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 homebox 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)F1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N1 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 NotI fragment of mouse cDNA, was labeled with α[32P]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 EcoRI-digested C57BL/6J DNA and a major fragment of 9.6 kb was detected in EcoRI-digested M. spretus DNA. The presence or absence of the 9.6 kb EcoRI 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 (Lhgr) 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 RNAsase treatment were performed as described previously (Kessel and Gruss, 1991). T3 or T7 RNA polymerase in vitro transcribed sense or antisense 35S-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 107 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 4.5 kb 3' flanking 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).
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) F1 × 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 EcoRI 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 Sox1, Six2, Msh2 and Lhcg (Fig. 2). Six3 mapped 2.0 cM distal of Sox1 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. 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 N2 animals is presented over the total number of N2 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.
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

![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.](image)

![Six3 expression is more anterior than that of other forebrain homeobox genes. 35S-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.](image)
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 evaginates 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 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), Six3 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, Six3 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
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).

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 (J,L) 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.

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, Pax6 function is either not necessary or redundant, and therefore Six3 expression remains normal. However, in the tissues that are clearly affected and missing in the mutants (eyes, nose), it is obvious that Pax6 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 Six3 and Pax6. Several alternative explanations remain: it could be possible that Six3 and Pax6 participate in different and independent regulatory pathways, or that Six3 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 (Berlet 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 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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