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Six6 (*Optx2*) is a novel murine *Six3*-related homeobox gene that demarcates the presumptive pituitary/hypothalamic axis and the ventral optic stalk☆

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Abstract

We report on the isolation of a murine homeobox-containing gene, *Six6* (*Optx2*), that shows extended identity in its coding region with *Six3*, the only member of the mammalian *Six* gene family known to be expressed in the optic primordium. Phylogenetic analysis demonstrates that *Six6* and *Six3* belong to a separate group of homeobox-genes that are closely related to the recently identified *Drosophila optix*. Earliest *Six6* expression was detected in the floor of the diencephalic portion of the primitive forebrain, a region predicted to give rise to the neurohypophysis and to the hypothalamus. Later on, *Six6* mRNA was found in the primordial tissues giving rise to the mature pituitary: the Rathke's pouch and the infundibular recess. In the optic primordium, *Six6* demarcates the presumptive ventral optic stalk and the ventral portion of the future neural retina. In the developing eye, *Six6* expression was detected in the neural retina, the optic chiasma and optic stalk, but not in the lens. When compared to *Six6*, *Six3* expression pattern was highly similar, but with a generally broader transcripts distribution in the brain and in the visual system. We finally show that *Six6* does not require *Pax6* for its expression in the optic primordium, suggesting that *Six6* acts on a parallel and/or independent pathway with *Pax6* in the genetic cascade governing early development of the eye.

Keywords: Optic primordium; Eye; Retina; Neurohypophysis; Rathke's pouch; Hypothalamus; *Sine oculis*; *Optix*; Mouse; Mammal

1. Introduction

The vertebrate eye is a complex sensory organ, specialized in photodetection and image formation. Interestingly, light-detection cells and organs can be found throughout the metazoan, suggesting either the existence of a common genetic program or a phenomenon of convergent evolution for photoreceptor cells development (see for review Halder et al., 1995a; Oliver and Gruss, 1997). Genetic studies in *Drosophila* have allowed the identification of several genes that are required for the formation of the compound eye and/or of the entire visual system. In the recent years, murine homologues of some of these *Drosophila* genes were identified and were reported to be expressed in the developing eye (Oliver et al., 1995a; Xu et al., 1997; Hammond et al., 1998; Caubit et al., 1999). The most striking example of evolutionary conservation is coming from the *Drosophila eyeless* gene. *Eyeless* is a member of the paired-box and homeobox-containing gene family (PAX) of transcription factor and is essential for the entire development of the fly visual system (Quiring et al., 1994). *Pax6* is the murine homologue of *eyeless* and is expressed during eye development (Walther and Gruss, 1991). Heterozygous mouse mutants for *Pax6* present the *small eye* phenotype and the homozygous mutants show a complete absence of eyes at birth (Hogan et al., 1986; Hill et al., 1991). Humans carrying mutations in the *PAX6* gene suffer from Aniridia, which is primarily a failure of iris development (Ton et al., 1991). Most strikingly, mis-expression of either the *eyeless* or *Pax6* genes in transgenic fly leads to the formation of ectopic eyes on the legs, wings and antennas (Halder et al., 1995b). Accordingly, *Pax6* has been proposed to be a master regulator of eye development in metazoan (Halder et al., 1995b).

The *Drosophila sine oculis* (*so*) gene is known to be important for the entire development of the fly visual system

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(Cheyette et al., 1994; Serikaku and O'Tousa, 1994). By homology screening using the *so* gene, three *so*-related mouse genes (*Six1*, *Six2* and *Six3*) have been isolated (Oliver et al., 1995a,b), leading to the identification of a novel homeobox-containing gene family in vertebrates. The *Six* gene family is a group of transcription factors which are related to each other by two conserved domains. A homeodomain that is predicted to mediate DNA interaction and a *Six* domain that might modulate DNA-binding specificity.

Subsequently, two additional *Six* genes have been found in the mouse: AREC (*Six4*) and *Six5* (Kawakami et al., 1996a, b), as well as in many other species including the *Xenopus Six3* (Zuber et al., 1997); the medaka *Six3* (Loosli et al., 1998); the zebrafish *Six3*, *Six6*, *Six7* and *Six8* (Kobayashi et al., 1998; Seo et al., 1998a,b,c); and the chicken *Six3* and *Six4* (Bovolenta et al., 1996, 1998). Although all the murine *Six* genes share sequence homology to *so*, only *Six3* is expressed in the optic primordium (Oliver et al., 1995a). Injection experiments of a murine *Six3* expressing vector in medaka have demonstrated that *Six3* is able to promote ectopic lens formation (Oliver et al., 1996) showing its importance and conserved biological function in vertebrate eye development. Paradoxically, among the mammalian *Six1*, *Six2* and *Six3* proteins, *Six3* shares the lowest amino-acid sequence identity with *Drosophila so* (Oliver et al., 1995a), raising questions about the phylogenetic origin of the mammalian *Six* genes and/or the relationship between structural and functional conservation. The recent discovery in zebrafish of two *Six3*-related genes expressed in the optic primordium, *ZSix3* and *ZSix6*, has attracted our attention (Seo et al., 1998a). Zebrafish *Six3* is the structural orthologue of mouse *Six3*, while zebrafish *Six6* is a new member of the *Six* family, opening the possibility that a second *Six3*-related gene might exist in the mouse genome.

We have cloned a new member of the mouse *Six* gene family that is closely related to mouse *Six3*. A search in the public database using the BLASTP program showed that this novel mouse gene was slightly more related to zebrafish *Six3* than to zebrafish *Six6*, suggesting that it was not the zebrafish *Six6* orthologue. During the course of this work a novel *Six3* related cDNA, *Optx2*, has been isolated in chicken and partially in the mouse and in the fly (Toy et al., 1998). Sequence analysis showed that the gene we isolated was identical to mouse *Optx2*. Because this gene is an obvious member of the *Six* gene family and is the sixth member isolated to date in mammals, we will refer to it as *Six6*. Our phylogenetic analysis showed that mouse *Six6* and *Six3* are orthologues of *Drosophila optix*. During mouse development, *Six6* is mainly expressed in the primordial tissues that give rise to the pituitary/hypothalamic axis, the ventral optic stalk and the neural retina. Our results suggest that *Six6* is involved in the early steps governing pituitary and visual system development in mammals.

2. Results

2.1. Isolation and characterization of the cDNA

A DNA fragment of 173 base pairs (bp) was obtained by polymerase chain reaction (PCR) amplification of cDNA from mouse embryonic carcinoma cells using *Six3*-related degenerated oligonucleotides. This DNA fragment, whose sequence was highly similar but not identical to mouse *Six3* sequence, was used as a probe to screen a 11.5 day post coitum (dpc) mouse eye cDNA library (see Section 4). Eight independent clones were obtained and purified. After sequence analysis, seven clones revealed to be *Six3* and one of them to contain 800 bp of a novel *Six3* related sequence. By Northern blot analysis, a single band of approximately 1.75 kilo bases (kb) was detected using the 3′ region of the cDNA as probe (Fig. 1C). A 15.5 dpc total embryo cDNA library was screened with the same probe leading to the isolation of a 1705 bp full length cDNA clone. *Six6* cDNA codes for a protein of 246 amino acids (aa) with a stop codon in all three reading frames upstream of the first methionine. *Six6* contains a N-terminal *Six* domain of 126 aa (Fig. 1A, underlined) that is common to the *Six* gene family and a C-terminal DNA-binding homeodomain of 60 aa (Fig. 1A, bold) related to the superfamily of homeobox-containing genes (Cheyette et al., 1994). Alignment of the *Six6* and *Six3* protein sequences revealed that they are highly related, sharing 98% of identity at the amino acid level in their homeodomain and 86% identity and 93% similarity in their *Six* domain (Fig. 1D). In addition, *Six6* first N-terminal 11 aa, which are outside the *Six* domain, display 90% identity with the *Six3* N-terminal portion. The main difference found between the two proteins resided in the N-terminal Glycine-rich region of *Six3*, which is missing in *Six6,* and in the Serine-rich N-terminal region of *Six6*, which does not share significant similarity with the *Six3* N-terminal portion. A phylogenetic analysis using the homeodomain of all the known mammalian and *Drosophila Six* proteins demonstrated that *Six3* and *Six6* belong to a sub-group of homeoproteins (Fig. 1B) that are more related to *Drosophila optix* (Toy et al., 1998) than to *Drosophila sine oculis*.

2.2. Six6 expression in the visual system

We performed whole-mount RNA in situ hybridization on stage 8–12.5 dpc embryos using the 3′ portion of *Six6* cDNA as riboprobe. This particular region was chosen in order to avoid cross-reactivity with the endogenous *Six3* mRNA (see Section 4). *Six6* expression was first detected in 8 dpc embryos (4–5 pairs of somites, according to Kaufman, 1992) faintly in the floor of the diencephalic portion of the prospective forebrain (not shown). Based on the avian fate map (Couly and Le Douarin, 1988) this region gives rise to the hypothalamus (wall of the diencephalon) and to

the neurohypophysis. More intense staining was observed around 8.25 dpc (5–6 pairs of somites) (Fig. 2A,B), when the forebrain outgrowth is more obvious. Starting at the midline, a faint bilateral staining was observed in the ventral portion of the diencephalon (Fig. 2B). On the basis of the expression pattern at 9.5 dpc (Fig. 3C), this bilateral staining at 8.25 dpc probably corresponds to the first genetically detectable appearance of the optic stalk primordium. After sectioning, we observed that *Six6* expression domain at 9.5 dpc was restricted to the presumptive ventral optic stalk and to the ventral portion of the presumptive neural retina (Figs. 2F,G and 3A,C). In contrast, *Six3* signal was present in the entire retinal plate and partially in the presumptive ventral neural retina (Fig. 2E,G). In the developing eyes, *Six6* expression was detected in the optic stalk and the entire neural retina, but never in the lens or the lens placode (Fig. 4). This result is different from what has been reported for chicken *Optx2*, where strong expression was observed in the lens placode and in the lens (Toy et al., 1998), suggesting either cross-reactivity with endogenous chicken *Six3* or

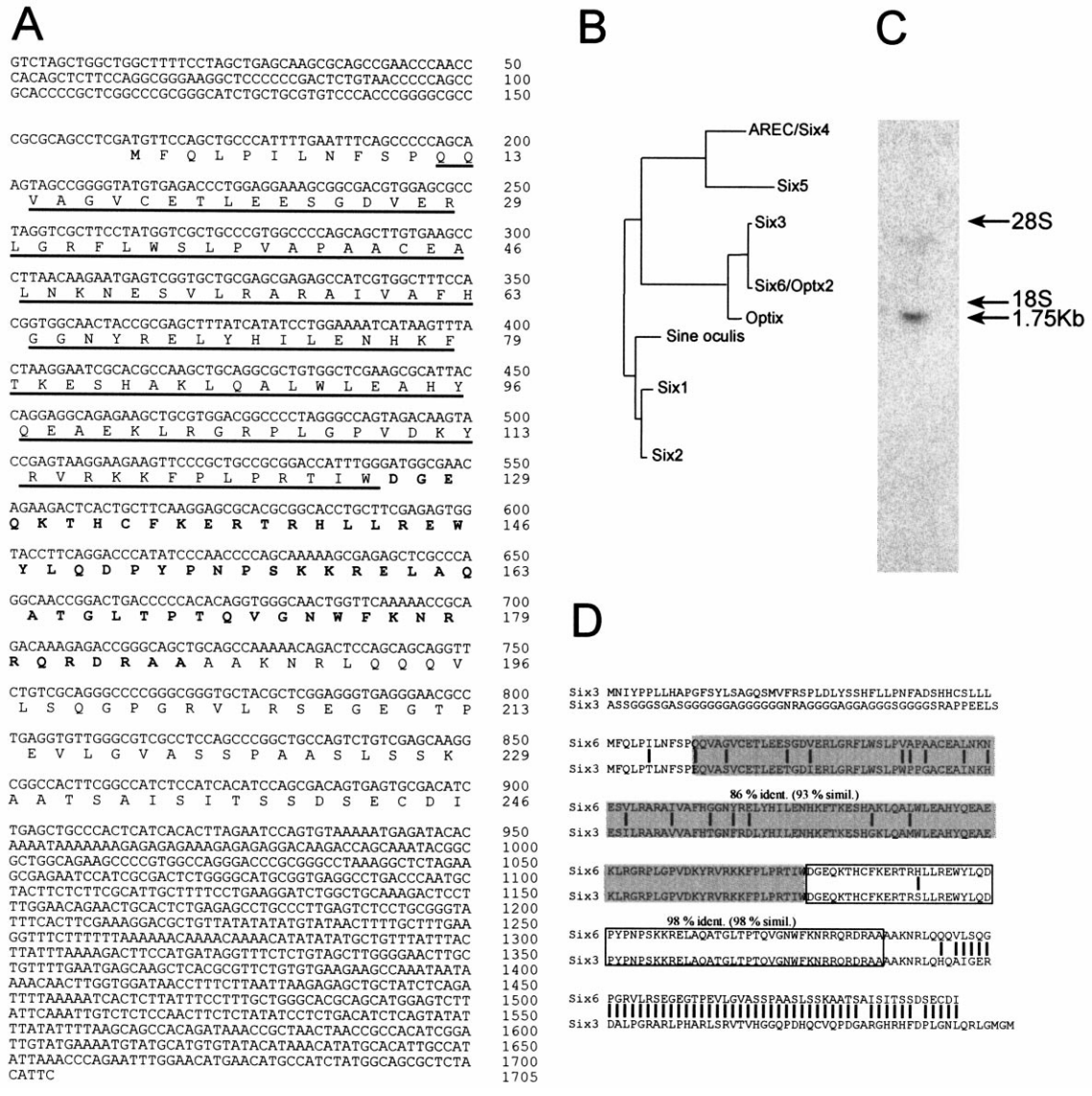

Fig. 1. Molecular characterization of *Six6*. (A) Nucleotide and predicted amino-acid sequence of *Six6*. Underlined amino-acid sequence corresponds to the conserved *Six* domain and the bolded one to the homeobox. The *Six6* nucleotide sequence contains only one putative first methionine with upstream stop codon in all three reading frames. (B) Phylogenetic analysis of the *Drosophila* and the mouse *Six* gene family using the amino-acid sequence of the conserved homeodomain. *Six6* and *Six3* proteins are more related to *Drosophila optix* than to *Drosophila sine oculis* and belong to a sub-group within the *Six* family of homeoproteins. The length of each branch of the tree corresponds to the relative phylogenetic distance between the protein sequence (see Section 4.5). (C) Northern blot analysis of *Six6* transcript. A single transcript of approximately 1.75 kb was detected on total mouse eye RNA. (D) *Six6* and *Six3* amino-acid sequence comparison. Dashed lines represent non-identical amino-acid. Boxed amino-acid corresponds to the homeodomain and shadowed one to the *Six* domain of both proteins. *Six3* encodes a larger protein than *Six6* with extra amino-acid in N-terminal. The proteins also diverge in their C-terminal portion.

significative species-specific variations. *Six6* expression was also observed at 13.5 dpc by radioactive RNA in situ hybridization in the optic stalk (not shown), in the region of the optic chiasm and in the entire neural retina (Fig. 5A–C) up to 17.5 dpc (not shown).

In comparison, *Six3* is expressed in the same area as *Six6* in addition to the lens placode (starting from 9.5 dpc, not shown), the lens and the retinal pigmented epithelium (RPE) at 13.5 dpc as shown by radioactive RNA in situ hybridization. We used albino mouse embryo sections to avoid light refraction by the natural pigment of the RPE (Fig. 5E). We could also observe the silver grain deposit in the RPE by bright field microscopic observation. The fact that *Six3* expression in the RPE was not reported before (Oliver et al., 1995a) might be explained by the relatively low level of *Six3* transcript in these cells.

2.3. Six6 expression during pituitary and hypothalamus development

The pituitary gland originates from two distinct embryonic sources, an ectodermal diverticulum from the roof of the stomatodaeum (the Rathke's pouch) and a downgrowth from the floor of the diencephalic portion of the future forebrain (Kaufman, 1992). These two tissues give rise to the neural (the neurohypophysis) and non-neural (the adenohypophysis) components of the pituitary. *Six6* expression was observed at 10.5 and 11.5 dpc on transverse sections in the wall of the diencephalon (hypothalamus), the infundibular recess (future neurohypophysis) of the diencephalon (in the region adjacent to the Rathke's pouch) and in the most dorsal part of the Rathke's pouch lumen ectoderm (future adenohypophysis). *Six6* expression was also detected in the

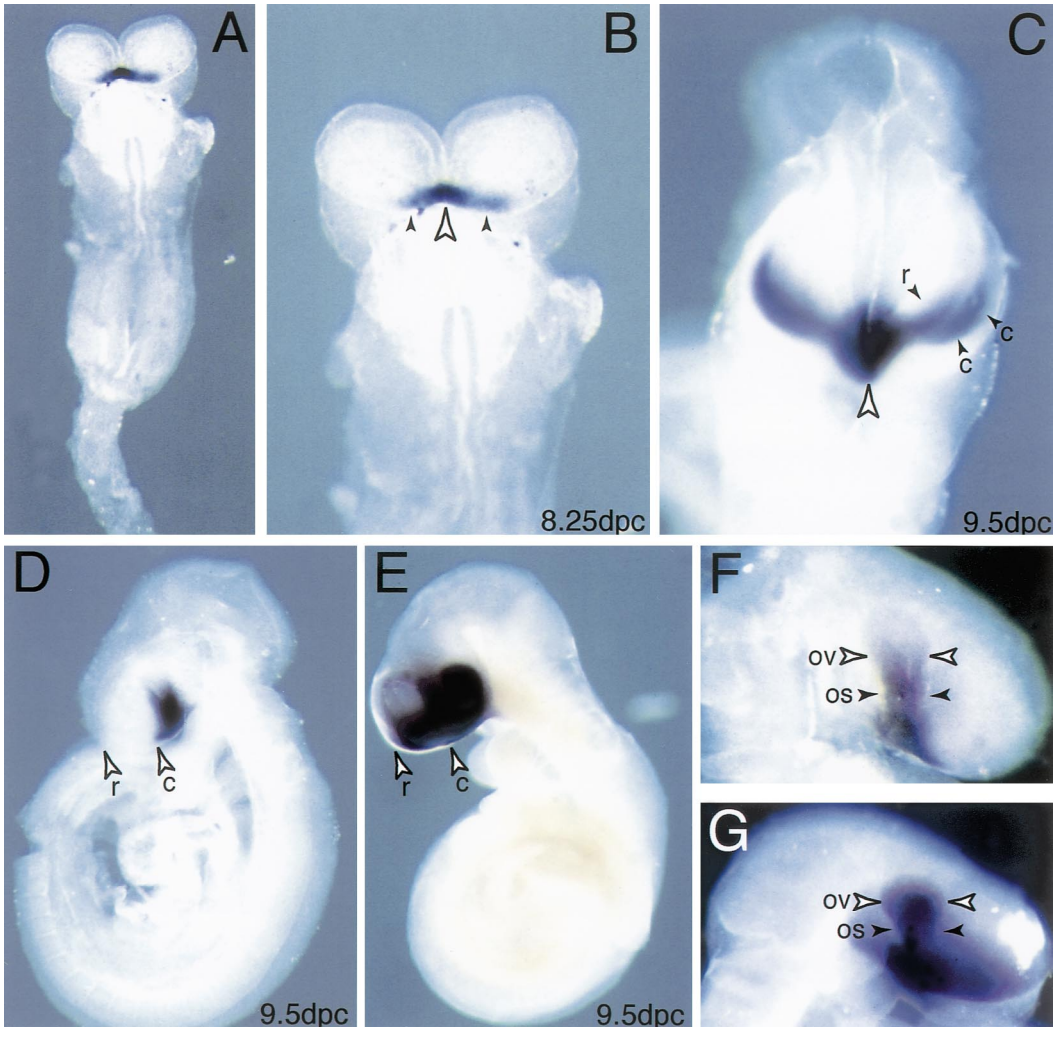

Fig. 2. *Six6* expression demarcates the presumptive pituitary/hypothalamic axis. (A) *Six6* is expressed in the ventral forebrain region (five pairs of somites, ventral view). (B) Higher magnification of (A) shows restricted expression in the floor of the diencephalic portion of the primitive forebrain (white arrowhead) and in the presumptive ventral optic stalk (black arrowhead). (C) Frontal view at 9.5 dpc. Strong expression is present in the presumptive pituitary/hypothalamic axis (white arrowhead) and in the rostral (r) and caudal (c) ventral portion of the optic vesicle.(D) *Six6* expression does not extend in the rostral portion of the forebrain (telencephalon) in contrast to *Six3* (E) and is limited to the ventral portion of the optic vesicle and to the optic stalk (F) in contrast to *Six3* which is expressed in the entire optic vesicle and optic stalk (G). Ov, optic vesicle; Os, optic stalk.

olfactory placode. On sagittal sections at 11.5 dpc, *Six6* expression was detected in the hypothalamus but not in the telencephalon, being limited at the level of the rostral neural pore (Fig. 6E). In contrast, *Six3* mRNA is present in both structures (Oliver et al., 1995a). At 15.5 dpc, when the pituitary has started to mature, *Six6* and *Six3* expression was detected in the hypothalamus, in the residual lumen of the anterior lobe of the pituitary (Rathke's pouch) and in the neural component of the pituitary (Fig. 7).

2.4. Expression in the Pax6 mutants

Pax6 homozygous mutant embryos show a complete absence of eyes at midgestation, but they form an optic vesicle that is morphologically abnormal (Grindley et al., 1995). This allowed us to ask whether *Six6* transcription is dependent on *Pax6* in the optic primordium at the optic vesicle stage. We performed whole-mount RNA in situ hybridization on 9.5 dpc *Pax6*$^{-/-}$ embryo (St-Onge et al., 1997) and their wild type littermates using *Six6* (*Optx2*) and *Vax1* as riboprobes. *Vaxl* is a homeobox-containing gene of the *Not* and *Emx* gene families that shows expression pattern similarities in the ventral stalk with *Six6* (Hallonet et al., 1998). In *Pax6* homozygous mutant embryos, *Six6* expression was still present in the presumptive ventral optic stalk region and in the ventral portion of the presumptive retina (Fig. 8A). Upon sectioning (not shown) no particular abnormalities in transcript distribution were observed in comparison to the normal *Six6* expression pattern. *Vax1* transcript distribution was also unaffected in the *Pax6* mutants (Fig. 8B). This experiment demonstrates that *Pax6* is not essential for the transcription of these two genes in the optic primordium. These results are comparable to what has been found for *Vax1* and *Six3* expression in the brain of the *Pax6* mutants (Oliver et al., 1995a; Hallonet et al., 1998).

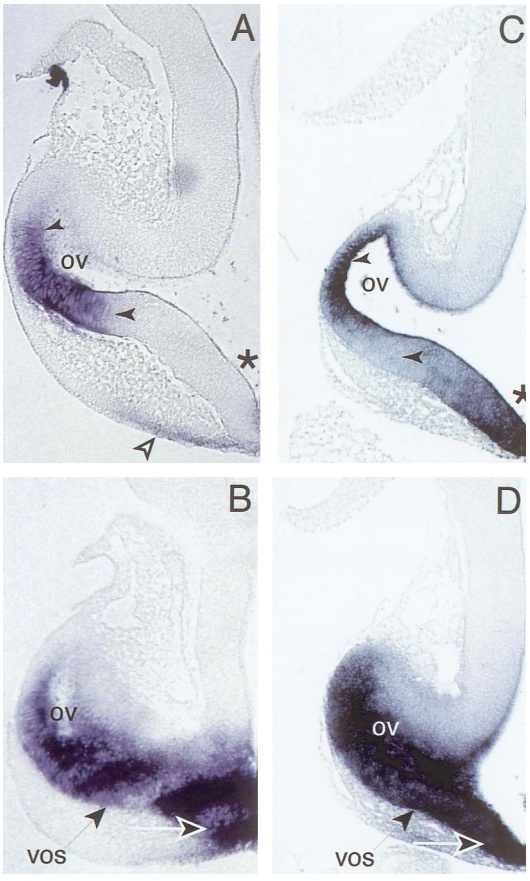

Fig. 3. *Six6* expression at the optic vesicle stage. (A–D) Coronal paraffin sections at 9.5 dpc. (A) *Six6* expression is limited to the ventral portion of the presumptive neural retina (black arrowhead) and partially overlaps with *Six3* expression domain (C) but is not present in the ventral rostral forebrain (asterix). Both *Six6* (B) and *Six3* (D) are expressed in the presumptive ventral optic stalk (vos) and in the pituitary/hypothalamic axis (arrow). *Six6* is also expressed in the olfactory placode – white arrowhead in (A). Ov, optic vesicle. (A,C) Rostral sections. (B,D) Caudal sections.

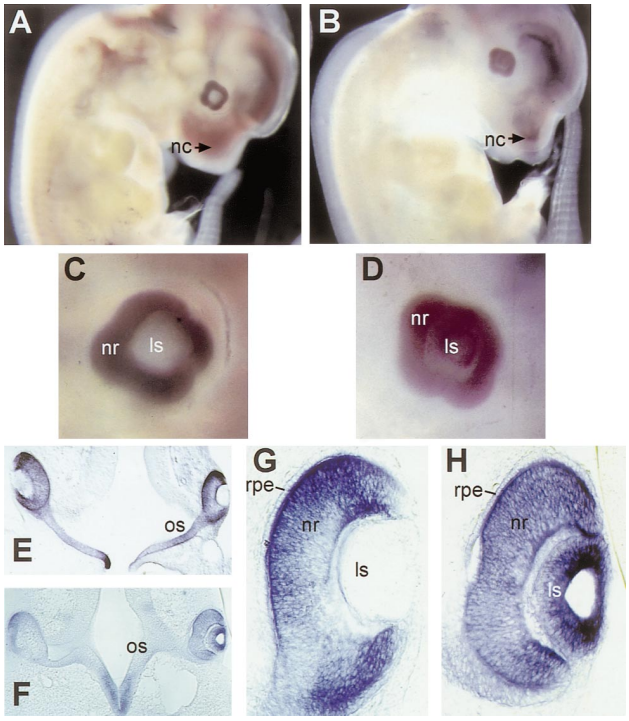

Fig. 4. *Six6* expression in the visual system by whole-mount RNA in situ hybridization. (A,C,E,G) *Six6* is expressed in the neural retina (nr) and the optic stalk (os) but not in the lens (le) and the retinal pigmented epithelium (rpe). (B,D,F,H) *Six3* is expressed in the whole visual system, including the lens. Nc, nasal cavity. (E–H) Coronal sections. Stage 11.5 dpc.

3. Discussion

We have reported on the cloning and expression analysis of a new mouse gene of the *Six* family that shares an extended nucleotide and amino-acid identity with *Six3*. *Six6* is, together with *Six3*, the only member of the mammalian *Six* gene family known to be expressed in the optic primordium. Although we found nested expression patterns of both genes, we also discovered distinct expression domains during eye and pituitary/hypothalamus develop-

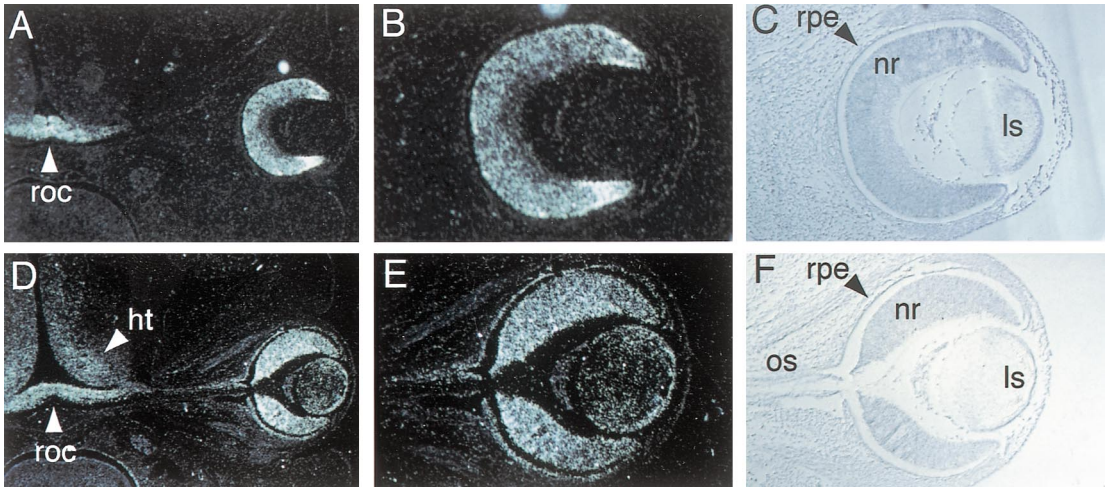

Fig. 5. *Six6* expression in the visual system by radioactive RNA in situ hybridization. (A–C) *Six6* expression is detected in the region of the optic chiasm (roc), in the neural retina (nr), the optic stalk and the hypothalamus (not shown). No expression was seen in the lens (ls) and in the retinal pigmented epithelium (rpe) in contrast with *Six3* in (D–F). Albino mouse embryos were here used to avoid dark field light refraction by the natural pigment of the retinal pigmented epithelium. (A,B,D,E) Dark field illumination. (C,F) Bright field illumination. Ht, hypothalamus; Os, optic stalk. Stage 13.5 dpc, coronal sections.

ment. Our results suggest that *Six6* acts at a high level in the genetic cascade controlling eye and pituitary development in mammals.

3.1. The Six gene family

The recent isolation of a novel *so*-like gene in the fly, *optix*, and the isolation of its vertebrate counterparts, *Six6* (*Optx2*) (Toy et al., 1998; and this article) and *Six3* (Oliver et al., 1995a), allows new insights for our understanding of the phylogenetic evolution of the *Six* gene family: *Six6* and *Six3* appeared to be orthologues of *Drosophila optix*; *Six1* and *Six2* of *Drosophila sine oculis*; and *AREC/Six4* and *Six5* of a still unknown common ancestor. Despite the fact that *so* is required for the development of the entire visual system of the fly (Cheyette et al., 1994), its mammalian orthologues (*Six1* and *Six2*) are not expressed in the optic primordium (Oliver et al., 1995b). This could mean that part of the genetic cascade and known molecular interactions involved in the development of the fly visual system can not be

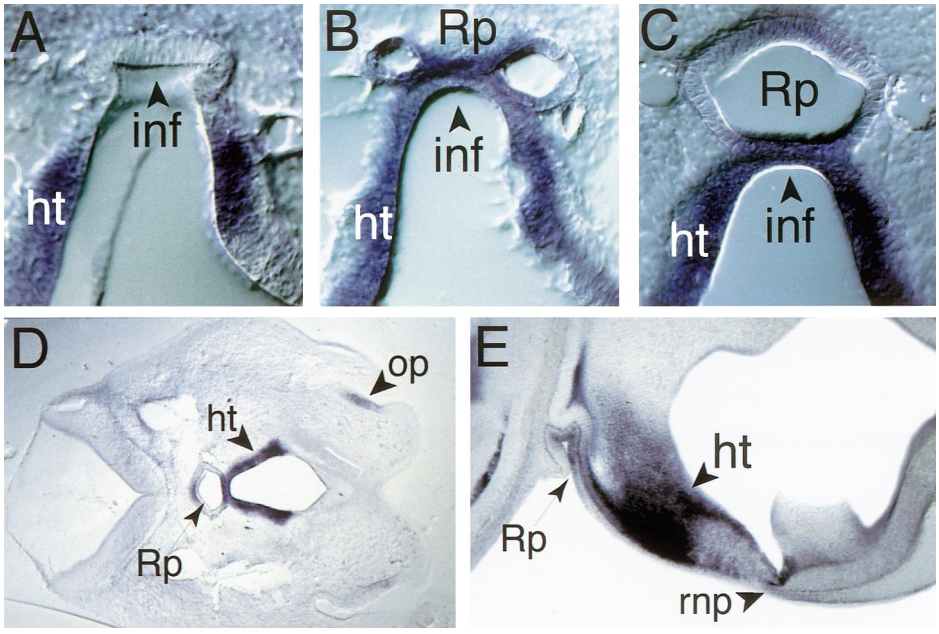

Fig. 6. *Six6* expression in the developing pituitary/hypothalamic axis. (A–C) Transverse serial sections of a 10.5 dpc embryo hybridized with a *Six6* riboprobe (anterior to posterior sections). Expression was observed in the infundibular recess (inf) – the future neural component of the pituitary, in the Rathke's pouch (Rp) – the future non-neuronal component of the pituitary, and in the hypothalamus (ht). (D) Transverse section at 11.5 dpc, low magnification. Expression is also observed in the olfactory placode (op). (E) Sagittal sections, 11.5 dpc. *Six6* expression in the forebrain do not extend outside the rostral neural pore (rnp).

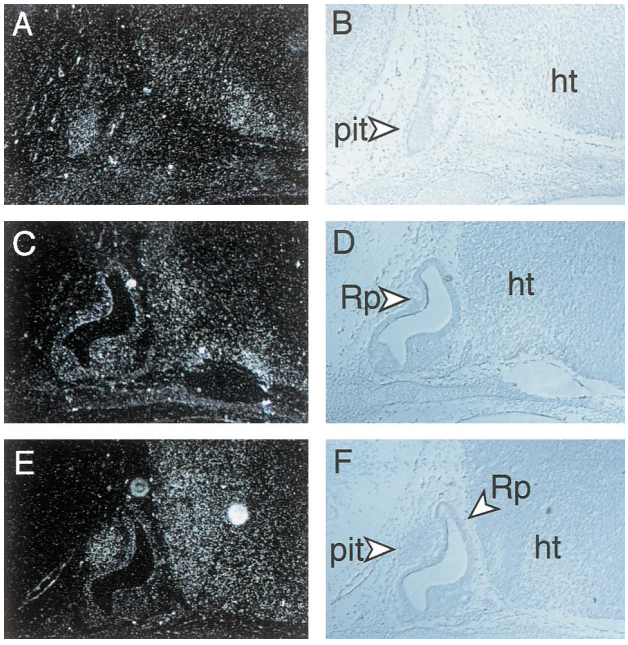

Fig. 7. *Six6* expression in the pituitary. Radioactive RNA in situ hybridization on sagittal sections of 15.5 dpc embryos. (A–D) *Six6* expression is present in the neural component of the pituitary (pit) and in the residual lumen of the anterior lobe of the pituitary (Rp, Rathke's pouch). (E,F) *Six3* expression is also present in both components of the pituitary. Ht, hypothalmus. (A,C,E) Dark field illumination. (B,D,F) Bright field illumination.

applied to mammals. For example, molecular interaction between *so* and *eyes absent* (Pignoni et al., 1997) would not be relevant in mammals for specification of the eye field. However, one cannot exclude the possibility that additional *so* orthologues expressed in the developing eyes still remain to be isolated in mammals. Thus, from the actual known mammalian *Six* gene family members, only *Six6* and *Six3* are expressed in the optic primordium and only these two genes can be involved in early eye development.

3.2. Six6 in eye and pituitary development

We have shown that *Six6* is expressed in the primordial tissues that will give rise to the ventral optic stalk, the neural retina, the hypothalamus and the pituitary. Interestingly, the *SIX6* (*OPTX2*) gene is localized on the human chromosome 14q22-q23 (Toy et al., 1998), a region that is associated with congenital failure of eye development and severe pituitary abnormalities (Bennett et al., 1991; Elliott et al., 1993). If *SIX6* (*OPTX2*) is indeed the gene mutated in these patients, this would mean that *SIX6* (*OPTX2*) is haploinsufficient, a situation reminiscent of PAX6 and PAX2 mutations in human (Ton et al., 1991; Sanyanusin et al., 1995). Additional evidence for a direct involvement of *Six6* in neural retina determination comes from transfection experiments on cultured cells where chicken *Six6* (*Optx2*) could convert pigmented epithelium to express neural retina and photoreceptor specific markers (Toy et al., 1998). Such effects were not obtained using either *Six3*, *Pax6* or Eya2. These experiments are in agreement with *Six6* expression in the retina, but not in the RPE and support the notion that *Six6* and *Six3* are not functionally redundant.

3.3. Six6 and Six3 in the specification of the optic field

In vertebrates, few genes have been shown to be expressed in the anterior neural plate of the embryo, a region previously referred to as the eye field (Adelmann, 1936). The anterior neural plate gives rise, in addition to other structures, to the primitive forebrain from which the optic vesicle and optic stalk originates (Couly and Le Douarin, 1988). In mammals, only *Rx*, *Six3*, *Pax6* and *Otx2* genes are known to be expressed in the prospective forebrain and in the optic sulcus, a structure that corresponds to the first morphological appearance of the optic vesicle (Walther and Gruss, 1991; Simeone et al., 1993; Oliver et al., 1995a; Furukawa et al., 1997; Mathers et al., 1997). Evidence from gene targeting experiments have shown that *Rx* is essential for optic sulcus formation. As a consequence, *Rx* homozygous mutant animals have no eyes (Mathers et al., 1997). In addition, the injection of *Rx* RNA into *Xenopus* blastomeres leads to the formation of ectopic RPE cells between the eyes and the neural tube, suggesting that *Rx* can recruit competent cells into the RPE fates. Only two other genes (both encoding homeopro-

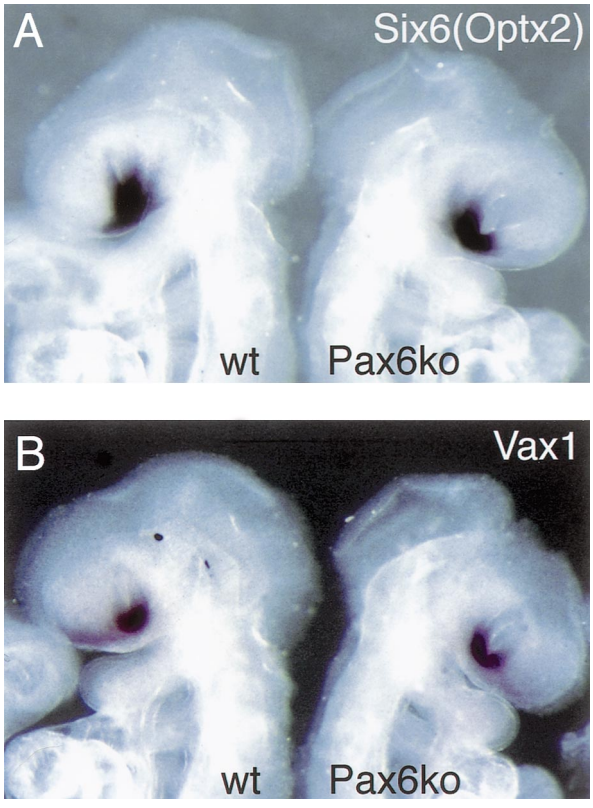

Fig. 8. *Six6* expression in the *Pax6* mutants. Whole-mount RNA in situ hybridization on 9.5 dpc wild type (wt) and *Pax6* homozygous mutants embryos (*Pax6*ko). (A) *Six6* and (B) *Vax1* expression is unaffected in the optic primordium of the *Pax6* mutants embryos.

teins) are known to be absolutely required for the development of the eyes in mammals: *Lhx2* mutant embryos form a hypocellular optic vesicle but never form an optic cup (Porter et al., 1997). *Pax6* has been shown to be essential for optic vesicle formation and for surface ectoderm differentiation in a cell autonomous manner (Hill et al., 1991; Fujiwara et al., 1994; Grindley et al., 1995). Mouse embryos homozygous for a null mutation in either *Pax6* or *Lhx2* showed a complete absence of eyes at birth (Hill et al., 1991; Porter et al., 1997). We show here that *Six6* demarcates the presumptive ventral optic stalk (8 dpc) and is later expressed (9–9.5 dpc) in the ventral portion of the prospective neural retina. Thus, the expression of *Six6* in the eye primordium is later and more restricted than *Six3*, which is expressed in the most anterior portion of the neural plate and later in the entire prospective ventral forebrain (Oliver et al., 1995a). In this respect, *Six6* expression would not correspond to the definition of a gene that can specify the eye field (Furukawa et al., 1997) in contrast to *Six3*, *Rx* and *Pax6*. However, *Six6* would be expressed early enough to specify the cells at the midline of the anterior neural plate that give rise to the anterior pituitary and the suprachiasmatic nucleus (Eagleson and Harris, 1990; Eagleson et al., 1995). From our expression analysis, we predict that *Six6* is one of the earliest markers of the presumptive pituitary/hypothalamic axis and that the optic stalk originates, or requires inducing factors from this particular region. *Pax2* and *Vax1* are two homeobox genes expressed in the presumptive optic stalk and are good candidates for the specification of this structure. In support of this, *Pax2* homozygous mutant embryos show agenesis of the optic chiasma, aberrant expression of RPE cells into the optic stalk and absence of closure of the optic fissure (Torres et al., 1996). It is therefore interesting to suggest that *Pax2* and *Vax1* might cooperate with *Six6* and *Six3* in the specification and formation of the optic stalk. How this genetic network is regulated remains to be resolved. We showed here that *Six6* expression in the optic primordium is not dependent on *Pax6*. This result is not surprising considering that *Pax6* expression is not present in the ventral optic stalk, but mainly in the optic vesicle, the lens placode, the spinal cord and the forebrain (Walther and Gruss, 1991). It is possible, however, that *Six6* expression in the prospective neural retina, which occurs later than *Pax6* during development, is directly or indirectly regulated by *Pax6*. This issue cannot be resolved using the *Pax6* mutants since they fail to develop an optic cup.

In conclusion, our analysis of *Six6* expression during mouse development suggests that this gene is involved in the specification and formation of the ventral optic stalk and of the pituitary/hypothalamic axis. In addition, it appears that *Six6* has a later role in the determination and/or differentiation of the neural retina. The generation of mouse mutants for *Six6* should shed light on the biological function of this novel *Six* family member in early eye development.

4. Materials and methods

4.1. Isolation of the cDNA clones

Five hundred mouse eyes were dissected, frozen in a dry ice/ethanol bath and stored at −70°C. Total RNA was extracted using TRIzol (GibcoBRL, Cat. No. 15596-026). Reverse transcription of the mRNA was done using oligo-dT primers. The rest of the procedure was performed according to the manufacturer instructions (Zap-cDNA Synthesis Kits Stratagene #200 450). Using the 173 bp DNA fragment isolated by RT-PCR, hybridization was performed at 65°C in hybridization buffer (10× Denhardt's, 5× SSC, 0.1% SDS, 0.1 mg/ml salmon sperm DNA). Filters were washed three times with 2× SSC, 0.1% SDS and twice with 0.5× SSC, 0.1% SDS at 65°C. Hybridization of the mouse 15.5 dpc total embryo random-primed λgt10 cDNA library (GibcoBRL) was performed using a 665 bp DNA fragment corresponding to the 3′ UTR end of the *Six6* cDNA (position 1044–1706) using the procedure described.

4.2. Northern blot analysis

RNA was extracted from 11.5 dpc NMRI mouse embryos using TRIzol. Approximately 10–15 μg of the total RNA was electrophoresed in a 1.2% agarose-formaldehyde gel and transferred on a Nylon Plus (Qiagen) membrane. Hybridization was performed with a 665 bp DNA probe (position 1044–1706) at 65°C using Church's buffer (1 mM EDTA, 0.5 M Sodium Phosphate Buffer pH 7.2, 7% SDS). Washes were done at 65°C in 0.1× SSC, 0.1% SDS.

4.3. In situ hybridization

Embryos were dissected, fixed overnight in 4% paraformaldehyde at 4°C and embedded in Paraplast (Monoject Scientific). Sections (10 μm) were cut and dried onto chromalum-gelatin slides. All the steps of high-stringency hybridization and washing were done as described previously (Kessel and Gruss, 1991). ^{35}S-labelled RNA probe using SP6 or T7 RNA polymerase were done with Boehringer enzyme according to the directive of the company. Exposure time for the radioactive RNA in situ hybridization was 20 days. Whole-mount preparation were probed with digoxigenin-labelled RNA probe and visualized with alkaline phosphatase-coupled anti-digoxigenin antibody (Boehringer) and NBT/BCIP substrate (Boehringer). Riboprobes corresponding to the 3′ UTR portion of *Six3* cDNA sequence (position 1044–1706) and to the 3′ UTR portion of *Six3* cDNA sequence (position 888–1402; Accession no.: X 90871) were used for radioactive and non-radioactive RNA in situ hybridization. The full length *Six3* cDNA sequence (1402 bp) was also used as riboprobe for non-radioactive RNA in situ hybridization, giving similar results as obtained with the shorter riboprobe.

4.4. Animals

NMRI mouse embryos were dissected out according to the day of vaginal plug. *Pax6*$^{-/-}$, heterozygous and wild type littermates embryos were genotyped by Southern blot analysis using the yolk sac as DNA source (St-Onge et al., 1997).

4.5. Phylogenetic analysis

Pairwise distances calculation (Tables 1 and 2) within the aligned homeobox amino-acid sequences of the *Six* genes was performed using the DISTANCES program (GCG). The phylogenetic tree construction was performed using the GROWTREE program (GCG), WPI (Wisconsin Package Inc.).

Table 1
Pairwise distances matrix

Gene	Accession no.	Amino acid
1. optix.pep	AF050132	Homeobox only
2. sinehomeo.pep	L31626	214–272
3. six1homeo.pep	X80339	110–168
4. six2homeo.pep	D83147	146–204
5. six3homeo.pep	X90871	224–282
6. six4homeo.pep	D50416	212–270
7. six5homeo.pep	D83146	139–197
8. six6homeo.pep	–	127–186

Table 2
Text representation of the tree[a]

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1	0	146	57	102	21	167	102	19
2		0	194	26	231	39	69	194
3			0	108	39	231	122	39
4				0	146	51	44	137
5					0	345	156	5
6						0	48	257
7							0	137
8								0

[a] ((((*optix.pep*:-6.76, (((*sinehomeo.pep*:-0.32,*six4homeo.pep*:39.43):39.94, *six2homeo.pep*:-20.83):27.89, *six5homeo.pep*:4.55):97.39):20.58, *six1homeo.pep*:20.96):15.51, *six3homeo.pep*:6.33) -0.53, *six6homeo.pep*:-0.53):0.00.

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