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Dysgenesis of cephalic neural crest derivatives in *Pax7*$^{-/-}$ mutant mice

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SUMMARY

***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 *Pax7* during mouse embryogenesis, we used the homologous recombination technique in embryonic stem cells and generated *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 *Pax7* and *Pax3* is discussed.**

Key words: *Pax7*, homologous recombination, mouse, neural crest, ES cells

INTRODUCTION

The mouse *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 *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, *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 *Pax1* and *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 *Pax* genes play in embryonic development, is documented by three mouse mutants with mutations in three corresponding *Pax* genes (for review see Hastie, 1991; Gruss and Walther, 1992; Chalepakis et al., 1993; Mansouri et al., 1994). Furthermore, *Pax* genes are also mutated in three human syndromes (Hill and Van Heyningen, 1992; Strachan and Reed, 1994; Sanyanusin et al., 1995) and *Pax6* is mutated in *Drosophila 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 *Pax* genes.

The mutation of *Pax3* in *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, *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, *Pax* genes can be subdivided into subgroups which share common expression domains. *Pax3* and *Pax7* form such a paralogous group. Although the *Pax3* function is revealed by the *Splotch* mutation, no spontaneous mutant is available which indicates the role of *Pax7*. Therefore, we wanted to mutate the *Pax7* gene in mice. Using the approach of homologous recombination in embryonic stem cells, we generated mice with a null allele for *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 *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 *Eco*RI 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 *Xho*I and the 1.7 kb neo insert containing the PGK promoter and bovine growth hormone Poly(A) was ligated into the unique *Sal*I site in the first exon of the paired box. The PGKneo and the *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 *Kpn*I 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 1.1kb. 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). 10^7 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 + gancyclovir 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 *Eco*RI 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

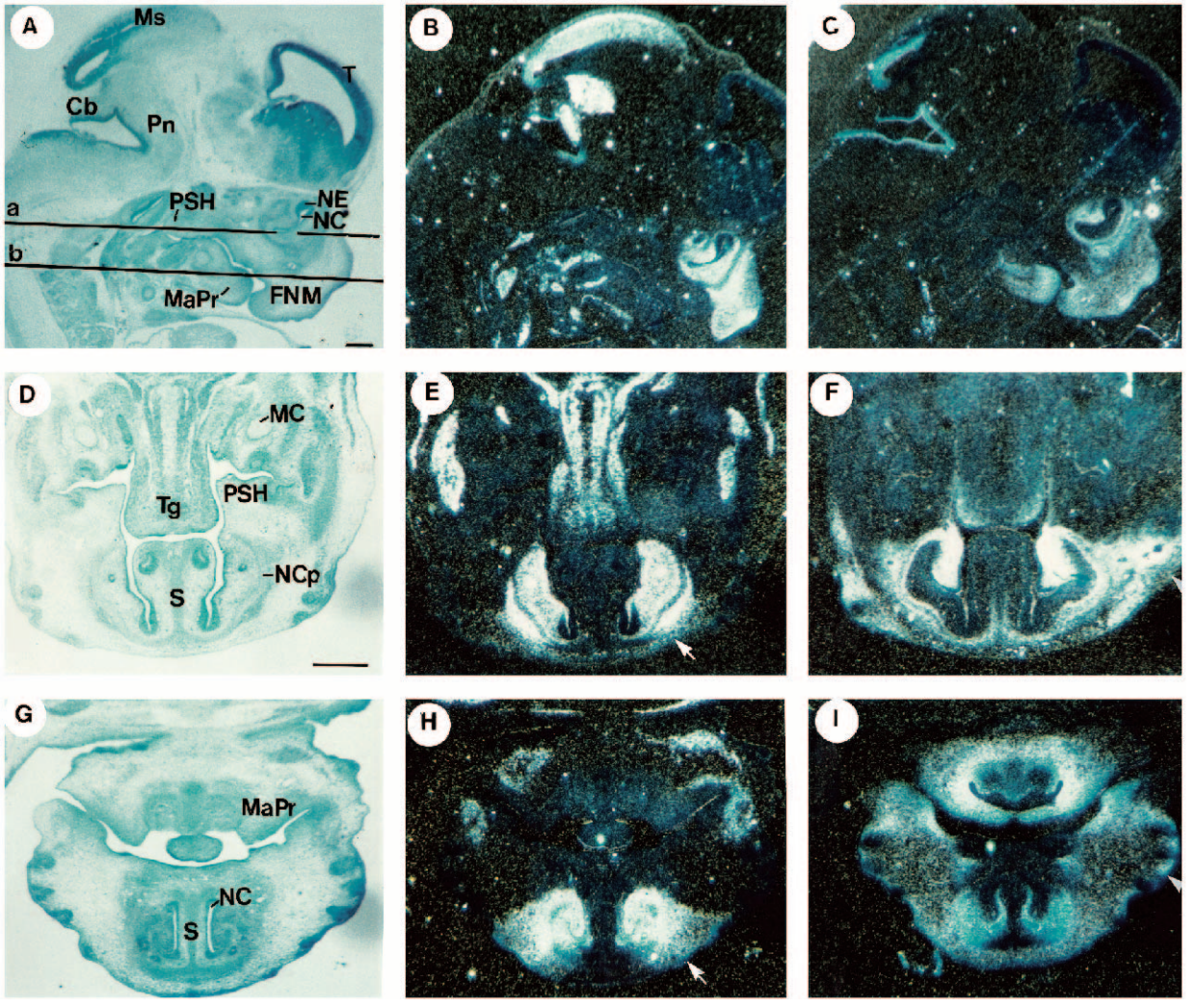


Fig. 1. Differential expression of Pax3 and Pax7 in the head of day 13.5 embryo. (A-C) Adjacent sagittal sections hybridized with *Pax7* (B) and *Pax3* (C) antisense RNA respective probe. (A) Bright-field; (B,C) dark-field illumination. D-F and G-I are transverse sections through the head respectively at level a and b as shown in A. (D,G) Bright-field; (E,F,H,I) dark-field illumination; (E,H) hybridized with *Pax7*; (F,I) hybridized with *Pax3* RNA probe. Note that unlike *Pax3* (arrowhead in F,I), *Pax7* is not expressed in the mandibular (B,C,H,I) and lateral frontonasal mass (E-I) but is expressed in the medial region (arrow in E,H). Cb, cerebellum; FNM, frontonasal mass; MaPr, mandibular process; MC, Meckel's cartilage; Ms, mesencephalon; NC, nasal cavity; NCp, Nasal capsule; NE, nasal epithelium; Pn, Pons; PSH, vertical palatinal shelf of maxilla; S, nasal septum; Tg, tongue. Bars, 100 μm.

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 commisure (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 *Pax3* is detected in both the medial and lateral parts of this region (Fig. 1F,I, white arrowhead). Furthermore, *Pax3* 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

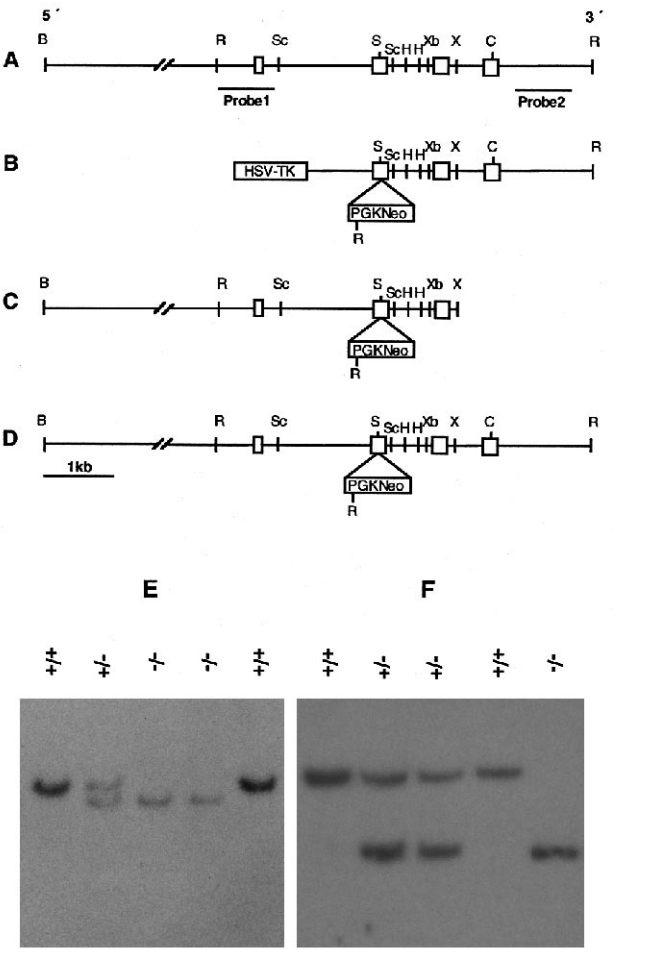


Fig. 2. Strategy for targeted disruption of Pax7. (A) Schematic representation of wild-type *Pax7* allele. Boxes: *Pax7* exons. (B) First and second targeting construct (constructed using genomic clones from Balb/c or isogenic DNA from the 129Sv strain). (C) Third targeting construct. (D) Mutated *Pax7* allele. The PGKneo expression cassette, which also contains a Poly(A) site and introduces a novel *Eco*RI restriction site. HSV-TK expression cassette for negative selection. The internal and external probes that lies outside the region of homology are shown as probe1 and probe2. B, *Bam*HI; R, *Eco*RI; Sc, *Sac*I; S, *Sal*I; H, *Hin*dIII; Xb, *Xba*I; X, *Xho*I; C, *Cla*I; kb, kilobase. (E,F) Southern analysis of genomic DNA isolated from mouse tails from heterozygous matings and digested with *Eco*RI; (E) hybridized with probe 2 – the upper band represents the normal allele (5.4 kb) and the lower band, the mutated allele (4.9 kb); (F) hybridized with probe 1 – the upper band represents the normal allele (5.4 kb) and the lower band the mutated allele (2.3 kb). +/+, wild-type; +/–, heterozygous; –/– homozygous. The electroporation of the first targeting vector (B), constructed using a λ genomic clone for *Pax7* isolated from a Balb/c genomic library, failed to yield any targeted clone. Therefore isogenic DNA was used for the second and a third targeting construct (B,C). The second construct (B), which is almost identical to the first one (B) yielded only one targeted clone in 400 screened double resistant clones. Therefore a third construct (C) was used which contains a much longer stretch of homology than the first two constructs. Upon electroporation, this vector resulted in a targeting frequency of 1 in 25 G418-resistant screened clones. Oligonucleotides used for RT-PCR: 5′-GTGGGGTCTTCATCAACGGTC. 5′-GCAGCGGTCCCGGATTTCCCAGC.

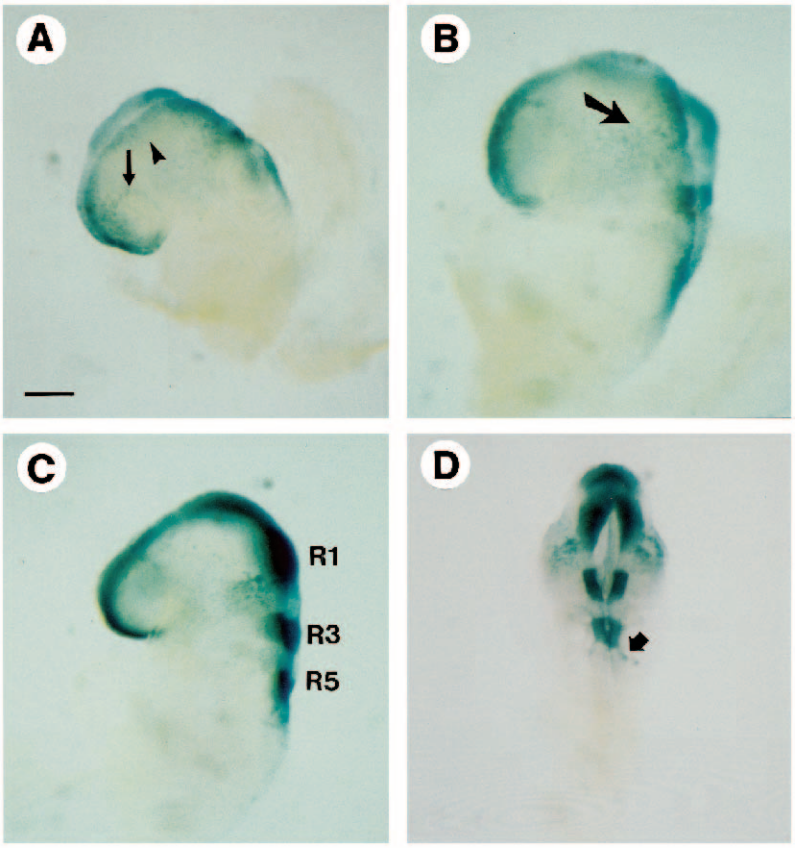
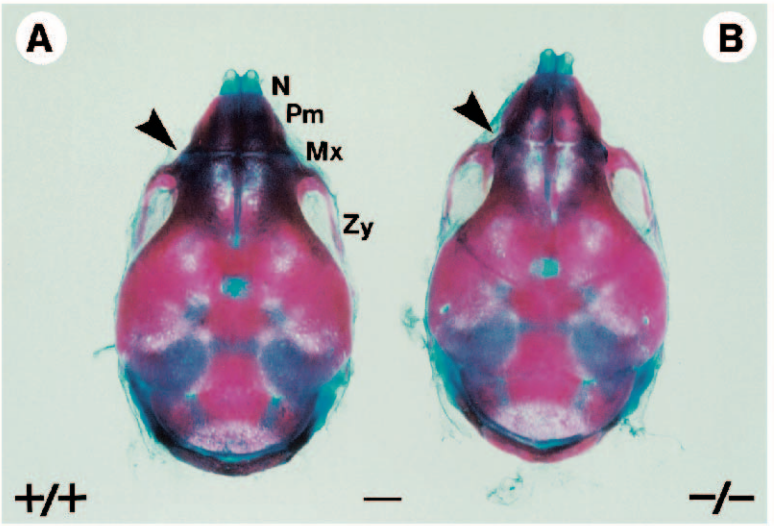


Fig. 3. Expression of *Pax7* in cephalic neural crest. Whole-mount staining for β-galactosidase of 8.5 d.p.c. (A,B) and 9.5 d.p.c. (C,D) heterozygous ($Pax7^{+/-}$) embryos. (C) Lateral view; (D) dorsal view; (A) The arrow indicates cephalic neural crest around the optic vesicle; the arrowhead indicates 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.



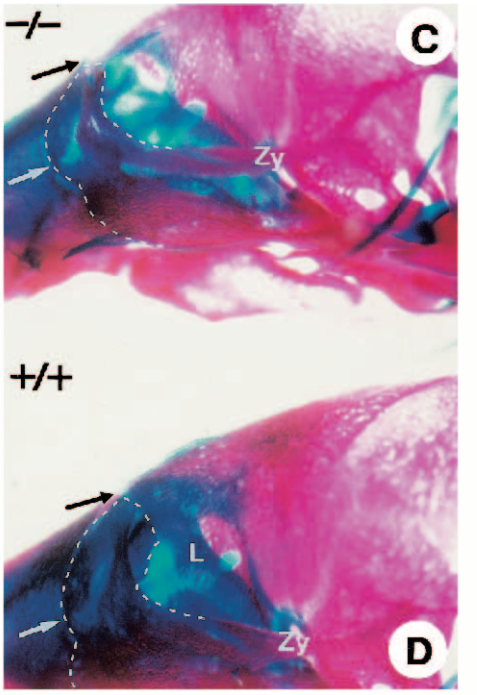


Fig. 4. Phenotype of $Pax7^{-/-}$ mice in the maxilla. Whole-mount alizarin red/alcian blue stained skeletal preparations (head) of new born $Pax7^{+/+}$ (A) and $Pax7^{-/-}$ mice. (A,B) Dorsal view and (C,D) lateral view of the wild-type and homozygous animals. The arrowhead indicates the reduced size 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.

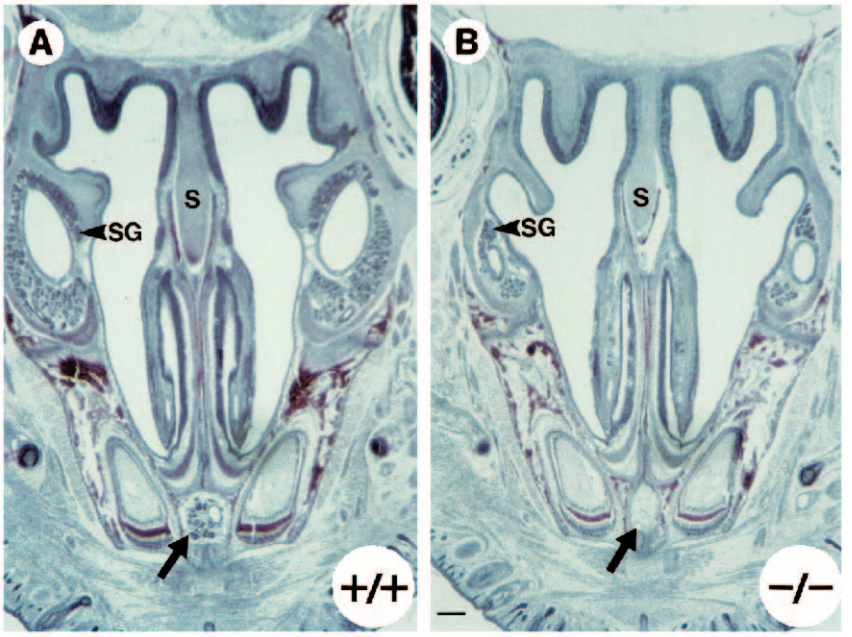


Fig. 5. Reduced number of tubules of serous glands in the nose of *Pax7*$^{-/-}$ mice. Transverse sections through the head of new born animals at the level of the nasal cavity. (A) wild-type; (B) homozygous animal. SG, serous gland; S, nasal septum; arrowhead indicates serous glands in the lateral part; arrow indicates serous glands in the inferior part. Bar, 200 μm.

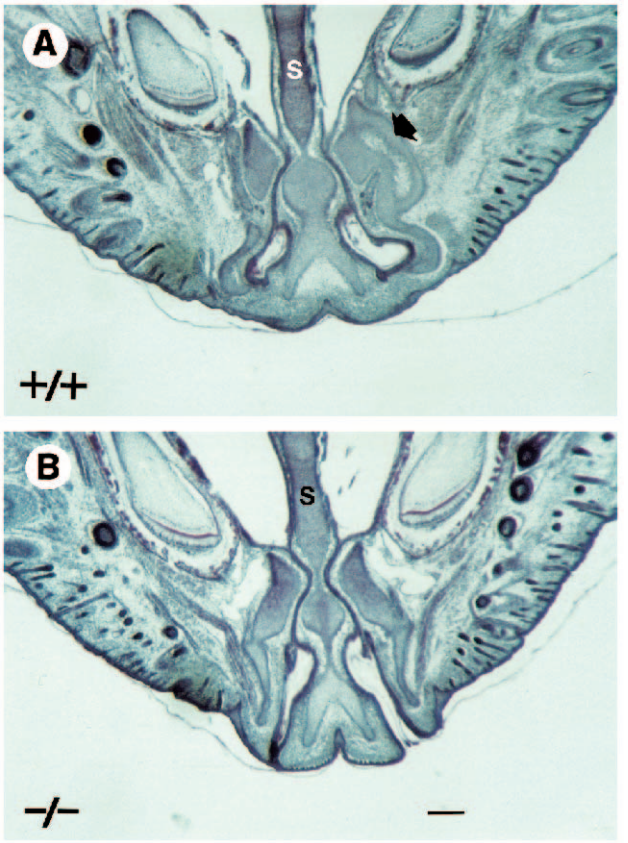


Fig. 6. In *Pax7*$^{-/-}$ mice the inferior lateral nasal capsule is missing. Transverse sections through the head of new born animals at the level of the nasal cavity. (A) Wild-type; (B) homozygous animal. S, nasal septum; the inferior lateral bone of the nasal capsule, which is missing in *Pax7*$^{-/-}$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

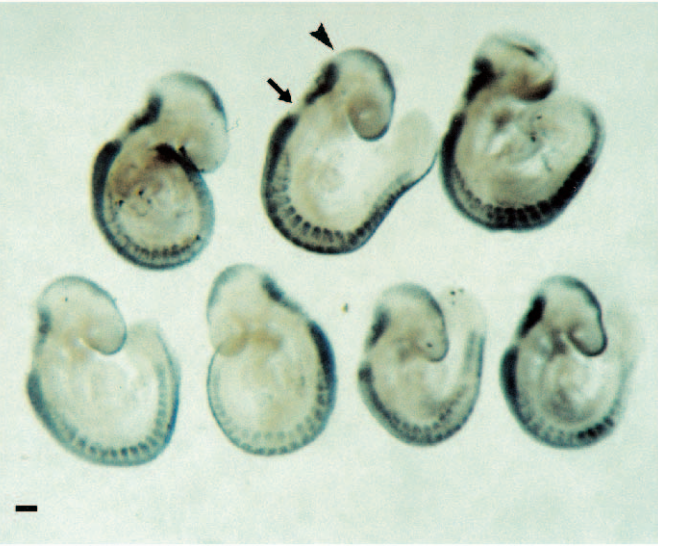


Fig. 7. Expression of *Pax3* at 9-9.5 d.p.c. Whole-mount in situ hybridization of *Pax3* expression using a riboprobe. The rostral part of the hindbrain, with lower expression, is indicated by a black arrowhead and the other domain of lower expression, in rhombomere 5 is indicated by a black arrow. Bar, 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 germline transmission. Chimeras were generated by blastocyst injection and morulae aggregation. Heterozygous *Pax7* mice appeared healthy and fertile. RT-PCR performed on RNA from 12.5-day embryos, using primers from the first and the second exon of the paired box (see legend to Fig. 2), revealed no *Pax7* RNA in *Pax7*$^{-/-}$ mice (data not shown) which demonstrates that 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 dilatations in the small intestine and appendix.

Phenotype analysis of the *Pax7*$^{-/-}$ mice

New born animals were histologically analyzed. Examinations of eosin-hematoxylin stainined 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*$^{-/-}$ mice. 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 Capecchi, 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; Le Douarin, 1982; Couly and Le Douarin, 1985; Couly et al., 1993; Noden, 1978, 1983), mouse (Serbedzija et al., 1992; Osumi et al., 1994) and rat (Tan and Morriss-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 rostral part of the rhombencephalon also contributes to the formation of the maxilla and the frontonasal mass (Lumsden et al., 1991; Osumi-Yamashita et al., 1994). Therefore this defect observed in *Pax7*$^{-/-}$ mice cannot be overcome by the expression of *Pax3*, as it is is not present in the most rostral hindbrain. In other words, as in the dermomyotome and possibly the neural tube, in the hindbrain *Pax3* and *Pax7* exhibit different expression patterns which could lead to the observed phenotypes. In this context, the possible significance of the lower expression of *Pax3* in rhombomere 5 remains unclear. Taken together these results strongly suggest that the development of a sub population of cephalic neural crest cells is regulated by *Pax7*. Further experiments are needed to elucidate the respective functions of *Pax3* and *Pax7* during this process. Both genes may, directly or indirectly, influence the specification of these cells.

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