XSEB4R, a novel RNA-binding protein involved in retinal cell differentiation downstream of bHLH proneural genes

Sébastien Boy[1,*], Jacob Souopgui[2,3,*], Marcos A. Amato[1], Maurice Wegnez[1], Tomas Pieler[2] and Muriel Perron[1,†]

[1]Laboratoire d'Embryologie Moléculaire et Expérimentale, UMR CNRS 8080, Bâtiment 445, Université Paris XI, 91405 Orsay, France
[2]Universität Göttingen, Institut für Biochemie und Molekulare Zellbiologie, Abteilung Entwicklungsbiochemie, Justus von Liebig Weg 11, 37077 Göttingen, Germany.
[3]Department of Biochemistry, Faculty of Science, University of Yaoundé I, PO Box 812, Yaoundé, Cameroon
*These two authors have contributed equally to this work
†Author for correspondence (e-mail: muriel.perron@emex.u-psud.fr)

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Summary

RNA-binding proteins play key roles in the post-transcriptional regulation of gene expression but so far they have not been studied extensively in the context of developmental processes. We report on the molecular cloning and spatio-temporal expression of a novel RNA-binding protein, XSEB4R, which is strongly expressed in the nervous system. This study is focused on the analysis of *Xseb4R* in the context of primary neurogenesis and retinogenesis. To study *Xseb4R* function during eye development, we set up a new protocol allowing in vivo lipofection of antisense morpholino oligonucleotides into the retina. The resulting XSEB4R knockdown causes an impairment of neuronal differentiation, with an increase in the number of glial cells. By contrast, our gain-of-function analysis demonstrates that *Xseb4R* strongly promotes neural differentiation. We also showed a similar function during primary neurogenesis. Consistent with this proneural effect, we found that in the open neural plate *Xseb4R* expression is upregulated by the proneural gene *XNgnr1*, as well as by the differentiation gene *XNeuroD*, but is inhibited by the *Notch/Delta* pathway. Altogether, our results suggest for the first time a proneural effect for a RNA-binding protein involved in the genetic network of retinogenesis.

Key words: Retina, RNA binding proteins, Morpholinos, Proneural genes, *Xenopus*

Introduction

Dissecting genetic cascades responsible for the development of the nervous system has helped to understand some of the molecular mechanisms involved in the genesis of distinct neuronal subtypes. The best-known molecular network implicated in neural cell fate decisions involves proneural and differentiation genes, which encode bHLH and other transcription factors, as well as neurogenic genes belonging to the Notch/Delta signalling pathway (Bertrand et al., 2002). The retina has been used as a model to study the role of these genes during retinal histogenesis because of its simple organisation and the limited number of neuronal types that it contains (Cepko, 1999; Perron and Harris, 2000; Vetter and Brown, 2001). The order of expression of several proneural and neurogenic genes has been studied in the retina, focusing on the proliferative ciliary marginal zone (CMZ). The CMZ is a region at the peripheral edge of the retina where cells are spatially ordered with respect to their development, with stem cells closest to the periphery, retinoblasts in the middle and differentiating precursors at the central edge (Wetts et al., 1989; Dorsky et al., 1995). In the CMZ, neurogenic and proneural genes are activated first in retinoblasts, followed by differentiation genes in differentiating precursors, reflecting a genetic hierarchy among these genes (Perron et al., 1998; Perron et al., 1999b). Overexpression or loss-of-function experiments of differentiation genes in the retina affects retinal cell type distribution (Perron and Harris, 2000; Cepko, 1999). For example, loss of *ath5* (*atoh7* – Zebrafish Information Network; *Atoh7* – Mouse Genome Informatics) function in zebrafish and mouse prevents the differentiation of ganglion cells. Conversely, overexpression experiments show that *ath5* promotes ganglion cell production at the expense of other cell types in *Xenopus*. It has therefore been proposed that *ath5* is essential for retinal ganglion cell differentiation (Kanekar et al., 1997; Kay et al., 2001; Morrow et al., 1999; Brown et al., 2001). Recently, several lines of evidence have converged to propose that a combinatorial code of bHLH and homeobox proteins is responsible for the specification of the correct neuronal subtypes. For example, co-expression of the mouse bHLH genes *Math3* (*Neurod4* – Mouse Genome Informatics) and *NeuroD* (*Neurod1* – Mouse Genome Informatics) with the homeobox genes *Pax6* or *Six3* significantly increases their ability to promote amacrine cell genesis (Inoue et al., 2002). As the various retinal cell types are born in a sequential order, it has also been proposed that the Notch/Delta pathway could

generate cell diversity by controlling when a cell is released from lateral inhibition (Dorsky et al., 1995; Dorsky et al., 1997; Perron and Harris, 2000). If a neuroblast is released during early retinogenesis, it gives rise to an early born retinal cell type, whereas if it is released during late retinogenesis it gives rise to a late born retinal cell type.

Interactions among neurogenic, proneural and differentiation genes have been extensively studied during primary neurogenesis in *Xenopus* allowing the establishment of a genetic cascade (Ferreiro et al., 1993; Turner and Weintraub, 1994; Bellefroid et al., 1996; Ma et al., 1996; Chitnis and Kintner, 1996; Perron et al., 1999b). These interactions encompass mainly transcriptional regulation. However, this genetic network probably requires other levels of gene regulation. For instance, it has recently been found that XNeuroD function during primary neurogenesis and retinogenesis can be inhibited by glycogen synthase kinase 3β (Marcus et al., 1998; Moore et al., 2002). This post-translational phosphorylation regulation is crucial for the proper function of XNeuroD (Moore et al., 2002). Post-transcriptional regulation at the mRNA level, involving RNA binding proteins, is also known to play a key role in gene regulation (Burd and Dreyfuss, 1994; Perrone-Bizzozero and Bolognani, 2002). Once mRNAs are transcribed, RNA-binding proteins can control all subsequent maturation steps from splicing and translation, to mRNA transport and stability (Harford and Morris, 1997). According to the motif contained in RNA-binding proteins, one can distinguish several families. The largest family of RNA-binding proteins is characterised by the presence of RNA recognition motifs (RRM), domains composed of 90-100 amino acids that are only moderately conserved with two consensus sequences (an octamer and a hexamer sequence called RNP1 and RNP2, respectively). The number of RRMs per protein varies from one to four (Burd and Dreyfuss, 1994). Recent advances in the analysis of several RNA-binding proteins during development have increased the perspectives in this developmental biology field.

In the nervous system, a large number of genes are regulated post-transcriptionally via the interaction of their mRNAs with specific RNA-binding proteins. At present, we know several RNA-binding proteins involved in the development and plasticity of the central nervous system (CNS). However, little is known about their precise role and their RNA targets. During neurogenesis for example, the Staufen protein mediates *prospero* mRNA localisation, which is important for neuroblast asymmetric division in early *Drosophila* embryogenesis (Matsuzaki et al., 1998). In mammals, the two homologues of Staufen (Stau1 and Stau2) are involved in mRNA transport in dendrites, and they also interact with ribosomes, suggesting an additional role in translation regulation. However, vertebrate RNA targets of Stau1 and Stau2 have not yet been identified (Duchaine et al., 2002; Kiebler and DesGroseillers, 2000). Another conserved RNA-binding protein family is the Musashi family. In mammals, two members, Musashi1 (Msi1) and Musashi2 (Msi2), are expressed in neural precursor cells. Antisense ablation experiments suggest that Msi1 and Msi2 are cooperatively involved in the proliferation and maintenance of CNS stem cell population (Sakakibara et al., 2002). Concerning the targets of these genes, Msi1 represses the translation of Numb, an antagonist of Notch (Okabe et al., 2001), and it has recently been suggested that Msi1 also mediates the post-transcriptional regulation of the microtubule-associated protein Tau (Cuadrado et al., 2002). The ELAV/Hu proteins belong to a RNA-binding protein family largely conserved across species. In vertebrates, most members are neuron specific, and have been shown to be essential for nervous system development and function through the regulation of the stability of their mRNA targets, including GAP43, Tau or MYCN (Beckel-Mitchener et al., 2002; Aranda-Abreu et al., 1999; Manohar et al., 2002; Perrone-Bizzozero and Bolognani, 2002). These examples emphasise the important role of post-transcriptional factors during neurogenesis.

With the aim of advancing our knowledge of the genetic network involved in retinal cell fate determination, we have characterised a novel RNA-binding protein and we have studied its function during retinogenesis. We present the cloning and spatio-temporal expression of *Xseb4R*, which encodes a putative RNA-binding protein containing a single RRM. A related gene, *Xseb4*, has been previously isolated in *Xenopus* (Fetka et al., 2000). While *Xseb4* is mainly expressed in muscles, *Xseb4R* is strongly expressed in neural tissues. We show here that overexpression of *Xseb4R* during primary neurogenesis or in the retina has a proneural effect. Blocking *Xseb4R* function using morpholino oligonucleotides leads to the opposite effect. Using classical overexpression experiments in the early *Xenopus* embryo, we demonstrate that *Xseb4R* is responsive to *neurogenin*, *NeuroD* and the Notch/Delta signal transcription cascade. In the *Xenopus* nervous system, several RNA-binding proteins have been identified previously but their functions remain elusive (Good et al., 1993; Gerber et al., 1999; Perron et al., 1999a). Our present data suggest that the RNA-binding protein XSEB4R has a proneural function during *Xenopus* neurogenesis.

Materials and methods

Cloning of *Xseb4R*

Large-scale whole-mount in situ hybridisation was performed for screening a tadpole head (ZAP express phage) cDNA library, as described by Souopgui et al. (Souopgui et al., 2002). Briefly, single recombinant phages were eluted in 96-well microplates. Fluorescein-labelled antisense RNA probes were transcribed from templates obtained by PCR amplification of cDNA inserts for these single phages. Four sets of flat-bottom 24-well devices for simultaneous whole-mount in situ hybridisation were used per round of screening. cDNA clones with an interesting expression pattern were sequenced and matched with the DDBJ/EMBL/GenBank sequence information. GenBank Accession Number: AY289193.

Oligonucleotides and mRNA

Two antisense morpholino oligonucleotides (Mo) against *Xseb4R* were designed (sequences complementary to AUG are underlined), Mo1 (GTG<u>CAT</u>GGTCACAGGCAAATTCACC) and Mo2 (starting 2 nucleotides after AUG; AAAGTTGTGTCTTTTTGCACGGTGT), as well as a Mo against GFP cloned into the pCS2 plasmid (TCCTTTACT<u>CAT</u>GGTGGATCCTGCA). The standard control morpholino (cMo: CCTCTTACCTCAGTTACAATTTATA) was used as a control (Genetools). Two kinds of Mo have been used: crude Mo (Mo1 and cMo) for blastomere injections and Special Delivery Mo (Mo1, Mo2, MoGFP and cMo), where the non-ionic crude morpholinos are paired to a complementary 'carrier' DNA in order to be transfected (Morcos, 2001; Ohnuma et al., 2002).

Plasmid construction

The full-length *Xseb4R* cDNA was cloned into the pCMV vector, and the open reading frame (ORF) subcloned into the pCS2 vector. The flag-tagged version was engineered by subcloning the ORF into the pCS2-Flag vector. Another construct, called *Xseb4R*-5′UTR, has been subcloned into pCS2. This construct contains the ORF as well as the region of the 5′UTR complementary to *Xseb4R* Mo1. *Xseb4R-GR* was generated by subcloning the ORF into pCS2-GR, a vector initially generated by inserting the GR coding sequence into the *Xho*I and *Xba*I sites within the multiple cloning sites of the pCS2 vector.

In vitro RNA synthesis and microinjection

Capped *Xseb4R, Xngn1* (a gift from C. Kintner), *XneuroD* (a gift from E. Bellefroid), *XNotch ICD* (a gift from E. Bellefroid), *Xseb4* (a gift from R. Rupp) and *NLS lacZ* RNAs were prepared from CS2 plasmids after *Not*1 linearisation using mMessage mMachine kit (Ambion). RNAs were injected in a volume of 5 nl, at a concentration of 50 pg/nl (when not notified), into a single blastomere of embryos at the two-cell stage. 5-20 ng of Mo1 or control Mo were injected into a single blastomere of embryos at the two-cell stage. *lacZ* mRNA was co-injected as a marker. Histochemical staining for β-galactosidase activity was performed to visualise the distribution of the co-injected *lacZ* mRNA. Embryos were collected at the neurula stage and subjected to in situ hybridisation as described below. *Xseb4R-GR*-injected embryos were continuously treated, or not, from the stages indicated in the results with 10 μM, final concentration, of dexamethasone.

RT-PCR

The Qiagen Rneasy mini kit was used for RNA isolation from oocytes or embryos of different developmental stages. All RNA preparations were treated with DNase I (Qiagen) and checked with 32 cycles of histone H4-specific PCR (Niehrs et al., 1994) for DNA contamination. RT-PCR was carried out using the Gene Amp RNA PCR kit (Perkin-Elmer). The following primers, annealing temperatures and cycle numbers were used:

histone H4, forward (F) 5′-CGGGATAACATTCAGGGTATCACT-3′, reverse (R) 5′-ATCCATGGCGGTAACTGTCTTCCT-3′, 58°C, 25 cycles; and

Xseb4R, (F) 5′-GGAACCTGCAGAGCGCATTTACTA-3′, (R) 5′-GTCAGGCTGGAGCTGTTGAGGCTG-3′, 60°C, 33 cycles.

PCR products were separated on 2% agarose gels.

In situ hybridisation

Digoxigenin (DIG)-labeled antisense RNA probes were generated for *Xseb4R* according to the protocol of the manufacturer (Roche). For analysis of expression in the whole embryo during development, in situ hybridisation was performed as previously reported (Souopgui et al., 2002). For analysis of expression in the retina, whole-mount in situ hybridisation was performed as described previously (Shimamura et al., 1994), apart from that embryos were bleached (Broadbent and Read, 1999) just before the proteinase K step. After NBT/BCIP (Roche) staining, embryos were then vibratome sectioned (50 μm).

BrdU staining

BrdU was injected intra-abdominally, and the animals were allowed to recover for 2-8 hours post-injection. BrdU was detected using the BrdU labeling kit (Roche) after a 45-minute treatment in 2N HCl. For double staining, the mRNA was first detected by whole-mount in situ hybridisation (as described above). Embryos were then cryostat sectioned and BrdU immuno-stained.

Immunohistochemistry

Immunohistochemistry was performed on 4% paraformaldehyde-fixed tissues. Cryostat sections (12 μm thick) were incubated with primary antibodies, anti-Islet1 (a gift from S. Thor), anti-Flag (Stratagene), anti-CD2 (Serotec) or anti-BrdU (Roche), and visualised using anti-mouse fluorescent secondary antibodies (Alexa, Molecular Probes). To visualise the nuclei, sections were incubated for 5 minutes in Hoechst solution (10 μg/ml) and washed three times in PBS.

In vivo lipofection

DNA was transfected into the presumptive region of the retina of stage 18 embryos, as previously described (Holt et al., 1990; Ohnuma et al., 2002). Mo (10 ng) were similarly transfected. Embryos were fixed at stage 41 and cryostat sectioned (12 μm). GFP-positive cells were counted and cell types were identified based upon their laminar position and morphology, as previously described (Dorsky et al., 1995).

Anti-XSEB4R polyclonal antibody

Polyclonal antibodies against XSEB4R have been raised by Eurogentec. This antibody is directed against the N-terminal peptide of XSEB4R: HTVQKDTTFT.

Mo microinjection, embryo extracts and western blotting

Capped synthetic *Xseb4R* mRNA containing the part of the 5′UTR against which Mo1 is directed was prepared from pCS2 plasmids after *Not*1 linearisation using mMessage mMachine kit (Ambion). 500 pg of this RNA and 5 ng of Mo (Mo1 or Control Mo) were injected into both blastomeres of embryos at the two-cell stage. Embryos were harvested at stage 10, frozen in liquid nitrogen and stored at –80°C until further analysis. For preparation of extracts, frozen embryos were homogenised in 10 μl of extraction buffer (50 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, pH 7.3) with proteases inhibitor cocktail (complete Mini, Roche), centrifuged for 15 minutes, and the supernatants collected. Proteins were then separated on 4% stacking and 10% resolving SDS-polyacrylamide gels (PAGE), as described by Laemmli (Laemmli, 1970). The separated polypeptides were electrophoretically transferred from gels to nitrocellulose membranes and processed for immunoblotting. Blots were then incubated for two hours with the primary antibody against XSEB4R, diluted 1:100 in 10% dried skimmed milk in Tris-buffered saline-Tween buffer [TBST: 1.37 M NaCl, 0.2 M Tris (pH 7.5), 1% Tween-20]. The peroxidase conjugated anti-rabbit (Vector) was used as secondary antibody at a dilution of 1:5000 in 5% dried skimmed milk in TBST buffer, for a two hour incubation. Blots were developed using the chemoluminescence kit (Amersham) and the reactivity was visualised on hyperfilm ECL (Amersham).

Apoptosis detection

Apoptotic cells were detected by TUNEL methods using the ‘In situ cell death detection kit, TMR red’ (Roche) on 12 μm cryostat sections of stage 34-41 lipofected embryos.

Results

Xseb4R cloning and predicted protein structure

During embryogenesis in *Xenopus*, XDelta1, XNotch1 and its downstream targets define a synexpression group (Niehrs and Pollet, 1999). Their specific expression pattern in the form of stripes in the open neural plate (Coffman et al., 1990; Chitnis et al., 1995; Koyano-Nakagawa et al., 1999; Lamar et al., 2001) offers a reliable criterion to search for novel candidate genes belonging to the Notch signalling pathway. The clone JS124 was identified using this strategy in a large random expression pattern screen of a tadpole head cDNA library by whole-mount in situ hybridisation (Souopgui et al., 2002). Nucleotide sequence analysis revealed that it encodes a *Xenopus* homologue of the vertebrate and invertebrate RNA-binding protein SEB4. It is clearly distinct from the previously known

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                                                    RNP2                                              RNP1
xSEB4R   M.........................HTVQKDTTFTKIFVGGLPYHTTDASLRKYFEVFGDIDEAVVITDRQTAKSRGYGF
 mSEB4   -...LLQPACSPSVFPRPSAAPSAM-GSR-----------------------------G----E----------G------
hSEB4B   -...Q.........YNRRFVNVVPTFGKK-G---------------------------G----E----------G------
 C.e-X   -YGQVQDPLVHAAAAALAQSTNAEPVVGSR--M------------S-KT-HE---Q----E--------N-Q------
 xSEB4   -.........................--T-----Y--------------S------------E----------G------

xSEB4R   VTMSDRAAAERACKDPNPIIDGRKANVNLAYLGAKPRNLQSAFTIGVQQLH.......PAFIQRPFGLTPQYIYPPAIVQ
 mSEB4   ---A-----D--------------------------S--TG-AV------.......-TL---TY----H---------
hSEB4B   ---A-------------------------------WC--TG-A-------.......-TL---TY----H---------
 C.e-X   ---K---S----------------------------TNVQLAALAAG-VQLPLTTQLQ-LF-PRM--PQM-.--TVT..
 xSEB4   ---A--------------------------------IM-PG-AF----I-.......--LV---Y-IPAH-V--Q-Y--

                                             CD1
                                        <------------------------->
xSEBR    PSMVIP...TPIPSLQSPYIDYNAATQAYTHYTTAA...YEQYPYAASPATG..YM....GYGY..TSPVQQPLSTTTGA
 mSEB4   --V---..A--V---S---LE-TP-SP--AQ-PP-T...-D---------A-TSFV....----..PAA-P-A--AAA.P
hSEB4B   --V---..AA-V---S----E-TP-SPV-AQ-PP-T...-D----------ADSFV....----..PAA-H-A--AAA.P
 C.e-X   .GL-N-LMAQQGAAA-QQLF----LAAVMNPQAA--YGIQS-GKF--A-T-IEQ-AAYPA---LVPQ-AYNIGNPQLQQL
 xSEB4   QGL---HVQQTAAAST------T..SA--AQ-AA--AAA-D----------.TG-VT.AA----AVPQ-LTAATPG-AA-

                CD2
         <---------------->    Amino acids   overall similarity (%)   RRM similarity (%)
xSEB4R   PPTAYIQYQPQQLQPDRMQ       214                 -                       -
 mSEB4   AG-TFV---AP--------       238                71                      99
hSEB4B   AG-TFV---AP--------       229                70                      99
 C.e-X   AAAQ......---EHQ-V.       249                44                      93
 xSEB4   AAA-FA--------A----       224                73                      98
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Fig. 1. XSEB4R is an RRM-type protein. Alignment of XSEB4R (Accession Number AY289193) with its homologues in mouse (mSEB4, Accession Number NP062420), human (hSEB4B, Accession Number CAA53064) and *C. elegans* (C. e-X, Accession Number T33034), and with the *Xenopus* muscle specific XSEB4 protein (Accession Number AF223427). Dashes represent identical amino acids; dots represent gaps. The RNA recognition motif (RRM) is shaded in grey and the characteristic RNP consensus motifs (RNP1 and RNP2) are boxed. Two additional conserved domains (CD1 and CD2) are indicated. Sequence comparison is indicated as percentage (%) of amino acid similarity.

XSEB4 (Fetka et al., 2000), and closely related to murine SEB4, human SEB4B, and to a *Caenorhabditis elegans* hypothetical protein (Fig. 1). To distinguish the two SEB4 genes identified in *Xenopus laevis*, we designate the new gene as XSEB4R ('R' for related). XSEB4R is one of the few members of the RRM protein family, containing only one RRM located at the N terminus. The vertebrate SEB4 protein also shows two other conserved domains in the C-terminal portion (Fig. 1).

Xseb4R expression during development

The role of a given gene during development is reflected in its specific spatio-temporal pattern of expression. To investigate what role *Xseb4R* plays during *Xenopus* development, we examined the tissue distribution of its transcripts by RT-PCR and whole-mount in situ hybridisation techniques at different stages of development. The first method revealed the presence of *Xseb4R* transcripts at all stages from the oocyte (indicating a maternal expression of this gene) to late tadpole stage (not shown). However, by whole-mount in situ hybridisation, *Xseb4R* transcripts are first detected at stage 10.5, broadly around the blastopore (Fig. 2A). This discrepancy probably comes from a difference in sensitivity of the techniques. The

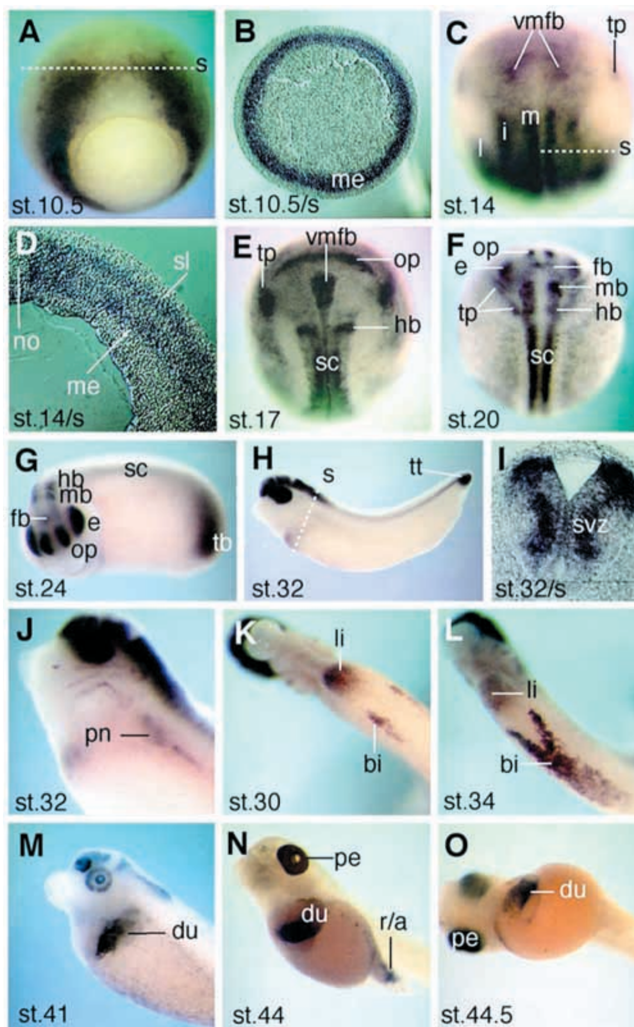


Fig. 2. *XSeb4R* is strongly expressed in the developing central nervous system. (A) Embryo (stage 10.5) presented in a posterior view, with dorsal up, shows a ring-like expression around the blastopore. (B) Transverse section (as indicated in A) showing *Xseb4R* in the mesoderm (me). (C) Embryo (stage 14) presented dorsally, with anterior up, revealing three bilateral stripes, medial (m), intermediate (i) and lateral (l), of *Xseb4R* expression in the open neural plate, as well as expression in the trigeminal placode (tp) and in the presumptive ventral midbrain/forebrain (vmfb) area. (D) Transverse section (as indicated in C) showing *Xseb4R* signals in both the sensorial layer (sl) of the neuro-ectoderm and in the mesoderm (me) as well as the notochord (no). (E,F) Embryos (stage 17 and 20, respectively) presented dorsally, with anterior up, showing additional *Xseb4R* expression in the olfactory placode (op), eye (e), forebrain (fb), midbrain (mb), hindbrain (hb) and spinal cord (sc). (G,H) Embryos (stage 24 and 32, respectively) placed laterally with anterior left showing *Xseb4R* expression in the tail bud (tb) and tail tip (tt). (I) Transverse section (as indicated in H) showing *Xseb4R* signal in the subventricular zone (svz) of the neural tube. (J) Lateral view showing a weak expression of *Xseb4R* expression in the developing pronephros (pn). (K) Two bilateral stripes of *Xseb4R* expression in cells associated with blood islands (bi), and a strong signal in the liver (li). (L) Expression during the characteristic Y-shape formation of blood islands. (M) *Xseb4R*-expressing cells in the area associated with duodenum (du) formation. (N,O) Embryos presented in the ventral view showing pigmented eye (pe) and *Xseb4R* expression in the presumptive rectum/anus (r/a).

expression of *Xseb4R* before stage 10.5 must be too low to be detectable by in situ hybridisation. At this stage, *Xseb4R* expression is exclusively localised in the mesoderm (Fig. 2B). As development proceeds, *Xseb4R*-expressing cells arise in three bilateral longitudinal stripes lateral to the dorsal midline within the open neural plate (Fig. 2C). Transverse sections of embryos from developmental stage 14 show a double layer of *Xseb4R*-positive cells, one corresponding to the mesoderm and the other one to the sensorial layer of the ectoderm where primary neurons are born (Fig. 2D). As neurogenesis proceeds, the same pattern of expression is maintained, as the lateral stripes of *Xseb4R*-expressing cells converge towards the dorsal midline during the process of neural tube folding (Fig. 2E). At embryonic stage 20, *Xseb4R* expression clearly follows the formation of the central nervous system (CNS), including the expression in the area designated to form the eye, olfactory placodes, forebrain, midbrain, hindbrain and spinal cord (Fig. 2F). This correlation of *Xseb4R* expression and CNS formation becomes more evident from tail bud stage 24 onwards. At this stage, *Xseb4R*, like many members of Delta/Notch pathway, is further expressed in the tail bud (Fig. 2G). In swimming tadpole stage 32 embryos, *Xseb4R* is strongly expressed in the CNS, and particularly in the subventricular zone of the neural tube, an area containing specified neuroblasts (Fig. 2I).

The expression of *Xseb4R* in the other germ layers is quite dynamic. As mesodermal derivatives, *Xseb4R*-expressing cells are found in a group of cells associated with the pronephros and the blood islands (Fig. 2J,L). *Xseb4R* expression is also detected in endodermally derived structures, i.e. in the liver progenitors (Fig. 2K). As development proceeds, *Xseb4R* expression fades out from the developing liver but is observed in an area associated with the duodenum (Fig. 2M,O), and in a more posterior group of cells that may contribute to rectum/anus formation (Fig. 2N). This expression is lost at around stage 44.5 (Fig. 2O). Beyond embryonic stage 46, no *Xseb4R* signal is detected in the endodermally derived structures (data not shown).

Expression of *Xseb4R* in the developing neural retina

To better study *Xseb4R* expression during retinal development, we examined sectioned embryos after whole-mount in situ hybridisation and analysed more carefully its expression in the developing eye. At stages 28 and 32, when most cells in the optic vesicle are proliferating, *Xseb4R* expression is distributed throughout the neural retina (Fig. 3A,B). From stage 34 onwards, *Xseb4R* expression is no longer observed in the central retina, where neurons start to differentiate. It rather becomes restricted to the margins, where retinoblasts continue to proliferate, and to the lens (Fig. 3C). At stage 40, when all cells in the central retina are postmitotic, *Xseb4R* expression is observed in the ciliary marginal zone (CMZ), the only region of the retina where retinogenesis is still occurring (Fig. 3D). *Xseb4R* expression is, however, not detected in the most peripheral region of the CMZ (Fig. 3D), where stem cells are present (Dorsky et al., 1995).

In order to determine whether *Xseb4R* is only expressed in proliferating precursors, we performed in situ hybridisation experiments combined with anti-BrdU immunohistochemistry. In the CMZ, *Xseb4R* is not expressed in stem cells but is expressed in proliferating cells in the middle region of the CMZ (Fig. 3E,G). In addition, some expression of *Xseb4R* is detected in a few postmitotic cells in the most central region of the CMZ (Fig. 3G). This expression pattern in the CMZ is very similar to that of neurogenic and proneural genes (Perron et al., 1998; Perron et al., 1999b).

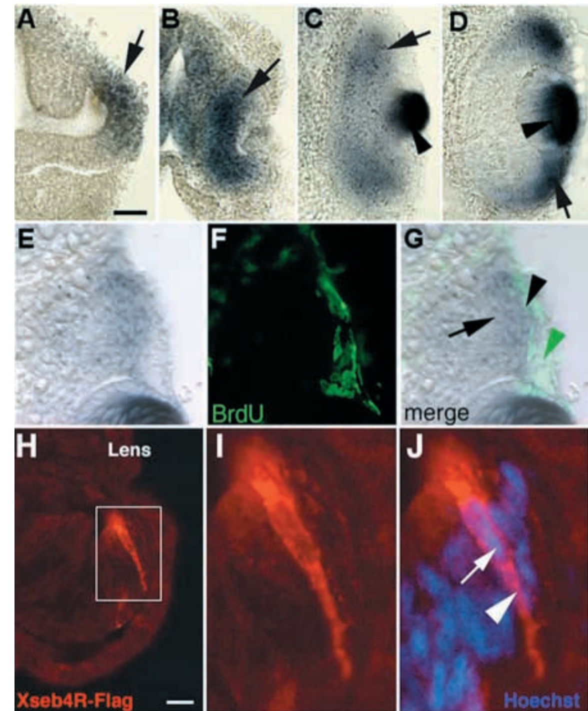


Fig. 3. In the retina, *Xseb4R* is expressed in retinoblasts and undifferentiated postmitotic neurons. (A-D) In situ hybridisation showing the spatio-temporal expression pattern of *Xseb4R* on retinal sections at various developmental stages. (A) At stage 28, *Xseb4R* is expressed in the whole presumptive neural retina that contains dividing retinoblasts (arrow). (B) At stage 32, *Xseb4R* is expressed in the whole neural retina containing mainly dividing precursor cells (arrow). (C) At stage 34, *Xseb4R* is most strongly expressed in the lens (arrowhead) and in the peripheral region of the neural retina containing dividing precursors (arrow). (D) At stage 37, *Xseb4R* expression is restricted to the CMZ (arrow) and the lens (arrowhead). (E-G) Staining for BrdU uptake (green; F) and *Xseb4R* expression (blue; E) at stage 39. Double staining (G) shows that BrdU-positive cells in the peripheral CMZ are *Xseb4R* negative (green arrowhead). In the central CMZ, BrdU-positive cells also express *Xseb4R* (black arrowhead). Some cells are BrdU negative and stained with *Xseb4R* (arrow). Scale bar in A: 50 μm. (H-J) Retinoblasts of stage 34 embryos were transfected at stage 18 with *Xseb4R*-Flag DNA. On sections, immunostaining was performed with an anti-FLAG antibody revealing the presence of XSEB4R-FLAG (red; H). (I) Higher magnification of this retinoblast. Hoescht staining was performed to visualise the nucleus. (J) Double staining showing that XSEB4R-FLAG is mainly cytoplasmic. The nucleus (arrow) and cytoplasm (arrowhead) are indicated. Scale bar in H: 30 μm.

As a RNA-binding protein can play a role in the nucleus, in the cytoplasm, or in both, we wanted to determine the subcellular localisation of XSEB4R protein. With this aim, we constructed a Flag epitope-tagged form of XSEB4R (XSEB4R-FLAG). We co-transfected this construct into retinoblasts at stage 18 using the in vivo lipofection technique (Holt et al., 1990). We then analysed its subcellular localisation

by immunostaining with an anti-Flag antibody in the retina. We found that XSEB4R-FLAG is concentrated in the cytoplasm of retinoblasts (cells where the endogenous gene is expressed, see above), whereas only a faint staining is detected in the nucleus (n=177 examined cells; Fig. 3H,J). The subcellular localisation of XSEB4R-FLAG thus suggests that XSEB4R is involved in RNA metabolism regulation at a cytoplasmic level.

Targeted expression of *Xseb4R* in retinal progenitor cells promotes early differentiation

During retinal neurogenesis, the different cell types of the retina are born in a sequence that is conserved across species. Retinal ganglion cells are born first, bipolar cells and Müller glial cells last (Holt et al., 1988). As *Xseb4R* is expressed coincidentally with bHLH genes involved in retinoblast determination and differentiation, it may also play an important role in regulating the determination/differentiation of these cells. To test this hypothesis, we misexpressed *Xseb4R* in the developing retina by in vivo lipofection of *Xseb4R* DNA into the optic vesicles of stage 18 embryos. GFP DNA was co-transfected, allowing identification of transfected cells in stage 41 retina, when most cells in the central retina are postmitotic and fully differentiated (Holt et al., 1988). The analysis of retinal sections transfected with *Xseb4R* and GFP shows that GFP-positive cells are present mainly in the ganglion cell layer and in the photoreceptor layer, while very few positive cells are formed in the inner nuclear layer (Fig. 4B). This is very different from a control retina transfected only with GFP, where inner nuclear layer cells are the most represented cells (Fig. 4A).

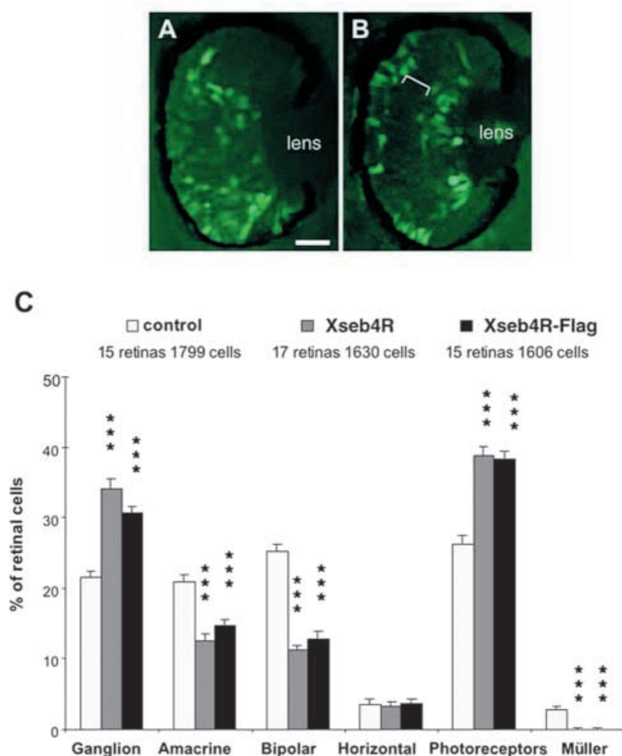


Fig. 4. *Xseb4R* overexpression promotes early differentiation of retinal cells. (A,B) Typical sections of retinas co-transfected with *GFP* plus a control plasmid *pCS2* (A), or *GFP* plus *Xseb4R* (B). The white bracket in B indicates the inner nuclear layer, where very few *Xseb4R* transfected cells are present compared with the control. (C) Percentage of retinal cell types observed in retinas co-lipofected with *GFP* plus *pCS2*, *GFP* plus *Xseb4R*, or *GFP* plus *Xseb4R-flag*. Statistical analysis was performed using the Student's t-test. ***, P<0.0001. Scale bar in A: 50 μm.

We wondered whether the decrease of cells in the inner nuclear layer (amacrine, bipolar, horizontal and Müller cells) after overexpression of *Xseb4R* was due to massive apoptotic cellular death. To test this hypothesis, we performed a TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) assay at various developmental stages (34, 38 and 41) and counted the number of apoptotic cells. At stage 34, we counted apoptotic cells in the whole neuroepithelium and found an average of 3.8 apoptotic cells per control retina (n=32 retinas) and 3.7 apoptotic cells per retina transfected with *Xseb4R* (n=34 retinas). At stage 38 and 41, where cells are organised in layers, we counted apoptotic cells in each of the three cell layers and calculated the percentage of apoptotic cells in the inner nuclear layer. We found that 56% (n=308 cells in 15 retinas) or 49% (n=65 cells in 4 retinas) of apoptotic cells in control retinas reside in the inner nuclear layer compared with 55% (n=578 cells in 18 retinas) or 48% (n=60 cells in 4 retinas) in retinas transfected with *Xseb4R* at stage 38 or 41, respectively. Therefore this suggests that there is no significant increase in apoptosis following *Xseb4R* transfection in cells in the inner nuclear layer.

Another hypothesis is that cells supposed to be in this layer have changed their cellular fate in favor of ganglion or photoreceptor cells. To analyse this hypothesis quantitatively, we counted the different types of cells transfected with *Xseb4R*. We indeed found that overexpression of *Xseb4R* leads to a significant increase of ganglion cells and photoreceptors at the expense of amacrine, bipolar and Müller cells (Fig. 4C). We confirmed that *Xseb4R* transfected cells observed in the ganglion cell layer are indeed differentiated ganglion cells by staining with an anti-islet1 antibody (a ganglion cell marker, data not shown). We found that the number of horizontal cells also had a tendency to decrease but this effect was rarely significant (probably due to the low number of horizontal cells) and highly variable from one experiment to another. Nevertheless, our results suggest that overexpression of *Xseb4R* leads to a proneural-like effect, pushing progenitor cells to differentiate prematurely as ganglion or photoreceptor cells at the expense of late born cells (bipolar and Müller cells). We found exactly the same phenotype when we overexpressed *Xseb4R-Flag* (Fig. 4C), which demonstrates that the FLAG epitope does not alter the function of the XSEB4R protein, which strengthens the subcellular localisation of XSEB4R-FLAG (see above). *Xath3* is a bHLH gene that leads to a very similar effect when lipofected in the retina (Perron et al., 1999b). To compare the strength of their effects, we lipofected side-by-side *Xath3* and *Xseb4R* in the same batch of embryos. *Xath3* and *Xseb4R* both increase photoreceptors and ganglion cells by the same magnitude (data not shown). The XSEB4R homologue, XSEB4, shows a high degree of similarity to XSEB4R (Fig. 1). We therefore wondered whether the specificity of XSEB4R arises from its expression in neural tissue (*Xseb4* being mostly expressed in muscle) or from protein-functional differences. We therefore lipofected side-by-side *Xseb4R* and *Xseb4*. We found that these two genes cause the same effects in the retina (data not shown),

suggesting that XSEB4, at least when overexpressed, can interact with the same targets than XSEB4R.

Experimental set up of in vivo lipofection using morpholino oligonucleotides

To further characterise XSEB4R function during retinogenesis, we tried to block its function during retinogenesis. We therefore set up a new protocol to transfect morpholinos (Mo) into retinoblasts in vivo. Mo are antisense oligonucleotides that block the translation of a target gene with a high specificity (Ekker and Larson, 2001; Heasman, 2002). Recently we have shown that Mo can be efficiently lipofected in retinoblasts and that they do not interfere with retinogenesis under a certain threshold (Ohnuma et al., 2002). In order to determine whether lipofected Mo could indeed block the translation of a target gene, we first tested this technique with Mo directed against the mRNA encoding GFP (GFP Mo). We therefore co-lipofected GFP Mo, or a standard control Mo, plus a GFP plasmid and then analysed the intensity of the GFP fluorescence in the retina. To prevent any subjectivity in the analysis, we added a filter that decreased the fluorescence light of the microscope, implying that low fluorescent cells would be below the detectable threshold. As a positive control, we also co-transfected a plasmid encoding the CD2 protein. CD2 is a membrane protein (Brown et al., 1987) for which a very good antibody is available. We then analysed GFP fluorescence among lipofected retinas (CD2 positive). If the GFP intensity is low (below the detectable threshold), then cells would be only CD2 positive, whereas if the GFP intensity is normal or high, cells would be both CD2 and CFP positive. We found that GFP intensity in retinas transfected with GFP plus GFP Mo is strongly diminished compared with control retinas transfected with GFP plus a control Mo (Fig. 5A). This experiment thus shows that lipofected Mo can specifically and effectively reduce translation of their target genes in such lipofection experiments, and can therefore be used to block the function of a given gene in the retina.

Blocking XSEB4R function by morpholino lipofection in retinal progenitor cells delays retinal cell differentiation

To block XSEB4R function, we decided to lipofect in vivo *Xseb4R* Mo (Mo1) into retinoblasts. We first tested the specificity of *Xseb4R* Mo1. For this purpose, we raised a polyclonal antibody against the N-terminal region of XSEB4R (see Materials and methods). On western blots, this antibody indeed recognises the XSEB4R protein, which migrates at 22 kD (Fig. 5B). In order to test the specificity of *Xseb4R* Mo1, we co-injected into two-cell stage embryos *Xseb4R* mRNA and *Xseb4R* Mo1, or a control Mo. We then analysed at stage 10, by western blot, the presence of XSEB4R protein. We found that *Xseb4R* Mo1 inhibits XSEB4R protein expression but not that of a control protein, α tubulin (Fig. 5B). This result shows that *Xseb4R* Mo1 indeed specifically and efficiently blocks the translation of *Xseb4R* mRNA.

In order to obtain a knock down of *Xseb4R* during retinogenesis, we used two different Mo directed against *Xseb4R*, namely *Xseb4R* Mo1 and *Xseb4R* Mo2. Some authors have reported that some Mo can have unspecific effects at a certain threshold concentration that is Mo dependent (Heasman, 2002). These effects may complicate the study of

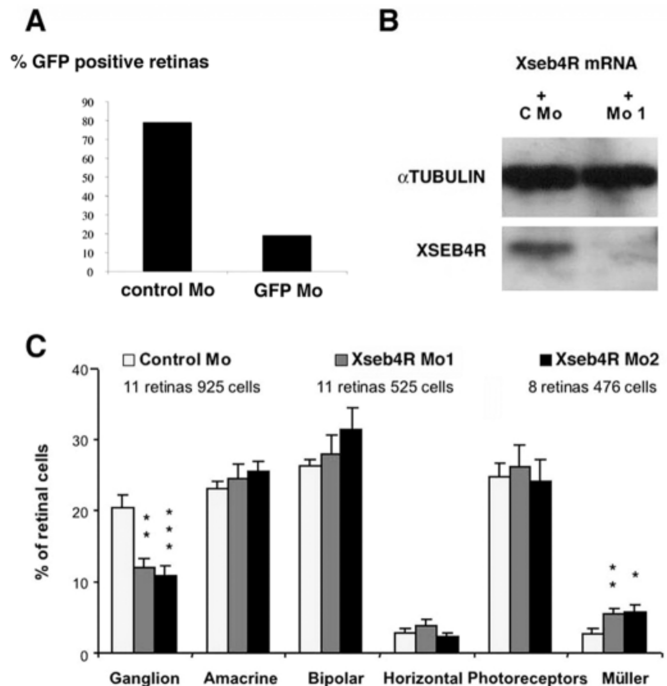


Fig. 5. Morpholino oligonucleotides lipofection in the retina. (A) Retinal lipofection of GFP Mo interferes with GFP translation. Embryos were lipofected at stage 18 with GFP and a control Mo plus CD2, or with GFP and GFP Mo plus CD2. In stage 41 embryos, the intensity of GFP fluorescence in retinal cells was analysed. We used a filter to decrease the fluorescence light of the microscope so that low GFP fluorescent cells are below the detectable threshold. We then counted the number of lipofected retinas (CD2 positive) that also contain GFP-positive cells. While 79% (n=43 retinas) of CD2-positive retinas also contained GFP-positive cells when lipofected with a control Mo, only 19% (n=42 retinas) contained GFP-positive cells when lipofected with GFP Mo. (B) *Xseb4R* Mo block the translation of *Xseb4R* mRNA. Synthetic *Xseb4R* mRNA (containing the complementary sequence of Mo1) was injected into two-cell stage embryos together with *Xseb4R* Mo1 or control Mo. Stage 10 embryo lysates were then analysed by western blotting with a polyclonal anti-XSEB4R antibody and a monoclonal anti-αtubulin antibody (control). In the presence of control Mo, the anti-XSEB4R antibody recognises the 22 kD XSEB4 protein. The presence of *Xseb4R* Mo1 specifically abolishes *Xseb4R* translation. (C) *Xseb4R* loss-of-function delays differentiation of retinal cells. The proportion of retinal cell types observed in retinas co-lipofected with GFP plus a control Mo, GFP plus *Xseb4R* Mo1, or GFP plus *Xseb4R* Mo2 was determined. Both Mo give the same results. The statistical analysis was performed using the Student's t-test. *, $P<0.05$; **, $P<0.001$; ***, $P<0.0001$.

a not yet characterised gene because it is impossible to distinguish between an unknown loss-of-function phenotype and an unspecific effect. We therefore used two Mo directed against two different regions of *Xseb4R* mRNA sequences (see Materials and methods) and compared their effects. We have previously studied the optimum concentration of a control Mo that does not lead to any toxic effect in the retina (Ohnuma et al., 2002). We therefore targeted a subcritical concentration of *Xseb4R* Mo1 or *Xseb4R* Mo2 into the developing retina by in vivo lipofection of optic vesicles in stage 18 embryos together with *GFP* DNA as a tracer (Fig. 5C). Control embryos were co-lipofected with *GFP* DNA and a standard control Mo.

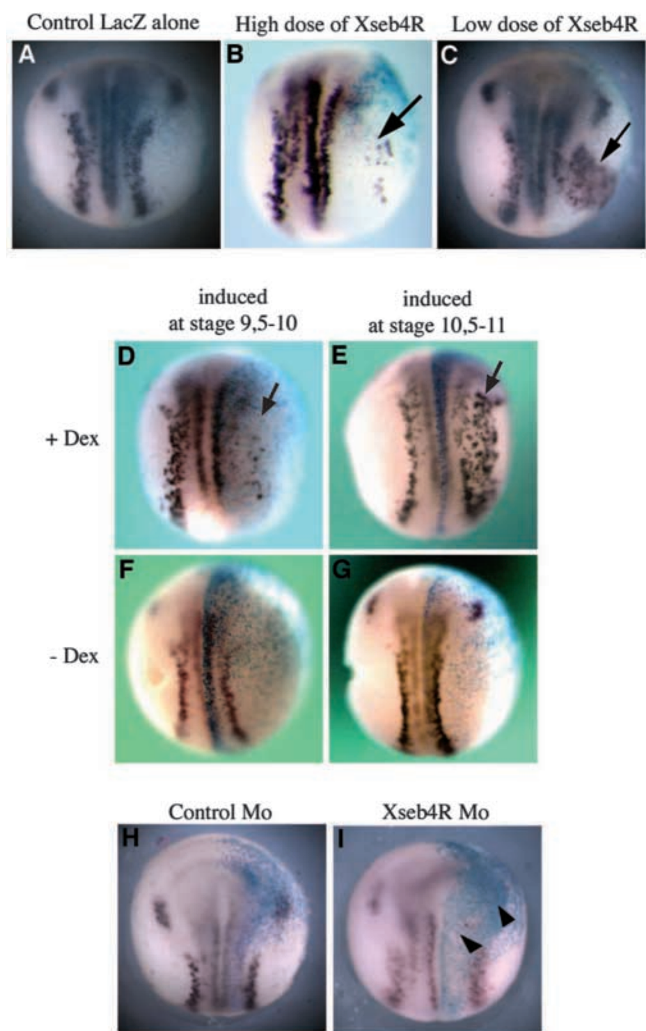
Transfected retinal cells were counted on stage 41 embryos. Consistent with our overexpression data, the two *Xseb4R* Mo lead to the same phenotype, characterised by a significant increase of Müller cells at the expense of ganglion cells (Fig. 5C), indicating a change of cell fate specification in which the latest born cells of the retina are promoted at the expense of the earliest born cell type. To rule out the possibility that the decrease of ganglion cells was due to apoptosis, we performed a TUNEL assay at stages 34 and 38, and counted the number of apoptotic cells. At stage 34, we found an average of 3.2 apoptotic cells per control retina (*n*=37 retinas) and 2.9 apoptotic cells per retina transfected with *Xseb4R Mo1* (*n*=40 retinas). At stage 38, we found that 25% (*n*=539 cells in 19 retinas) of apoptotic cells in the retina reside in the ganglion cell layer compared with 24% (*n*=461 cells in 17 retinas) in retinas transfected with *Xseb4R Mo1*. Therefore, this suggests that there is no significant increase in apoptosis following *Xseb4R* Mo1 transfection in cells in the ganglion cell layer.

Overexpression of *Xseb4R* during primary neurogenesis promotes neuronal differentiation

We then wondered whether *Xseb4R* could also have a proneural-like effect during primary neurogenesis. We therefore injected transcripts encoding XSEB4R unilaterally into two-cell stage embryos, using *lacZ* mRNA as a tracer. The embryos were collected at stage 15 and the expression of *N-tubulin*, a neuronal-specific marker, was analysed by whole-mount in situ hybridisation. Surprisingly, overexpression of high concentrations of *Xseb4R* RNA (>100 pg) inhibits *N-tubulin* expression (Fig. 6B; 100%, *n*=85 embryos). However, to the contrary, low dose of *Xseb4R* RNA (50 pg) promoted the formation of ectopic *N-tubulin*-positive neuroepithelial cells in the lateral ectoderm (74%, *n*=35 embryos; Fig. 6C). The lateral band of *N-tubulin* is indeed expanded laterally. Ectopic *N-tubulin*-positive cells were never observed in control *lacZ*-injected embryos (Fig. 6A).

As high doses of *Xseb4R* RNA were associated with gastrulation defects, we attributed the resulting suppression of neuronal differentiation to an early function of this gene and this hypothesis is supported by the expression of *Xseb4R* in the involuting mesoderm during gastrulation (Fig. 2A,B). To circumvent this handicap, we generated a *Xseb4R-GR* construct by fusing the ligand-binding domain of the glucocorticoid receptor (GR) to the C-terminal end of *Xseb4R* cDNA. RNAs prepared from this plasmid were microinjected at different concentrations and activation of the fusion protein was investigated at a range of developmental stages from blastula stage 9.5 to neurula stage 13.

When 100 pg of *Xseb4R-GR* transcripts were injected into one or two animal blastomers of 4- to 8-cell stage embryos, and then treated with dexamethasone at developmental stages between 9.5 and 10, a high number (80%, *n*=34/42 embryos) of the injected embryos still showed a significant reduction of *N-tubulin* expression (Fig. 6D). However, a high proportion (53%, *n*=29/55 embryos) of the injected embryos induced at a



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	quantity ng	% of embryos with a decrease in Ntub expression	total embryos
Control Mo	20	12	25
Xseb4R Mo1	20	84	25
Control Mo	10	12	18
Xseb4R Mo1	10	64	25
Control Mo	5	13	16
Xseb4R Mo1	5	10	11

Fig. 6. Xseb4R is involved in the regulation of primary neurogenesis in *Xenopus* embryos (A-C) Dorsal views of embryos injected with control *lacZ* mRNA (A), 200 pg of *Xseb4R* mRNA plus *lacZ* mRNA (B), or 50 pg of *Xseb4R* mRNA plus *lacZ* mRNA (C), and stained for *N-tubulin* expression (dark blue). β-galactosidase activity is shown in light blue. Whereas the high dose (200 pg) of *Xseb4R* represses *N-tubulin* expression on the injected side (arrow in B), the low dose (50 pg) leads to the expansion of the *N-tubulin* domain (arrow in C). (D-G) Dorsal views of embryos after injection of the fusion construct *Xseb4R-GR*. Induction with dexamethasone was performed between stage 9.5 and 10 (D), or between stage 10.5 and 11 (E). F and G show non-induced control embryos. Whereas induction of *Xseb4R-GR* between stage 9.5 and 10 represses N-tubulin expression in the injected side (arrow in D), induction of *Xseb4R-GR* between stage 10.5 and 11 leads to expansion of the *N-tubulin* domain (arrow in E). (H,I) Dorsal views of embryos after injection of control Mo (H) or *Xseb4R* Mo1 (I). *Xseb4R* Mo1 leads to a repression of *N-tubulin* expression in the injected side (arrowheads in I). (J) Effects of various doses of control Mo and *Xseb4R* Mo1 on *N-tubulin* expression. Injections of 10 or 20 ng of *Xseb4R* Mo1 lead to a decrease of *N-tubulin* expression compared with control embryos.

developmental stage of between 10.5 and 11 showed a significant increase of *N-tubulin*-positive cells within the territory of primary neurogenesis (Fig. 6E), whereas no increase was observed in the control (*n*=52) (Fig. 6G). This result strongly suggests that when activated between stage 10.5 and 11, *Xseb4R* is able to upregulate the process of neuronal differentiation. However, a low proportion of the injected embryos showed no phenotype (10/55, 18%) or showed a reduction (16/55, 29%) of *N-tubulin* expression (data not shown), indicating that some of the dexamethasone-treated embryos were not at the right competence to signal the proneural activity of XSEB4R.

Reduced function of *Xseb4R* during primary neurogenesis inhibits neurogenesis

To gain more insight into the role of *Xseb4R* during primary neurogenesis, we decided to analyse the effects of its knock down and to compare them with our overexpression data. Various amounts of *Xseb4R* Mo1 were injected into one cell of a two-cell stage embryo. The embryos were collected at stage 15 and the expression of *N-tubulin* was analysed by whole-mount in situ hybridisation. Overexpression of the lowest dose, 5 ng of *Xseb4R* Mo1, results in a phenotype that is not significantly different from that observed in embryos injected with a control Mo (Fig. 6J). However, 10 or 20 ng of *Xseb4R* Mo1 significantly inhibits *N-tubulin* expression (Fig. 6I,J). Taken together, these results suggest that *Xseb4R* plays an important role in primary neurogenesis

Xseb4R acts downstream of *XNgnr1* and *XNeuroD* during primary neurogenesis and is regulated by lateral inhibition

As we reported above, *Xseb4R* is expressed in the CMZ of the retina, as well as during primary neurogenesis in the region where many regulators of retinogenesis and primary neurogenesis (such as proneural, neurogenic and differentiation genes) are also expressed. In addition, our functional studies during retinogenesis and primary neurogenesis suggest that *Xseb4R* is involved in neuronal differentiation. *Xseb4R* expression is therefore likely to be responsive to proneural and neurogenic signalling pathways. We addressed this question directly in vivo by analysing the transcriptional regulation of *Xseb4R* in response to the activated proneural and neurogenic pathways. mRNAs encoding XNgnr1 or its downstream target, *XNeuroD*, which also encodes a transcriptional regulator, were injected into one of the two blastomeres of two-cell stage embryos. *lacZ* mRNA was co-injected as a tracer. Embryos were fixed at neurula stage and stained by X-gal treatment for probe distribution control. Results obtained by whole-mount in situ hybridisation show that *Xseb4R* expression is ectopically activated by *XNgnr1* (100%, *n*=135), suggesting that this gene functions downstream of the neuronal determination bHLH factor (Fig. 7A,B). Similar results were obtained with *XNeuroD* (78%, *n*=86; Fig. 7C,D). We then injected 100 pg of *Xseb4R-GR* transcripts into one of the blastomeres of 4-cell stage embryos, treated the embryos with dexamethasone between stage 10.5 and 11, and analysed the expression of *XNgnr1* and *XNeuroD*. Under these conditions, although *N-tubulin* expression is upregulated (see above), no ectopic expression of *XNgnr1* or *XNeuroD* was observed (data not shown). Altogether these results indicate that *Xseb4R* functions downstream of these bHLH factors.

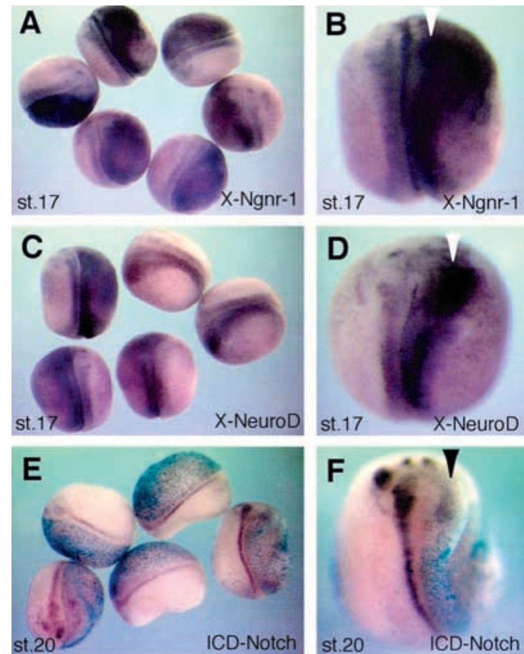

Fig. 7. *Xseb4R* is regulated by proneural and neurogenic genes. (A,C,E) Dorsal views of embryos after injection of the indicated genes and stained for *Xseb4R* expression (dark blue). β-galactosidase activity is shown in light blue. (B,D,F) One representative embryo for each injection series is shown at high magnification. (A,B) *XNgnr1* strongly activates *Xseb4R* transcription in neural and non-neural ectoderm. (C,D) *XNeuroD* equally induces ectopic *Xseb4R* expression. (E,F) Activation of Notch signalling by injection of *ICD-Notch* reduces the expression of *Xseb4R*.

Xseb4R expression, though broadly detected in the open neural plate, exhibits a 'salt and pepper'-like pattern, indicating that *Xseb4R*-expressing cells might be subject to lateral inhibition. To address this question, we analysed *Xseb4R* expression in *ICD-Notch* (a constitutively active form of XNotch1)-injected embryos. Our results reveal a reduction of *Xseb4R* transcription (89%, *n*=110; Fig. 7E,F), indicating that *Xseb4R* is regulated, directly or indirectly, by the Delta/Notch signalling pathway. Hence, *Xseb4R* is an additional component of the proneural and neurogenic signalling pathways involved in neuronal cell differentiation during *Xenopus* development.

Discussion

We have identified a new putative RNA-binding protein, XSEB4R, which contains a single RRM domain. *Xseb4R* is strongly expressed in the nervous system during development. Focusing our study on the retina, we showed that *Xseb4R* expression is restricted to proliferating retinoblasts and postmitotic differentiating precursors. Overexpression experiments, as well as loss-of-function analysis, converge to suggest that XSEB4R is the first known RNA-binding protein displaying a proneural effect during retinogenesis. We also showed a similar function during primary neurogenesis.

Finally, we have positioned *Xseb4R* in a genetic cascade involved in neurogenesis.

XSEB4R is a novel RNA-binding protein

Xseb4R encodes a putative RNA-binding protein containing a single RRM. Some RRM-containing proteins also contain other RNA-binding domains. For example, Vg1-RBP/vera has two RRMs as well as four KH domains (reviewed by Yaniv and Yisraeli, 2002). However, in the XSEB4R protein sequence, besides the RRM, we have not detected any other consensus sequences revealing the presence of another known domain. The C-terminal two-thirds of XSEB4R is somewhat enriched with proline residues, known to be involved in protein-protein interactions (reviewed by Williamson, 1994). A single RRM is known to be sufficient to bind RNA (Scherly et al., 1989), but better target specificity has been demonstrated when several RRMs are present (Kuhn and Pieler, 1996). These data suggest that XSEB4R could act through a multi-protein complex, conferring a higher specificity. The two additional conserved domains, observed in the C-terminal half of the vertebrate SEB4 protein sequence, could be involved in the formation of this protein complex.

RNA-binding proteins can regulate the expression of their mRNA targets at different steps of mRNA processing and translation (Harford and Morris, 1997). We have shown that an epitope-tagged XSEB4R protein is mainly cytoplasmic in retinal progenitors. We therefore propose that this protein could regulate the transport, the stability or the translation of its mRNA targets in the cytoplasm.

Xseb4R is expressed in neural progenitor cells

We found that *Xseb4R* is strongly expressed in the developing nervous system. As we have not detected any expression in cranial ganglia, *Xseb4R* expression in the nervous system seems to be restricted to the CNS, at least during early neurogenesis. However, its expression is not restricted to the nervous system. *Xseb4R* expression is multiphasic and arises in (1) the mesoderm during gastrulation, (2) the neuroectoderm during neurulation, and (3) different organs of the endoderm during organogenesis. Expression of the related *Xenopus* gene *Xseb4*, as *Xseb4R*, is not restricted to a single tissue. *Xseb4* is indeed mainly expressed in muscle but also in the lens (Fetka et al., 2000). These genes seem therefore to be involved in different developmental processes.

In the retina, *Xseb4R* is expressed in retinoblasts of the developing optic vesicle. To compare its expression with that of other genes involved in retinogenesis, we took advantage of the specific properties of the CMZ. According to the expression of various genes and to cell division activity, the CMZ has been divided into four different regions from the peripheral to the central retina (Perron et al., 1998). Proneural and differentiation genes are not expressed in the first zone of the CMZ, which contains retinal stem cells, but in proliferating retinoblasts and some postmitotic neurons (Perron et al., 1998). We showed that *Xseb4R* is also expressed in the CMZ, in both proliferating retinoblasts and postmitotic neurons, and is excluded from the most peripheral region. This expression correlates nicely with the expression of *Xseb4R* in the subventricular zone of the neural tube during primary neurogenesis, where neurons are in the transition step between proliferation and differentiation. Some differentiation genes, such as *XNeuroD* or *Xath3*, are also expressed in a subset of neurons in the central retina (Perron et al., 1998). *Xseb4R* expression, however, is restricted to the CMZ in the mature retina, like some proneural genes such as *Xash1*, *Xash3* and *XNgnr1*, or the *atonal*-like gene *Xath5*. The expression of neurogenic genes belonging to the Notch/Delta signalling cascade is also similarly restricted to the CMZ. The expression of *Xseb4R* in this region of the CMZ together with several proneural and neurogenic genes suggests that it is involved in crucial steps of retinogenesis, where precursor cells become determined and differentiate into a particular type of retinal neuron or glial cell.

Xseb4R is involved in neurogenesis during both primary neurogenesis and retinal development

In *Xenopus*, several strategies to reveal a loss-of-function effect have been used. For example, fusion constructs between the DNA-binding domain of a transcription factor and an activator domain (VP16), or a repressor domain (Engrailed), have been used extensively (e.g. Mariani and Harland, 1998). Ectopic expression of dominant-negative variants is another alternative. This is often the case for transmembrane receptors (e.g. McFarlane et al., 1996). Researchers working on RNA-binding proteins belonging to the ELAV family have constructed dominant-negative versions of these proteins. This was possible because ELAV-type proteins contain three RRMs (Robinow et al., 1988). It has indeed been proposed that truncated constructs, missing one or two RRMs, behave as dominant-negative constructs (Akamatsu et al., 1999; Kasashima et al., 1999; Anderson et al., 2000). However, because XSEB4R only contains one RRM we could not use such a strategy.

Mo have recently been used in developmental studies in a wide range of model organisms to block the translation of a target mRNA (Ekker and Larson, 2001; Heasman, 2002). So far, in *Xenopus*, Mo have been used in blastomere injection experiments. In order to target a single tissue where we expect a loss-of-function phenotype, we set up a protocol to lipofect Mo in vivo into specific regions (Ohnuma et al., 2002). In this paper, we demonstrate the efficiency of this Mo lipofection strategy in the retina. Our results with *Xseb4R* Mo suggest that this gene is required for ganglion cell production. Whether *Xseb4R* specifically promotes ganglion cells or simply promotes neurogenesis remains to be determined. Indeed, because ganglion cells are the first cells to be born in the retina, one cannot distinguish between these two hypotheses. Nevertheless, it is important to note that the *Xseb4R* knockdown phenotype reflects the opposite phenotype, as obtained in the gain-of-function experiments. Indeed when we overexpress *Xseb4R* in retinoblasts, they tend to differentiate precociously. It has indeed been shown previously that when precursors are forced to adopt an early fate, an increase in ganglion and cone photoreceptor is observed (Dorsky et al., 1997). When they are forced to differentiate slightly later an increase in the number of cone and rod photoreceptors is observed (Dorsky et al., 1997). Although overexpression of *Xseb4R* induces a severe phenotype, significantly affecting almost all cell types, the *Xseb4R* knockdown phenotype is less severe, as photoreceptor cells, amacrine and bipolar cells are not affected. This suggests that the Mo strategy does not lead to a complete loss of function, but rather reduces the amount

of XSEB4R protein in vivo. Alternatively, this could be due to a redundant function of another RNA-binding protein partly compensating for the absence of XSEB4R. Nevertheless, our knockdown experiment, together with our gain-of-function experiment, strongly suggest that *Xseb4R* promotes neurogenesis during retinal development.

So far, mainly transcription factors have been found to have such an effect in retinogenesis. Indeed, overexpression of *Xath3* in the retina leads to exactly the same phenotype as *Xseb4R* overexpression (this study) (Perron et al., 1999b). This paper thus provides the first example of an RNA-binding protein displaying proneural properties in the retina.

During primary neurogenesis, we found that *Xseb4R* could also have a proneural-like effect, i.e. activation of *N-tubulin* expression when overexpressed, only at a particular dose or at a particular time during development. Such discrepancy in *Xseb4R* expression pattern (Notch/Delta-like pattern) and resulting proneural-like function has already been observed with the *Hes6* gene (Koyano-Nakagawa et al., 2000). Furthermore, functional characteristics of XBF1, an anterior neural plate-specific winged helix transcription factor, just like the data that we report on *Xseb4R* overexpression, revealed ectopic *N-tubulin* expression at low doses and inhibition of this same marker at high doses (Hardcastle and Papalopulu, 2000). These differential effects of XBF1 were found to correlate with its role in cell proliferation. In the context of our studies, we did not know which early functions could be mediated by *Xseb4R*. However, because we saw gastrulation defects when a high dose of *Xseb4R* was injected, one hypothesis is that it interferes with early development, preventing us from analysing its specific effect during neurogenesis. By using an inducible construct we managed to overcome this problem and to analyse the effect of *Xseb4R* on early neurogenesis. We indeed found that when activated towards the end of gastrulation, i.e. between stage 10.5 and 11, just like with low doses of *Xseb4R*, *Xseb4R-GR* promotes ectopic neurogenesis as well. This effect is in accordance with the *Xseb4R* knockdown effect, which inhibits neuronal differentiation. Therefore, these results suggest that during primary neurogenesis *Xseb4R* has, as in the retina, proneural properties.

Xseb4R is regulated by proneural genes

As a result of *Xseb4R* expression in the CMZ and of our functional analysis, we wanted to identify genes belonging to the genetic cascade involved in neurogenesis that could regulate *Xseb4R* expression. We found that the proneural gene *XNgnr1* is able to induce strong ectopic expression of *Xseb4R* in the whole ectoderm. We have also shown that the *atonal*-like gene *XNeuroD* (a differentiation gene) also induces ectopic expression of *Xseb4R*. In addition, our analysis reveals that Notch/Delta signalling negatively regulates *Xseb4R* expression. This is similar to the inhibition found for several bHLH proneural genes, such as *XNgnr1* (Chitnis and Kintner, 1996). Altogether, these results suggest that *Xseb4R* is a component of the genetic cascade involved in neurogenesis. It would be interesting now to characterise genes that function downstream of *Xseb4R* during neurogenesis and that may be post-transcriptionally regulated by this cytoplasmic RNA-binding protein.

It has recently been reported that *Xenopus NeuroD* is regulated post-translationally by the kinase GSK3β (Moore et al., 2002). This observation, together with ours, illustrates the fact that post-transcriptional and post-translational regulators may have a crucial role in neurogenesis. These regulatory mechanisms, in contrast to transcriptional gene regulation, have been poorly studied so far during vertebrate neurogenesis.

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