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Six3, a murine homologue of the *sine oculis* gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development

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SUMMARY

The *Drosophila sine oculis* homeobox-containing gene is known to play an essential role in controlling the initial events of pattern formation in the eye disc and is also required for the development of other parts of the fly visual system including the optic lobes. In this paper, we report the isolation of a sequence-related gene referred to as *Six3*. Based on its amino acid sequence, this gene can be included in the new *Six/sine oculis* subclass of homeobox genes. Early on, *Six3* expression is restricted to the anterior neural plate including areas that later will give rise to ectodermal and neural derivatives. Later, once the longitudinal axis of the brain bends, *Six3* mRNA is also found in structures derived from the anterior neural plate: ectoderm of nasal cavity, olfactory placode and Rathke's pouch, and also the ventral forebrain including the region of the optic recess, hypothalamus and optic vesicles. Based on this expression pattern, we conclude that *Six3* is one of the most anterior homeobox gene reported to date. The high sequence similarity of *Six3* with the *Drosophila sine oculis*, and its expression during eye development, suggests that this gene is the likely murine homologue. This finding supports the idea that mammals and insects share control genes such as *eyeless/Pax6* (Halder, G., Callaerts, P. and Gehring, W. J. (1995) *Science* 267, 1788-1792), and also possibly other members of the regulatory cascade required for eye morphogenesis. In *Small eye* (*Pax6*) mouse mutants *Six3* expression is not affected. Finally, based on the chromosomal localization and the expression pattern of the mouse *Six3* gene, the human *Six3* cognate could be a good candidate to be at least one of the genes affected in patients with holoprosencephaly type 2 due to an interstitial deletion of 2p21-p22. This region shares a homology with the distal region of mouse chromosome 17 where *Six3* has been mapped.

Key words: homeobox, eye development, anterior neural plate, *sine oculis*, optic recess, forebrain, mouse.

INTRODUCTION

Since the first experimental analysis of an inductive effect of one single tissue (optic vesicle) over another (ectoderm) performed by Spemann in *Rana temporaria* (1901), eye development and particularly lens formation, has been used as a model for the study of inductive mechanisms in vertebrate embryos. In *Drosophila*, the development of the compound eye has also been extensively studied and many of the genes regulating cell fate determination in the late larval eye disc have been identified (for a review see Cagan and Zipursky, 1992). It has recently been shown that the paired-box and homeobox-containing gene *eyeless* plays an early and fundamental role during *Drosophila* eye development (Quiring et al., 1994). Mutations in this gene produce the *eyeless* phenotype. Even more remarkable has been the finding that targeted expression of the *eyeless* gene is able to induce ectopic eyes in structures deriving from the fly imaginal discs (Halder et al., 1995). Its mammalian homologue, the *Pax6* gene, is also known to be expressed during eye development (Walther and Gruss, 1991) and disruptions of the murine gene are responsible for the *Small eye* mutation (Hill et al., 1991) in which the homozygous embryos are unable to form eyes.

In this paper, we describe the identification of a new member of the recently described *Six/sine oculis* subclass of homeobox genes (Oliver et al., 1995). This gene has been named *Six3* and, similar to the previously described *Six1* and *Six2* (Oliver et al., 1995), exhibits a strong amino acid sequence homology with the *Drosophila sine oculis* (*so*) gene (Cheyette et al., 1994). In contrast to *Six1* and *Six2*, *Six3* is expressed during mouse eye development.

The analysis of *Six3* early expression has also revealed that its mRNA demarcates the most anterior border of the developing mouse neural plate. The anterior early expression of *Six3* in

the neural plate, restricted to regions that later will give rise to the most rostral non-neural (Rathke's pouch, olfactory placodes) and neural (optic recess, hypothalamus, optic vesicles) derivatives, as well as its later expression in the most anterior part of the neural tube, suggests that this gene is involved in a process by which positional information is established at the anterior boundary of the developing mouse embryo.

MATERIALS AND METHODS

Isolation of *Six3* cDNA

Using an 800 bp fragment containing the homeobox of the previously described *Six1* cDNA (Oliver et al., 1995), a mouse E14.5 brain cDNA library (Wijnholds et al., 1995) was screened. Hybridization was performed at 65°C in hybridization buffer (10× Denhardt's, 5× SSC, 0.1% SDS). Filters were washed three times with 2× SSC, 0.1% SDS and twice with 0.5× SSC, 0.1% SDS at 65°C. The longest cDNA of 1.8 kb containing the new *Six3* gene was further characterized. We found that the first 350 bp of this cDNA did not correspond to the *Six3* gene; instead, they probably correspond to another gene and this chimeric product was originated during the library preparation. The majority of the isolated clones corresponded to the *Six3* gene. A few other clones showing a different restriction pattern are currently being characterized.

Chromosomal localization of *Six3*

Interspecific backcross progeny were generated by mating (C57BL/6J × *M. spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N_2 mice were used to map the *Six3* locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization were performed essentially as described (Jenkins et al., 1982). All blots were prepared with Hybond-N^+ nylon membrane (Amersham). The probe, an 650 bp *Not*I fragment of mouse cDNA, was labeled with $\alpha[^{32}P]$dCTP using a random primed labeling kit (Stratagene); washing was done to a final stringency of 0.5× SSCP, 0.1% SDS, 65°C. A major fragment 16.5 kb was detected in *Eco*RI-digested C57BL/6J DNA and a major fragment of 9.6 kb was detected in *Eco*RI-digested *M. spretus* DNA. The presence or absence of the 9.6 kb *Eco*RI *M. spretus*-specific fragment was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to *Six3* including *son of sevenless* homolog 1 (*Sos1*), *sine oculis*-related homeobox 2 (*Six2*), mut S homolog 2 (*Msh2*) and luteinizing hormone/choriogonadotropin receptor (*Lhcgr*) has been reported previously (Fishel et al., 1993; Oliver et al., 1995). Recombination distances were calculated as described (Green, 1981) using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

Northern blot analysis

RNA was extracted from E10.5 NMRI mouse embryos using the lithium chloride-urea method described by Auffray and Rougeon (1980). Total RNA was poly(A) selected by fractionating total RNA on columns containing Poly(U) Sepharose4B (Pharmacia). Poly(A) RNA (5 μg) was electrophoresed in a 1.2% agarose-formaldehyde gel and transferred to a nylon membrane (Qiagen). Hybridization with the *Six3* probe was performed at 65°C in 50% formamide, 5× SSC, 5× Denhardt's, 0.02% sodium pyrophosphate, 0.1% SDS, and 50 μg/ml salmon sperm DNA. Washes with increasing stringency were performed, the last being at 65°C in 0.1× SSC, 0.1% SDS.

In situ hybridization

Embryos were dissected, fixed overnight in 4% paraformaldehyde and embedded in Paraplast (Monoject Scientific). Sections (8 μm) were cut and dried onto chromalum-gelatin slides. All the steps of high-stringency hybridization, washing and RNAse treatment were performed as described previously (Kessel and Gruss, 1991). T3 or T7 RNA polymerase in vitro transcribed sense or antisense ^{35}S-labelled RNA probes were generated from various Bluescript KS II^+ subclones containing different coding regions of *Six3*. The exposure time was approximately 10 days. Whole-mount preparation were probed with digoxigenin-labelled RNA probes, visualized with alkaline phosphatase-coupled anti-digoxigenin antibody and NBT/BCIP substrate (Boehringer) and sectioned as described previously (Bober et al., 1994).

RESULTS

Isolation and characterization of *Six3* cDNA

In the initial screening of an E8.5 mouse cDNA library using the *Drosophila so* homeobox-containing gene, two novel murine genes of this type were identified and named *Six1* and *Six2* (Oliver et al., 1995). The expression of these genes was found to be restricted mainly to head and body mesenchyme, limb muscle and tendons. In an attempt to identify additional *Six*-related homeobox genes specifically expressed in the CNS, an E14.5 brain cDNA library was screened at low stringency using the previously isolated *Six1* cDNA as probe. Approximately 10^6 plaques were screened and the positive clones were further purified. The largest cDNA insert isolated from these clones was of 1.8 kb (see Materials and Methods). Sequence analysis showed that this cDNA codes for a new gene belonging to the *Six/sine oculis* subclass of homeobox genes therefore, we named it *Six3* (Fig. 1A). Northern blot analysis (Fig. 1B) of E10.5 poly(A) mRNA showed that *Six3* produces two transcripts of approximately 3.2 and 2.7 kb. This indicates that the isolated *Six3* cDNA is not full length; however, sequence analysis reveals that it contains the complete coding region. As can be seen in Fig. 1A, two putative translation initiation codons are present in the same open reading frame as the homeodomain. Stop codons in all three frames are found 5′ of them. Both of these putative translation initiation sites show a weak similarity to the consensus sequence proposed by Kozak (1989), including the absence of the highly conserved purine at position −3. Therefore, we cannot yet be certain which of the two initiation codons is the functional translational start site. The stop codon of the predicted *Six3* protein is also indicated in Fig. 1A. The predicted amino acid sequence of *Six3* shows a striking similarity with *Six1, Six2* and *so*. The sequence conservation is not only restricted to the homeodomain but includes also approximately 110 amino acids of the 5′ flanking region (the *Six* domain) (Fig. 1C,D). The presence of this additional conserved domain is unique for the *Six/sine oculis* subclass of homeobox genes. In most other cases, homology is restricted to the homeodomain and some amino acids on the flanking regions. Interestingly, of the three *Six* genes known, *Six3* is the one exhibiting the lowest degree of amino acid homology in both the homeodomain and the flanking *Six* domain when compared with *so*. However, similar to the *so* gene, *Six3* is very glycine rich in the 5′ flanking region (Fig. 1C,D). Another interesting feature of this new subclass of homeobox-containing genes is the presence of a lysine at position 50 of the homeodomain recognition helix. A similar residue is only found in the genes expressed in the anterior region such as *bicoid, goosecoid* and *orthodenticle* from *Drosophila* and vertebrates and in the gene *EgHbx 4* from flatworms (Oliver et al., 1992; Bürglin, 1994).

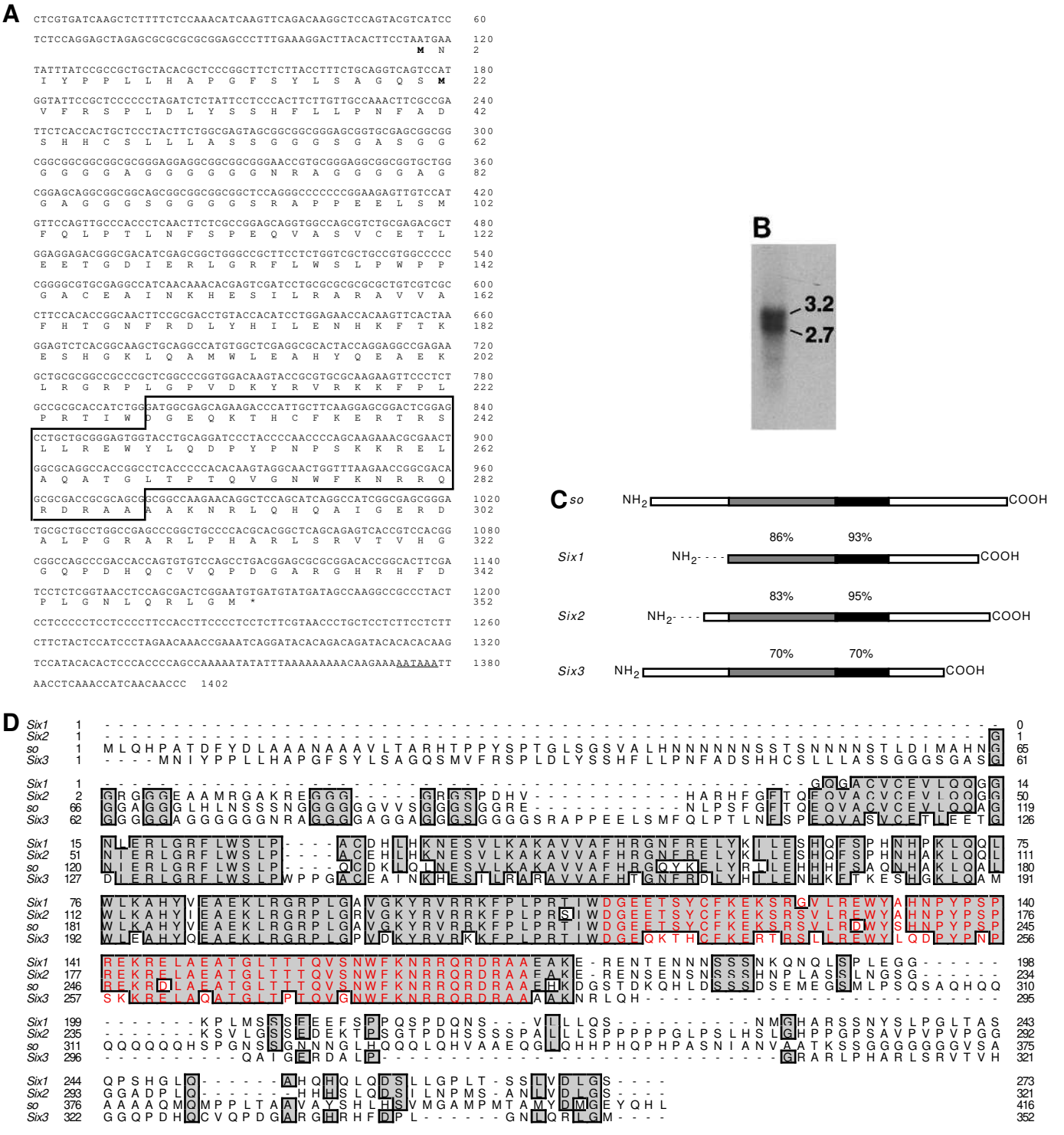

Fig. 1. Molecular characterization of *Six3*. (A) Nucleotide and predicted amino acid sequence of the *Six3* cDNA. Boxed amino acid sequence corresponds to the homeodomain. The underlined sequence is the putative polyadenylation signal. Possible initiation methionines are bold. (B) Northern blot analysis of *Six3* transcripts. The size of the transcripts is indicated on the right and was estimated by comparison with the RNA ladder (Boehringer) (not shown). (C) Diagrammatic representation of the percentage of amino acid homologies between the three different mouse *Six* genes when compared with the *Drosophila sine oculis*. The homeodomain is shown in black (60 amino acids), while the conserved upstream *Six* domain is in grey (110 amino acids). Dashed lines represent the missing 5′ coding sequence of *Six1* and *Six2*. (D) The amino acid conservations along the coding sequences of the identified members of the *Six/sine oculis* subclass of homeobox genes is indicated by boxed blocks. The region of the homeodomain is indicated in red. High homology is also found between the four genes in the 5′ flanking region (*Six/sine oculis* domain). For *Six1* and *Six2*, the coding sequence is still incomplete in the 5′ region. The homologies are only indicated when they are at least three matches. Dashed lines represent gaps included for better alignment. *Six3* has at position 141, an insertion of four amino acids which are not present in any of the other genes. Accession number: X 90871 (*Six3*).

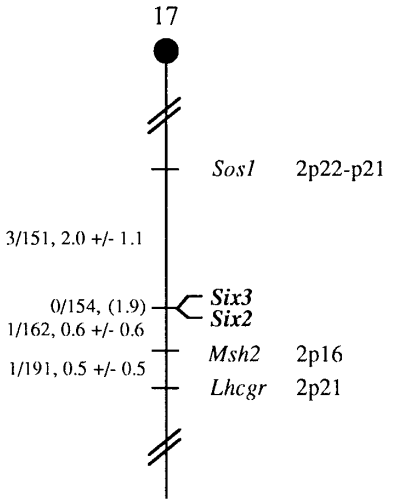

Fig. 2. *Six3* maps in the distal region of mouse chromosome 17. *Six3* was placed on mouse chromosome 17 by interspecific backcross analysis. The number of recombinant N_2 animals is presented over the total number of N_2 animals typed and the recombination frequencies between loci in centimorgans (± standard error) are shown to the left of the chromosome between each pair of loci. No double or multiple recombination events were observed. When no recombination between loci was observed, the upper 95% confidence limit of the recombination distance is given. The positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).

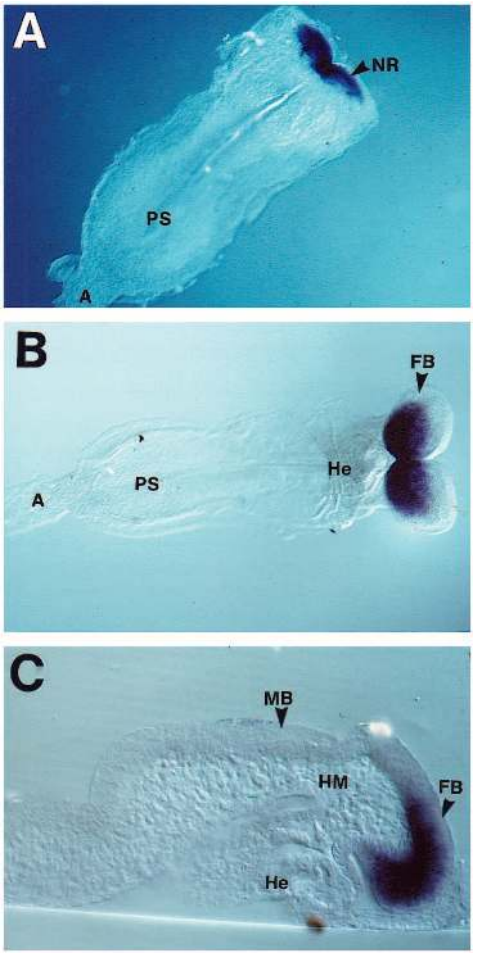

Fig. 3. *Six3* early expression demarcates the anterior neural plate. (A) Ventral view of an embryo at stage E8.2 showing *Six3* positive cells in the most rostral border of the neural plate, which at this stage is represented by the neural ridge (NR). The remainder of the embryo is devoid of staining. PS, primitive streak; A, allantois. (B) At E8.5 (ventral view), the region with positive cells has expanded and now covers part of the area corresponding to the prospective forebrain (FB). He, heart. (C) Sagittal section of an embryo at approximately a similar stage to that in B showing the very localized anterior expression. MB, midbrain; HM, head mesenchyme.

Chromosomal mapping of *Six3*

The mouse chromosomal location of *Six3* was determined by interspecific backcross analysis using progeny derived from matings of (C57BL/6J × *Mus spretus*) F_1 × C57BL/6J mice. This interspecific backcross mapping panel has been typed for over 1900 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a mouse cDNA probe. The 9.6 kb *Eco*RI *M. spretus* RFLP (see Materials and Methods) was used to follow the segregation of the *Six3* locus in backcross mice. The mapping results indicated that *Six3* is located in the distal region of mouse chromosome 17 linked to *Sos1*, *Six2*, *Msh2* and *Lhcgr* (Fig. 2). *Six3* mapped 2.0 cM distal of *Sos1* and does not recombine with *Six2* in 154 animals typed in common, suggesting the two loci are within 1.9 cM of each other. The tight linkage and sequence relatedness between the *Six2* and *Six3* genes suggest that the loci arose by a tandem gene duplication event.

We have compared our interspecific map of chromosome 17 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (provided from Mouse Genome Database, a computerized database maintained at The Jackson Laboratory, Bar Harbor, ME). *Six3* mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in this locus (data not shown). The distal region of mouse chromosome 17 shares a region of homology with human chromosome 2p22-p16 (summarized in Fig. 2). It is interesting to note that a dominant human disorder with a phenotype that might be consistent with a defect on *Six3,* holoprosencephaly type 2 (HPE 2), maps to 2p21 (Grundy et al., 1989; Hecht et al., 1991). Patients have craniofacial malformations including cyclopia, midfacial defects and absence of nasal bones. Given the mouse map position and its expression pattern in forebrain, eyes and nose, *Six3* is an attractive candidate for HPE2. Furthermore, the fact that another member of this family, *Six2*, also maps in that region of chromosome 17 and is strongly expressed in the mouse facial mesenchyme (Oliver et al., 1995), could suggest that maybe both *Six2* and *Six3* are affected in this form of holoprosencephaly. This notion is currently being tested by mapping *Six3* and *Six2* in human chromosomes and examining the structure and expression of both genes in families carrying HPE2.

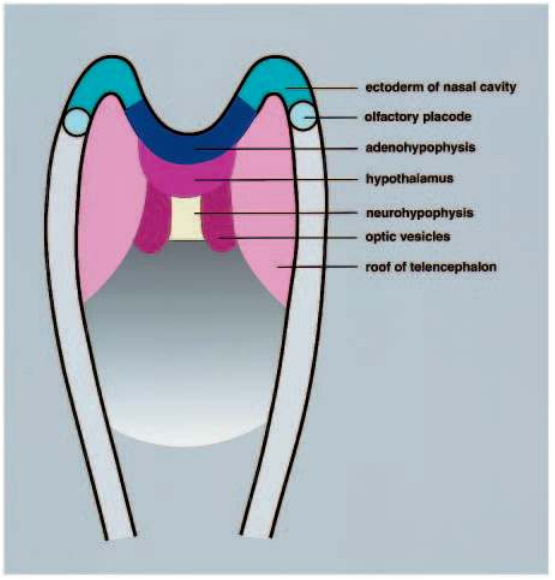

Fig. 4. Diagrammatic representation of an avian fate map of the anterior neural primordium at the 3- to 4-somite stage (redrawn from Couly and LeDouarin, 1988). The main areas in which *Six3*-positive cells are found in the early mouse embryo are here indicated.

Analysis of *Six3* expression pattern

We detect the first signal of *Six3* expression by whole-mount in situ hybridization at approximately E6.5 days of mouse development (before headfold appearance). The signal is faint and located at the anterior border of the embryo (not shown). Signal becomes stronger at around E8.2 and is restricted to the most anterior border of the neural plate (Fig. 3A). Chicken fate map analysis (Couly and LeDouarin, 1988) has shown that the most anterior region of the avian neural plate (neural ridge) at the 3- to 4-somite stage will later give rise to non-neural ectodermal structures, i.e. the adenohypophysis, ectoderm of nasal cavity and olfactory placodes (Fig. 4). A few hours later (E8.5), the *Six3*-expressing region has expanded further. At this time, *Six3* mRNA is not only located over the anterior neural ridge, but is also seen over the adjacent region of the neural plate (Fig. 3B,C). In the chick, and according to fate map analysis (Couly and LeDouarin, 1988), this anterior region has a neural fate and later will give rise to the most anterior neural derivatives (hypothalamus, optic vesicles, ventral forebrain, neurohypophysis) (Fig. 4). As shown below, *Six3* expression is later found in the tissues derived from those earlier expression domains. Based on this early expression analysis, we can conclude that *Six3* is a molecular marker for the anterior neural plate.

We compared the *Six3* expression pattern with those of the homeobox-containing genes *Otx2* (Simeone et al., 1993) and *Pax6* (Walther and Gruss, 1991). Both genes are known to be expressed in anterior CNS, ventral forebrain and also during eye development. At E8.2, all three mRNAs are located in the anterior headfold; however, *Six3* is the one having the strongest silver grains accumulation in the most rostral part (Fig. 5). Thus, *Six3* is one of the few homeobox genes with such an anterior expression pattern during early mouse development.

The comparative analysis with these three homeobox probes was continued using adjacent sections of E9.5 mouse embryos (Fig. 6). At this stage, *Six3* expression is found in the diencephalic part of the ventral forebrain (Fig. 6B), in the optic vesicles and olfactory placodes (Fig. 7A,B), and Rathke's pouch (not seen). Therefore, these expression domains of *Six3* are in agreement with the predictions based on the chick fate map (Couly and LeDouarin, 1988). They correspond to structures derived from the anterior neural ridge (prospective non-neural ectodermal derivatives as olfactory placodes, ectoderm of nasal cavity and Rathke's pouch), and the adjacent prospective neural derivatives (ventral forebrain including optic recess, hypothalamus and optic vesicles). Furthermore, the expression domain seen in the ventral forebrain is mostly restricted to the region of the optic recess (the anatomical ventral landmark

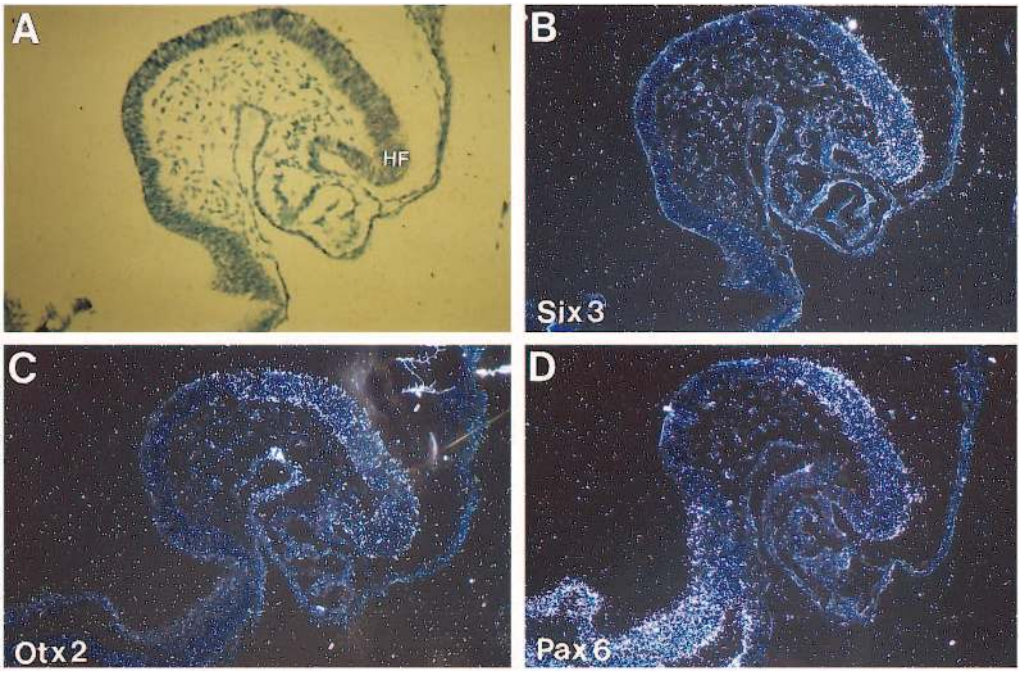

Fig. 5. *Six3* expression is more anterior than that of other forebrain homeobox genes. ^{35}S-labelled in situ hybridization on sagittal adjacent sections of an E8.2 embryo. (A) Bright field (HF, headfold). (B) Dark field showing that *Six3* expression is stronger in the most anterior region of the head fold. (C) The *Otx 2* expression domain partially overlaps that of *Six3* over the posterior forebrain. Silver grain accumulation decreases in the most anterior border. (D) The expression of *Pax6*, another homeobox gene expressed during eye development, is also not so strong as *Six3* in the anterior head fold.

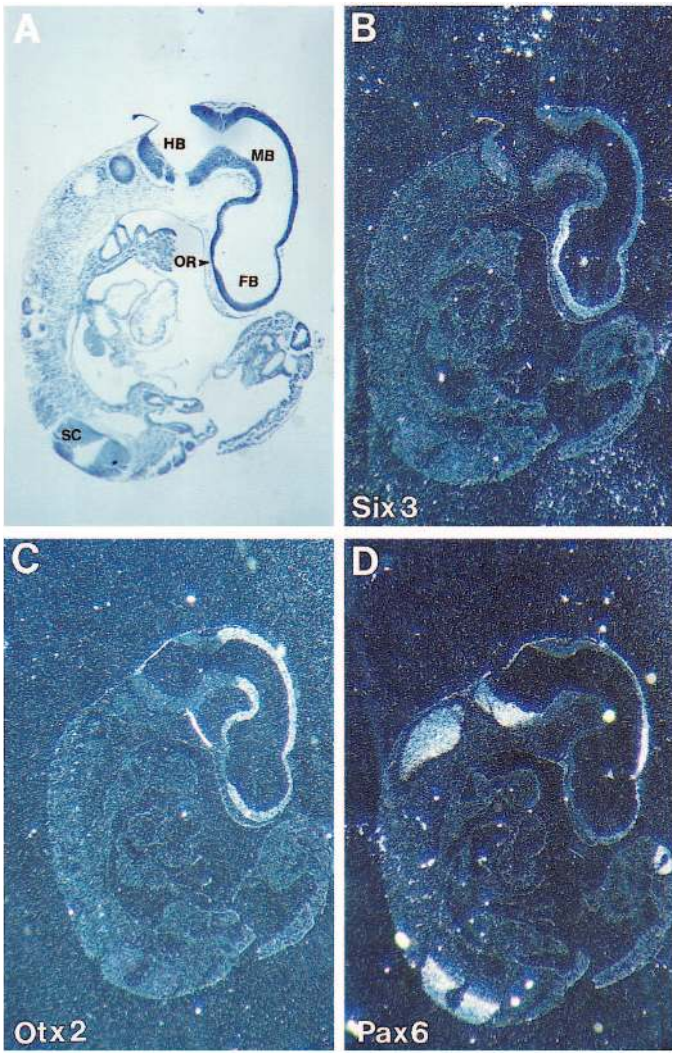

Fig. 6. *Six3* mRNA is localized in the ventral forebrain at the level of the optic recess. ^{35}S-labelled adjacent sections of E9.5 mouse embryos showing: (A) bright field; (B) *Six3* silver grain accumulation in the region of the ventral forebrain, mostly restricted to the optic recess (OR); (C) *Otx2* expression seems to specifically escape the optic recess region and is found to be located in the rest of the forebrain (FB) and midbrain (MB); (D) *Pax6* is found in some parts of the forebrain, midbrain, hindbrain (HB) and spinal cord (SC).

connecting with the optic stalks). According to Puelles and Rubenstein (1993), this region of the ventral forebrain, which includes the medial aspect of the retrochiasmatic hypothalamus corresponds to the vertebrate most rostral end of the neural tube, from which the telencephalic and eye vesicle evaginations develop. They propose that once the brain bends at the neural tube flexure, the most anterior border of this longitudinal embryonic axis is represented by the medial aspect of the tuberal and retrochiasmatic hypothalamus, which are in the vicinity of the optic recess and which correspond in their model to prosomere 6 (Puelles and Rubenstein, 1993).

Both *Otx2* and *Pax6* mRNAs are absent from the region of the optic recess (Fig. 6C,D). This is more obvious with *Otx2* mRNA, which appears to specifically leave a gap in this region (Fig. 6C). In the ventral forebrain, *Otx2* expression is also

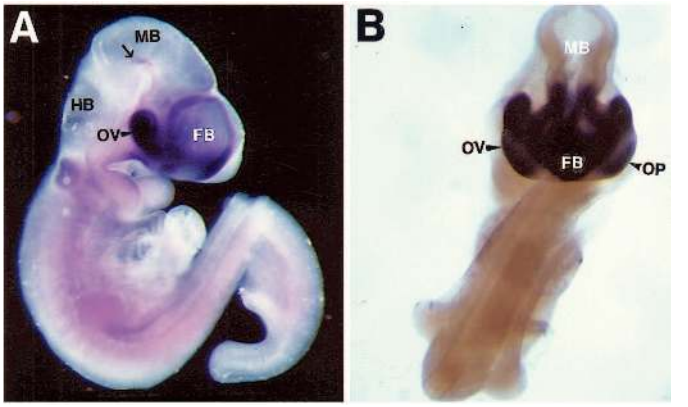

Fig. 7. *Six3*-positive cells are now strongly seen in the developing eye. Whole-mount in situ hybridization of E9.5 mouse embryo. (A) Strong labelling in the optic vesicle (OV) and in the ventral forebrain (FB). Weak expression is also detected in the midbrain tegmentum (arrow). (B) Embryo photographed from the top of the head region showing the staining in the optic vesicles, forebrain and also in the olfactory placodes (OP). HB, hindbrain; MB, midbrain.

present in the region proposed for prosomere 6 (Puelles and Rubenstein, 1993) and has its anterior boundary just posterior to the optic chiasma (Simeone et al., 1993). The *Dlx2* homeobox gene, which is also not found in the optic recess and retrochiasmatic hypothalamus, has been reported to be expressed in the alar component of prosomere 6 (Puelles and Rubenstein, 1993). From these data, it appears that *Six3* is not only one of the most anteriorly expressed homeobox genes to date, but it is also one of the few to be expressed in the anterior regions of the neural plate and, later, of the body axis (optic recess).

At approximately E11.5, and similarly to what has been reported for *Pax6* (Walther and Gruss, 1991), *Six 3* expression is also found in the eye, specifically in the neural retina, lens and optic stalk (Fig. 8A). Also, like *Pax6*, at E12.5 *Six3* labelling is seen in the epithelium of the nasal cavities (Fig. 8B). In sections of E12.5 embryos, *Six3* mRNA is detected in the ventral thalamus and is clearly visualized in the hypothalamus, midbrain tegmentum, posterior part of the pretectum (Fig. 9) and Rathke's pouch (not shown). Weak expression is also detected in the medial and lateral ganglionic eminence (Fig. 9).

In order to analyze whether the early lineage relations were also maintained at later stages, we then analyzed *Six3* expression at E14.5. We found that label remained in Rathke's pouch (pituitary), hypothalamus and eyes; has become more intense in the midbrain tegmentum and ganglionic eminence, and is also very strong in the septum and the region of the optic chiasma (Fig. 10). Later in development, expression fades away and at E18.0, some weak expression remains only in the nasal cavities (not shown) and eyes (Fig. 11).

Six3 expression in the visual system and in *Small eye* mutants

The dynamic of *Six3* expression during eye development is shown in detail in Fig. 11. Starting at E9.5, *Six3* mRNA is found in the optic vesicles and optic stalks (Fig. 11A). Later on, and up to approximately E13.5, *Six 3* is strongly expressed in the eye neural retina and lens (Fig. 11C,E). At E13.5 and E14.5 (Fig. 11G,I), the labelling remains in the neural retina and lens, but starts to present a differential distribution. In the

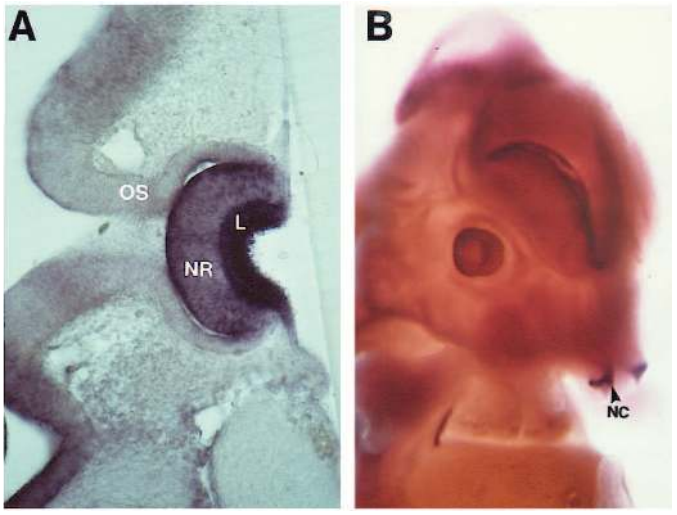

Fig. 8. *Six3* expression in the sensory regions. (A) Vibratome section of a whole-mount hybridization stained E11.5 embryo showing *Six3* staining in the neuroretina (NR) and lens (L). The optic stalks (OS) are weakly stained. (B) At E12.5 *Six3* is found over the ectoderm of the nasal cavities (NC).

first tissue, expression is stronger in the inner neuroblastic layer but it is starting to fade away in the outer neuroblastic one. In the lens, expression in the fibers is also fainter and becomes stronger in the anterior epithelial part. Finally, at E18.0, weaker *Six3* expression is only remaining in the inner neuroblastic layer of the eye retina (Fig. 11K).

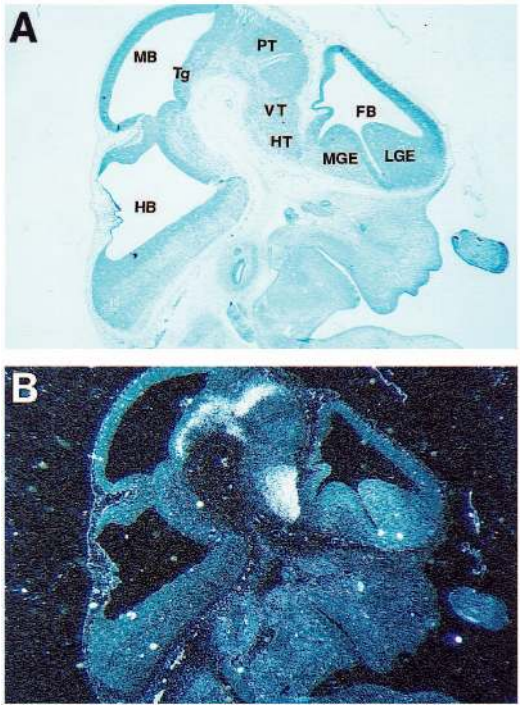

Fig. 9. *Six3* expression in the developing brain. ^{35}S-labelled sagittal section of an E12.5 embryo. (A) Bright field; (B) dark field showing silver grain accumulation in the midbrain tegmentum (Tg), posterior pretectum (PT) and ventral thalamus (VT) and hypothalamus (HT). HB, hindbrain; FB, forebrain; LGE, lateral ganglionic eminence; MB, midbrain; MGE, medial ganglionic eminence.

The high amino acid sequence similarities, together with its expression during eye development, suggests that *Six3* could be the mouse functional homologue of the *Drosophila so* gene, which in the fly is required for eye formation (Cheyette et al., 1994).

In mice, *Pax6* is known to be implicated in some of the steps leading to eye formation and mutations in this gene lead to the *Small eye* phenotype (Hill et al., 1991). In *Small eye* homozygous mice, eye formation is almost completely affected (Hogan et al., 1986). In normal embryos, *Pax6* expression is found in the developing eye, pituitary and nasal epithelium, hypothalamus, hindbrain and spinal cord (Walther and Gruss, 1991; Stoykova and Gruss, 1994). The expression pattern that we reported here for *Six3* overlaps with many of the regions expressing *Pax6,* with the main exception being the surface ectoderm of the optic placodes, dorsal midbrain and spinal cord. Therefore, based on the possibility that both genes might participate in the same regulatory pathway at least during vertebrate eye development, we decided to see whether *Six3* expression was affected in *Small eye* mutants. Our results shown in Fig 12 indicate that this is not the case. *Six3* expression is unaffected in the brain of E12.5 *Small eye* mouse embryos, showing the normal pattern in the hypothalamus, Rathke´s pouch and septum. Some of the areas in which we found normal *Six3* expression, such as the hypothalamus and developing pituitary, are tissues in which *Pax6* expression is also present (Walther and Gruss, 1992).

DISCUSSION

Six3 during eye development

The newly identified *Six3* gene is the third member of a variant subclass of homeobox genes that we have named *Six/sine oculis* and from which we have previously reported the *Six1* and *Six2* genes (Oliver et al., 1995). The *Six/sine oculis* subclass of homeobox genes has the interesting characteristic of having a lysine at position 50 of the recognition helix like the anterior-expressed genes *bicoid, orthodenticle* and *goosecoid.* Another feature of this subclass is the high percentage of amino acid homology in the homeodomain 5′ flanking region. This suggests, that this region (*Six* domain) may be of functional importance and therefore has been conserved as a domain during evolution. Similar to *Six3*, the two previously described members of this subclass (Oliver et al., 1995) also share a high sequence homology with *so*. However, neither one is expressed during mouse eye development. It is interesting to note that, from the three murine *Six* genes, the newly identified *Six3* is the one exhibiting the lowest percentage of sequence homology with *so*. Nevertheless, two major lines of evidence support the idea that *Six3* could be the mouse functional homologue of the *Drosophila so* gene. First, both genes share extensive amino acid sequence identity and, second, *Six3* is also found to be expressed during mouse eye development. The analysis of *Six3* expression during eye development has shown that contrary to *Pax6* (Walther and Guss, 1991), *Six3* mRNA is not detected early, before the lens has formed, in the ectoderm overlying the optic vesicle. Later on, from approximately E9.0 onwards, *Six3* expression is progressively detected in optic stalk, optic vesicle, neuroretina and lens. Expression was also found in other structures of the visual system such as the optic chiasma.

At the beginning, *Six3* mRNA seems to have a more or less uniform distribution in the neural retina and lens. This starts to change at around E13.5 when *Six3* transcripts become more abundant in the inner neuroblastic layer of the neuroretina. Similarly, at this stage, expression in the lens is stronger in the epithelium of the anterior part rather than in the lens fibers. It is well known that the inner neuroblastic layer of the retina gives rise to the ganglion cells and supporting cells. Later in development, these ganglion cells sprout axons, which later form a fiber layer that lines the inner surface of the retina. Therefore, according to the expression pattern, *Six3* may participate in some of the steps leading to visual system development in vertebrates and, most probably, after some of the initial inductive steps occurring during gastrulation and neurulation have already taken place. This could be accomplished by providing positional informational clues to the forebrain, so that the optic vesicles will be formed at the right place. It may also be involved in the process of optic vesicle formation itself.

In *Drosophila*, the *so* locus is required for the development of the entire visual system (Cheyette et al., 1994). Similarly, the fly gene *eyeless* (Quiring et al., 1994), which is the homologue of the vertebrate *Pax6* gene, is also required in this process. Loss-of-function mutations in both the insect and the mammalian *Pax6* genes are known to produce the reduction or absence of eye structures

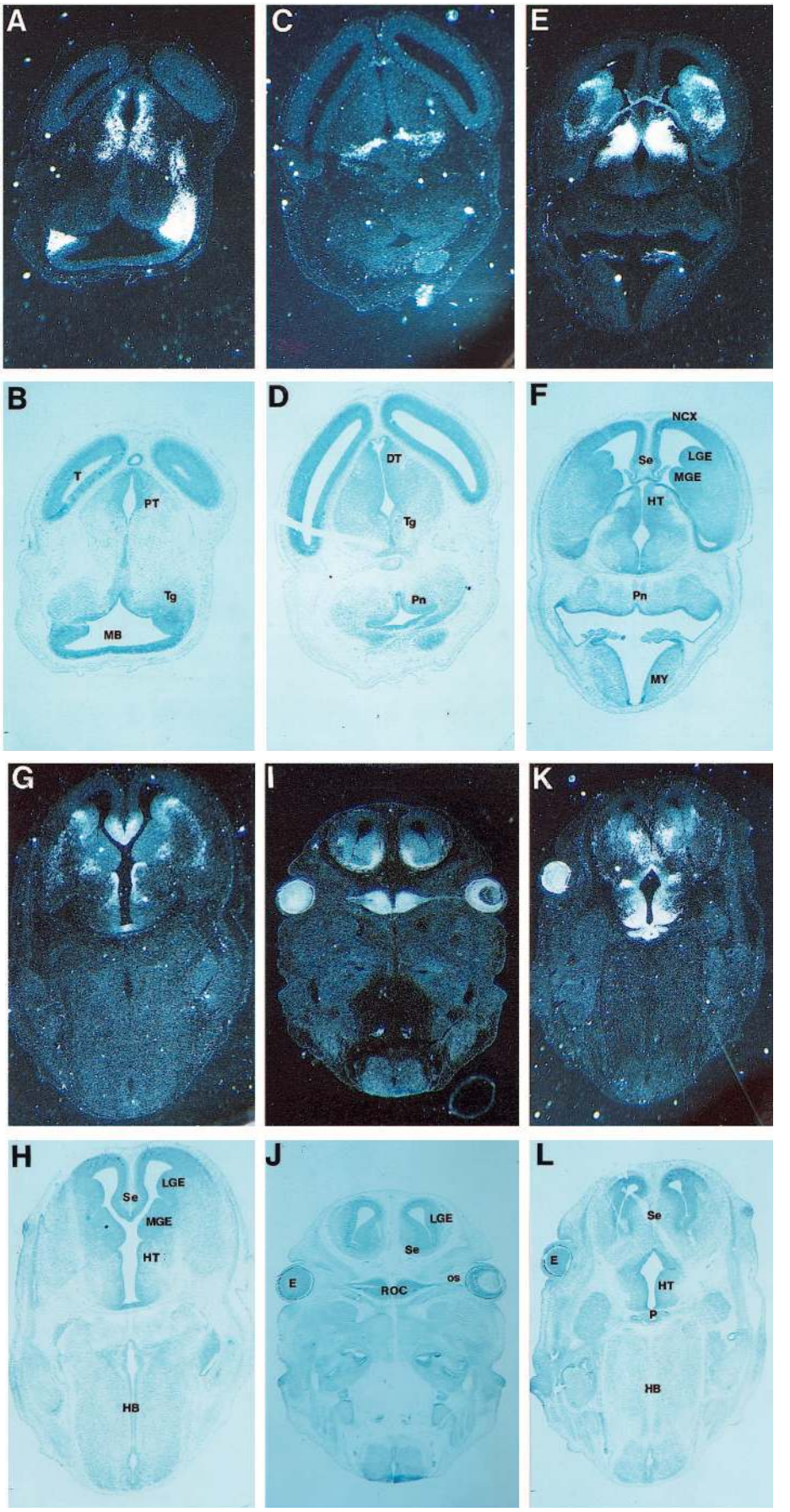

Fig. 10. Late *Six3* expression in the brain region. Adjacent transverse sections of a normal E14.5 mouse brain from rostral to caudal. (A,C,E,G,I,K) Dark field; (B,D,F,H,J,L) corresponding bright field. (A,B) *Six3* expression is seen in the pretectum (PT) and midbrain tegmentum (Tg). (C,D) In a more caudal section, expression remains in the tegmentum, while in E,F labelling is now also detected in the lateral (LGE) and medial (MGE) ganglionic eminence and in the hypothalamic region (HT). (G,H) *Six3* labelling still remains in the aforementioned regions but also is now detected in the septum (Se). (I,J) Strong labelling is seen in the eyes (E), in the region of the optic chiasma (ROC) and in the septum. (K,L) Silver grain accumulation is seen in the eye, hypothalamus, septum and pituitary (P). DT, dorsal thalamus; HB, hindbrain; MB, midbrain; NCX, neocortex; Pn, pons; T, telencephalon.

(eg. mouse *Small eye* and human *aniridia*) (Hill et al., 1991; Quiring et al., 1994). Furthermore, ectopic eye formation can be induced by targeted expression of *eyeless* in *Drosophila* embryos (Halder et al., 1995).

In the last few years, some examples of this type of functional parallelism between mouse and *Drosophila* have emerged. For example, the fly *tinman* gene (Bodmer, 1993) and the mouse *Nkx-2.5* (Lints et al., 1993) are homeobox genes

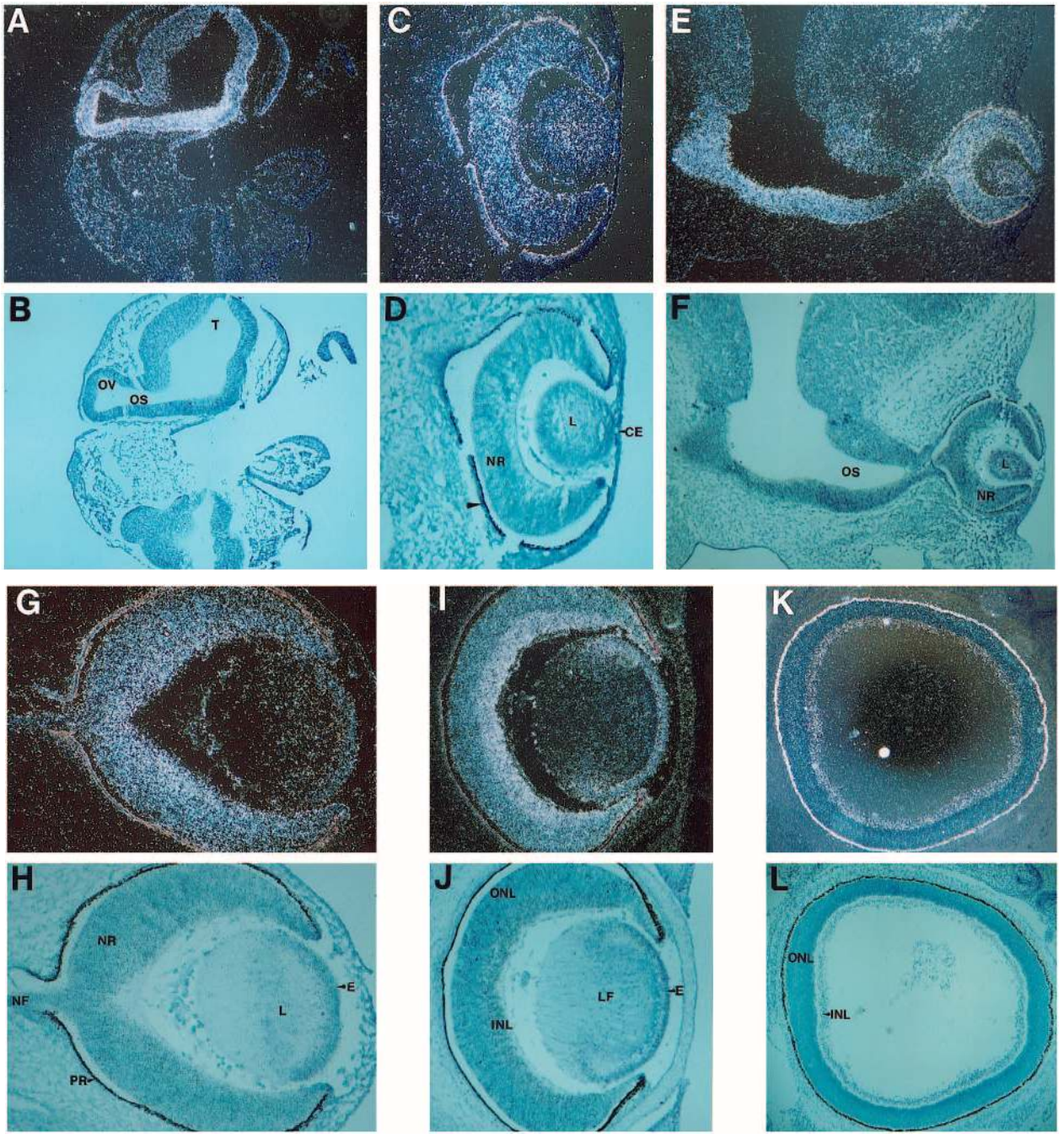

Fig. 11. *Six3* is expressed during the development of the visual system. (A,C,E,G,I,K) Dark field; (B,D,F,H,J,L) corresponding bright field. (A,B) At E9.5 mRNA accumulation is seen in the developing optic vesicle (OV) and connecting optic stalk (OS). (C,D) At E10.5, the neural retina (NR) and the lens (L) have formed and *Six3* expression is observed uniformly distributed in both tissues. No specific expression is seen in the pigmented layer of the retina (arrowhead) and in the corneal ectoderm (CE). At E11.5 (E,F) labelling remains in the lens, neural retina and optic stalk. At E13.5 (G,H) *Six3* mRNA starts to show a differential distribution in the neural retina and in the lens. In the first tissue, expression is stronger in the inner neuroblastic layer, while in the lens labelling in the fibers is weaker and is strong in the anterior epithelial layer (E). The nerve fibers (NF) in the optic stalk seems to have a weak expression above background. The pigmented layer of the retina does not express *Six3* (PR). At E14.5 (I,J) the expression in the neural retina shows a clear boundary and strong silver grain accumulation is observed in the inner neuroblastic layer (INL). Labelling is weaker in the outer neuroblastic layer (ONL). The anterior lens epithelium is also strongly labelled. Finally, at E18.0 (K,L) the only remaining expression is found in the inner retinal layer. LF, lens fibers.

that seem to be required for heart development in both of these organisms. This, and other evidence (Scott, 1994), support the notion that the evolutionary relationships between *Drosophila* and mouse appear to be closer than expected. Perhaps, *sine oculis/Six3* represents another example of this parallelism, with *sine oculis*/*Six3* required for the control of some of the steps leading to eye formation in flies and vertebrates.

The fact that the expression of the *eyeless* gene in *Drosophila* imaginal discs results in the induction of ectopic eyes has led to the suggestion that this gene could be a master regulator in this process (Halder et al., 1995). However, it could also be possible that, in order to obtain ectopic eyes, normal *so* expression is required in the *eyeless*-targeted imaginal discs. Perhaps, neither the *so* nor the *eyeless* products alone are capable of giving rise to eyes and cross-talk between both products is required. It is conceivable that in vertebrate embryos also an interaction between the product of both genes (*Six3* and *Pax6*) is necessary in order to produce a functional eye.

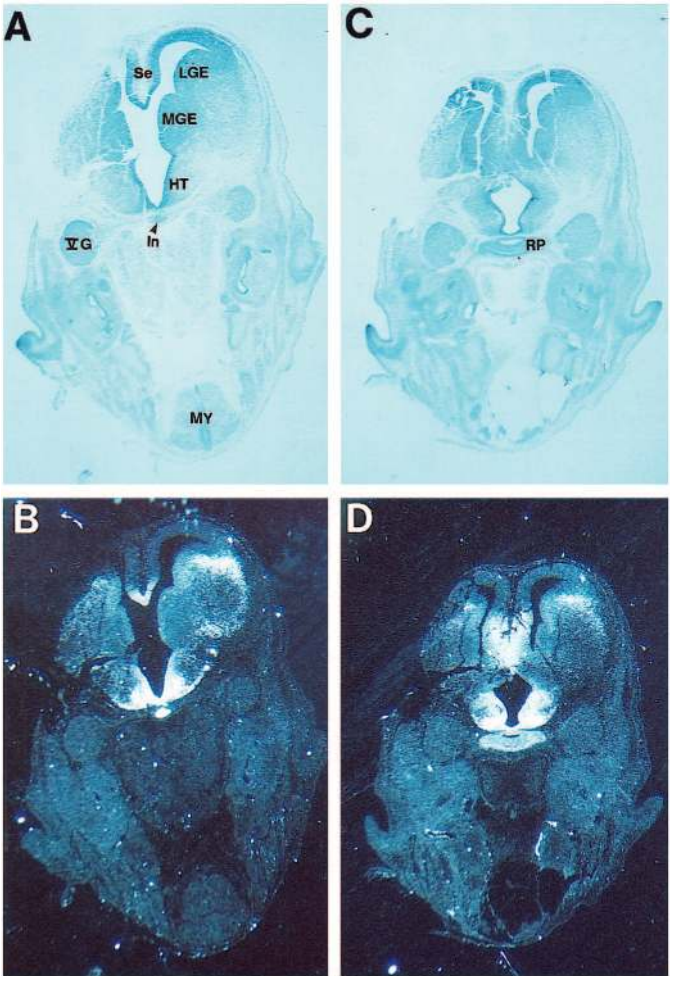

Fig. 12. *Six3* expression is unaffected in *Small eye* mutant embryos. Transverse sections of the head of E12.5 mutant embryos. (A,C) Bright fields. (B) *Six3* silver grain accumulation is observed in the septum (SE), lateral (LGE) and medial (MGE) ganglionic eminence, hypothalamus (HT) and infudibulum (In) of the developing pituitary (D). Expression is also found in similar areas than in B, with strong silver grain accumulation in Rathke's pouch (RP). VG, fifth cranial ganglia; MY, myelencephalon.

Early during vertebrate development, *Pax6* is expressed in the head surface ectoderm and, during eye formation, its product seems to be required for the process of lens placode formation. The expression pattern that we reported here for *Six3* during eye development suggests that this gene might also participate in the process of eye formation.

In *Small eye* mutants, the optic vesicles are formed but have an abnormal shape (Grindley et al., 1995). In these mutants, *Six3* expression was found to be unaffected in some brain tissues that are also known to express *Pax6* (hypothalamus, pituitary). From this analysis, we cannot conclude that the expression of both *Six3* and *Pax6* in these tissues co-localize in the same cells. However, we should mention that those tissues do not seem to have an obvious phenotype in *Small eye* mice. This suggests that in these structures, *Pax 6* function is either not necessary or redundant, and therefore *Six 3* expression remains normal. However, in the tissues that are clearly affected and missing in the mutants (eyes, nose), it is obvious that *Pax 6* is essential during their morphogenesis. As these affected tissues are missing in the mutant mice, we can not address the question concerning a regulatory hierarchy between *Six 3* and *Pax 6*. Several alternative explanations remain: it could be possible that *Six 3* and *Pax 6* participate in different and independent regulatory pathways, or that *Six 3* acts upstream of *Pax6*. Another interpretation could be that, in *Small eye* mutants, since the contact with the *Pax6*-expressing ectoderm is affected, there is no cross-talk between the product of both genes, thus the *Six3*-expressing optic vesicle degenerates and no eye is formed. Transgenic gain-of-function experiments for both genes may shed some light on this.

In any case, the finding of a *so* homologous gene that is, like *Pax6*, active during vertebrate and invertebrate eye development, indicates that at least some of the genes of the pathway involved in eye formation in invertebrates and vertebrates have been conserved during evolution.

Six3 is a marker for the anterior neural plate

The first detectable expression of *Six3* is restricted to the most rostral part of the neural plate. Interestingly, this expression was first confined to the very anterior neural ridge, which later will give rise to non-neural ectodermal derivatives such as adenohypophysis, ectoderm of nasal cavity and olfactory placodes. Subsequently, expression expands towards the adjacent neural region. Once the longitudinal axis of the brain bends at the neural tube flexure, *Six3* expression is localized in the regions predicted by the chick lineage mapping. i.e. the most rostral ectodermal-derived regions like Rathke's pouch and nasal placodes and, also, over the most anterior neural structures, which have been proposed to be located at the ventral forebrain (optic recess, optic vesicles, hypothalamus). It is conceivable that the different cell types of the anterior neural plate, which later will give rise to non-neural and neural derivatives, arise from a few common precursors located in the very early neuroepithelium (Couly and LeDouarin, 1988). In this case, it may be possible that *Six3* is a marker for those few common precursors.

The fact that *Six3* expression is found localized in the optic recess region is very interesting. According to some authors, this area of the ventral forebrain represents the tip of the longitudinal axis of the brain (Puelles and Rubenstein, 1993). Thus far, no other homeobox gene has been found expressed in that region, including the expression of some genes that are found in the forebrain (Finkelstein and Boncinelli, 1994). Also,

the *Sonic Hedgehog* gene, which has been shown to induce the differentiation of floor plate, spinal cord motor neurons and ventral forebrain neurons (Roelink et al., 1995; Ericson et al., 1995), does not seem to overlap in this *Six3*-expressing region in the ventral forebrain (not shown).

In *Drosophila,* the homeobox gene *bicoid* is known to be responsible for the positioning of morphological anterior landmarks in the gastrulating embryo and to subdivide the anterior part into distinct domains (Berleth et al., 1988). It is known that this gene is responsible through a diffusible gradient for the setting of the anterior embryonic boundaries. Similarly, for the establishment of the anterior positional information, a related mechanism should exist in other metazoans. Despite the fact that no *bicoid* homologues have been found in any other organism, homeobox genes are still good candidates for playing such a role in vertebrates. Based on our finding that the *Six3* homeodomain contains a lysine at position 50, similar to the anteriorly expressed genes *bicoid, orthodenticle* and *goosecoid*, and to its restricted expression pattern in the most anterior neural plate, it is tempting to speculate that this gene may be providing anterior positional information in vertebrates. Although we have not been able to detect *Six3* expression in very early embryos, as would be expected from a gene that, as *bicoid*, may participate in the establishment of positional information to the developing embryo, we cannot exclude that a low level of activity is present in at least a few cells.

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