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Conserved regulatory elements establish the dynamic expression of *Rpx/Hesx1* in early vertebrate development

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Abstract

The*Rpx/Hesx1* homeobox gene is expressed during gastrulation in the anterior visceral and definitive endoderm and the cephalic neural plate. At later stages of development, its expression is restricted to Rathke's pouch, the primordium of the pituitary gland. This expression pattern suggests the presence of at least two distinct regulatory regions that control early and late *Rpx* transcription. Using transgenic mice, we have demonstrated that regulatory sequences in the 5′ upstream region of *Rpx* are important for early expression in the anterior endoderm and neural plate and regulatory elements in the 3′ region are required for late expression in Rathke's pouch. We have found that the genetically required LIM homeodomain-containing proteins Lim1/Lhx1 and Lhx3 are directly involved in the regulation of *Rpx* transcription. They bind two LIM protein-binding sites in the 5′ upstream region of *Rpx*, which are required for *Rpx* promoter activity in both mice and *Xenopus*. Furthermore, we have found that a conserved enhancer in the 3′ regulatory sequences of *Rpx* is not only required, but is also sufficient for the expression of *Rpx* transgenes in the developing Rathke's pouch.

Keywords: *Rpx/Hesx1*; LIM-HD proteins; Homeobox; Rathke's pouch; Anterior visceral endoderm; Lhx3; Pitx2; Neural plate

Introduction

The *Rpx/Hesx1* gene encodes a homeodomain transcription factor expressed during gastrulation in the anterior visceral endoderm (AVE), anterior definitive endoderm, prechordal plate and the cephalic neural plate (Hermesz et al., 1996; Thomas and Beddington, 1996). At later stages of development, the expression of *Rpx* is restricted to Rathke's Pouch (RP), the primordium of the pituitary gland, and is no longer detectable in the neuroectoderm. *Rpx* is one of the earliest genes expressed in the developing RP. At E15.5, when pituitary-specific cells start to differentiate, *Rpx* is downregulated in the pouch (Hermesz et al., 1996).

Rpx function is essential for normal embryonic development as *Rpx* null mice generally die between E10.5 and E12.5 and display several developmental defects in structures derived from the anterior neural plate and RP. They typically have a reduced prosecephalon, anophthalmia and bifurcations in RP (Dattani et al., 1998). This phenotype resembles human septo-optic dysplasia (SOD). Two SOD families were found to be homozygous for the Arg53Cys missense mutation within the homeodomain of *Rpx*, leading to a loss of in vitro DNA binding (Dattani et al., 1998). Recently, a Alu-element insertion in the exon3 of *Rpx/Hesx1* was found in a patient with pituitary aplasia and retinal coloboma, and this insertion was predicted to generate severely truncated proteins (Sobrier et al., 2005). In the RP, the consistent expression of *Rpx*, driven

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by the α-glycoprotein subunit (α-GSU) promoter, leads to dwarf mice lacking lactotroph, somatotroph and gonadotroph cell types (KM, unpublished and Dasen et al., 2001). This suggests that the downregulation of *Rpx* is important for cell type differentiation in the anterior pituitary.

Several homeobox genes are coexpressed with *Rpx* and are known to be genetically required for its expression. The LIM homeodomain (LIM-HD) transcription factor *Lhx1*/*Lim1* is coexpressed with *Rpx* during gastrulation in the AVE (Shawlot and Behringer, 1995). *Lhx1* plays a crucial role in anterior head formation as the disruption of the *Lhx1* gene leads to a lack of forebrain, midbrain and some hindbrain elements (Shawlot and Behringer, 1995). At E8.5, *Rpx* and a second LIM-HD gene, *Lmx1b*, are coexpressed in the anterior neural plate (Yuan and Schoenwolf, 1999). Later, during pituitary development, *Lhx3*/*Lim3*, a third LIM-HD gene, is coexpressed with *Rpx* in RP (Sheng et al., 1996). *Lhx3* is essential for the differentiation and proliferation of pituitary cell lineages (Sheng et al., 1996). Both *Lhx1* and *Lhx3* are required for the proper expression of *Rpx* during embryonic development as *Rpx* expression is either lacking or not maintained in mutants of these genes (Sheng et al., 1996; Shimono and Behringer, 1999). Thus, these LIM-HD proteins are potentially direct regulators for the *Rpx* gene.

In addition to these LIM-HD genes, the bicoid homeobox transcription factor, *Pitx2*/*Ptx2* (*Otlx2*, *Rieg*) is also expressed in the developing pituitary gland (Mucchielli et al., 1997; Semina et al., 1996) and is a candidate for a direct regulator of *Rpx*. Like *Lhx3*, *Pitx2* is required for pituitary organogenesis past the committed Rathke's pouch stage (Gage et al., 1999; Lin et al., 1999) and is genetically required for expression of *Rpx* in the oral ectoderm and Rathke's pouch (Gage et al., 1999).

We have delineated the *cis*-elements required for *Rpx* expression by generating transgenic embryos bearing the *lacZ* reporter gene fused to different fragments of *Rpx* genomic sequences. Our previous studies suggested that there are at least two stages of *Rpx* regulation. The 5′ upstream regulatory sequences of *Rpx* were shown to be critical for the early expression pattern in the anterior endoderm, prechordal plate and anterior neural plate, but were unable to drive expression in Rathke's pouch (Hermesz et al., 2003). In this study, we demonstrate that regulatory elements located 3′ to the gene are both required and sufficient for the late expression of transgenes in Rathke's pouch. Furthermore, we show that LIM-HD proteins that are coexpressed with *Rpx* can directly activate the *Rpx* promoter. Two LIM-HD protein-binding sites are present in the *Rpx* 5′ regulatory region, and these sites are required for *Rpx* promoter activity in mouse embryos. We also map a minimal regulatory element in the 3′ region that is sufficient for activation of transgenes in Rathke's pouch. This conserved element can bind Pitx2 and GATA proteins, strongly suggesting that these proteins directly regulate *Rpx*.

To demonstrate the evolutionary conservation of *Rpx* gene regulation, we have examined expression of the murine regulatory elements in *Xenopus* embryos. The *Rpx* homolog in *Xenopus*, *Xanf*, shows a similar expression pattern as the mouse *Rpx* gene, and is expressed in the anterior neural folds and the pituitary primordium of the frog embryo (Mathers et al., 1995; Zaraisky et al., 1992). Injection of the *Rpx* reporter constructs into *Xenopus* embryos showed that the expression pattern of *Rpx* transgenes is similar to that of *Xanf* demonstrating the conservation of *Rpx* regulation between these two species.

Materials and methods

DNA constructs

For all of the Rpx-*LacZ* reporter constructs described in this paper, a 3.35-kb *Kpn*I–*Pst*I fragment, containing the *E. coli lacZ*-SV40 polyA cassette from plasmid pGT4.5A (Gossler et al., 1989) was fused in frame to the *Rpx* genomic sequence at the *Kpn*I site in the second exon. To align the reading frames of the *lacZ* and *Rpx* coding regions, a 1-bp deletion was generated in *Rpx* close to the fusion point by oligonucleotide directed mutagenesis. All constructs reported here carry the first exon, first intron and 200 bp from the second exon as well as varying amounts of sequence 5′ to the translation start site of *Rpx*. RpxA contains 1.5 kb of sequence upstream to the initiation methionine of *Rpx*. RpxD contains 570 bp of the *Rpx* upstream sequences. RpxW contains RpxA and a 3.3-kb *Pst*I–*Pst*I fragment from the *Rpx* downstream sequences. RpxD-mut was generated by mutating both the dTAAT and pTAAT sites in the context of RpxD-H3, which contains a *Hin*dIII site at −60 bp in RpxD (this *Hin*dIII site does not affect promoter activity and specificity in transgenic mice). RpxA + StyI, RpxA + H3, RpxA + H3H3 and RpxA + H3P constructs were derived from RpxW. The PCR products of 3′H3P, 3′H3P-1 + 2, 3′H3P-3 + 4, 3′H3P-4 and 3′H3P-4-25 were subcloned into Hsp68-lacZ.

For cell transfection experiments, the different fragments of Rpx upstream sequences, Rpx1300, Rpx570, Rpx390, Rpx 300 and Rpx200 were generated by PCR using *RpxA* as template. These PCR products were cloned into pGL2-basic (Promega) to generate luciferase reporter constructs. The 390 bp *Rpx* upstream sequences containing either single dTAAT or pTAAT mutations or both dTAAT and pTAAT mutations were generated by site-specific PCR mutagenesis. For cotransfection experiments, the *Lhx1/Lim1* (kindly provided by Dr. Richard Behringer), *Lmx1b* (kindly provided by Dr. Randy Johnson) and *Lhx3* (kindly provided by Dr. Denny Porter) full-length cDNAs were cloned into pcDNA3.1+ (Invitrogen).

Cell culture and transfection

F9 cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin and streptomycin. According to the manufacturer's protocol, 10^5 cells were transiently transfected by Lipofectamine (Gibco-BRL) with 500 ng of *Rpx* luciferase reporter constructs, 100 ng of LIM protein and 100 ng of CMV-*LacZ* expression vectors. Cell extracts were prepared 2 days after transfection. Luciferase activity was measured and normalized against β-galactosidase activity as an internal control. At least three independent transfections were performed.

Electrophoretic mobility shift assay (EMSA)

EMSA experiments were performed as described before (Scott et al., 1994). Double stranded oligonucleotides were labeled with α-^{32}P-dCTP. In vitro translated proteins were incubated with 1× binding buffer [25 mM HEPES (pH7.9), 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT and 5 mM MgCl2], DNA competitors and 1 mg poly-dIdC in 10% glycerol solution for 15 min on ice prior to adding probe. Probe was added to the reaction and allowed to bind for 30 min on ice, and then protein–DNA complexes were resolved by electrophoresis. Oligonucleotides used for EMSA: dTAAT-wt: 5′-GGATCCGGTGTAGCCATTAGTTGCTAATAAC-3′; dTAAT-mut: 5′-GGATCCGGTGTAGCCTCGAGTTGCTGCAGAC-3′; pTAAT-wt: 5′-GGATCCAGGATTTTAATTAGTGACTTTGG-3′; pTAAT-mut: 5′-GGATCCAGGATTTTGAATTCTGACTTTGG-3′; 3′H3P-4-1F: 5′-G GGGTACCTTAATCCACAGCTTAT-3′; 3′H3P-4-2F: 5′-GTAATTCCAGGCCTTTGTCTCC-3′; 3′H3P-4-3F: 5′-TCAGGACAAGGTGAACAGTAG-3′; 3′H3P-4-4F: 5′-CTAATGCAGTCAGGCTAATAC-