Natural antisense transcripts associated with genes involved in eye development

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Natural antisense transcripts (NATs) are a class of genes whose role in controlling gene expression is becoming more and more relevant. We describe the identification of eight novel mouse NATs associated with transcription factors (Pax6, Pax2, Six3, Six6, Otx2, Crx, Rax and Vax2) that play an important role in eye development and function. These newly identified NATs overlap with the mature processed mRNAs or with the primary unprocessed transcript of their corresponding sense genes, are predicted to represent either protein coding or non-coding RNAs and undergo extensive alternative splicing. Expression studies, by both RT–PCR and RNA in situ hybridization, demonstrate that most of these NATs, similarly to their sense counterparts, display a specific or predominant expression in the retina, particularly at postnatal stages. We found a significant reduction of the expression levels of one of these NATs, Vax2OS (Vax2 opposite strand) in a mouse mutant carrying the inactivation of Vax2, the corresponding sense gene. In addition, we overexpressed another NAT, CrxOS, in mouse adult retina using adeno-associated viral vectors and we observed a significant decrease in the expression levels of the corresponding sense gene, Crx. These results suggest that these transcripts are functionally related to their sense counterparts and may play an important role in regulating the molecular mechanisms that underlie eye development and function in both physiological and pathological conditions.

INTRODUCTION

Eye development is a complex process requiring a precise coordination and timing of morphogenetic and cell differentiation events that eventually lead to the formation of the mature organ. A large number of transcription factors that control, at different hierarchical levels, the proper regulation of the key events underlying eye development have been identified in the past 10–15 years, both in vertebrates and in invertebrates (1,2). The discovery of these molecules made it possible to elucidate some of the molecular mechanisms of this complex biological process. The expression of most of these transcription factors is restricted to the eye, or, at most, it is extended to a few other tissues. Owing to the specialized nature of the eye, a non life-essential organ, mutations in these molecules are tolerated and are responsible for a variety of eye developmental anomalies both in human and in mouse (3).

Although the number of transcription factors known to be involved in eye development has increased significantly, little is known about the transcriptional pathways they regulate or about the control of their expression levels, particularly in vertebrates. The complete sequencing of both the human and the murine genomes provided us with new tools and resources to gain insight into the complexity of gene regulation (4–6). In particular, recent evidence suggests that a relevant fraction of the mammalian transcriptome is composed of transcripts (both coding and non-coding) complementary to other endogenous RNAs, namely either to mature processed sense coding mRNAs or to their primary unprocessed transcripts (7–11). These transcripts are termed natural antisense transcripts (NATs) and can be transcribed in cis from opposing DNA
strands at the same genomic locus or in trans from separate loci, such as in the case of microRNAs (12). The functional role of cis-NATs in regulating gene expression was recognized in recent studies (13–15). Here, we report the identification and characterization of a subset of cis-NATs associated to eight transcription factor genes that are known to play a basic role in eye development in vertebrates. In particular, we determined their genomic structures in both mouse and human and studied their conservation between the two species. The analysis of their expression pattern both by RT–PCR and by RNA in situ hybridization demonstrated that these transcripts are specifically or predominantly expressed in retina. Moreover, we found evidences that these transcripts may be functionally related to their corresponding sense genes as we determined that the expression levels of Vax2OS (Vax2 opposite strand), are altered in the eyes of mice knockout for Vax2 and that CrxOS, is able, when overexpressed, to cause a significant decrease in the expression levels of Crx in vivo.

RESULTS

Identification and genomic characterization of eye transcription factor-related NATs

To identify putative NATs associated with transcription factor genes involved in eye development, we analyzed the UCSC Mouse Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway, February 2003 assembly). The list of genes to analyze (~50) was largely selected from a review by Horsford et al. (2). The genomic loci encompassing the selected genes were retrieved from the Mouse Genome Browser and analyzed for the presence of Expressed Sequence Tags (ESTs) and/or cDNAs with a minimal distance from the currently annotated gene borders not exceeding 20 kb and showing the following features: (a) transcription from the opposite strand with respect to the reference gene and (b) presence of introns. The direction of transcription was determined through the evaluation of the intron-exon splice junctions, which followed the GT-AG rule in all the transcripts described subsequently. As a result, we identified putative NATs for the following genes: Pax6, Six3, Six6, Vax2, Crx, Otx2, Pax2 and Rax, which were termed Pax6OS, Six3OS, Six6OS, Vax2OS, CrxOS, Otx2OS, Pax2OS and RaxOS, respectively (Fig. 1A), whereby OS stands for ‘opposite strand’. The genomic characterization of these transcripts was performed by using both in silico (subsequent rounds of BLAST searches versus EST databases followed by sequence alignment) and experimental procedures (RT–PCR and RACE–PCR). We performed a similar analysis of the human genome by analyzing the UCSC Human Genome Browser (July 2003 release) and found evidence for the presence of five of the identified NATs (CRXOS, OTX2OS, SIX3OS, SIX6OS and PAX6OS, Fig. 1B). A detailed description of all the identified NATs is available in Supplementary Material section. Here, we summarize some of their more remarkable features: (1) almost all undergo extensive alternative splicing, with the number of splice isoforms ranging from 1 (RaxOS) to 11 (Six3OS), (2) all of them show sequence complementarity, either in human or in mouse, to the mature sense mRNA (see red boxes in Fig. 1A) or to their primary unprocessed transcripts, (3) the degree of sequence similarity between murine and human NATs is very low, with the exception of Six6OS (~79% identity), and is in general restricted to segments (exons) not exceeding 150 bp in length for each NAT and (4) five of these NATs do not show any open reading frames (ORF) and may therefore represent non-coding RNA transcripts. Only the Six3OS, the Six6OS and the CrxOS cDNAs are predicted to encode putative protein products. Interestingly, sequence analysis of the murine CrxOS revealed an ORF of 738 bp that is predicted to encode a protein of 246 amino acids (GenBank accession no. XP_133196), characterized by the presence of two putative homeodomains (at position 13–75 and 123–185), as assessed by the SMART, Prosite and Pfam servers. Similar to its putative murine counterpart, the human CRXOS cDNA contains a long ORF, 1524 bp long, which is predicted to encode a protein of 508 amino acids containing a complete homeodomain sequence of the paired type. Remarkably, no sequence similarity could be found between the mouse CrxOS and the human CRXOS, neither at the nucleotide nor at the protein level, even in the homeodomains.

The antisense transcripts identified are predominantly expressed in retina both in human and in mouse

To study the expression pattern of the putative antisense transcripts and to compare it with the expression of the corresponding sense genes, we used reverse transcriptase (RT)–PCR on a panel of mouse and human tissues and RNA in situ hybridization on mouse embryos and tissue sections. We carried out RT–PCR experiments on a panel of mouse adult tissues (Fig. 2A) using primers specific to the eight antisense cDNAs identified in the course of this study (see Supplementary Material, Tables S1 and S2). In parallel, we performed the same analysis using oligonucleotide primers specific for the eight corresponding sense genes to compare the expression pattern of antisense and sense transcripts. We were able to obtain the expected amplification products for all the NATs analyzed with the exception of Pax2OS, probably due to lack of expression in the tissues analyzed. It must be noted that, besides the expected product, in the case of Pax6OS, Six3OS and Six6OS, additional RT–PCR products were obtained. Sequence analysis showed that these extra fragments were specific products derived from alternative splice events. The remaining seven antisense transcripts examined showed the strongest levels of expression in postnatal retina, similar to their corresponding sense genes (Fig. 2A). Expression was restricted to the retina for the Otx2OS, CrxOS, RaxOS, Pax6OS and Vax2OS transcripts, whereas it was detected in retina and brain for the Six3OS cDNA and in retina and skeletal muscle for the Six6OS cDNA. In general, the expression of the antisense cDNAs appeared to be more tissue restricted than that of the sense genes (compare for instance Pax6OS with Pax6). We also studied the expression profiles by RT–PCR of the five human antisense cDNAs identified in the course of this work (SIX3OS, SIX6OS, OTX2OS, CRXOS and PAX6OS) and of their corresponding sense genes. RT–PCR experiments carried out on a panel of 10 adult human RNAs demonstrated that the human antisense cDNAs are also predominantly
expressed in retina (CRXOS, SIX3OS, OTX2OS and PAX6OS) with a lower level of expression in the brain (OTX2OS, SIX3OS, SIX6OS and PAX6OS) or colon (SIX6OS) (Fig. 2B). Similar to its murine counterpart, SIX6OS displayed a high level of expression not only in retina but also in skeletal muscle (Fig. 2B). Overall, although they share no or very little sequence similarity, with the exception of Six6OS, the similarities in the expression pattern and localization suggest that the earlier described human and mouse antisense cDNAs represent homologous genes.

To better define the spatial and temporal profiles of expression in the developing and in the postnatal eye of the identified NATs, we carried out RNA in situ hybridization experiments on mouse whole embryos (E10.5) and sections
We detected the expression in adult retina (postnatal day 30, P30) of Six3OS, Six6OS, Otx2OS, Otx2, Pax6OS, CrxOS and RaxOS (Fig. 3 and data not shown). In general, these transcripts were all expressed at higher levels in the inner nuclear layer (INL) and in the ganglion cell layer (GCL). We found almost complementary patterns of expression for the Otx2–Otx2OS and the Crx–CrxOS pairs, with the sense genes expressed predominantly in the ONL and the corresponding OS genes in the INL and in the GCL (Fig. 3). Expression of the OS transcripts in prenatal stages was only seen in the case of Six3OS, which was detected in the optic cup at E10.5 (Fig. 3A and B) and in the neural retina at E14.5 (Fig. 3C and D) and of Six6OS, which was detected in the neural retina at E14.5 (Fig. 3E and F). No expression above background level was detected in any of the stages analyzed for the Vax2OS, Pax6OS and Pax2OS transcripts (data not shown), suggesting that these transcripts were expressed at lower levels in the stages analyzed.

**Vax2OS is down regulated in Vax2 knockout mice**

To establish whether the expression levels of the eye OS transcripts could be altered following the functional inactivation of the corresponding sense genes, we studied, by Real-Time RT–PCR, Vax2OS expression levels in the developing eye of mice carrying a targeted deletion of the Vax2 gene. We chose this animal model because, differently from knockout mice for the other NAT-related sense genes that are characterized either by complete absence of the eye or by remarkable anomalies affecting one or more retinal cell types (2), Vax2 homozygous mutant mice (Vax2<sup>−/−</sup>) have an almost normal eye structure and integrity of all retinal cell types (16,17). Vax2 inactivation was obtained by deleting the second coding exon of the transcript with a consequent shift of the ORF and the removal of the first two helices and part of the third helix of the homeodomain present in the Vax2 protein. As a result, a Vax2 mRNA is normally
detectable in Vax2−/− mice but is predicted to encode an aberrant protein entirely lacking the homeodomain (16,17).

We compared the expression levels of Vax2OS total RNA from head (E9.5 and E10.5) and from whole eye (E12.5, E13.5, E14.5, E16.5, P0, P6 and adult) between wild-type and Vax22/2 mice by Real-Time RT–PCR. For each developmental stage, the RNA analyzed derived from a pool of animals belonging to the same litter. This study revealed that Vax2OS expression levels were significantly reduced in Vax22/2 with respect to wild-type mice at all stages analyzed (Fig. 4A). The extent of Vax2OS reduction in Vax2−/− mice by Real-Time RT–PCR. For each developmental stage, the RNA analyzed derived from a pool of animals belonging to the same litter. This study revealed that Vax2OS expression levels were significantly reduced in Vax22/2 with respect to wild-type mice at all stages analyzed (Fig. 4A). The extent of Vax2OS reduction in Vax2−/− mice ranged from 2-fold (E10.5 and postnatal eyes) to over 6-fold (E9.5 and E16.5) (Fig. 4B). These findings, which were validated using RNAs derived from different litters, clearly indicate that in Vax2−/− mice there is an alteration in the expression levels of Vax2OS throughout eye development.

Overexpression of CrxOS in mouse postnatal retina is capable of altering the expression levels of the Crx sense transcript

To gain insight into the function of retinal NATs, we overexpressed in mouse adult retina one of the OS transcripts, the murine CrxOS cDNA and then determined Crx cDNA levels by Real-Time RT–PCR. We chose to study this transcript because (1) it is one of the three transcripts predicted to encode a protein product as opposed to the others NATs described here that appear more likely to represent non-coding RNAs and undergo extensive alternative splicing and (2) the Crx gene plays a major role in maintenance of the retina (18) and is expressed at high levels in the postnatal, fully differentiated retina thus facilitating the determination of its expression levels.

We took advantage of recombinant vectors on basis of adeno-associated virus (AAV) that represent an efficient tool for long term in vivo gene delivery (19). Vectors containing an AAV2 genome in an AAV5 capsid (AAV2/5) were previously reported to effectively transduce photoreceptors following subretinal injections (20). We therefore produced an AAV2/5 vector encoding the predicted murine CrxOS protein under the control of the cytomegalovirus (CMV) promoter. A mixture of AAV2/5-CMV-EGFP and AAV2/5-CrvOS was injected subretinally in the right eye of a cohort of C57Bl/6 mice (n = 15) of ~4–6 weeks of age. The contralateral left eye received a subretinal injection of the same viral dose of AAV2/5-CMV-EGFP. Indirect ophthalmoscopic evaluation 14 days after AAV2/5-CMV-EGFP administration allowed us to assess the efficiency of transduction. The eyes of the treated animals were harvested at day 15 and total RNA was extracted separately from each eye. We then carried out quantitative Real-Time RT–PCR experiments on these RNAs to identify differences in the expression levels.
of the Crx murine transcript between the right and the left eye for each animal. We found that Crx expression levels were significantly lower in the right eyes, injected with both the AAV2/5-CMV-CrxOS and the AAV2/5-CMV-EGFP vectors than in the controlateral left eyes (P < 0.001) that had received the AAV2/5-CMV-EGFP construct alone (Fig. 5A).

The average reduction of 26% in the expression levels of Crx in the CrxOS-injected eyes with respect to the controlateral ones was found to be statistically significant when compared with the values observed in the right and left eyes of a population of control, untreated mice (n = 13, P < 0.001, Fig. 5B).

To evaluate whether the gene expression alterations caused by the overexpression of CrxOS were not restricted to the Crx gene only but were more widespread, we sought to determine the expression levels of other genes expressed in photoreceptors in the CrxOS-injected eyes. In particular, we investigated three additional genes: Otx2, a transcription factor involved in photoreceptor cell fate determination (21), rhodopsin (Rho) and rhodopsin kinase (Rkok), two key players of the phototransduction cascade. We found a statistically significant reduction in the expression levels of the Otx2 transcript in the CrxOS-injected eyes when compared with the controlateral eyes (with an average reduction of 25%) whereas we did not find any significant variations in the expression levels of Rho and Rkok (data not shown). Finally, we did not observe any morphological or functional alterations, as assessed by electroretinographic (ERG) analysis, in the retinas of mice injected with CrxOS (data not shown).

Altogether, these results indicate that the overexpression of CrxOS in the postnatal retina of wild-type mice is able to down regulate, either directly or indirectly, the expression of...
the Crx and Otx2 transcription factors and that this effect is specific and not the result of a general depression of transcriptional activity.

DISCUSSION

The interest in the study of NATs in the mammalian transcriptomes has considerably grown in the past few years. Recent reports estimate that the total number of NATs in the human genome may be in the order of a few thousands (8–11). Previous studies have not identified neither particular families of genes preferentially associated with the presence of NATs (7) nor any significant correlation in terms of function, localization and biological process between the two members of the putative sense–antisense pairs found in the genome (8). Here, we focused our attention on the identification of a subgroup of NATs associated with transcription factor genes involved in eye development and function. Our search for eye transcription factor NATs was based on public EST data combined with the analysis of the Human and Murine Genome Browser resources at UCSC. We used very stringent criteria to select NATs, and in particular we chose to consider only ESTs and anonymous cDNAs spanning at least one intron with canonical splice junctions. This approach allowed us to firmly define the direction of transcription, in contrast to the difficulties inherent in establishing the correct orientation of unspliced ESTs and cDNAs. The use of these stringent selection criteria facilitated the identification of bona fide NATs but may have excluded NATs associated with other eye transcription factors.

The eight NATs described here do not appear to be homogeneous in terms of genomic organization and properties.

Figure 5. Crx mRNA levels are decreased in mouse eyes injected with an AAV2/5-CMV-CrxOS as determined by Real-time quantitative PCR analysis. (A) Differences in Crx expression levels between eyes injected with a mixture of AAV2/5-CMV-CrxOS and AAV2/5-CMV-EGFP (black bars) and the controlateral eyes (white bars) injected with AAV2/5-CMV-EGFP alone. x-axis: ID numbers of animals used for the experiment; y-axis: 2^(-ΔCt) values where ΔCt = Ct_CrxOS - Ct_reference mRNA. On average, 2^(-ΔCt) values are significantly lower (P < 0.001 at Student’s T-test) in eyes injected with the CrxOS construct than those in control eyes. The reference mRNA is β-actin for the values shown, which were confirmed in independent experiments using Hprt mRNA as reference. (B) Representation of the average variation in Crx mRNA expression levels between CrxOS-injected eyes (black column) and the controlateral eyes (white column) of treated mice (n = 15) and a collection of right (hatched column) and left (gray column) eyes from a population of untreated mice (n = 13). Crx mRNA expression is reduced on average of ~26% in CrxOS-injected eyes when compared with the corresponding controlateral eyes. This difference is statistically significant (P < 0.001) when compared with the values obtained in the population of control, uninjected eyes. Fold change variations in Crx expression levels are reported as 2^(-ΔCt) values (y-axis). (C) Fluorescence microscopy evaluation of EGFP 15 days after subretinal injection with AAV2/5-CMV-CrxOS and AAV2/5-CMV-EGFP. As previously reported, the AAV-transduced portion of the retina does not exceed 25–30% of the total retina and is mostly represented by the outer nuclear layer and by the retinal pigment epithelium (see magnification on the right). Thin bars, standard errors. Le, lens; Re, retina.
First, five of them (namely Otx2OS, Six3OS, Six6OS, CrxOS and RaxOS) display sequence complementarity to exonic sequences of their corresponding sense genes in either mouse or human (Fig. 1). The remaining three NATs (Vax2OS, Pax6OS and Pax2OS) are complementary only to intronic sequences of their corresponding sense genes (Fig. 1A). Secondly, on the basis of the evaluation of available ESTs as well as on the results of RT–PCR and RACE–PCR experiments, only three of the transcripts (CrxOS, Six3OS and Six6OS) are predicted to encode a putative protein product, whereas the others are more likely to correspond to non-coding RNA transcripts. Finally, most of the NATs described here undergo substantial alternative splicing whose extent seems to be much more significant than in the corresponding sense genes. This observation, which has been also reported for other NATs (22,23), suggests that the control of the levels of expression of different splice isoforms at different developmental stages may be important for the correct function of these transcripts.

We demonstrated by both RT–PCR and RNA in situ hybridization that seven of the NATs described in this paper are expressed predominantly in retina and are therefore coexpressed with their corresponding sense genes. In particular, five of the sense–antisense pairs described here (Six3, Six6, Crx, Otx2 and Rax) are coexpressed in at least one cell layer in adult retina (Fig. 3 and data not shown) suggesting that they may be functionally related. Although we cannot exclude that their expression, at the single cell level, is mutually exclusive, it is very likely that these sense–antisense pairs may form double-stranded RNAs (dsRNA), which may regulate their expression levels, either in the cytoplasm when they share sequence complementarity in exonic regions (24,25) or in the nucleus when they display complementarity only in intron sequences (26). However, it is unlikely that the NATs described here exert their function exclusively through the formation of dsRNAs with their corresponding sense genes, as suggested by the fact that the majority of the NAT splice isoforms do not seem to overlap the corresponding sense transcripts.

At least five of the murine antisense transcripts identified here (CrxOS, Six3OS, Six6OS, Otx2OS and Pax6OS) are conserved in human. However, it must be emphasized that there is a significantly lower number of human ESTs from retinal cDNA libraries, compared with mouse, which limits the identification and characterization of human retinal NATs. In most cases, the conservation was assessed more on the basis of genomic localization and expression pattern than the evaluation of sequence similarity. Overall, with the exception of Six6OS, the degree of sequence similarity of these transcripts between the human and the mouse is very low and high values of identity are restricted to small regions not exceeding 150 bp in size. It may be hypothesized that these small conserved regions may represent the functional domains of these transcripts. Alternatively, the function of these NATs may not be only dependent on their sequence properties but rather on the formation of secondary structures or on their transcription from those particular chromosomal regions with a consequent alteration of the chromatin structure of the adjacent regions.

The significant reduction of Vax2OS in Vax2 KO mice supports the idea that the proper expression of sense–antisense transcript pairs may be coordinated. Interestingly, in Vax2−/− mice, the Vax2 mRNA is still present and seems to be slightly more abundant than that in wild-type animals (data not shown) even if it encodes an aberrant protein completely lacking the homeodomain. It may be hypothesized that the down regulation of Vax2OS may be determined by either a reaction, via a negative feedback mechanism, to the increase in the expression levels of the Vax2 mRNA or alternatively by the absence of the functional Vax2 protein that may be required for the correct expression of Vax2OS. Furthermore, it cannot be excluded that the down regulation of Vax2OS may be determined by the removal of a Vax2OS enhancer localized in the 2 kb genomic fragment deleted in the Vax2 KO mouse. Regardless of the molecular mechanisms, the observation that Vax2OS expression levels are altered in Vax2−/− mice prompt us to investigate the potential role of Vax2OS in Vax2−/− mice. It is conceivable that the phenotype observed in the mutant animals, i.e. eye coloboma and anomalies of the establishment of the correct dorso-ventral axis of the eye (16,17), may be caused not only by the functional inactivation of Vax2 but, at least in part, also by the altered expression of Vax2OS. This hypothesis may be extended also to the other OS genes described in this report and to the interpretation of the previously described mouse knockout mutants generated for the corresponding sense genes (2).

Another indication that these OS transcripts may play a role in eye development derived from the misexpression experiments of the CrxOS transcript. We demonstrated that the AAV-mediated overexpression of CrxOS in mouse photoreceptors is able to interfere with the expression of Crx and Otx2 by decreasing their expression levels of ~25% in the whole eye. This effect did not seem to be the result of a general decrease of transcriptional activity in the CrxOS-injected eyes as no significant variations were observed in the expression levels of four other transcripts analyzed including two housekeeping genes (B-actin and Hprt) and two photoreceptor specific genes (Rho and RhoK). We hypothesize that the extent of Crx and Otx2 down regulation in the AAV-transduced region, which does not exceed 30% of the retina (Fig. 5C), as also previously reported (20), may be higher than that observed when analyzing the whole eye as we did in this report. Interestingly, the possibility that CrxOS could modulate Crx and Otx2 expression levels is also strengthened by the analysis of their expression patterns by RNA in situ hybridization in mouse adult retina. This analysis revealed that CrxOS on one hand and Crx and Otx2 on the other hand showed almost complementary expression patterns with the former predominantly expressed in the interneuron and ganglion nuclear layers and the latter in the photoreceptor nuclear layer (Fig. 3). Otx2 and Crx play major roles in photoreceptor development and maintenance and have been shown to interact as Otx2 was found to be able to transactivate Crx (21). Therefore, the finding of a concomitant variation in the expression levels of these two genes in CrxOS-injected eyes may not be surprising if one takes into account that the two genes are functionally related. Obviously, it is not possible to determine at this stage the molecular mechanisms underlying Crx and Otx2 down regulation by CrxOS overexpression.

The putative CrxOS ORF that was used for the overexpression experiments does not contain any sequence that overlaps
the Crx mature or unprocessed mRNA. Therefore, the mechanism underlying the down regulation of Crx by CrxOS, if direct, is very unlikely to be represented by the formation of dsRNA either in the nucleus or in the cytoplasm. Although they do not share any significant sequence similarity, both the human and the murine CrxOS transcripts are predicted to encode a putative protein characterized by the presence of homeodomain sequences. It is possible to hypothesize that this putative protein product may bind either Crx or Otx2 regulatory sequences (or both) and influence their transcription. On the other hand, it is not possible to exclude that the action of CrxOS in controlling the expression levels of Crx and Otx2 may be exerted primarily at the RNA level or both at the RNA and at the protein levels and further studies are needed to test these hypotheses.

The identification of NATs associated to transcription factors playing an important role in eye development and function may also have implications in the elucidation of the molecular basis of eye diseases. Mutations in all transcription factors reported here have been related to the pathogenesis of eye developmental anomalies either in human or in mouse (2, 3, 16, 21, 27–29). The evidence that the NATs associated with these genes may participate in the regulation of their expression and consequently in the processes underlying eye development suggests these antisense transcripts may also play a role in the pathogenesis of eye developmental diseases.

The role of NATs in the pathogenesis of human diseases was already suggested in the case of the SNURF–SNRPN sense/UBE3A antisense transcription unit in Prader–Willi and Angelman syndromes (30) and clearly demonstrated in an inherited form of α-thalassemia caused by the silencing of the α-globin gene via the generation of an aberrant cis-NAT (31). For all these reasons, we believe that a detailed functional analysis of the NATs described in this report will be instrumental to a more comprehensive understanding of the molecular pathways controlling eye development and function in both physiological and pathological conditions.

MATERIALS AND METHODS

Bioinformatic analysis

To identify NATs associated to eye transcription factor genes, we analyzed the Human (July 2003 assembly) and Mouse (February 2003 assembly) Genome Browser at UCSC (http://genome.ucsc.edu/cgi-bin/hgGateway) using the inclusion criteria described in Results. The genomic regions encompassing the NATs identified here were also retrieved and used as queries in BLASTN analysis (32) against the EST and non-redundant nucleotide databases at NCBI (http://www.ncbi.nlm.nih.gov/BLAST) to identify additional ESTs and/or anonymous cDNAs not yet annotated in the analyzed releases of Genome Browser. All retrieved cDNA sequences were assembled using the contig assembly program (33) at http://bio.ifom-firc.it/ASSEMBLY/assemble.html.

cDNA characterization

To extend the cDNA sequences obtained through the bioinformatic analysis, we carried out RT–PCR on cDNA derived from human and mouse total RNA using oligonucleotide primers specific for the identified NATs. RACE–PCR was performed on mouse 11 day embryo and human retina Marathon-Ready cDNAs (Clontech) following the manufacturer’s instructions.

Expression studies

Embryonic and adult murine tissues were obtained from C57BL/6 wild-type mice. Head and total eye tissues were also obtained from Vax2+/− mice (16) for the quantitative analysis of Vax2OS expression following Vax2 inactivation. Total RNA was extracted using TRIzol extraction kit (Gibco BRL) according to the manufacturer’s instructions. For the expression studies on human tissues, commercial total RNAs BD Biosciences Clontech were used except for the retina RNA that was obtained from the eyes of a 50-year-old male donor through the Eye Bank of Canada. Appropriate ethical approval was obtained for the use of this sample.

The SuperScript First Strand Synthesis System for RT–PCR (Invitrogen) was used to generate cDNA for PCR from 5 μg of total RNA. Identical aliquots of cDNA were used for amplification using gene-specific primers. The primers were designed to span introns. Their sequences, the temperature of annealing and the length of the RT–PCR products are shown in Supplementary Material Table S1 (mouse transcripts) and S2 (human transcripts).

RNA in situ experiments on whole embryos and eye sections were performed as described (34,35) with digoxigenin-labeled antisense RNA probes for Six3 (36), Otx2 (36) and Otx2OS (RIKEN cDNA clone A730014E05). ISH probes for Crx, Six6, CrxOS, RaxOS, Six3OS and Six6OS were obtained by RT–PCR on mouse adult retina cDNA using the oligonucleotides described in Supplementary Material Table S3.

Vector construction and virus production

For construction of pAAV-CrxOS, the entire predicted coding sequences of murine CrxOS was amplified from mouse adult retina cDNA by PCR with the oligonucleotide primers CrxOS-VF (5'-AGTCATGGAAAGCATCTCCACGTTC-3') and CrxOS-VR (5'-AGTCATGGAAAGCATCTCCACGTTC-3'). The obtained product was then cloned into the pAAV2-CMV-EGFP plasmid (20). Recombinant AAV2/5 viruses were produced according to protocols described elsewhere (37). For each viral preparation, physical titers [genome copies per milliliter (GC/ml)] were determined by PCR quantification using Taqman (38). AAV viral vectors were produced by the TIGEM AAV Vector Core.

Fifteen animals were injected subretinally with 2 μl of vectors corresponding to 2 × 109 genome copies (GC). Animals were anesthetized and intraocular injections of virus were performed as described previously (39). The same individual performed all the surgical procedure to minimize variability in injection technique. Indirect ophthalmoscopy to assess EGFP expression in vivo was performed as described elsewhere (20).

All procedures on animals have been approved by the relevant Local Committees for ‘Good animal experimental activities’.
Real-Time PCR analysis

Real-time PCR was carried out with the GeneAmp 7000 Sequence Detection System (Applied Biosystem). The PCR reaction was performed using cDNA, 12.5 μl SYBR Green Master Mix (Applied Biosystem) and 400 nM primer. Water was added to make a total reaction volume of 25 μl. The PCR conditions for all genes were as follows: preheating, 50°C for 2 min and 95°C for 10 min; cycling, 40 cycles of 95°C for 15 s and 60°C for 1 min. Quantification results were expressed in terms of the cycle threshold (Ct). The Ct values were averaged for each triplicate. Both the β-actin and the Hprt genes were used as endogenous controls (reference markers). Differences between the mean Ct values of tested genes and those of reference genes were calculated as ΔCt = Ctgene − Ctreference and represented as 2−ΔΔCt values (Figs 4 and 5).

Relative fold changes in expression levels were determined as 2−ΔΔCt. When we analyzed Crx, Otx2, Rho and RhoK gene expression in injected animals by AAV2/5

Sequence accession numbers

The sequences of all the mouse NATs and related alternatively spliced transcripts described in this report have been deposited in GenBank with the following accession numbers: Vax2OS: AK044313, BK005211, AY589787, AY589788, Pax6OS: BG802265, AK029183, AY589783, AY589784, AY589785, AY589786; Six5OS: NM_175267, CA527570, BE332550, AK053722, AY589790, AY589791, AY589792, AK590888, AY590889, CF729478; Six6OS: NM_029444, AK044729, BK005232, BK005233, AY576418, AK014811; Pax2OS: BK005212; CrxOS: BY736445, AY593966, BB642159, C76788, AY590890, XM_133196, Otx2OS: AK042665, BU504680, BE860198.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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REFERENCES