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Rpx: a novel anterior-restricted homeobox gene progressively activated in the prechordal plate, anterior neural plate and Rathke's pouch of the mouse embryo

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SUMMARY

We have isolated a new murine homeobox gene, *Rpx* (for Rathke's pouch homeobox), that is dynamically expressed in the prospective cephalic region of the embryo during gastrulation. Early expression is seen in the anterior midline endoderm and prechordal plate precursor. Expression is subsequently activated in the overlying ectoderm of the cephalic neural plate, suggesting that inductive contact with *Rpx*-expressing mesendoderm is required for this expression. Subsequently, *Rpx* expression is extinguished in the mesendoderm while remaining in the prospective prosencephalic region of the neural plate ectoderm. Ultimately, transcripts become restricted to Rathke's pouch, the primordium of the pituitary, which is known to be derived from the most anterior ectoderm of the early embryo. Down regulation of *Rpx* in the pouch coincides with the differentiation of pituitary-specific cell types. *Rpx* is the earliest known marker for the pituitary primordium, suggestive of a role in the early determination or differentiation of the pituitary. Since *Rpx* is expressed so dynamically and so early in the anterior region of the embryo, and since its early expression domain is much more extensive than the region fated to form the pituitary, it is likely that *Rpx* is involved in the initial determination of the anterior (prechordal) region of the embryo.

Key words: homeobox, pituitary, adenohypophysis, gastrulation, prechordal plate, anterior neural plate, endoderm, Rathke's pouch, mouse

INTRODUCTION

In recent years, the elucidation of the genetic program involved in vertebrate embryonic patterning has been initiated, in part, through the study of homeobox genes. These genes, originally identified as members of the homeotic gene clusters in *Drosophila* (*HOM-C*), occupy key positions in the regulatory gene hierarchy responsible for defining the embryonic body plan in the fly. It has become clear that members of the vertebrate *Hox* homeobox gene family are structurally and functionally equivalent to their *HOM-C* counterparts, and play analogous roles in defining positional identity along the rostrocaudal axis (reviewed by McGinnis and Krumlauf, 1992). In mice, both *Hox* loss-of-function and gain-of-function result in defects in morphogenesis and, in some cases, apparent homeotic transformation (for example see: Kessel et al., 1990, Ramirez-Solis et al., 1993). These genes do not provide spatial cues along the entire axis however, since they are not detectably expressed anterior to the hindbrain. Accordingly, the identification of regulatory genes that control the determination and specification of anterior structures of the vertebrate embryo, including the forebrain, is of fundamental importance, particularly since the forebrain is determined in the absence of the inductive influences of the notochord.

Recently, murine counterparts of several homeobox genes, expressed in the head structures in *Drosophila*, have been characterized that are promising candidates for cephalic regulatory genes. These include members of the *Distal-less (Dlx)* (Price et al., 1991; Robinson et al., 1991, 1994; Porteus et al., 1991; Simeone et al., 1994) and *Nkx* (Price et al., 1992) gene families, which are expressed prominently in the head and forebrain and show homology to the *Distal-less (Dll)* and *NK* genes of *Drosophila*. Murine relatives of the *Drosophila orthodenticle (otd)* and *empty spiracles (ems)* genes (*Otx1*, *Otx2* and *Emx1*, *Emx2*, respectively) display nested patterns of expression in the developing rostral central nervous system (CNS; Simeone et al., 1992, 1993). Since it has been established that *otd* and *ems* participate in a regulatory network required for head formation in *Drosophila* that is distinct from the *HOM-C* regulatory network at work in the embryonic trunk, it is highly likely that the conserved murine genes perform similar functions in the morphogenesis of the head and rostral CNS (reviewed by Finkelstein and Boncinelli, 1994).

While intriguing parallels exist between the *Drosophila* head segmentation genes and their vertebrate counterparts, with the exception of *Otx*2, which is expressed in the epiblast and becomes restricted to the anterior region of the embryo during gastrulation, the expression of the vertebrate genes is

detected relatively late in embryogenesis (i.e. after 8.5 days p.c.). Consequently, while these genes, like the *Hox* genes, may play a role in the specification or elaboration of regional identity, they are probably not involved in the very early determinative events which initiate patterning during gastrulation.

Several candidate vertebrate genes have been isolated that may play a role in these early events. These include putative transcription factors such as *Brachyury* (*T*) (Herrmann, 1992), the fork head genes *XFKH1* (Dirksen and Jamrich, 1992) and *HNF-3β* (Sasaki and Hogan, 1994; Ang et al., 1993), and the homeobox genes *goosecoid (gsc)* (Cho et al., 1991; Blum et al., 1992) and *lim-1* (Taira et al., 1992; Barnes et al., 1994; Fujii et al., 1994). Signalling molecules that appear to be directly involved in early inductive events, such as *noggin* (Smith and Harland, 1992), *nodal* (Zhou et al., 1993) and *sonic hedgehog* (Echelard et al., 1993), have also been identified. These genes are expressed in the Spemann's organizer region in the blastopore lip of *Xenopus* embryos, and in the comparable region in the node of murine embryos. Subsequent expression in the chordamesoderm of the notochord and/or prechordal plate, structures known to be required for both anteroposterior and dorsoventral patterning of the embryonic CNS, further implicates these genes as important developmental regulators.

During a screen for novel homeobox genes that are expressed during gastrulation, we identified a gene that is restricted in its expression to the anterior region of the mouse embryo. Early in gastrulation its expression is detected in the anterior endoderm and prechordal plate precursor, followed by activation in the overlying cephalic neural plate. Throughout early postimplantation development, this gene displays a very limited expression domain in the anterior neuroectoderm of the embryo, initially encompassing the prechordal area and prospective prosencephalon, and ultimately becoming restricted to Rathke's pouch, the primordium of the pituitary. This gene encodes a homeodomain that is similar to that of the *prd* gene of *Drosophila* and has been called *Rpx* for Rathke's pouch homeobox gene. We propose that *Rpx* is involved in the determination of the pituitary gland and that it may participate in establishing an early embryonic field in the anterior prechordal neural plate of the embryo that is later subdivided into smaller developmental units during organogenesis.

MATERIALS AND METHODS

Animals and embryos

Mice used were FVB/N or C57B/6J. Embryos were obtained from superovulated females. Noon of the day when the vaginal plug appeared was considered 0.5 days post coitum (p.c.).

Embryoid body formation

Embryoid bodies were generated by aggregation of D3 (Doetschman et al., 1985) embryonic stem (ES) cells according to Robertson (1987). Samples of aggregates were removed from continuous culture at various time points and total RNA was isolated.

PCR amplification of homeobox sequences

Template for PCR reactions was phage DNA prepared from a 7.5 day p.c. mouse embryo cDNA library (kindly provided by J. Rossant). PCR reactions were carried out according to Mackem and Mahon (1991). Degenerate primers that were derived from the predicted N and C terminus of the *Xenopus Mix-1* homeodomain (Rosa, 1989) were synthesized with linkers containing restriction sites (*Xho*I, *Sal*I, *Cla*I). The sequences were as follows (IUPAC code): TFFTQAQLD (5′-GACTCGAGTCGACATCGATACNARGCNCARCTNGA-3′) and IQVWFQN (5′-GACTCGAGTCGACATCGATTYTGRAAC-CANACYTGDAT-3′). Purified reaction products of 140 bp were cloned into the *Sal*I site of pGEM3Z (Promega), and the inserts were sequenced (Sanger et al., 1977). The insert from pMix7d1 contained the *Rpx* homeobox. A longer cDNA sequence was isolated by PCR from the library using a non-degenerate oligo homologous to a region from the homeobox in combination with a primer specific for the λgt10 phage. This clone, pPrd3, contained 400 bp of sequence from the 3′ end of the gene extending from the middle of the homeobox to the putative poly(A) tail.

RNA isolation and analysis

Total RNA was prepared with RNAzol (Tel-Test, Inc.) according to the manufacturer's instructions. Poly(A) RNA was prepared using

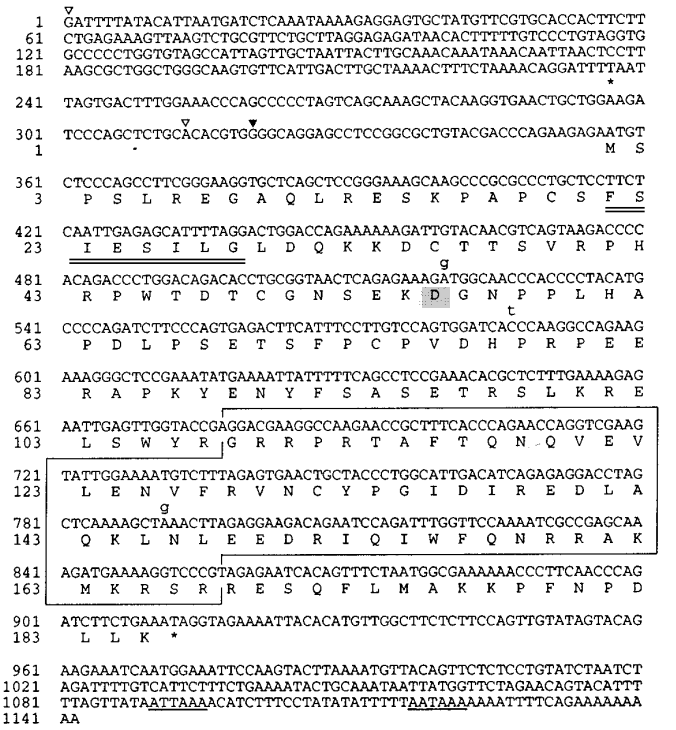

Fig. 1. Nucleotide sequence of the *Rpx* cDNA and its conceptual translation. Composite nucleotide sequence is derived from overlapping cDNA clones and from 5′ RACE reaction products. The 5′ terminus of the longest cDNA clone is indicated by the solid black arrowhead. The two termini derived from 5′ RACE are indicated by the open arrowheads. The most 5′ terminus, beginning at the first nucleotide, is believed to correspond to that of the longer, minor transcript seen on northern blots (see Fig. 3). The more 3′ terminus, at approximately position 310, corresponds to that of the shorter, major transcript. The homeodomain region is boxed. The octapeptide is double underlined. Possible polyadenylation signal sequences are underlined. In frame translational stop codons upstream of the predicted initiator methionine and at the end of the coding sequence are indicated by asterisks. Polymorphism between the cDNA and genomic sequence was observed at three sites, demarcated by small case letters over the relevant bases. Only one, at amino acid position 520 (shaded), resulted in a change in amino acid sequence from aspartic acid (cDNA, shown) to a glycine (genomic). These variations could be due to strain differences between mice used to make the libraries [i.e. outbred mice (genomic) versus FVB/N (cDNA)]. The GenBank accession no. is U40720.

A

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                        Helix-1         Helix-2          Helix-3
Rpx     GRRPRTAFTQNQVEVLENVFRVNCYPGIDIREDLAQKLNLEEDRIQIWFQNRRAKMKRSR
XANF-1  ---------RS-I-I--------S-----V--E--S--A-D-------------L---H  80%

ceh-10  K--H--I---Y-IDE--KE-QDSH--D-YA--V--G-TE-Q-----V--------CRQQL  55%
Mix.1   Q--K--F---A-LDI--QF-QT-M--D-HH--E--RHIYIP-S---V--------VR-QG  53%
al      Q--Y--T--SF-L-E--KA-SRTH--DVFT--E--M-IG-T-A---V--------WRKQE  52%
MHox    Q--N--T-NSS-LQA--R--ERTH--DAFV-----RRV--T-A-V-V--------FR-NE  52%
Phox2   Q--I--T--SA-LKE--R--AETH--D-YT--E--L-ID-T-A-V-V--------FRKQE  52%
S8      Q--N--T-NSS-LQA--R--ERTH--DAFV--E--RRV--S-A-V-V--------FR-NE  50%
Cart-1  K--H--T--SL-L-E--K--QKTH--DVYV--Q--LRTE-T-A-V-V--------WRKRE  50%
Pax-3   Q--S--T--AE-L-E--RA-ERTH--D-YT--E---RAK-T-A-V-V--S----RWRKQA  48%
prd     Q--C--T-SAS-LDE--RA-ERTQ--D-YT--E---RT--T-A---V--S----RLRKQH  48%

Antp    RK-G-QTY-RY-TLE--KE-HF-R-LTRRR-IEI-HA-C-T-RQ-K-------M-W-KEN  40%
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B

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M MSPSLREGAQLRESKPAPCSFSIESILGLDQKKDCTTSVRPHRPWTDTCGNSEKDGNPPLHAPD
X ---A-QK-SS-M-NRSP-S-S---H-----K-T-WLHHPLLSTTAVIE-SSKGVVNGTCWQI-V

M LPSET^SFPCPVDHPRPEERAPKYENYFSASETRSLKRELSWYRGRRPRTAFTQNQVELENVF
X IACDLPIQVHA-HRSEE--TKIRL-KC-GDEDRLTY----------------RS-I-I-----

M RVNCYPGIDIREDLAQKLNLEEDRIQIWFQNRRAKMKRSRRESQFLMAKKPFNPDLLK*
X ---S-----V--E--S--A-D-------------L---H------IV-DSLSSKIQE*
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Fig. 2.(A) Comparison of the *Rpx* homeodomain sequences to those of other *prd*-like proteins and the *Antennapedia (Antp)* sequence. All sequences are compared to the predicted amino acid sequence of the *Rpx* homeodomain (top) in descending order of relatedness. Dashes indicate sequence identities to *Rpx*. Percentage amino acid identity is indicated on the far right. The positions of the predicted homeodomain helices 1-3 are demarcated above the sequences. The residue at position 9 in the third helix, which is a glutamine in the *prd*-like homeodomains shown, but a serine in canonical *prd*-domain genes, is indicated by boldface type. References for the homeodomain sequences are found in the text. (B) Comparison of the predicted *Rpx* and *Xanf-1* polypeptides. The mouse *Rpx* sequence is on the top and the *Xenopus Xanf-1* on the bottom. Completely conserved residues are indicated in boldface type on the *Rpx* sequence and by dashes in the *Xanf-1* sequence. A gap in the sequence is indicated by ^, and the termination codon by an asterisk.

POLY(A)QUIK push columns (Stratagene). Northern blots of total RNA (50 μg/lane) were prepared according to standard methods and were hybridized to random-primed ^{32}P-labeled probes (Feinberg and Vogelstein, 1983) in Rapid-hyb buffer (Amersham Life Science) at 65°C. The probe used was a 538 bp cDNA fragment (nucleotides 604-1142). The sizes of the *Rpx* transcripts were determined by comparison to RNA markers of known size (BRL). Blots were stripped and reprobed with rat glyceraldehyde-3-phosphate dehydrogenase (GADPH) or actin to control for loading. Northern blots of embryoid body RNA were also reprobed with *Oct-3* (700 bp cDNA sequence generated by PCR) and *Endo-B* (pUC9B7, Singer et al., 1986) labeled restriction fragments.

Isolation of *Rpx* cDNA clones

Three oligo(dT)-primed λgt10 mouse embryo cDNA libraries representing different stages of development, 7.5 days p.c., 8.5 days p.c. (Fahrner et al. 1987) and 12.5 days p.c. (Joyner and Martin, 1987), were screened using standard methods (Sambrook et al., 1989) with either the 140 bp insert from pMix7d1 or the 400 bp insert from pPrd3 as probes. One *Rpx* cDNA was obtained from each library. A mixed-primed cDNA library was constructed using poly(A) RNA prepared from 8.5- to 9-day old embryos. First strand synthesis was primed with either random oligos or with oligo(dT). After second-strand synthesis, both sets of reaction products were ligated to *Eco*RI adapters, combined at a ratio of 2:1 (random:oligo(dT) primed), and cloned into λZAPII (Stratagene). Two positive clones were identified after screening with a 2.5 kb genomic DNA fragment overlapping with, and extending 5′ to, the known cDNA sequence. cDNA clone inserts were subcloned into plasmid Bluescript KS II and sequenced.

RACE PCR

The 5′ ends of the *Rpx* mRNAs were obtained by the modified RACE method termed SLIC (Edwards et al. 1991) using the 5′-AmpliFINDER RACE and the PCR Optimizer kits (Clontech). First strand cDNA was made by random priming 2 μg of poly(A)-enriched RNA isolated from 8.5- to 9-day old embryos. The single-stranded anchor oligonucleotide was ligated to the 3′ end of the cDNA by T4 RNA ligase and amplified using a gene-specific primer as partner. This PCR product was further amplified with a nested *Rpx*-specific primer. Southern analysis revealed two bands hybridizing to the 5′ coding end of *Rpx* which were purified and cloned into pCR vector (Invitrogen). Seven independent clones were sequenced, all of them overlapped with the *Rpx* cDNA sequence.

In situ hybridization

In situ hybridization with ^{35}S-labeled riboprobes was performed as previously described (Mackem and Mahon, 1991). Serial sections from several embryos for each stage were examined. Labeled sense and antisense riboprobes were synthesized from pPrd3 (*Rpx*), pSK75 (*Brachyury* or *T*) (Hermann, 1992), p3 (*gsc*) (Blum et al., 1992), and pmPit1 (*Pit-1*) (Camper et al., 1990). Sense control probes in all cases gave background labeling. Exposure to NTB-2 (Kodak) emulsion varied from 2-4 days for *Pit-1* and *T*, and 2.5 to 3 weeks for *Rpx* hybrids. After development, the slides were photographed in dark-field optics, stained in Geimsa and rephotographed in bright-field optics. In some cases, slides were stained in Hoechst and photographed under simultaneous dark-field and UV illumination according to the method of Sundin et al. (1990).

RESULTS

Isolation of cDNA clones containing *Rpx*

A PCR-based screen was utilized to isolate novel homeobox

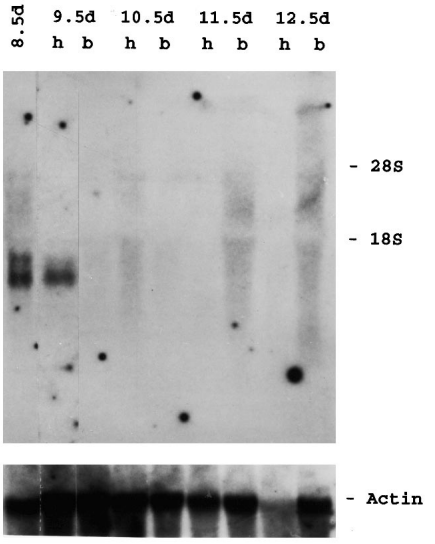

Fig. 3. Northern blot analysis of *Rpx* expression in early-mid gestation embryos. Total RNA (50 μg) isolated from 8.5 day conceptuses and from 9.5 to 12.5 day p.c. embryos that had been microdissected into head and body fractions, was loaded, blotted, and hybridized to *Rpx* cDNA probe. At 8.5 days, a prominent wide band at 910-950 and a larger, less abundant species at 1200 nucleotides is seen. By 9.5 days, only the smaller species is visualized. The positions of the 28S and 18S ribosomal RNA bands are indicated. The blot was stripped and reprobed with an actin probe as a control for loading.

genes expressed during gastrulation. Using a 7.5 day p.c. cDNA library as template with primers specific for the *Xenopus* homeobox gene *Mix-1,* one clone (pMix7d1) containing the novel *Rpx* homeobox sequence was isolated. A longer clone (pPrd3), containing sequences extending 3′ to the poly(A) tail was obtained by PCR from the cDNA library and was used to screen cDNA libraries for full-length clones.

A total of five overlapping cDNA clones were isolated from three different mouse embryo oligo(dT)-primed cDNA libraries and from one mixed random-primed/oligo(dT)-primed cDNA library. The sequence of the longest *Rpx* clone contained a single open reading frame that would encode a predicted polypeptide of 185 amino acids. The nucleotide sequence and conceptual translation deduced from this and other overlapping cDNA clones, as well as 5′ RACE analysis, is shown in Fig. 1. This cDNA represents the full length or near full length transcript since its size is close to that of the predominant message (910 nt) seen on northern blots (see below and Fig. 3), taking into account the addition of poly(A) tails. In addition, all three reading frames are terminated upstream of the putative initiation codon.

A RACE PCR amplification procedure was used to verify the position of the 5′ end of the message. Two distinct products were obtained. The major product terminates several bp from the 5′ end of the cDNA, supporting the contention that this cDNA represents the entire smaller mRNA transcript. The other RACE-PCR product ends 380 bp upstream of the putative AUG codon (1123 bp from the poly(A) signal), consistent with the predicted size of the less abundant, longer transcript visualized on northern blots. Both start sites are indicated in Fig. 1.

The putative amino acid sequence of *Rpx* contains a homeodomain near the C-terminal end which is similar to the *paired (prd)* class of homeodomain sequences of *Drosophila* (Bopp et al. 1986). Comparison with other known homeodomain proteins reveals that the *Rpx* homeodomain belongs to a subset of *prd*-like homeodomains (Fig. 2A) that differ from canonical

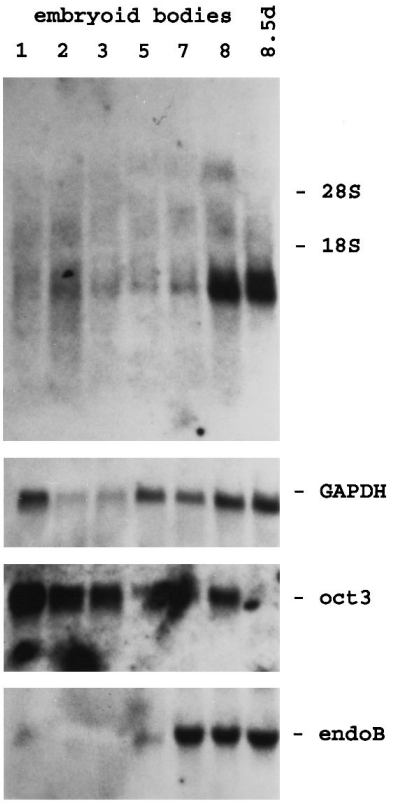

Fig. 4. Expression of *Rpx* in differentiating embryoid bodies. Northern blot analysis of total RNA (50μg/lane) extracted from embryoid bodies sampled at daily intervals after differentiation was induced by removal of feeder cells and LIF. The numbers 1-8 correspond to the day post-withdrawal. 50 μg of total RNA from 8.5 day embryos was included in the far right lane for comparison. Note the strong activation of *Rpx* at 8 days post-differentiation. the blot was stripped and reprobed consecutively with GAPDH as a control for loading, *Oct* 3 as a marker for undifferentiated cells, and *Endo B* as a marker for differentiated cells.

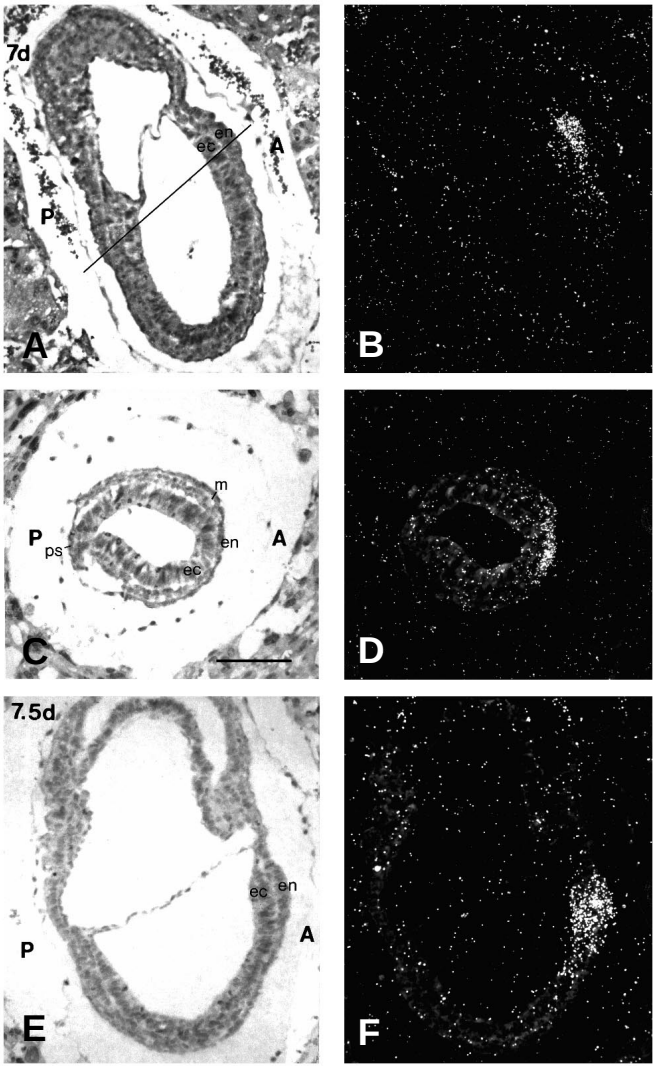

Fig. 5. Expression of *Rpx* during gastrulation. *Rpx* transcripts were localized by in situ hybridization to mouse embryo tissue sections. (A-D) At 7 days, *Rpx* is expressed in the mesendoderm (endoderm and prechordal plate) (en) at the cephalic end of the embryo. (A,B) Longitudinal section. (C,D) Cross section of a 7 day p.c. embryo. Plane of section is indicated in A. (E,F) A few hours later (7.5 days p.c.), expression is also seen in the ectoderm (ec) overlying the endoderm. (E) Bright-field optics,; (F) Dark-field optics. ps, primitive streak; en, endoderm/mesendoderm; m, mesoderm; A, anterior; P, posterior. Bar, 150 μm.

prd homeoproteins in two respects. First, the amino acid at position 9 of the recognition helix is a glutamine instead of a serine. This residue is known to be critical for the sequence specificity of DNA binding (Treisman et al., 1989). Second, *Rpx* lacks a *paired* box, another conserved DNA binding domain typically found N-terminal to the homeodomain in *prd* family members (Walther et al., 1991). In these respects, *Rpx* is more related to the *Xenopus* genes, *Mix-1* (Rosa, 1989) and *Xanf1* (Zaraisky et al., 1992); the mouse genes, *Chx10* (Liu et al., 1994), *S8* (Opstelten et al., 1991), *MHox* (Cserjesi et al., 1992), *Phox2* (Valarché et al., 1993), and *Cart-1* (Zhao et al., 1993); the *Drosophila* gene, *aristaless (al)* (Schneitz et al., 1993), and the *C. elegans* gene, *ceh-10* (Hawkins and McGhee, 1990). With the exception of *Xanf1*, which may be the *Xenopus* ortholog of *Rpx*, the homeodomains encoded by these genes are not extremely conserved and share only 50-55% homology.

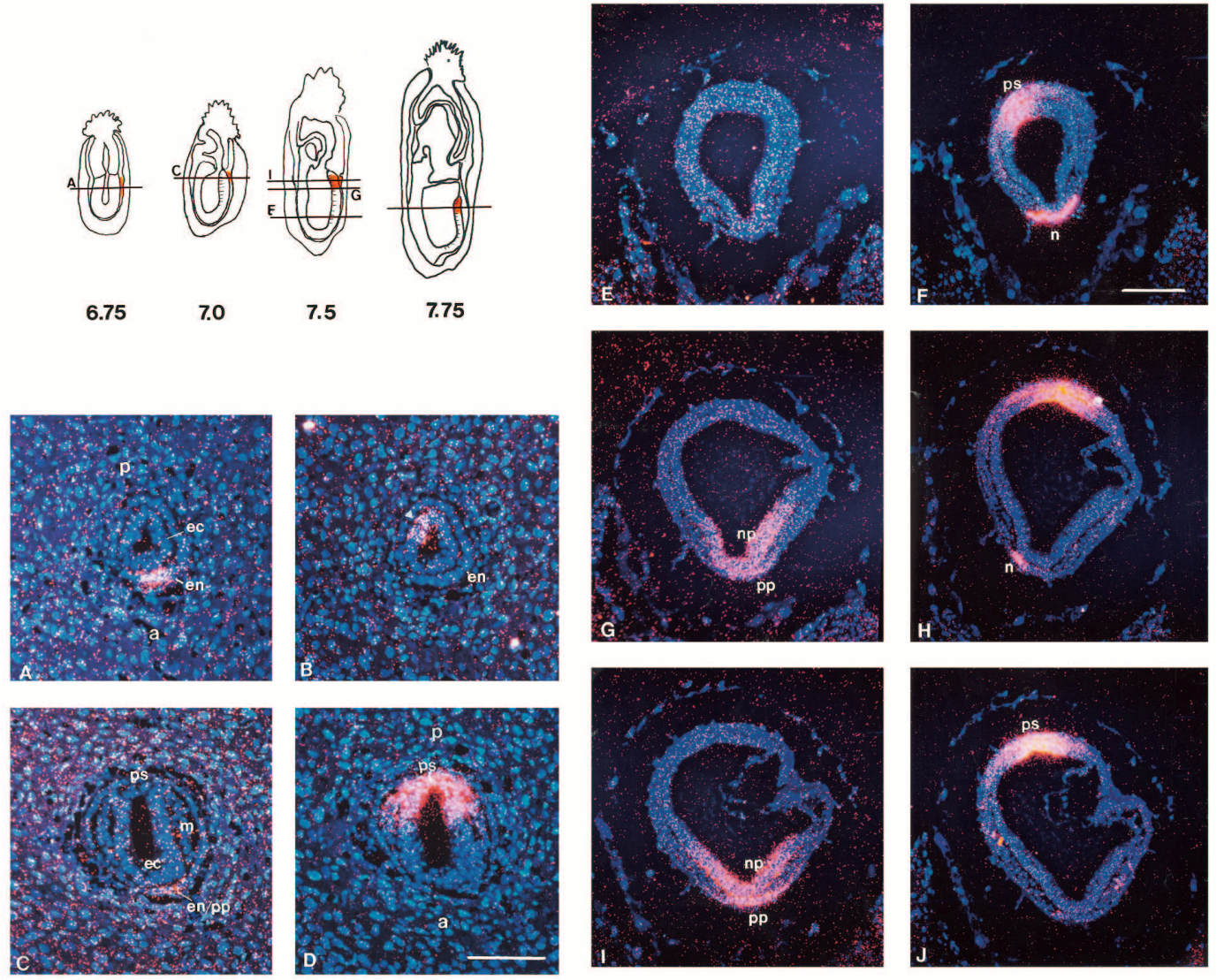

Fig. 6. Comparison of *Rpx* and *Brachyury* (*T*) expression during gastrulation. Adjacent transverse sections of mouse embryos at 6.75 (A,B), 7 (C,D), and 7.5 (E-J) days p.c. were hybridized to either *Rpx* (A,C,E,G,I) or *T* probes (B,D,F,H,J). Schematic view of embryos and approximate planes of sections are shown in top left corner (7.75 day embryo section is shown in Fig. 7 C,D). (A) At 6.75 days, the onset of gastrulation, *Rpx* expression is seen in the endoderm layer directly across from the ectodermal region (arrow) that expresses *T* (B). This *T*-expressing ectoderm marks the position of the primitive streak, although there is little evidence of delaminating mesodermal cells at this early stage. (C,D) *Rpx* expression in 7 day p.c. embryos is in the mesendoderm of the prechordal plate precursor (en/pp) (C). *T* expression is seen in the primitive streak and newly formed mesoderm directly across (and posterior) from the *Rpx* expression domain. (E-J) A series of transverse sections (distal to proximal) through a 7.5 day p.c. neural plate stage embryo hybridized to *Rpx* and *T* probes. (E,F) In the most distal sections, *Rpx* is not expressed (E), while *T* is strongly expressed in the primitive streak and the notochordal plate (F). (G,H) Slightly more proximal sections document the boundary between the *Rpx*-expressing prechordal plate (G) and the *T* expressing notochordal plate (H). The section in H was the most proximal section that showed *T* expression in the notochord, indicating its most rostral extent. Note that the *T*-positive cells do not overlap significantly with the *Rpx*-positive prechordal plate cells. (I,J) More proximal sections through the most rostral region of the embryo show expression of *Rpx* in the prechordal plate and cephalic neural plate (I). At this level, *T* is only expressed in the more posterior primitive streak. a, anterior; p, posterior; ec, primitive ectoderm; en, endoderm; m, mesoderm; ps, primitive streak; n, notochordal plate; np, neural plate; pp, prechordal plate. Bar, 100 μm in A-D; 200 μm in E-I.

The predicted *Rpx* protein also contains the octapeptide sequence present in some, but not all, *prd*-like homeodomain protein sequences. Interestingly, The *Rpx* octapeptide (FSIESILG) is more similar to the octapeptide domains of four non-*prd*-like homeobox genes (Noll, 1993) and the recently described *Chx10* (Liu et al., 1994) than to those of canonical *prd*-domain genes (consensus HSIDGILG) (Noll, 1993).

The homeodomain sequences of *Rpx* and *Xanf1* are 80% identical, and sequence comparison across the entire coding regions reveals additional regions of homology concentrated near the N terminus around the initiator methionine and the octapeptide sequence (Fig. 2B). These regions potentially encode domains involved in protein:protein interaction, such as transactivation or dimerization. The extended homology overlapping the octapeptide sequence in particular is rich in serine:threonine residues (9/38 residues). In addition, the relative position of these motifs within the coding regions and the size of the predicted proteins are practically identical between the two genes. Overall, the *Rpx* and *Xanf1* proteins show 48.1% sequence identity and 70.8% similarity, taking into account conservative changes. Although the homology is not extremely high, it is possible that *Rpx* is the ortholog of *Xanf1* (see Discussion).

Developmental profile of *Rpx* expression

Northern blot analysis of total RNA isolated from microdissected embryos was performed to ascertain when *Rpx* was expressed during embryogenesis. It was found that at 8.5 days p.c., the earliest time point analysed, there were at least two hybridizing bands: a predominant broad band of approximately 910 bases, and a minor species of 1.2 kb (Fig. 3). At later stages of development, only the smaller species is detected. At this level of analysis, transcripts were only detected up to 9.5 days p.c. and were specifically localized to the head but not the body.

These data indicated that *Rpx* was expressed early in postimplantation development. Since it was not technically feasible to isolate significant quantities of RNA earlier than day 8, northern analysis was performed on differentiating embryoid bodies to gain further insight into the developmental profile of *Rpx* expression (Fig. 4). Embryoid bodies are aggregates of ES cells that differentiate when cultured in the absence of feeder cells and the differentiation inhibitor, LIF. Differentiating embryoid bodies are believed to represent a good in vitro model system for the study of preimplantation and early postimplantation development (Doetschman et al., 1985). After one day postaggregation, *Rpx* expression was virtually undetectable. Expression was progressively activated after 2-3 days, but was dramatically upregulated at 8 days to levels roughly comparable to those seen in 8.5 day p.c. embryos.

Since the kinetics of in vitro differentiation attained by embryoid body aggregates can vary slightly depending on the passage number of the ES cells and other culture variables, the degree of differentiation was independently assessed by reprobing northern blots for expression of the POU domain gene *Oct-3* (Rosner et al., 1990; Scholer et al., 1990) and the *Endo B* cytokeratin gene (Singer et al., 1986), marker genes for undifferentiated and differentiating cells, respectively. *Rpx* is strongly upregulated as *Oct-3* begins to be downregulated, and after *Endo B* is activated. In vivo, *Oct-3* is expressed in preimplantation stage embryos and in the epiblast of postimplantation stage embryos, and is turned off during gastrulation as cells enter the primitive streak (Rosner et al., 1990; Scholer et al., 1990). By comparison, the hybridization data suggests that *Rpx* is activated at a stage comparable to gastrulation in these embryoid body cultures.

In situ hybridization analysis of *Rpx* expression during gastrulation and neurulation

The spatial pattern of expression of *Rpx* was examined by in situ hybridization to tissue sections of mouse embryos. Whole-mount in situ hybridization was also performed but, due to the low abundance of *Rpx* transcripts, was less reliable than hybridization of radiolabeled probes to tissue sections. Hybridization of *Rpx* probes to pre-gastrula stage embryos was negative, although transcripts could be detected by northern blot in undifferentiated ES cells (data not shown). Since ES cells are thought to resemble undifferentiated epiblast cells, it is conceivable that levels of transcripts below the limits of detection are present in these early embryos. *Rpx* transcripts were first detected by in situ hybridization at gastrulation (Figs 5, 6). At 7 days p.c., expression was detected in the endoderm/prechordal plate underlying the prospective cephalic neural plate (Figs 5A-D, 6). A few hours later (7.5 days p.c.), expression was detected in the ectoderm juxtaposed to the endoderm/prechordal plate, which will become the cephalic neural plate (Figs 5E,F, 6G,I, 7). This pattern of expression suggests that *Rpx* may be induced in the anterior neural plate by the adjacent *Rpx*-expressing mesendoderm of the prechordal plate. By the early head fold stage, *Rpx* expression has been extinguished in the underlying mesendodermal cells, and is limited to the anterior neuroectoderm (Fig. 7C,D), underscoring the dynamic nature of *Rpx* expression during this important period.

These data indicated that *Rpx* was expressed very early in an anterior-restricted domain along the developing embryonic axis. In order to define more precisely the temporal and spatial domain of *Rpx* expression, a comparative analysis of *Rpx* expression with that of *Brachyury* (*T*) (Fig. 6) and the homeobox gene *goosecoid* (*gsc*) was performed. The *T* gene is required for the proper development of the notochord, and has been shown to be strongly activated along the entire primitive streak at gastrulation and, at later stages, in the developing notochord (Herrmann, 1992). The murine *gsc* gene has been shown to be transiently expressed during gastrulation in the anterior-most region of the node (Blum et al., 1992) and, in other vertebrate species, in the prechordal plate (Izpisua-Belmonte et al., 1993; Steinbeisser and De Robertis, 1993).

During early gastrulation, 6.5-6.75 days p.c., *Rpx* expression was seen in a very restricted region of the endoderm, which in cross section was directly across from the *T* expression domain in the primitive streak. At the earliest stage examined (6.5 days; Fig. 6A,B), just as cells begin to delaminate from the primitive ectoderm to form mesoderm, *T* expression was visualized in the primitive ectoderm of the prospective primitive streak. The *Rpx*-expressing region (Fig. 6A) in the endoderm layer was clearly anterior – proximal to both *T* (Fig. 6B) and *gsc* (data not shown) expression domains in the ectoderm and, by inference from later expression patterns, may represent precursor cells of the prechordal plate or head process. Attempts to confirm this by colocalization with *gsc* transcripts, which are reported to be present in prechordal plate in both frog (Steinbeisser and De Robertis, 1993) and chick (Izpisua-

Belmonte et al., 1993) were not definitive, although hybridization slightly over background was seen in this region with *gsc* probes (data not shown). It may be that in the mouse, *gsc* is not expressed at very high levels in this region. It was clear, however, that the *Rpx* hybridization domain was anterior/proximal to the prominent *gsc* expressing region of the node. Alternatively, it is possible that *Rpx* expression at this early stage was in the primitive visceral embryonic endoderm, a cell layer that is essentially replaced by definitive endoderm during the process of gastrulation, and as such presages the prechordal plate primordium.

By 7-7.5 days p.c., *Rpx* was clearly expressed in the mesendoderm of the prechordal plate primordium (Fig. 6C,D), which has a raised, columnar appearance at the midline (Tam et al., 1982). Although at 7 days p.c. the *Rpx*-expressing region was distinctly separated from the domain of *T* expression in the primitive streak and notochordal plate, by 7.5 days, the *Rpx* expression domain in the prechordal plate directly abutted the *T* expression domain of the notochordal plate (Fig. 6E-J). By this stage, the *Rpx* gene was activated in the ectoderm overlying the prechordal plate (Figs 6G,I, 7A,B).

By 8-8.5 days p.c., *Rpx* expression was limited to the neuroectoderm of the prospective prosencephalon (Fig. 8). In 8.5 day embryos, expression was also detected in the oral ectoderm and in a very small region of the foregut endoderm that briefly lies in contact with the oral ectoderm at this stage of development (Fig. 8C,D). Expression in the foregut endoderm was extremely transient and, because it was seen only in an area of direct contact with ectoderm, may result from inductive signals emanating from the juxtaposed *Rpx*-expressing ectoderm.

Expression in the developing pituitary

Dramatic restriction of *Rpx* expression was seen at 9 days p.c. Expression was no longer detectable in the neuroectoderm, but was exclusively restricted to the layer of ectodermal cells that will give rise to Rathke's pouch, the primordium of the adenohypophysis, which ultimately becomes the anterior and intermediate lobes of the pituitary (Fig. 9A,B). Rathke's pouch originates from a cohort of cells within the oral ectoderm that come to lie in contact with the floor of the diencephalon (Schwind, 1928). Classical embryological experiments in several vertebrate species suggest that reciprocal inductive interactions between the embryonic brain and the pituitary primordium in this region of contact are crucial for the development and proper differentiation of the adenohypophysis (derived from Rathke's pouch), and of the median eminence of the hypothalamus (Pehlemann, 1962; Etkin, 1967; Wantanabe, 1982a,b; Daikoku et al., 1982, 1983). Subsequently, the neurohypophysis, derived from an outpocketing of the diencephalic neuroectoderm adjacent to the pouch, gives rise to the posterior or neural lobe of the pituitary, which contains the axon terminals of neurosecretory neurons which reside in the hypothalamus. Thus, the developmental programs of these essential components of the hypothalamic-pituitary axis are interdependent.

The cells of Rathke's pouch primordium invaginate and eventually detach from the oral ectoderm to form a definitive pouch structure by 11.5 days p.c. *Rpx* is strongly expressed in virtually all of the cells of the pouch at this stage (Fig. 9C,D). Around 12.5-13 days, definitive pituitary cell types begin to differentiate in the developing pouch in a stereotypical spatial and temporal order (reviewed in Simmons et al., 1990; Japón et al., 1994), beginning with corticotrophs that appear in the ventral pouch at 12.5 days and thyrotrophs in the rostral tip at 13.5 days, followed by melanotrophs at 14.5 days, and somatotroph, lactotrophs, and gonadotrophs at 15.5-16 days p.c. *Rpx* was found to be downregulated spatially and temporally coincident with this differentiation, such that by 15.5 days, expression was no longer detectable (Fig. 9E,F).

The progressive downregulation of *Rpx* occurs in a distinct ventral to dorsal direction (Fig. 10), mirroring the general direction of differentiation of the pouch, beginning at 12.5-12.75 days within the cells of the so-called rostral tip (Fig. 10A), which produce a transient population of thyrotroph cells early in development (Lin et al., 1994). *Rpx* transcripts disappear from the entire ventral portion that will give rise to the anterior lobe by 13.5 days (Fig. 10C,D). At 14.5 days p.c., only sparse labeling was seen in the dorsal part of the pouch (the incipient intermediate lobe), particularly in areas in contact with the neuroectoderm of the diencephalon or posterior lobe as well as in cells lining the lumen of the pouch (Fig. 10E).

The extinction of *Rpx* expression in the anterior lobe coincided temporally and spatially with the activation of the pituitary-specific POU domain transcription factor *Pit*-1 (Fig. 10D), which has been shown to occur at 13.5 and 15 days of embryogenesis in the mouse and rat, respectively (Dollé et al., 1990; Simmons et al., 1990). *Pit-1* is required for the expression of growth hormone (GH), prolactin (PRL) (Ingraham et al., 1990; Fox et al., 1990; Castrillo et al., 1991), and β-thyroid stimulating hormone (β-TSH) genes (Lin et al., 1994). In addition, *Pit-1* plays a poorly understood role in the proliferation and/or maintenance of pituitary cell types in development, for the *Snell dwarf (dw)* mutant mouse, which lacks *Pit-1* gene function (Li et al., 1990; Camper et al., 1990), has a severely hypoplastic pituitary that lacks three pituitary cell lineages – thyrotroph, somatotroph, and lactotroph (Slaughbaugh et al., 1981).

Comparative in situ hybridization of neighboring sections revealed that *Pit*-1 expression was first detectable in the ventral region of the developing anterior pituitary where *Rpx* expression had turned off at 13.5 days (Fig. 10D). By 14.5 days p.c., *Pit-1* was almost uniformly expressed in the ventral anterior lobe, where *Rpx* expression was no longer detectable (Fig. 10F). These reciprocal expression domains may indicate potential regulatory relationships between *Rpx* and *Pit-1*.

DISCUSSION

We report here the isolation and characterization of a new murine homeobox gene that is expressed dynamically during gastrulation in the future cephalic region of the embryo. Because this gene is ultimately restricted in its expression to Rathke's pouch, the precursor of the mammalian pituitary gland, the gene has been called *Rpx* for Rathke's pouch homeobox. *Rpx* is a member of a subfamily of homeobox genes related to the *prd* homeobox gene of *Drosophila* that differ most notably in that they lack the *paired* box found in *Drosophila prd, gooseberry* and vertebrate *Pax* genes.

Expression of *Rpx* is initially detected during gastrulation in mouse embryos in the midline endoderm/mesendoderm that

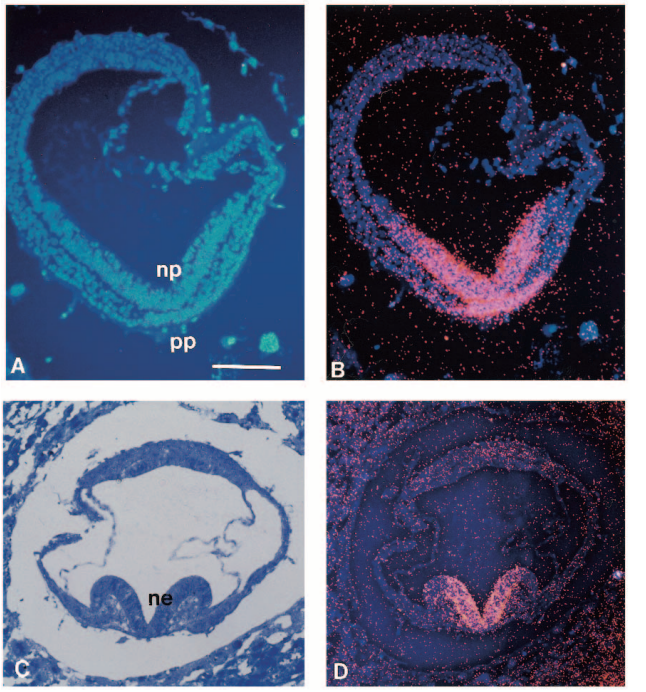

Fig. 7. Expression of *Rpx* during late gastrulation and neurulation. (A,B) The section of a 7.5 day embryo from Fig. 6I shown at higher magnification. (A) Hoechst-stained section viewed with epifluorescence optics only. (B) Same section simultaneously viewed with epifluorescence and dark-field optics. Note thickened appearance of prechordal plate cells and lack of detectable expression in the mesodermal cells on either side of the midline. (C,D) In head fold stage embryos (7.75 days p.c.; plane of section shown schematically in Fig.6), expression is only evident in the cephalic neuroectoderm, but not in underlying mesendoderm of the prechordal plate or head mesoderm. (C) Geimsa stained section. (D) Section viewed under dark-field illumination. np, neural plate; ne, neuroectoderm. Bar, 100 μm in A,B; and 250 μm in C,D.

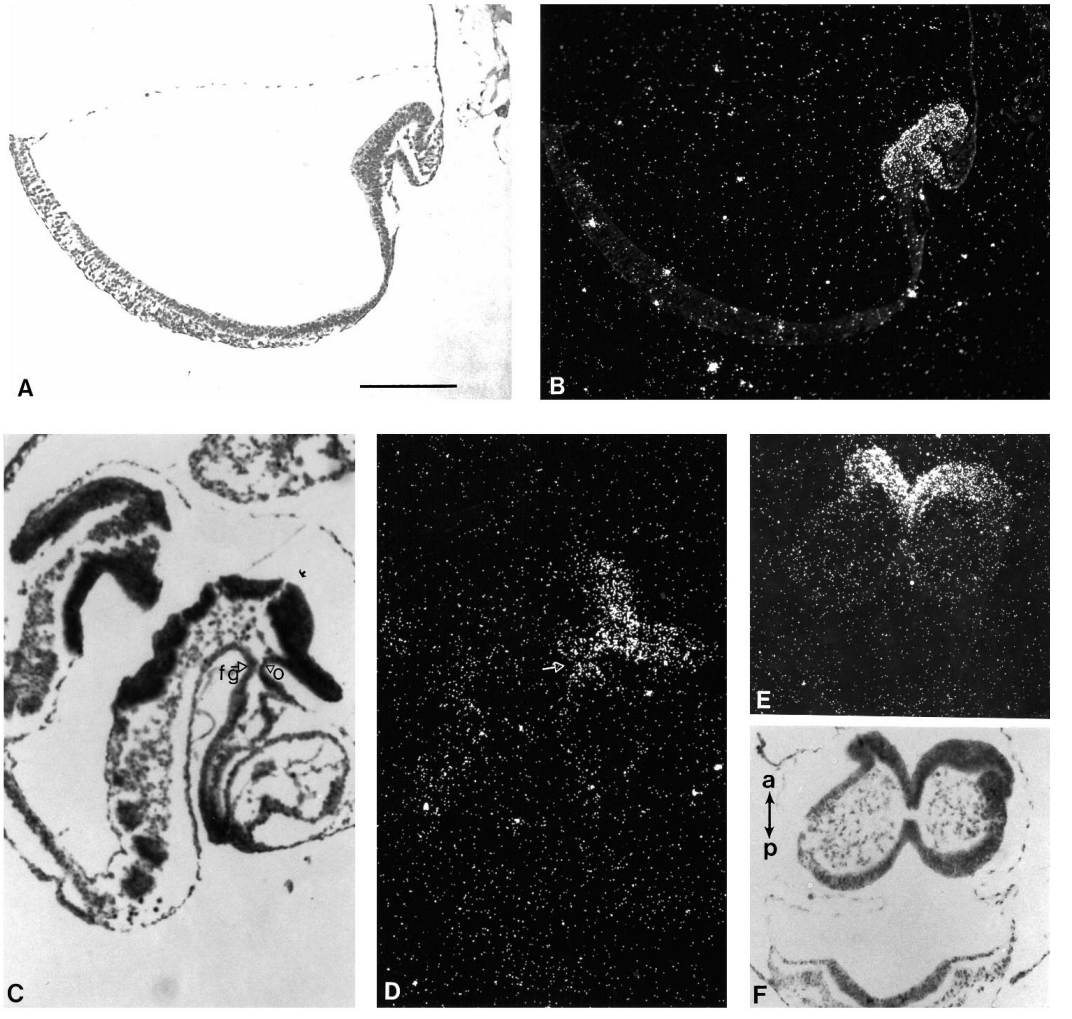

Fig. 8. Expression of *Rpx* in 8-8.5 day p.c. embryos. (A,B) In situ hybridization of *Rpx* probe to a slightly oblique longitudinal section of an 8 day p.c. four-somite embryo shows expression is restricted to the cephalic neuroectoderm. (C-F) Expression in 8.5 day p.c. embryos. (C,D) Longitudinal section demonstrating expression domain in the rostral neuroectoderm corresponding to the prosencephalic region. Note that expression is also seen in the oral ectoderm and in a small region of the rostral foregut endoderm (open arrow) that is in contact with the *Rpx*-expressing oral ectoderm. (E,F) A transverse section through the cephalic region of an 8.5 day embryo showing that expression is confined to the most rostral neuroectoderm of the prospective prosencephalic region. a, anterior; p, posterior; o, oral ectoderm; fg, foregut endoderm. Bar, 200 μm in A,B; 170 μm in C-F.

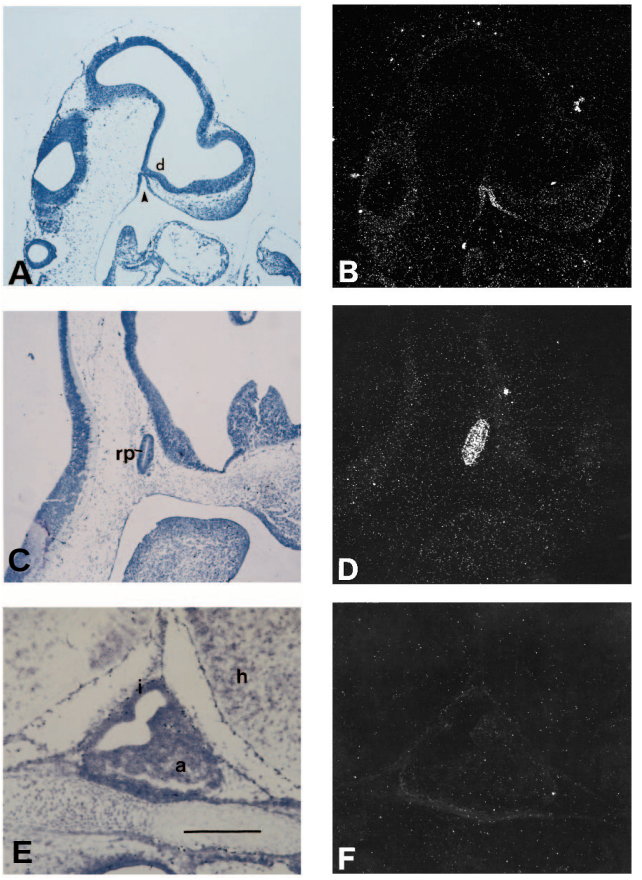

Fig. 9. Expression of *Rpx* during pituitary development. *Rpx* is transiently expressed during the formation of the anterior and intermediate lobes of the pituitary. (A-F) Sagittal sections of mouse embryos hybridized to *Rpx* probes. (A,B) At 9.5 days p.c., transcripts become restricted to the ectodermal placode that gives rise to Rathke's pouch (arrowhead). (C,D) Expression is still strong throughout Rathke's pouch at 11.5-12 days. (E.F) By 15.5 days, only background levels of transcripts are seen. a, anterior lobe; rp, Rathke's pouch; d, diencephalon (future hypothalamus), h, hypothalamus; i, intermediate lobe. Bar, 500 μm in A-D. and 400 μm in E and F.

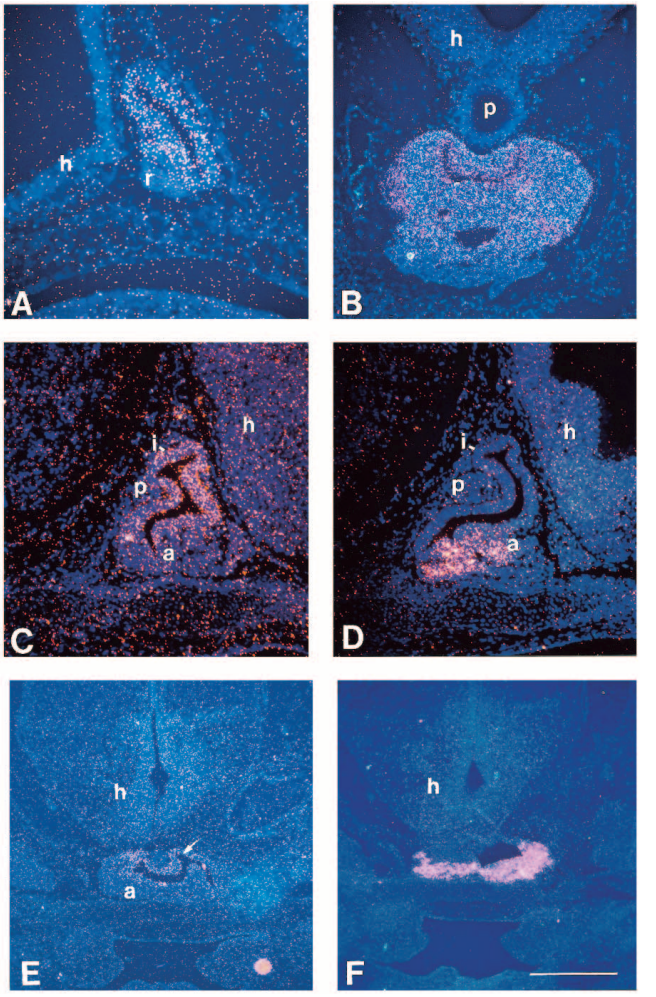

Fig. 10. Downregulation of *Rpx* in the pituitary. (A,B) *Rpx* is progressively downregulated in a ventral to dorsal direction, beginning with the rostral tip of the incipient anterior lobe at 12.5-12.75 days. (C) By 13.5 days, expression is not detected in the ventral region of the pouch (the incipient anterior lobe), but is still expressed in the dorsal region of the pouch (incipient intermediate lobe). (D) The pituitary-specific transcription factor *Pit-1* is activated in the ventral region of the developing anterior lobe coincident with the downregulation of *Rpx*. (E) By 14.5 days, expression of *Rpx* only persists in the dorsal region of the pouch (arrow) that is in contact with the floor of the diencephalon (hypothalamus) and posterior lobe. (F) *Pit-1* is expressed throughout the anterior lobe at 14.5 days. Plane of sections: A,C,D, sagittal; B,E,F, coronal. r, rostral tip; a, incipient anterior lobe; p, incipient posterior lobe; i, incipient intermediate lobe; h, hypothalamus. Bar, 200 μm in A-D and 500 μm in E and F.

forms the prechordal plate precursor. The prechordal plate presumably is involved in the induction of anterior head structures, including the forebrain, and prechordal plate precursor cells have been shown to possess neural inducing activity in chick embryos (Izpisua-Belmonte et al., 1993). Other potential developmental regulatory genes have been reported to be expressed in the prechordal plate, including *lim-1* (Barnes et al., 1994) and *Otx2* (Simeone et al., 1993; Ang et al., 1994), *HNF-3β* (Sasaki and Hogan, 1993; Ang and Rossant, 1993; Weinstein et al., 1994) and *gsc* (Izpisua-Belmonte et al., 1993). However, *Rpx* is, to our knowledge, the only gene that is exclusively expressed in this region at 6.5-7 days. Most of these other genes are also simultaneously expressed, often at higher levels, in the notochord and primitive streak (*lim-1*, *HNF-3β*) or in the ectoderm (*Otx2*). *Gsc* is expressed in the prechordal plate in both chick and frog embryos, but our attempts to colocalize *gsc* with *Rpx* transcripts indicate that the expression of *gsc* in this region in the mouse is extremely low. The zebrafish gene *hlx-1* is expressed in the early prechordal plate (Fjose et al., 1994), but expression of the apparent mouse ortholog (*Dbx*) has not been reported there (Lu et al., 1992).

The amino acid sequence of *Rpx* is most similar to that of *Xenopus Xanf1*, a homeobox gene reported to be expressed in the anterior neural folds of the frog embryo (Zaraisky et al., 1992). These two genes may be orthologs, for they show 48.1% total homology and 80% homology in the homeodomain. Since the homology is not extremely high relative to other orthologous homeobox genes, it is also possible that there may be another gene that is more similar. However, it has been found

that *Xanf1* and a very similar *Xenopus* gene, *Xanf2* are both expressed in anterior neural plate and in the pituitary primordium in the frog (Mathers et al., 1995).

Several features of the *Rpx* expression pattern suggest that it is responsive to inductive interactions. At approximately 7.5 days p.c., corresponding to the neural plate stage, transcripts of *Rpx* can first be detected in the rostral ectoderm overlying the *Rpx*-expressing prechordal plate in a manner suggesting that *Rpx* expression in the ectoderm is induced by signals emanating from the *Rpx*-expressing cells in contact with it. It has, for example, been shown that grafted *gsc*-expressing cells are capable of inducing *gsc* expression in host tissues (Izpisua-Belmonte et al., 1993). This can be experimentally addressed in the mouse through the use of recombinant tissue explants of gastrulation stage embryos (Ang and Rossant, 1993).

By extrapolation from the limited fate map available for the gastrulating mouse embryo, the *Rpx* expression domain in the cephalic neural plate of the 7.5 day embryo is limited to the prospective prosencephalic region and perhaps part of the mesencephalon (Tam, 1989; Tam and Beddington, 1992). Certainly by 8.5 days, expression is restricted to the prosencephalon, and serves as the most anterior restricted marker for this region. *Rpx* expression is also detected in the oral ectoderm at this stage and in a region of the rostral foregut endoderm that lies in contact with it, again suggestive of inductive interactions.

Restriction of expression to Rathke's pouch occurs at 9-9.5 days p.c. Fate mapping in chick (Couly and Le Douarin, 1988), frog (Eagleson and Harris, 1989; Kawamura and Kikuyama, 1992), and mouse (Osumi-Yamashita et al., 1994) has shown that the primordium of the adenohypophysis is derived from the anterior ridge of the neural plate. This assignment is consistent with the *Rpx* expression domain, which becomes progressively anteriorly restricted within the cephalic neural plate during development. Alternatively, expression of *Rpx* may be biphasic, with the early prechordal plate/neural plate expression being distinct mechanistically from the later expression in the pouch.

The adenohypophysis does not form in chick embryos after surgical removal of the anterior neural ridge, suggestive of commitment to pituitary development prior to neural tube closure (elAmraoui and Dubois, 1993). *Rpx* is the earliest known gene expressed in the ridge and pituitary primordium, suggesting involvement in the early determination or differentiation of this organ. Its expression precedes that of the α-glycoprotein subunit gene (*αGSU*), the first hormone gene reported to be expressed in the developing pituitary. *αGSU* is expressed in the placode that gives rise to Rathke's pouch by day 11 in the rat (10.5 days p.c. in mouse), and is thought to be a marker for most or all of the progenitor cells of the pituitary (Simmons et al., 1990). Most obviously, the *Rpx* gene could be required for the proper differentiation of the adenohypophysis. Alternatively or in addition, *Rpx* expression could be crucial for the development of other tissues known to interact with the pouch via cell-cell contact, such as the median eminence of the hypothalamus and associated posterior lobe of the pituitary, by regulating the expression of signaling molecules in the pouch.

Rpx expression in the developing pituitary is transient and is extinguished as differentiated pituitary cell types appear. In the anterior lobe, this extinction coincides temporally and spatially with the transcriptional activation of the *Pit-1* gene, raising the possibility that *Pit-1*, or one of its regulators, may negatively control *Rpx* expression in this part of the developing gland. This possibility is being investigated by the analysis of the transcriptional regulation of the *Rpx* gene.

The role *Rpx* plays in development is currently under analysis through the generation of both gain-of-function and loss-of-function mouse models. However, since the expression of *Rpx* is detected several days before the formation of a definitive Rathke's pouch, and since the initial *Rpx* expression domain encompasses a much larger region than that fated to form Rathke's pouch, it seems likely that *Rpx* plays a role in the early establishment of the anterior region of the embryo. *Rpx* may be instrumental in defining a larger embryonic field that is eventually divided into smaller developmental units through the interaction with other developmental control genes. In this respect, it is interesting that the *Otx2* expression domain overlaps with that of the *Rpx* gene in the anterior neural plate at early stages, and it has been shown that this expression depends on signals from the anterior mesendoderm (Ang et al., 1994). *Rpx* potentially may regulate, or be regulated by, these signals. As development proceeds and *Rpx* expression becomes more anterior-restricted to Rathke's pouch, *Otx2* expression regresses from its most anterior extent, and is localized to discrete regions of the forebrain (Simeone et al., 1992), raising the possibility that *Rpx* and *Otx2* may be part of an interactive regulatory gene network involved in defining anterior cell fates.

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Note added in proof

While this manuscript was in press, it came to our attention that the sequence of *Rpx* is the same as that of the *Hesx1* gene recently reported by Thomas et al., *J. Biol. Chem.* **270**, 3869-3875 (1995).

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