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Expression of *Six3 Opposite Strand (Six3OS)* during mouse embryonic development

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Abstract

Recently, sequence analyses have identified a large number of opposite strands transcripts in the vertebrate genome. Although the transcripts appear to be spliced and polyadenylated, many of them are predicted to represent noncoding RNAs. High levels of noncoding transcripts of the *Six3 opposite strand (Six3OS)* were recently identified in the embryonic and postnatal retina of the mouse. In this study, we expanded those initial expression analyses, elucidated in detail the developmental expression profile of mouse *Six3OS* in the brain and visual system, and compared it with that of *Six3*. Our results show that *Six3OS* expression overlaps extensively with that of *Six3* and is not altered in *Six3*-null embryos.

Keywords

Six3OS; *Six3*; homeobox; mouse; expression analysis; brain development

1. Results and Discussion

In the last few years, large-scale expression sequence tag (EST) sequencing performed particularly in the mouse, identified thousands of putative spliced transcripts that do not appear to be translated into protein (Numata et al., 2003; Katayama et al., 2005). Similarly, public database analyses suggested the presence of approximately 1,600 sense-antisense gene pairs in the human genome (Yelin et al., 2003). These studies concluded that the occurrence of loci transcribed from both DNA strands in the mammalian genome is a common phenomenon; however, insights about their putative functional relevance are limited.

A group of newly identified transcripts originating from the opposite strand of different homeobox-containing genes was shown to be expressed in the embryonic and postnatal retina (Blackshaw et al., 2004; Alfano et al., 2005). One of these, *Vax2OS*, corresponds to the antisense of the homeobox gene *Vax2* and its expression level was found to be significantly reduced in *Vax2*-null mice (Alfano et al., 2005). The same group has also reported that adenoviral-mediated overexpression of *CrxOS* in the mouse adult retina leads to a significant decrease in the expression levels of the corresponding sense gene, the homeobox gene *Crx*. These results suggested that at least some of these opposite strand transcripts are functionally related to their sense counterparts (Alfano et al., 2005).

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Six3, a homologue of the *Drosophila* homeobox gene *Optix*, plays a key role during vertebrate forebrain and visual system development (Oliver et al., 1995; Seimiya and Gehring, 2000; Lagutin et al., 2003; Liu et al., submitted). In the mouse, functional inactivation of *Six3* abolishes telencephalon formation, a phenotype most likely resulting from the abnormal anterior expansion of posteriorizing *Wnt* activity (Lagutin et al., 2003). In *Xenopus*, *Six3* activity participates in the specification of the anterior neural plate by promoting cell proliferation and inhibiting BMP4 expression (Gestri et al., 2005). In medaka fish, *Six3* acts as an antagonist of the replication-initiation inhibitor Geminin to control the balance between proliferation and differentiation during retina formation (Del Bene et al., 2004). In addition, it has been shown that ectopic expression of *Six3* promotes retina formation in zebrafish embryos (Loosli et al., 1999), and optic vesicle-like structures in the hindbrain-midbrain region of mouse embryos (Lagutin et al., 2001). Additionally, in humans, mutations in the coding region of *Six3* are associated with holoprosencephaly, a syndrome that results from defective mid-line cleavage of the embryonic forebrain (Wallis & Muenke, 2000; Dubourg et al., 2004; Pasquier et al., 2005).

Eleven endogenous transcripts originating from the *Six3* opposite strand (*Six3OS*) were identified from the mouse public database by Alfano et al., (Alfano et al., 2005). One of these transcripts (*Six3OS1*), contains a putative short open reading frame (ORF) predicted to encode a 200 amino acids long protein (GenBank accession no. NM175276). The other ten opposite strand transcripts either did not contain any putative ORFs, or, according to predictions, could only be translated into proteins smaller than 100 amino acids (Alfano et al., 2005); therefore, they are defined as non-coding RNAs.

In addition to those eleven transcripts, we identified an additional 12th 4-kb non-coding opposite strand transcript from the NCBI EST database (GenBank accession no. BC065087; Fig. 1). This transcript was also originally named *Six3OS1* by the NCBI database; however, to be consistent with the nomenclature and to avoid further confusion, we have renamed this transcript as *Six3OS12*. Twelve putative ORFs were identified in *Six3OS12* by DNA sequence analysis (data not shown); the longest one could be translated into a 120 amino acids long protein.

Expression of *Six3OS* was previously identified in the optic cup of embryonic day 10.5 (E10.5) mouse embryos and also in the adult chicken and mouse retina using a probe that partially overlaps with most of the reported alternatively spliced forms of *Six3OS* (Blackshaw et al., 2004; Alfano et al., 2005). However, a detailed spatial and temporal expression analysis of *Six3OS* has not yet been conducted. In this paper we describe the expression profile of *Six3OS* during mouse embryonic development by *in situ* hybridization analysis and compare it with that of *Six3*.

1.1. *Six3OS* and *Six3* show extensive overlap in their expression profile in the developing mouse brain

To gain further insight about the *Six3OS* transcripts, Northern blot analysis was initially performed by using Poly(A) RNA prepared from either E10.5 heads or adult retinas. Two different probes were designed for this analysis. The first probe included the first three exons of *Six3OS3* and partially overlapped with all of the previously reported *Six3OS* transcripts (Fig. 1; Alfano et al., 2005). The second probe recognized *Six3OS11* and *Six3OS12* (Fig. 1 and data not shown; Alfano et al., 2005). This analysis demonstrated that both probes recognized the same 3 major RNA transcripts of 4.7, 5.5 and 7.0 kb in RNA isolated from E10.5 heads (Fig. 2A). A similar set of bands was detected when using Poly(A) RNA isolated from adult retina (data not shown). No bands were detected when using total RNA isolated from E10.5 tails (Fig. 2A). These bands are larger than those previously reported by Alfano et al., (2005) based on the assembly of available EST databases.

To characterize the spatial and temporal expression pattern of *Six3OS* during mouse embryonic development, we performed a detailed spatial and temporal *in situ* hybridization analysis on whole mouse embryos (from E7.5 to E10.5) and on mouse sections (E12.5, E14.5, and E18.5). The *in situ* probe was *in vitro* transcribed from the same *Six3OS3* cDNA fragment used for the Northern analysis that, as already indicated, detects all of the reported *Six3OS* transcripts. In contrast to *Six3* (Oliver et al., 1995), no *Six3OS* expression was detected in the anterior neural ectoderm at E7.5 (data not shown). Expression was also not observed in the anterior neural ridge or any other embryonic region at E8.0 (Fig. 2B); in contrast, *Six3* expression was strongly expressed in the anterior neural ridge and anterior neuroectoderm at this stage (Fig. 2F). Starting at around E8.5 (12 somite stage), *Six3OS* (Fig. 2C) was detected in the same regions (although at lower levels) than *Six3* (Fig. 2G); mainly in the ventral forebrain and optic vesicles. The overlap in expression of *Six3OS* (Fig. 2D,E) and *Six3* (Fig. 2H,I) in these embryonic regions was also observed at E9.5 and E10.5. At later stages (E14.5-E18.5), abundant regions of overlap in the expression of *Six3OS* and *Six3* are also detected in different regions of the developing forebrain such as telencephalon (ganglionic eminence and septum), diencephalon (ventral thalamus and hypothalamus), and pituitary (Fig. 3). In the midbrain, both *Six3OS* and *Six3* are expressed in the tectum and in the tegmentum at E14.5 (Fig. 3A-D); however, the weak expression of *Six3OS* in the tegmentum has disappeared by E18.5 (compare Fig. 3E-H). In the septum and hypothalamus, *Six3OS* expression is restricted to the ventricular zone; instead, that of *Six3* is detected in both the ventricular zone and the mantle zone (compare Fig. 3C and D). Similarly, in the E12.5 hypothalamus *Six3OS* is only expressed in a subpopulation of *Six3* positive cells that is negative for MAP2 (Fig. 3I-K), a marker of terminal neuronal differentiation (Garner et al., 1988). In conclusion, starting from E8.5, the expression of *Six3OS* largely overlaps with that of *Six3* in the developing mouse prenatal brain.

1.2. *Six3 OS* expression is independent of *Six3*

The relatively similar pattern of expression we described for *Six3* and *Six3OS* during mouse embryonic development suggested that the expression of one of the transcripts could regulate the activity of the other. To determine whether *Six3OS* expression could be influenced by *Six3*, we analyzed *Six3OS* expression in standard and conditional *Six3*-null mice (Fig. 1; Lagutin et al, 2003; Liu et al, submitted). As described above, at E9.5 *Six3OS* is expressed in the ventral forebrain and the optic vesicles (Fig. 4A). Although reduced due to the absence of the telencephalic region, *Six3OS* expression was still detected in the remaining ventral part of the posterior diencephalon of standard *Six3*-null embryos at this stage (Fig. 4B). This result suggests that *Six3OS* expression (at least in that embryonic territory) is independent of *Six3* activity; however, using this approach, we cannot completely exclude the possibility that subtle changes in the levels of *Six3OS* expression take place following *Six3* inactivation.

As most of the *Six3OS* expressing-territories (telencephalon and eye) were truncated in the standard *Six3*-null embryos, we could not conclude whether *Six3OS* expression in those regions was also normal following the removal of *Six3* functional activity. To address this question, we took advantage of conditional *Six3*-null embryos generated by crossing this conditional mouse strain with an available α -*Cre* (*Pax6*) transgenic strain that exhibits distal neuroretina specific Cre activity starting at around E10.5 (Marquardt et al., 2001; data not shown). As conditional *Six3* heterozygous mice are identical to wild type, *Six3*^{+/f}; α -*Cre* embryos were used as controls in this analysis. Normally, *Six3OS* expression largely overlaps with that of *Six3* in the neuroretina and lens of E14.5 wild-type embryos (Fig. 4C, E, G). As shown in Fig. 4D, only a few *Six3*-expressing cells remain in the distal neuroretina of E14.5 *Six3*^{Δ/f}; α -*Cre* embryos (*Six3*-null in the distal neuroretina; Liu et al., submitted). However, the expression of *Six3* in the lens and in the central portion of the neuroretina was unaffected (Fig. 4D, H). No obvious changes in the domains of expression or in the expression levels of *Six3OS* were

observed in *Six3*^{Δf}; *α-Cre* neuroretina, although *Six3* was absent from the distal part (compare Fig. 4E and F).

These results indicated that the 5' upstream region, first intron and exon 1 of *Six3* that were deleted in *Six3* conditional null embryos are not required for *Six3OS* expression; loss of *Six3* activity did not affect the expression of *Six3OS*.

2. Experimental Procedures

2.1. Probe preparation

The *Six3OS3* probe was obtained by RT-PCR using total RNA isolated from the E10.5 heads and the following primers: 5'-GGCCGCGCCTTGTAAGCGCTA-3' (forward) and 5'-GTTGAGAATCAGTCTGGGGTCGGC-3' (reverse), to amplify a 400bp fragment containing the first three exons of *SixOS3*. 50 ng of the purified PCR product were used for labeling (Megaprime DNA labeling system, Amersham Biosciences). The purified PCR product was cloned into pGEM-T easy vector (pGEM-T easy vector system I, Promega), linearized with *NcoI*, and *in vitro* transcribed with digoxigenin RNA labeling mix and SP6 RNA polymerase (Roche).

To prepare the *Six3OS12* probe, part of the Exon 3 of *Six3OS12* (Image Clone 6850501, purchased from Open Biosystems) was amplified with primers: 5'-TCTAACCTCGACACACAGGACCTTGG-3' (forward) and 5'-CCCCTTCCTGCGAAATACCGG-3' (reverse). The PCR product was gel purified and used for northern blot analysis as described above.

2.2. Northern blot analysis

For RNA preparation, the telencephalic region anterior to the isthmus (head), and the tail were isolated from E10.5 mouse embryos. The retina was isolated from adult mice. RNA was extracted using Trizol (GIBCO-BRL). Total RNA was poly(A)- selected using PolyA Tract mRNA isolation systems (Promega). PolyA RNA (0.5μg) was electrophoresed in a 1% agarose-formaldehyde gel and transferred to a nylon membrane (Amersham). Hybridization was performed at 65°C in 7% SDS, 0.5M sodium phosphate buffer. Washes with increasing stringency were performed at 65°C (Sambrook and Russell, 2001).

2.3. In situ hybridization

Embryos were fixed in 4% paraformaldehyde on ice for 1 hour and processed as described for whole-mount *in situ* hybridization (Belo et al., 1997). Alternatively, embryos were fixed in 4% paraformaldehyde overnight at 4°C, cryoprotected in 30% sucrose and embedded in TissueTek (Sakura). Following cryosection (14μm), *in situ* hybridization was performed on sections as described (Schaeren-Wiemers and Gerfin-Moser, 1993). In same cases, immunohistochemistry was applied on top of the *in situ* hybridization reaction using rabbit anti-*Six3* antibody (1:500) and diaminobenzidine as a substrate (ABC kit, Vector).

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References

Alfano G, Vitiello C, Caccioppoli C, Caramico T, Carola A, Szego MJ, et al. Natural antisense transcripts associated with genes involved in eye development. *Hum. Mol. Genet* 2005;14:913–923. [PubMed: 15703187]

- Belo JA, Bouwmeester T, Leyns L, Kertesz N, Gallo M, Follettie M, De Robertis EM. Cerberus-like is a secreted factor with neutralizing activity expressed in the anterior primitive endoderm of the mouse gastrula. *Mech. Dev* 1997;68:45–57. [PubMed: 9431803]
- Blackshaw S, Harpavat S, Trimarchi J, Cai L, Huang H, Kuo WP, et al. Genomic analysis of mouse retinal development. *PLoS Biol* 2004;2:E247. [PubMed: 15226823]
- Del Bene F, Tessmar-Raible K, Wittbrodt J. Direct interaction of geminin and Six3 in eye development. *Nature* 2004;427:745–749. [PubMed: 14973488]
- Dubourg C, Lazaro L, Pasquier L, Bendavid C, Blayau M, Le Duff F, et al. Molecular screening of SHH, ZIC2, SIX3 and TGIF genes in patients with features of holoprosencephaly spectrum: Mutation review and genotype-phenotype correlations. *Hum. Mutat* 2004;24:43–51. [PubMed: 15221788]
- Garner CC, Brugg B, Matus A. A 70-kilodalton microtubule-associated protein (MAP2c), related to MAP2. *J. Neurochem* 1988;50:609–615. [PubMed: 3121794]
- Gestri G, Carl M, Appolloni I, Wilson SW, Barsacchi G, Andreazzoli M. Six3 functions in anterior neural plate cell proliferation and inhibiting Bmp4 expression. *Development* 2005;132:2401–2413. [PubMed: 15843413]
- Katayama S, Tomaru Y, Kasukawa T, Waki K, Nakanishi M, Nishida H, et al. Antisense transcription in the mammalian transcriptome. *Science* 2005;309:1564–1566. [PubMed: 16141073]
- Lagutin O, Zhu CC, Furuta Y, Rowitch DH, McMahon AP, Oliver G. Six3 promotes the formation of ectopic optic vesicle-like structures in mouse embryos. *Dev. Dyn* 2001;221:342–349. [PubMed: 11458394]
- Lagutin OV, Zhu CC, Kobayashi D, Topczewski J, Shimamura K, Puelles L, et al. Six3 repression of Wnt signaling in the anterior neuroectoderm is essential for vertebrate forebrain development. *Genes Dev* 2003;17:368–379. [PubMed: 12569128]
- Liu W, Lagutin OV, Mende M, Streit A, Oliver G. Six3 activation of *Pax6* expression is essential for mammalian lens induction and specification. 2006Submitted
- Loosli F, Winkler S, Wittbrodt J. Six3 overexpression initiates the formation of ectopic retina. *Genes Dev* 1999;13:649–654. [PubMed: 10090721]
- Marquardt T, Ashery-Padan R, Andrejewski N, Scardigli R, Guillemot F, Gruss P. *Cell* 2001;105:43–55. [PubMed: 11301001]
- Numata K, Kanai A, Saito R, Kondo S, Adachi J, Wilming LG, et al. Identification of putative noncoding RNAs among the RIKEN mouse full-length cDNA collection. *Genome Res* 2003;13:1301–1306. [PubMed: 12819127]
- Oliver G, Mailhos A, Wehr R, Copeland NG, Jenkins NA, Gruss P. 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. *Development* 1995;121:4045–4055. [PubMed: 8575305]
- Pasquier L, Dubourg C, Gonzales M, Lazaro L, David V, Odent S, Encha-Razavi F. First occurrence of aprosencephaly/atelencephaly and holoprosencephaly in a family of a SIX3 gene mutation and phenotype/genotype correlation in our series of SIX3 mutations. *J. Med. Genet* 2005;42:e4. [PubMed: 15635066]
- Sambrook, J.; Russell, DW. *Molecular Cloning: A laboratory Manual*. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 2001.
- Schaeren-Wiemers N, Gerfin-Moser A. A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. *Histochemistry* 1993;100:431–440. [PubMed: 7512949]
- Seimiya M, Gehring WJ. The *Drosophila* homeobox gene *optix* is capable of inducing ectopic eyes by an *eyeless*-independent mechanism. *Development* 2000;127:1879–1886. [PubMed: 10751176]
- Wallis D, Muenke M. Mutation in holoprosencephaly. *Hum. Mutat* 2000;16:99–108. [PubMed: 10923031]
- Yelin R, Dahary D, Sorek R, Levanon EY, Goldstein O, Shoshan A, et al. Widespread occurrence of antisense transcription in the human genome. *Nat. Biotechnol* 2003;21:371–372. [PubMed: 12665819]

Fig 1.

Schematic representation of the wild-type *Six3* locus, the standard, and the conditional (floxed, f) and delta (Δ) *Six3* targeted alleles. The *Six3OS* transcripts are indicated below. In this scheme, 5' end of the 42 kb genomic fragment of *Six3* is labeled as position 1. In the *Six3* standard knockout, *Six3* was inactivated by the in-frame insertion of *lacZ*, (blue box). In the *Six3* conditional knockout, a 3 kb genomic fragment containing 2kb of *Six3* 5' upstream region, Exon1 and part of intron 1, were flanked by two *loxP* sites (red triangles) (Liu et al., submitted). The *Six3* mRNA is shown above the *Six3* locus and three of *Six3OS* transcripts (*Six3OS1*, 3, and 12) are shown below the *Six3* conditional knockout allele. Exons are represented as boxes whereas introns are depicted as lines. Open reading frames are in purple. Exon(s) used as the template to generate hybridization probe are in green.

Fig 2.

Six3OS is expressed in the optic vesicle and the ventral forebrain during early mouse development. (A) Northern blot analysis of *Six3OS*. Poly(A) RNA isolated from E10.5 forebrain (lanes 1 and 3), or total RNA isolated from E10.5 mouse tails (lanes 2 and 4) was hybridized with the *Six3OS3* probe (lanes 1 and 2), or the *Six3OS12* probe (lanes 3 and 4). The size of the transcripts is indicated on the left side and was estimated by comparison with a RNA ladder (Fermentas) and ribosomal RNA. (B-I) Expression pattern of *Six3OS* compared with that of *Six3*. No *Six3OS* expression was detected in E8.0 mouse embryos; *Six3* is strongly expressed in the anterior neural ridge at this stage (arrows in B, F). The expression of *Six3OS* starts to be detected at around E8.5 (12 somite stage) in the optic vesicles and ventral forebrain and continues to be expressed in these regions at later stages (C-E). At these same stages, *Six3* is expressed in identical regions (G-I). Arrows indicate the optic vesicles and arrowheads the ventral forebrain.

Fig 3. *Six3OS* is expressed in the developing mouse brain. (A-D) *Six3OS* and *Six3* are expressed in identical regions of the mouse brain at E14.5. However, the expression of *Six3OS* is restricted to the ventricular zone, as compared with that of *Six3* (see hypothalamus (H) or septum (S) in (C, D)). (E-H) At E18.5, *Six3OS* expression remains in the forebrain and tectum (arrowheads); whereas in the tegmentum (arrows) it is no longer detected. (I-K) *Six3OS* expression is restricted to the ventricular zone of E12.5 hypothalamus. (I) *Six3OS* *in situ* hybridization and *Six3* immunohistochemistry using diaminobenzidine as a substrate. The dark purple staining indicates the co-expression of *Six3OS* (blue) and *Six3* (brown). *Six3* co-localizes with the MAP2 positive domain in the mantle zone (K). (J) *Six3* and MAP2 (K) immunofluorescence. GE, ganglionic eminence; VT, ventral thalamus; TC, tectum; S, septum; H, hypothalamus; TG, tegmentum; P, pituitary; MZ, mantle zone; VZ, ventricular zone.

Fig 4.

The expression of *Six3OS* appears to be independent of *Six3*. (A, B) Frontal view of E9.5 mouse embryos. *Six3OS* is expressed in the floor plate of the posterior diencephalon of standard *Six3*-null embryos (arrow in B). (C, E, G) At E14.5, *Six3* and *Six3OS* are co-expressed in the retina and the lens of control embryos. (D, H) In conditional *Six3*-null retina, few if any *Six3* positive cells could be detected in the distal neuroretina; the expression of *Six3* in the lens and the central portion of the retina remains unaffected. No obvious changes of *Six3OS* expression could be detected in conditional *Six3*-null retina (F, H) compared with that in the control retina (E, G). (G, H) *Six3OS* *in situ* hybridization and *Six3* immunohistochemistry using diaminobenzidine as a substrate.