arrowhead) and rhombencephalon (Fig. 3B, arrow and Fig. 3C,D). These neural crest cells migrate rostrally and laterally as shown in Fig. 3 for 8.5 and 9.5 d.p.c. embryos. Some cells migrate caudally as shown in Fig. 3D for rhombomere 5 in a 9.5 d.p.c. embryo (see arrow). These migratory cells are cephalic neural crest cells (Serbedzija et al., 1992; Osumi-Yamashita et al., 1994; for review see Bronner-Fraser, 1993). Some of these β-galactosidase-positive cells also migrate into the first and second branchial arches (data not shown).

**Generation of Pax7 null mutant mice**

To inactivate the Pax7 gene in ES cells the neomycin gene was inserted into the first exon of the paired box (the paired box is encoded by three exons) (Fig. 2). This was expected to abolish the DNA-binding activity of the paired domain as has been demonstrated by Chalepakis et al. (1991, 1994). We used replacement targeting vectors (see legend to Fig. 2). Two different ES cell lines, D3 and R1, were used for electroporation and from each cell line two targeted clones have given different ES cell lines, D3 and R1, were used for electroporation targeting vectors (see legend to Fig. 2). Two demonstration by Chalepakis et al. (1991, 1994). We used the DNA-binding activity of the paired domain as has been encoded by three exons) (Fig. 2). This was expected to abolish premature death. The few null mutant mice were probably generated.

**Pax7 mutation leads to postnatal lethality**

Pax7−/− mice develop to term and appear normal. They represent 23% of the offspring. After a few days, homozygous embryos can already be recognized by their growth retardation and frequent lethality. Most of the Pax7−/− mice (97%) die within 3 weeks after birth, without an obvious reason for premature death. The few Pax7−/− animals that survived to adulthood appeared very unhealthy. They were usually killed and examined and we found that many of them exhibited dilations in the small intestine and appendix.

**Phenotype analysis of the Pax7−/− mice**

New born animals were histologically analyzed. Examinations of eosin-hematoxylin stained sagittal and transverse sections of whole embryos revealed no obvious defect. Domains where Pax7 has been found to be strongly expressed, namely the mesencephalon, hindbrain, neural tube and adult brain (Jostes et al., 1991; Stoykova et al., 1994), appear morphologically normal. No obvious abnormality could be detected in neuronal derivatives of cephalic neural crest.

Homozygous animals were also analyzed by whole mount in situ hybridization using three myogenic markers (Myf5, MyoD and Myogenin; Weintraub, 1993) to examine the dermomyotome and the myotome where Pax7 has also been shown to be expressed. Analysis was done using embryos between 9 and 11.5 d.p.c. Examination of whole-mount staining for the RNA of these myogenic markers revealed no obvious changes in their pattern of expression in mutant embryos (data not shown). This indicates that the morphology of these somitic structures is not affected in Pax7−/−. At later stages of development and in new born mice histological analysis revealed no obvious changes in the intercostal muscles.

However, skeletal preparations from new born Pax7−/− animals analyzed using alizarin red and alcian blue staining (shown in Fig. 4) revealed that Pax7−/− mice have reduced maxilla. The maxilla is shortened in anterior/posterior direction (Fig. 4, arrowhead). This is more obvious in a lateral view of the same specimen (Fig. 4C,D). This skeletal phenotype has been found in all analyzed homozygotes. Transverse sections of the head of new born animals at the level of the nose also revealed some phenotypic changes. As shown in Fig. 5B, the tubules of serous glands, which are associated with the lateral wall of the middle meatus (see arrowhead) and those associated with the nasal septum (see arrow) are very reduced in number as compared to wild-type animals.

In further transverse sections, the inferior lateral part of the nasal capsule was not formed in Pax7−/− mice. This missing structure is normally associated with the cartilage which lines the anterior nasal cavity. Therefore, homozygous animals appear to have a pointed snout which distinguishes them phenotypically from the wild-type and heterozygous animals. This is shown in Fig. 6B (see arrow in Fig. 6A). Analysis of embryos at 14.5 d.p.c. revealed that this part of the nasal capsule is not formed in Pax7−/−.

**DISCUSSION**

The analysis of Pax7−/− mice revealed defects in the maxilla, nasal tubules of serous glands and in the anterior part of the nasal capsule. The neural tube, the brain and the skeletal muscle are not obviously affected.

In the developing neural tube, Pax7 and Pax3 expression overlaps in the alar plate. At this level, Pax3 is already detected at the neural plate stage and precedes that of Pax7 which starts after neural tube closure (Goulding et al., 1991; Jostes et al., 1991). Therefore, Pax3 could complement the function of Pax7 in the neural tube of Pax7−/− mice. In contrast, normal Pax7 expression does not compensate for all the functions of Pax3 in the neural tube of Splotch mice since they exhibit an open posterior neuropore. However, this latter phenotype involves only the most dorsal neural tube where Pax7 is not expressed and thus cannot compensate for the absence of Pax3. Alternatively, Pax7 may not rescue the lack of Pax3 because it is expressed slightly later.

The dermomyotome of Pax7−/− mice is normal morphologically, as demonstrated by the expression of myogenic markers. This clearly indicates that the Pax7 mutation does not affect this somitic derivative. In Splotch mice, the development of the limb muscle and the associated shoulder is affected, the dermomyotome is disorganized and the limbs devoid of Pax3-expressing cells (Franz et al., 1993; Bober et al., 1994; Goulding et al., 1994). Again Pax3 and Pax7 share extensive overlapping expression domains in the dermomyotome, and Pax7 cannot substitute for the function of Pax3 in the limb formation of Splotch mice. This may be the consequence of the slightly different dynamics of expression of both genes in
the dermomyotome. In the most lateral part of the dermomyotome, which gives rise to the precursors of the limb muscles (Ordahl and Le Douarin, 1992), Pax3 is more strongly expressed, while Pax7 is more prominent in the medial part. Therefore partial redundancy may explain the lack of phenotype in the skeletal muscles of Pax7-/- mice and in the axial muscles of the Pax3-/- mice. However, while Pax3 expression is down regulated upon activation of the myogenic markers, Pax7 is still strongly expressed (Jostes et al., 1991; Goulding et al., 1991, Williams and Ordahl, 1994). The function of Pax7 in the myotome still remains unclear.

Partial redundancy has been reported recently for a number of genes. Only the generation of compound mutations revealed the phenotype, as anticipated by expression studies. For the MyoD family, the inactivation of MyoD, Myf5 and myogenin individually does not affect muscle development (Braun et al., 1992; Rudnicki et al., 1992). However, when mice carry null alleles for both the MyoD and myf5 genes they have no muscle, and myogenin is not transcribed (Rudnicki et al., 1993). Redundancy has also been described for Hox (Kostic and Capecci, 1994; Horan et al., 1995) and retinoic acid receptor genes (Lohnes et al., 1994; Mendelsohn et al., 1994). Recently functional redundancy for engrailed 1 (En-1) and engrailed 2 (En-2) in the mouse has been very elegantly demonstrated by expressing En-2 under the promoter of En-1 by using the knock-in technique to rescue En-1 mutants (Hanks et al., 1995). Accordingly, we propose that the function of Pax3 and Pax7 may be in part redundant.

The structures affected by the inactivation of Pax7 are neural crest cell derivatives. This has been demonstrated in birds (Le Lievre, 1978; De Leourain, 1982; Couly and De Leourain, 1985; Couly et al., 1993; Noden, 1978, 1983), mouse (Serbedzija et al., 1992; Osumi et al., 1994) and rat (Tan and Morris-Kay, 1986; Matsuo et al., 1993). Does functional redundancy also occur in the cephalic neural crest in Pax7-/- mice? Pax3 is expressed in neural crest, and Splotch mice exhibit defects in neural crest-derived structures such as dorsal root and cranial ganglia and Schwann cells (Moase and Trasler, 1989; Franz, 1990; Tremblay et al., 1995). These defects cannot be compensated for by Pax7, as it does not appear to be expressed in caudal neural crest (Mansouri and Gruss, unpublished data). The origin of the nasal serous glands is not documented.

In the hindbrain, Pax7 expression is confined to rhombomeres 1, 3 and 5. Pax3 is found in all of the hindbrain except at the most rostral level, and in rhombomere 5 (the area facing the otic vesicle), where the expression is lower (Fig. 7, see arrow and arrowhead). The neural crest from the most facing the otic vesicle), where the expression is lower (Fig. 7, see arrow and arrowhead). The neural crest from the most lateral part of the dermomyotome gives rise to the precursors of the limb muscles (Ordahl and Le Douarin, 1992), Pax3 is more strongly expressed, while Pax7 is more prominent in the medial part. Therefore partial redundancy may explain the lack of phenotype in the skeletal muscles of Pax7-/- mice and in the axial muscles of the Pax3-/- mice. However, while Pax3 expression is down regulated upon activation of the myogenic markers, Pax7 is still strongly expressed (Jostes et al., 1991; Goulding et al., 1991, Williams and Ordahl, 1994). The function of Pax7 in the myotome still remains unclear.

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