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Segment-Specific Expression of the *neuronatin* Gene during Early Hindbrain Development

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The developing hindbrain is segmented in a series of repetitive bulges called neuromeres or rhombomeres. In the mouse, first molecular evidence for segmentation of the hindbrain came from rhombomeres 3- and 5-specific expression of the *Krox-20* gene. The hindbrain segments are linked with the expression of different Hox genes which have a role in patterning the hindbrain and branchial region of the vertebrate head. Here we identified by subtractive hybridization a gene, mouse *neuronatin*, that is downregulated in P19 embryo carcinoma cells that have undergone a partial differentiation process. *Neuronatin* encodes putative transmembrane proteins of 54, 55, and 81 amino acids that might serve as protein ligands, cofactors, or small cell adhesion molecules. The *neuronatin* gene is transiently expressed in rhombomeres 3 and 5 during early hindbrain development and in the floor of the foregut pocket. In addition, expression is observed in the early Rathke's pouch, in the derived adenohypophysis, and in the developing inner ear. During later embryogenesis the *neuronatin* gene is strongly expressed in the major part of the central and peripheral nervous system. These results suggest that *neuronatin* participates in the maintenance of segment identity in the hindbrain and pituitary development and maturation or maintenance of the overall structure of the nervous system. © 1995 Academic Press, Inc.

INTRODUCTION

The developing hindbrain reveals a segmental organization (for review see Vaage, 1969). The segments, called neuromeres or rhombomeres, are associated with certain cranial ganglia and neurons (Lumsden and Keynes, 1989). In addition, the rhombomeric segmentation plays a role in the patterning of the cranial neural crest (Chisaka *et al.*, 1992; Hunt *et al.*, 1991; Krumlauf *et al.*, 1993; Lufkin *et al.*, 1991; Lumsden, 1991).

Several regulatory genes are reported to be expressed in the hindbrain. The transcription factor-encoding gene *Krox-20* was the first gene shown to be expressed in specific rhombomeres (r), r3 and r5 (Wilkinson *et al.*, 1989a). The *Hox* genes are expressed in the spinal cord and show anterior limits of expression at rhombomeric borders in the hindbrain (for review see Krumlauf *et al.*, 1993). In the hindbrain, *Hox-b1* (Murphy *et al.*, 1989) and the receptor–protein tyrosine kinase (RTK) *eck* are specifically expressed in r4 (Ruiz and Robertson, 1994), whereas another RTK, *sek*, is expressed in r3 and r5 (Nieto *et al.*, 1992). In a subtractive hybridization screen, we identified a novel mouse gene called *neuronatin* which is downregulated in a P19 embryo carcinoma (E.C.) cell line that has gone through a partial differentiation process. Recently, the rat homologue of mouse *neuronatin* encoding a putative transmembrane protein of 81 amino acids was cloned as a gene expressed in newborn rat brains and subsequently downregulated during the process of senescence (Joseph *et al.*, 1994). Here, we report three alternatively spliced mouse *neuronatin* cDNA sequences, the deduced proteins, and the expression pattern during embryogenesis and adulthood. The mouse neuronatin gene codes for putative proteins of 54, 55, and 81 amino acids. The neuronatin proteins have identical basic hydrophilic carboxyl ends that might serve as protein cofactors, ligands, or protein–protein interaction domains and hydrophobic aminoterminal ends that might serve as a signal peptide for translocation into the membrane (Briggs and Gierasch, 1986). We show that *neuronatin* is expressed in r3 and r5 during early hindbrain development, suggesting an early role in the maintenance of segment identity in the hindbrain. In addition, our results suggest that neuronatin participates in the development of the adenohypophysis and maturation of the nervous system.

MATERIALS AND METHODS

Cell Culture

P19 E.C. cells were stably transformed with pGKNeo and the heat shock vector pHS (pool P19B cells) or with pGKNeo

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and the pHSPax6 vector encoding the complete coding sequence of *Pax6* (Walther and Gruss, 1991) under the control of the *hsp68* promoter (pool P19A cells). Transfected cells were grown in Dulbecco's medium containing 10% serum and 400 μg/ml G418. Single P19A and P19B colonies (P19A cell line 1 and P19B cell line 1) were picked and the cells were heat shocked for 1 hr at 42°C and allowed to recover for 2 hr at 37°C. mRNA was isolated using the urea/LiCl procedure followed by purification on oligo(dT) columns (Pharmacia). P19B cell line 1, which has similar P19 E.C. cell morphology and cell growth to P19A cell line 1 or normal P19 cells, has gone through a partial differentiation process, as is shown with markers described in this report.

Subtraction and Differential Screening Procedure

The subtraction procedure of Wang and Brown (1991) was combined with a differential screening method. Briefly, cDNA from P19A and P19B mRNA was synthesized, digested with *Alu*I or *Alu*I/*Rsa*I, and adaptor-ligated. The adaptor-ligated cDNA in the range from 200 to 2000 bp was isolated from low melting point agarose (BRL) and amplified by PCR. Each pool of PCR-amplified linker-ligated cDNA (5 μg) was hybridized with an excess of biotinylated driver cDNA (100 μg) from the other cDNA pool, and the biotin-containing double- and single-stranded cDNA was removed by binding streptavidin to the biotinylated cDNA followed by phenol–chloroform extractions (Sive and St. John, 1988). The P19A cDNA, after six subtractions (P19A-6 cDNA), was cut with *Eco*RI and subcloned into pBluescript (Stratagene), and 1500 clones were plated on a 24 × 24-cm Nunc disc. A replica filter was made and hybridized with radiolabeled full-length *Pax6* cDNA to identify the *Pax6* cDNA clones. Subsequently, the same filter was hybridized for 2 days at 65°C with a mixture of 500 ng random primed radiolabeled subtracted P19A-6 cDNA plus 100 μg unlabeled P19B-6 cDNA and 100 μg unlabeled P19B-1 cDNA prehybridized for 1 hr at 65°C in 5× SSC in a volume of 125 μl. Washings were performed twice for 20 min at 65°C in 3× SSC, 0.3× SSC and 0.1× SSC containing 0.1% SDS. Subsequently, the same filter was hybridized with a mix of 500 ng radiolabeled subtracted P19B-6 cDNA plus 100 μg unlabeled P19A-6 cDNA and 100 μg unlabeled P19A-1 cDNA. From this differential screen we used 109 clones for further analysis, excluding 18 *Pax6* clones. Plasmid DNA was isolated, 2–4 μg was digested with *Eco*RI, and duplicate Southern blots were made. Each of the Southern membranes (Qiagen) were hybridized with 500 ng random primed radiolabeled nonsubtracted P19A-0 cDNA or P19B-0 cDNA. From this differential screen 50 clones were further analyzed on a Northern blot containing 5 μg of total RNA from P19A or P19B E.C. cells. From the 1500 clones analyzed in this way, 29 were differentially expressed.

Screening of cDNA Libraries

cDNA libraries were synthesized from 14.5-days p.c. brain or from heat-shocked P19A E.C. cell poly(A)$^+$ using random hexamers or oligo(dT) for the synthesis of the first strand cDNA, respectively. Approximately 8 × 10^5 clones of the 14.5-days p.c. brain cDNA library in λExcell or 8 × 10^5 clones of the P19A E.C. cell cDNA library in LambdaZAP were transferred to Qiagen membranes and hybridized with *neuronatin* PCR fragments (P19A6.42 and P19A6.89) obtained from the differential screening. Hybridization was performed overnight at 65°C in 3× SSC, 0.1% SDS, 10× Denhardt's, 0.1 mg/ml denatured salmon sperm DNA. After hybridization the membranes were washed twice for 20 min at 65°C with 3× SSC, 0.3× SSC, 0.1× SSC containing 0.1% SDS. Plasmids from the phage clones were released according to the instructions from Pharmacia (pExcell) or Stratagene (pBluescript).

In Situ Hybridization

RNA *in situ* analysis was performed on 8- to 10-μm-thick paraffin tissue sections. Single stranded ^{35}S-labeled sense and antisense RNA probes were generated by *in vitro* transcription of a plasmid clone containing a *Eco*RI/*Xho*I (residues 158–575) *neuronatin* cDNA fragment. It contains the complete coding region and part of the 3′ noncoding region and encodes the 55-amino-acid protein form. Identical expression patterns were observed using a 3′ probe *Xho*I/*Eco*RI (residues 576–1169), although this probe gave higher nonspecific background. Embryos from NMRI/B6D2 mice used for *in situ* hybridization were staged as Day 0.5 at noon of the day of the vaginal plug.

Whole-Mount Hybridization

Whole-mount *in situ* hybridization was performed as described using digoxigenin-labeled *in vitro* transcribed RNA (Wilkinson, 1992). Single-stranded sense or antisense digoxigenin-labeled *neuronatin* RNA probes were generated from the same plasmid as described above. After whole-mount hybridization, the embryos were cleared in a series of 30, 50, and 80% glycerol/PBT for 1 day for each step. For sectioning, embryos were passed into PBS, fixed for 2 hr in 4% paraformaldehyde in PBS, washed for 5 min in PBS, washed for 5 min in 0.86% NaCl, and dehydrated in an ethanol/PBS series (30, 50, 70, 80, 90, 96, 100%; 5 min each) and soaked in isopropanol for at least 10 min, in toluene for 5 min, and subsequently for at least 2 hr in paraffin wax (paraplast plus) at 60°C. After oriented embedding in paraffin wax, 8-μm sections were prepared on gelatin-coated slides. Sections were dewaxed for 3 min in xylene and mounted in Eukitt.

RESULTS

Isolation of a Gene, neuronatin, Downregulated in a Partially Differentiated P19 Cell Line

A cDNA subtraction protocol was used to identify the genes differentially expressed in two P19 E.C. cell lines,

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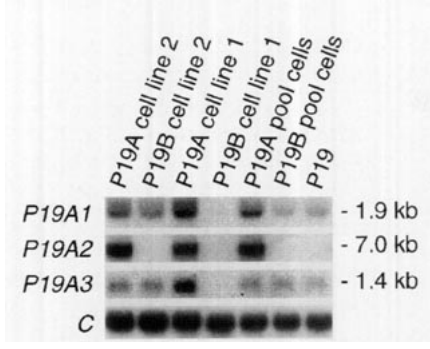

FIG. 1. Northern blots showing three differentially expressed genes (*P19A1*, *P19A2*, and *P19A3*) in P19 cells. Total RNA (10 μg) from two different cloned P19A or P19B cell lines or from pools of P19A or P19B cells or normal P19 cells were loaded in each lane as indicated.

P19A cell line 1 and P19B cell line 1. From 1500 subtracted clones analyzed, we obtained 29 cDNA clones that detected a more abundant mRNA transcript in P19A than in P19B cells. These differentially expressed clones could be divided into three groups on account of the length of the transcript (Fig. 1). The first group contained one clone that detected a 1.9-kb transcript from gene *P19A1*. The second group contained four overlapping cDNA clones that detected a 7-kb transcript from gene *P19A2*. The third group comprised 24 partially overlapping cDNA clones that detected a 1.4-kb transcript from gene *P19A3*.

All of the three identified genes were differentially expressed in the cloned P19A cell line 1 compared to that in the P19B cell line 1. However, when total RNA from pools of P19A and P19B cell lines was analyzed by Northern blotting we observed a different result. Genes *P19A1* and *P19A2*, but not gene *P19A3* were, as expected, differentially expressed in pools of P19A compared to that in P19B E.C. cells (Fig. 1). The further characterization of genes *P19A1* and *P19A2*, which are candidate Pax6 target genes, will be described elsewhere. The lower level of expression of the *P19A3* gene in P19B cell line 1 compared to that in the pool of P19B cell lines and P19B cell line 2 is most likely due to spontaneous initiated differentiation of P19B cell line 1. We could not detect morphological or growth differences between the P19A and P19B cell lines 1 or normal P19 E.C. cells, indicating that the P19B cell line 1 has gone through a partial differentiation process. *P19A3* was chosen for further characterization since the gene showed an interesting transcript distribution during embryogenesis indicating that the gene products might participate in early hindbrain and adenohypophysis development or differentiation.

Twenty-two cDNA fragments for clone *P19A3* were sequenced and compared with the EMBL DNA sequence library. These cDNAs showed extensive homology (94%) with different regions of a rat cDNA called *neuronatin* (Joseph *et al.*, 1994) and with rat and human expressed sequence tags (Adams *et al.*, 1993; Lomri *et al.*, 1989). Therefore, we renamed our clone *P19A3* as mouse *neuronatin*. Hereafter, we will report the cloning of the mouse *neuronatin* gene and its expression pattern during embryogenesis and will discuss the probable processes in which this gene participates.

Alternative Splicing of neuronatin mRNAs

To obtain the coding sequences of the *neuronatin* gene, mouse cDNA libraries from 14.5-days p.c. brain and P19A E.C. cells were screened for full-length cDNA. Due to alternative splicing at the 5′ end, we isolated three different cDNAs with three different open reading frames using two different ATGs (Fig. 2A). The first alternative splice form, *neuronatin-1*, was isolated from the P19A E.C. cell cDNA library. The two other alternative splice forms, *neuronatin-2* and *neuronatin-3*, were isolated from the 14.5-days p.c. brain library. Each of the open reading frames start at the first 5′ ATG, and the surrounding sequence shows good homology to the consensus Kozak translation initiation sequence (Kozak, 1991). Three putative mouse *neuronatin* proteins of 54, 55, and 81 aa were deduced and compared with the published rat 81 aa protein and a human 55 aa protein deduced from the human expressed sequence tag (Fig. 2B). These five proteins contain conserved hydrophobic NH2-terminal ends of 23 amino acids and strongly basic COOH-terminal ends of 28 amino acids.

neuronatin Expression during Development and in Adult Tissues

The tissue distribution of *neuronatin* in the adult mouse was examined by Northern blot analysis using poly(A)$^+$ RNA from several tissues. A transcript of 1.4 kb was detected at high levels in the brain and ovary, with weaker expression in the rest of the analyzed tissues (Fig. 3A). A Northern blot containing poly(A)$^+$ RNA from mouse embryos revealed that the *neuronatin* gene is expressed from 10.5–17.5 days p.c. (Fig. 3B).

neuronatin Is Expressed in Specific Segments of the Developing Hindbrain

The expression analysis was extended by using *in situ* hybridization and whole-mount *in situ* histochemistry on mouse embryos. In the hindbrain at 8.5 days p.c., expression was detectable in r3 only and slightly later also in r5 (Figs. 4A and 4C, and data not shown). Around 9.0 days p.c., strong expression was detected in both rhombomeres (Fig. 4B). Around 9.5 days p.c., expression in r3 retracted to the ventral half of the rhombomere, whereas from 9.5 days onward expression in the ventral ventricular zone of the other rhombomeres increased (Figs. 4F, 4G, 5A, 6A, 6C, and 6E). From 9.75–10.0 days p.c., the expression in the ventricular zone of r5 retracted to the ventral half (Figs. 5A and 5D). In the hindbrain, the newly forming intermediate zone of the hindbrain started to express *neuronatin* (Figs. 5C, 5D, 6A,

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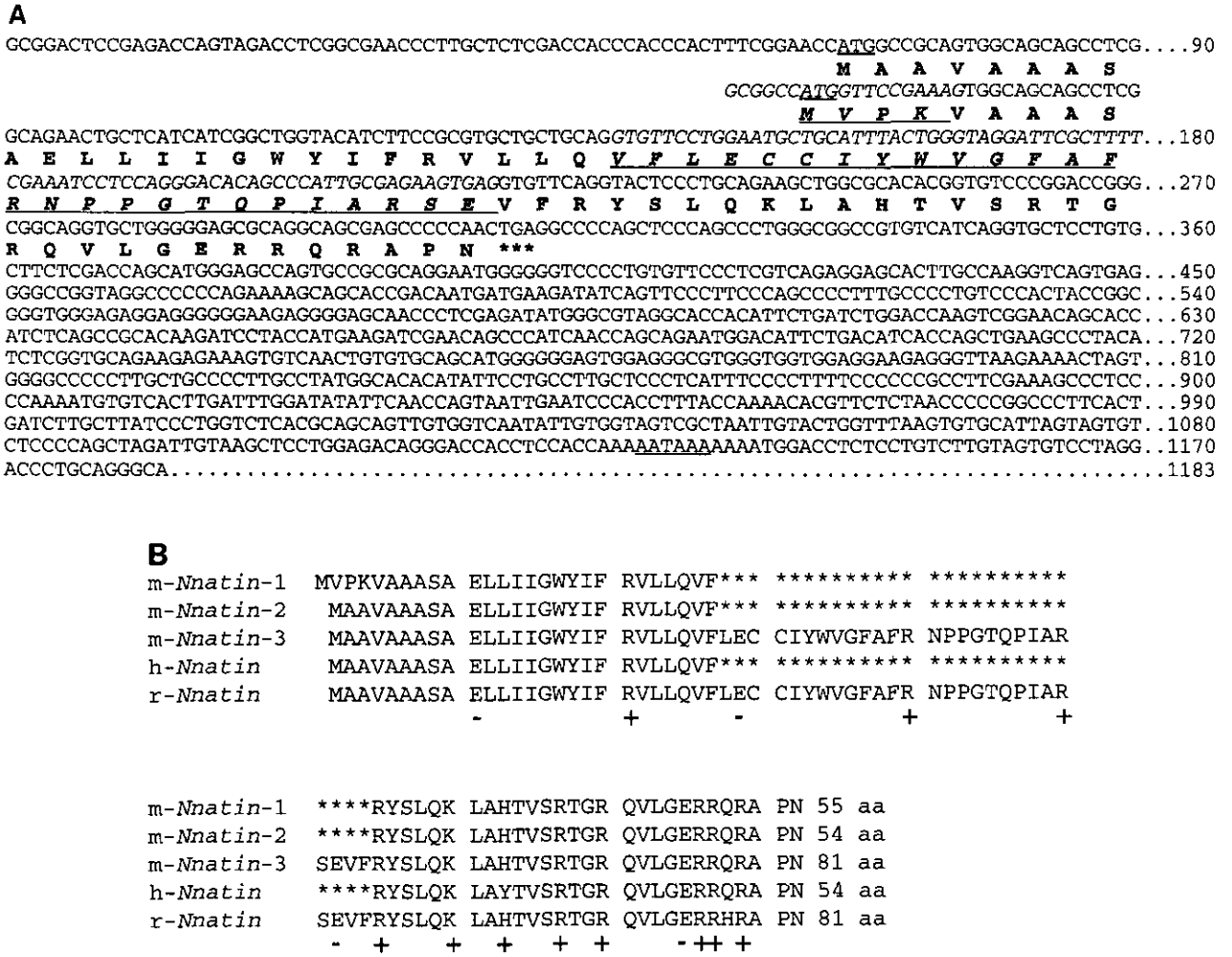

FIG. 2. The cDNA sequences of *neuronatin* and deduced proteins. (A) Structure of the *neuronatin* cDNAs and deduced proteins. Amino acid sequences are shown in single letter code, the three stars indicate the stop codon, and the putative polyadenylation site is underlined. The numbered lines belong to the longest alternative splice form for *neuronatin* (clone 3). Clone 2 lacks one exon (second italic sequence). Clone 1 starts with a different exon (first italic sequence) and also lacks the downstream exon (second italic sequence). (B) Comparison of the predicted proteins for the three alternatively spliced *neuronatin* cDNA clones from mouse (m-*Nnatin*-1, -2, and -3), rat (r-*Nnatin*; Joseph *et al.*, 1994) and human (h-*Nnatin*; Adams *et al.*, 1993). Positively or negatively charged amino acids are indicated with + and −, respectively. The EMBL accession numbers for the three mouse cDNAs in the sequence database are X83568 (*neuronatin*-1), X83569 (*neuronatin*-2), and X83570 (*neuronatin*-3).

6C, and 6E). During the subsequent periods of embryogenesis that we examined (up to 17.5 days p.c.), strong expression was observed in the myelencephalon and metencephalon (Figs. 7A, 7B, 7G, and 7H and data not shown).

neuronatin Expression in the Developing Central Nervous System

Expression in the ventral part of the undifferentiated ventricular zone of the spinal cord was observed around 9.5 days p.c. rostrally and also at 10.5 days p.c. in the more caudal part (Figs. 4F, 4G, and 5A). From 10.5 to 17.5 days p.c. we detected very strong expression in the intermediate zone of the neural tube (Figs. 7C, 7G, and data not shown).

From 9.75 days p.c., *neuronatin* transcripts were detected at the central midline of the forebrain in the lamina terminalis (Figs. 5A–5D). No expression in the midbrain was detected at this stage. At 10.5 days p.c., expression could be detected in the ventral diencephalon, the optic chiasma, the lamina terminalis, and the intermediate zone of the telencephalon (Figs. 4G and 6A–6D). Expression at this timepoint was also detected in the floor of the midbrain (Fig. 4G). At later stages, the detected expression was stronger in postmitotic regions of the brain than in mitotically active

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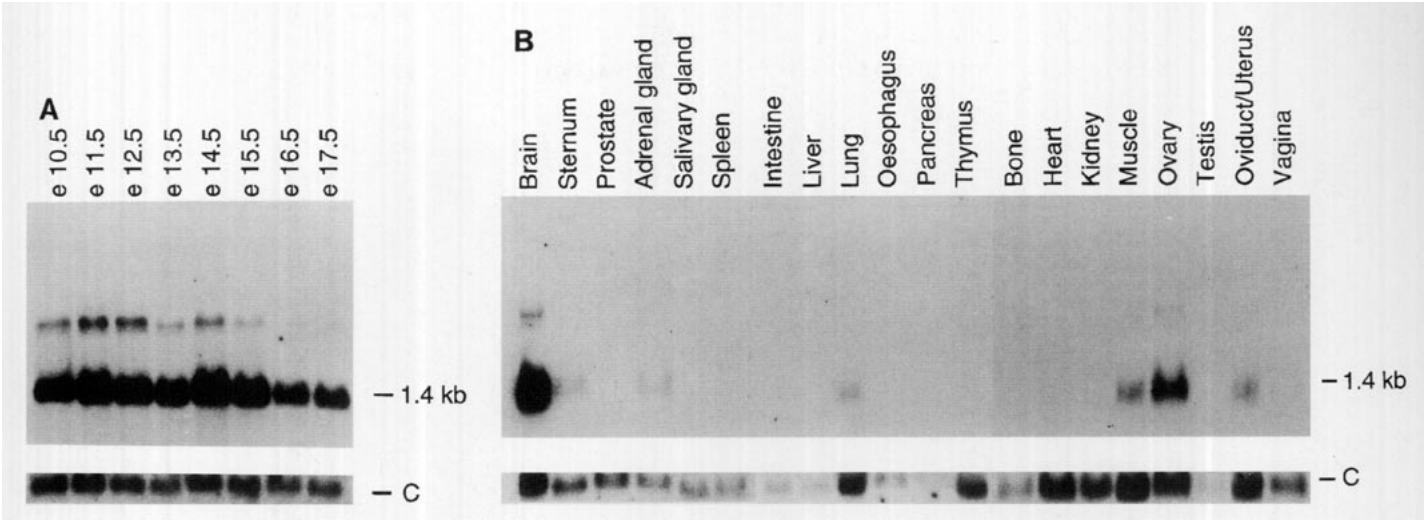

FIG. 3. Northern blot analysis of *neuronatin* expression. (A) Northern blot analysis of *neuronatin* poly(A)$^+$ RNA in mouse embryos from 10.5–17.5 days p.c. (B) Northern blot analysis of *neuronatin* poly(A)$^+$ RNA in adult tissues of 3-month-old mice. A *neuronatin* transcript of 1.4 kb is detected; the upper band represents unspliced transcript. Poly(A)$^+$ (10 μg) from the indicated tissues was loaded in each lane as indicated.

regions (Figs. 7A–7J). No expression was found in the plexus chorodei. *Neuronatin* transcripts were detected in the retina between 13.5–17.5 days p.c. in the inner neural layer (Figs. 7B, 7C, and data not shown). At 10.5 days p.c. onward, transcripts were also detected in the lateral epithelium of the otic vesicle. Expression of *neuronatin* was still observed in neural epithelium of the cochlea at 15.5 days p.c. (Figs. 7K and 7L).

neuronatin Expression in the Tip of the Foregut Pocket and the Developing Pituitary

The adenohypophysis forms from a placode in the midline ectoderm at the anterior tip of the neural plate. This placode invaginates to form Rathke's pouch. The posterior pituitary is formed from an extension of the infundibulum that invaginates from the base of the brain in close association with the anterior pituitary (Jacobson *et al.*, 1979; Rathke, 1838). The ectoderm in contact with the base of the brain is the roof of Rathke's pouch and the ectoderm in contact with or near the tip of the foregut is the floor of the pouch. The floor of the pouch extends to the oral plate. The lateral aspects of the ectoderm are the walls of the pouch and the area where the ectoderm contacts the prechordal plate is the tip of the pouch. The floor, tip, and part of the wall of the pouch are derived from the stomodeal ectoderm (Jacobson *et al.*, 1979). The area of the anterior foregut pocket, also called preoral gut or Seessel's pocket in chicken (Seessel, 1877), lies in close association with the surface ectoderm (stomodeum) that forms the oral plate and the floor of Rathke's pouch.

From 8.5–9.0 days p.c., the *neuronatin* gene was expressed in the endodermal floor of Seessel's pocket next to the oral plate and the floor of Rathke's pouch (Figs. 4A, 4B, 4D, and 4E). Weaker signals were detected in surrounding mesodermal cells and the presumed oral plate (Fig. 4D). After 9.5 days p.c. no signals could be detected anymore in the foregut. At 9.0 days p.c. strong signals were detected in the floor and roof of Rathke's pouch (Fig. 4B). The expres-

FIG. 4. Expression of *neuronatin* in 8.5- to 10.5-days p.c. mouse embryos. Whole-mount *in situ* hybridization. (A) Lateral view of an 8.5-days p.c. embryo (7 somites). (B) Lateral view of a 9.0-days p.c. embryo (10–12 somites). (C–E) Transverse sections of the 8.5-days p.c. embryo in A. (F) Lateral view of a 9.5-days p.c. embryo (17–19 somites). (G) Lateral view of a 10.5-days p.c. embryo (34 somites). d, diencephalon; f, foregut pocket; fl, forelimb; h, heart; hb, hindbrain; hl, hindlimb; lt, lamina terminalis; nt, neural tube; r3, rhombomere 3; r5, rhombomere 5; rp, Rathke's pouch; s, somite; sp, splachnic mesoderm; V, trigeminal ganglion.

FIG. 7. Localization of *neuronatin* RNA in 13.5- to 15.5-days p.c. mouse embryos. *In situ* hybridization. (A–C) Photomicrographs of transverse sections of Day 13.5 p.c. embryos under dark-field illumination. (D–F) Photomicrographs of the same series of sections under bright-field illumination. (G, H, K) Photomicrographs of sagittal sections of Day 15.5 p.c. embryos under dark-field illumination. (I, J, L) Photomicrographs of the same series of sections under bright-field illumination. (K, L) An enlargement of a section around the cochlea. c, cochlea; drg, dorsal root ganglia; d, diencephalon; e, ear pinnae; me, mesencephalon; my, myencephalon; nt, neural tube; oc, optic chiasma; p, pons; r, retina; rp, Rathke's pouch; sg, sympathetic trunk ganglion; t, telencephalon; tg, tongue; V, trigeminal ganglion; VII, facial ganglion; VIII, acoustic ganglion; IX, glossopharyngeal ganglion. Scale bar indicates 50 μm (A–F), 100 μm (G–J), or 50 μm (K,L).

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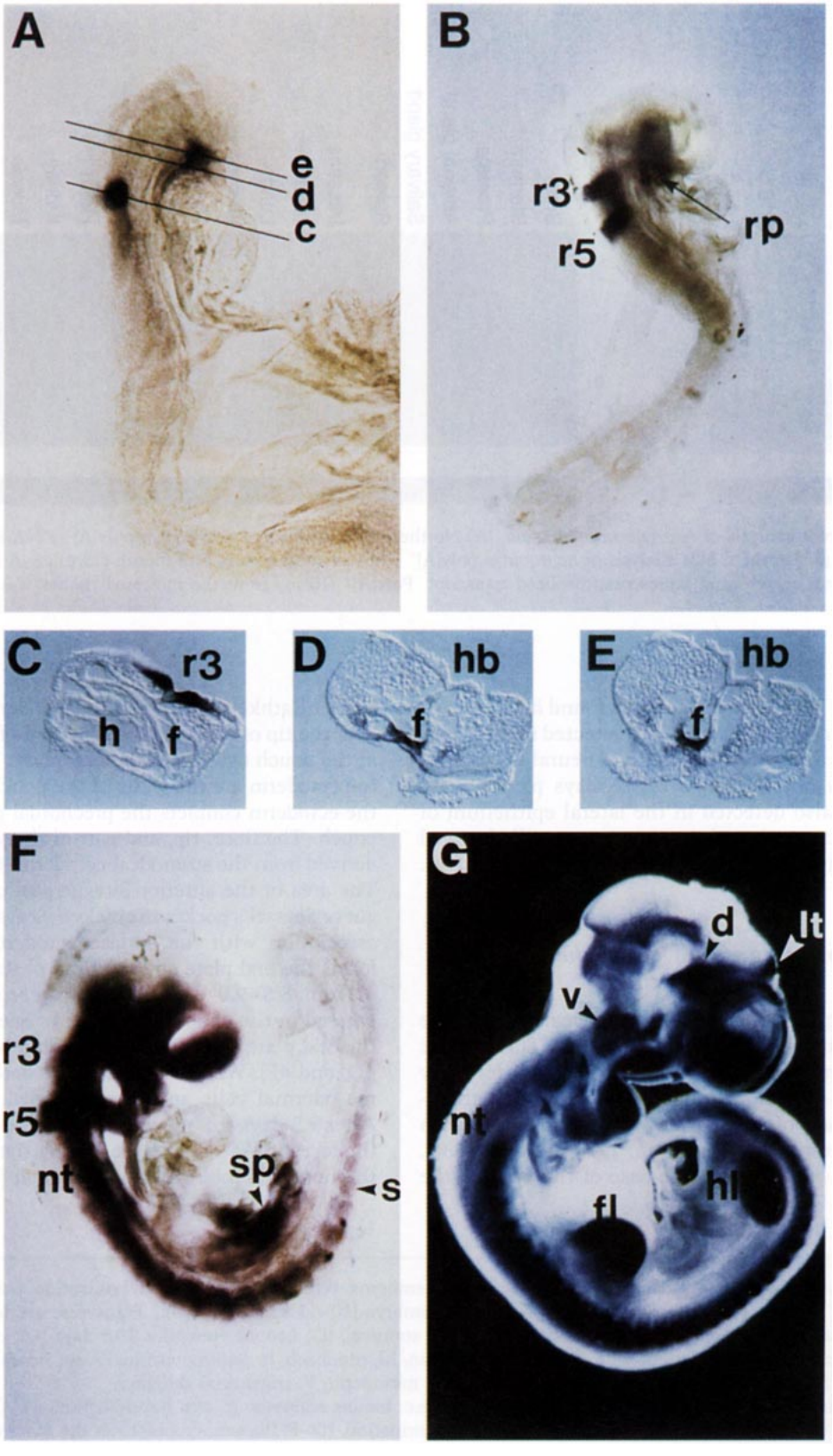

FIGURE 4

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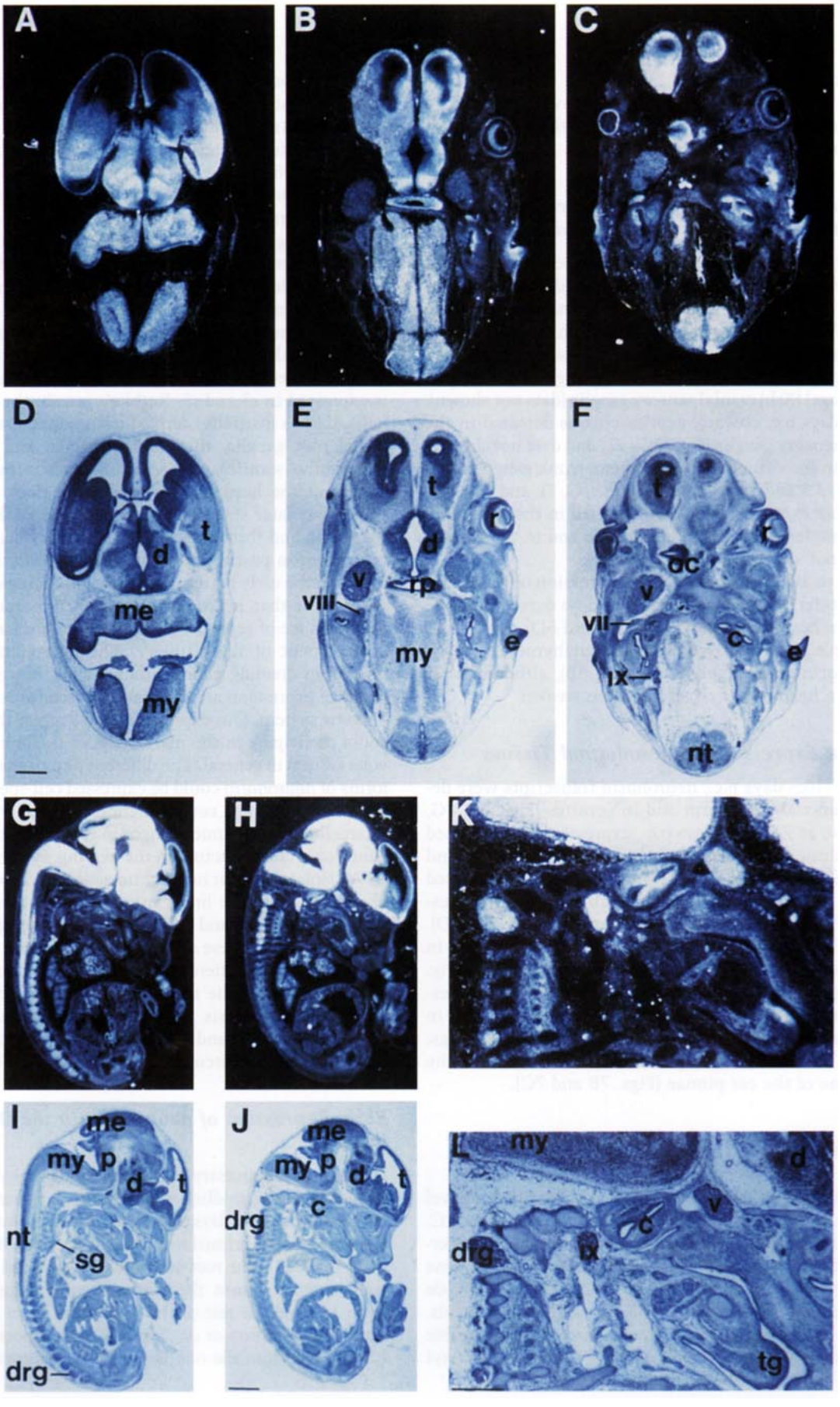
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FIGURE 7

sion in Rathke's pouch could be detected until 17.5 days p.c. (Figs. 5B, 5C, 6C, 7B, and data not shown). In addition to Rathke's pouch, the ventral midline surface ectoderm expressed *neuronatin* at 9.5 days p.c. (Figs. 5A and 5B).

neuronatin Expression in the Peripheral Nervous System and Neural Crest Cell-Derived Tissues

Neuronatin expression is associated with the development of some neural crest-derived tissues from different origins. From 9.75–10.0 days p.c., transcripts were first detected in the trigeminal (Vth) and facioacoustic (VII–VIIIth) cranial sensory ganglia (Figs. 5C, 5D, 7B, 7C, 7E, 7F, 7K, and 7L). At 10.5 days p.c., expression was also detected in the glossopharyngeal (IXth) and vagal (Xth) cranial sensory ganglia (data not shown). From 10.5 days p.c. onward, expression was detected in the dorsal root sensory ganglia (Figs. 7G–7L, and data not shown). Expression in the autonomic sympathetic trunk ganglion was detected at 13.5 and 15.5 days p.c. (Figs. 7G, 7I, and data not shown). Weak expression was also detected in the myenteric ganglion complex of the gut and stomach and in the adrenal gland (data not shown).

From 9.5 to 10.5 days p.c., strong expression of hindbrain neural crest-derived mesenchyme could be detected in the branchial arches (Figs. 4F, 4G, 5A, 5B, and 5D). From 9.5–10.0 days p.c., crest-derived head mesenchyme expressed the *neuronatin* gene (Figs. 4F, 5A, and 5B), although later the signal in head mesenchyme became weaker.

neuronatin Expression in Mesodermal Tissues

From 9.5–10.5 days p.c., *neuronatin* transcripts were detected in paraxial mesoderm and in somites (Figs. 4F, 4G, and 5E). Also at 9.5–10.5 days p.c., expression was detected in the splachnic mesoderm of the hindgut (Figs. 5B, 8A, and 8B). From 9.75 days p.c. onward, expression was detected in the limb mesenchyme (Fig. 4G). At 10.5 days p.c., expression was observed in the preocular muscle mass (Fig. 6D). By Days 13.5 and 15.5 p.c., transcripts were detected in mesoderm of the mandibula, maxilla, tongue, and limb (Fig. 7K). No expression was detected in the notochord. Expression in mesenchyme surrounding the neural epithelium in the cochlea was observed from 13.5–15.5 days p.c. (Figs. 7C and 7K). In addition, expression was detected in the mesenchyme of the ear pinnae (Figs. 7B and 7C).

DISCUSSION

In a subtractive hybridization screen we identified a novel mouse gene, *neuronatin*, that is expressed in normal P19 E.C. cells but is downregulated in P19B cell line 1 which has undergone a partial differentiation process. The *neuronatin* gene codes for three alternatively spliced mRNAs which encode three different putative proteins of 54, 55, and 81 amino acids. The three deduced protein products share a hydrophobic amino terminal end and a strongly basic hydrophilic carboxyl end. It is conceivable that the strongly basic carboxyl end serves as protein ligand, cofactor, or protein–protein interaction domain and that the amino terminal end functions as a signal peptide for translocation into membranes (Briggs and Gierasch, 1986). Recently, the rat homologue of mouse *neuronatin*, coding for the 81-amino-acid form, was isolated in a screen for genes expressed in newborn rat brains but downregulated during the process of senescence (Joseph *et al.*, 1994). These authors proposed that neuronatin might function in signal transduction and terminal brain differentiation.

We examined the expression of *neuronatin* in adult mice and during mouse embryogenesis by Northern blot analysis, *in situ* hybridization on sections and whole-mount hybridization histochemistry. During early embryogenesis *neuronatin* is expressed in r3 and r5; Rathke's pouch; the lamina terminalis; tissues (partially) derived from neural crest such as the dorsal root ganglia, the cranial ganglia, and the branchial arches; the somites; the splachnic mesoderm; the mesenchyme of the head and limb; and the floor of the foregut pocket. At later stages, strong expression is also detected in the retina and throughout the central nervous system but it is stronger in postmitotic than in mitotically active regions. Based on the early pattern of expression during embryogenesis we suggest that *neuronatin* plays an important role in the maintenance of segment identity in the hindbrain and in the development of the pituitary. The *neuronatin* gene is expressed in dividing neuroepithelial cells; however, very high levels of expression are detected in postmitotic regions of the nervous system. Our expression data suggest that *neuronatin* could participate in the maturation of the brain and the nervous system in general. The different putative transmembrane forms of *neuronatin* could be expressed cell-specifically in the nervous system and could function in signal transduction, in intercellular communication, or in the maturation or maintenance of overall structure in the nervous system. Joseph *et al.* (1994) isolated the rat *neuronatin* gene and showed high levels of expression in the brain around birth as well as in young rats (3 months old) and downregulation of the gene in old rats (33 months old). These authors suggested a role for *neuronatin* in terminal brain differentiation and signal transduction. Our results show that the *neuronatin* gene is expressed strongly during embryogenesis, suggesting an important role in the early development and differentiation of the central and peripheral nervous system.

Early Expression of neuronatin in the Developing Pituitary

The anterior pituitary gland or adenohypophysis forms from a placode in the midline ectoderm at the anterior tip of the neural plate. At 8.5 days p.c., invagination of the oral ectoderm at the roof of the primitive mouth gives rise to Rathke's pouch (Rathke, 1838). The roof of Rathke's pouch and portions of its walls originate from the ventral neural ridge, whereas the floor, tip, and the rest of the walls form from the stomodeal ectoderm (Jacobson *et al.*, 1979). Rathke's pouch detaches at 12 days p.c. from the oral epithelium to become an indepen-

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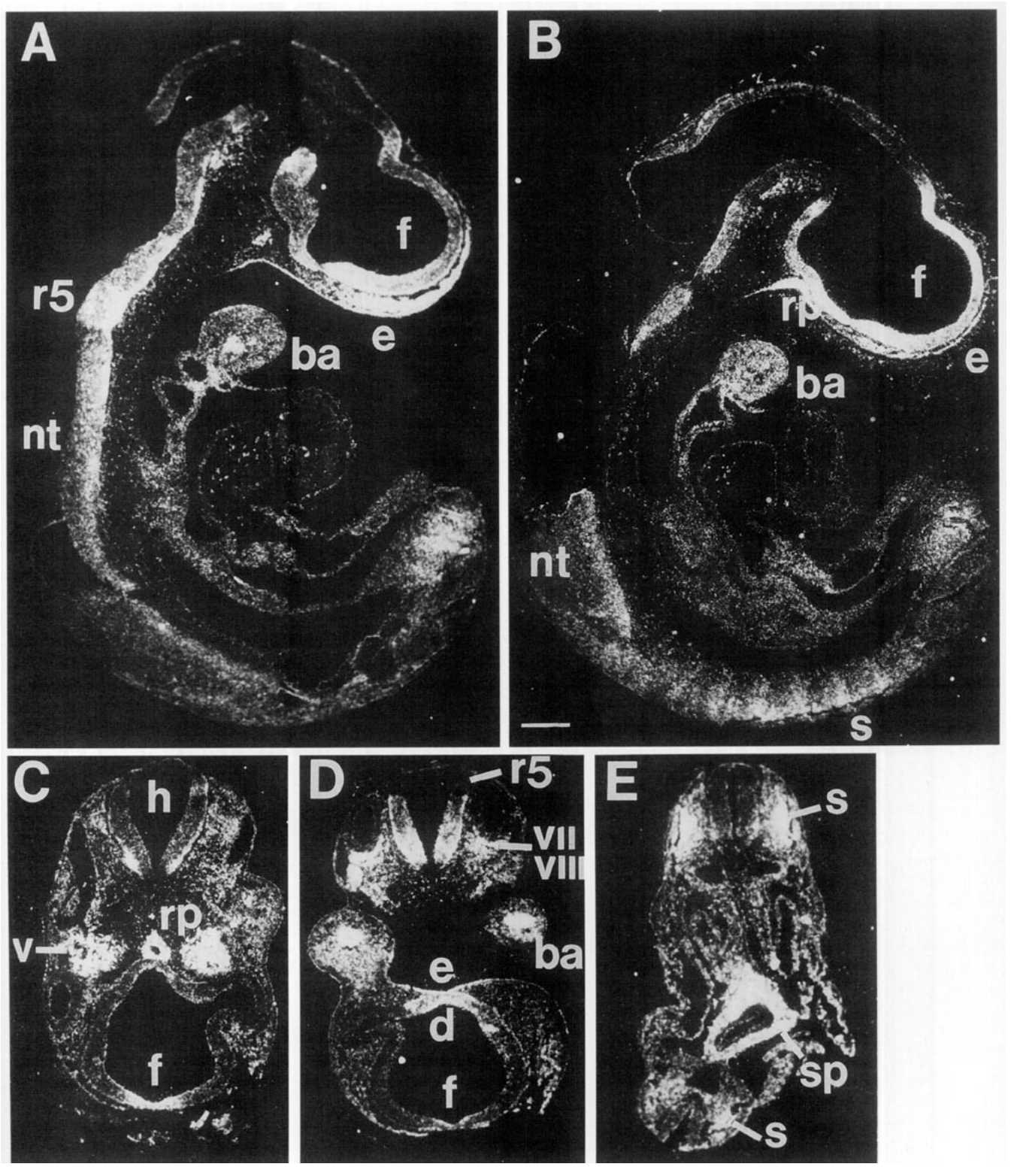

FIG. 5. Localization of *neuronatin* RNA in 9.75-days p.c. mouse embryos. *In situ* hybridization on sections. (A–E) Photomicrographs of sections under dark-field illumination. (A, B) Sagittal and parasagittal sections. (C, D) Transverse sections at the level of the hindbrain and optic stalk. (E) A transverse section at the level of the hindgut. ba, branchial arch; d, diencephalon; e, head surface ectoderm; f, forebrain; h, hindbrain; nt, neural tube; r5, rhombomere 5; rp, Rathke's pouch; s, somite; sp, splachnic mesoderm; V, trigeminal ganglion; VII–VIII, facloacoustic ganglion complex. Scale bar indicates 20 μm.

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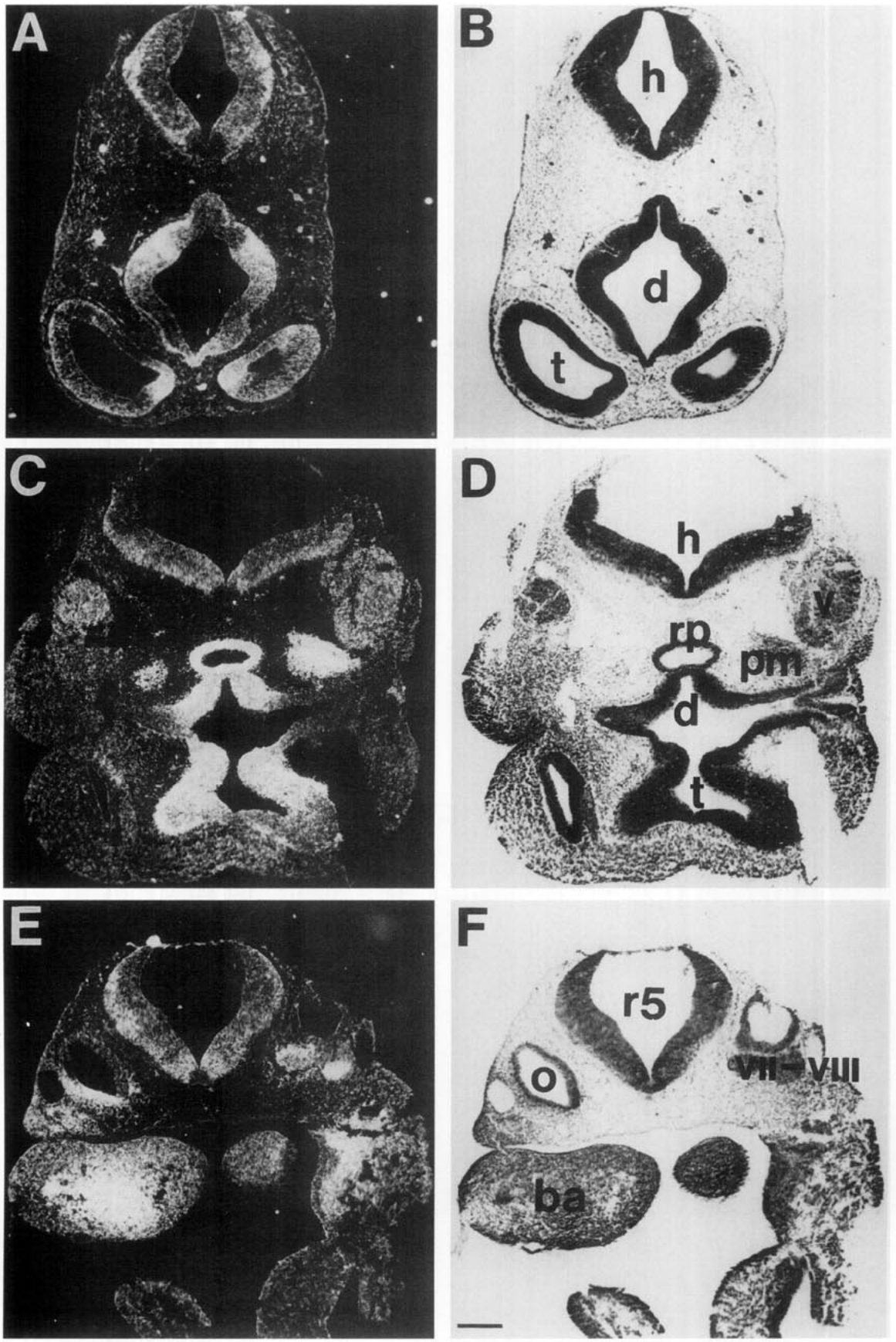

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dent structure. The posterior pituitary is formed from an extension of the infundibulum that invaginates from the base of the brain in close association with the anterior pituitary (Jacobson *et al.*, 1979; Rathke, 1838).

The ectodermal Rathke's pouch cells undergo proliferation and differentiation to generate at least five distinct cell types: the corticotrophins, producing proopiomelanocortin; the thryrotrophins, producing the β-subunits of thyroid-stimulating hormone (β-TSH); the gonadotrophins, producing the β-subunits of luteinizing and follicle-stimulating hormones (β-LH and β-FSH); the somatotrophins, producing growth hormone; and the lactotrophins, producing prolactin. The expression of these hormones in the rat pituitary starts around 13.5–17.5 days p.c. (Simmons *et al.*, 1990), after the formation of a definitive Rathke's pouch. Also, the expression of the pituitary-specific transcription factor Pit-1 is not detectable before 13.5 and 15.5 days p.c. in mouse and rat, respectively (Dollé *et al.*, 1990; Simmons *et al.*, 1990). The α-glycoprotein subunit (α-GSU) common to β-TSH, β-LH, and β-FSH is the earliest marker for adenohypophysis development. In the rat at 11 days p.c., α-GSU is expressed in a single layer placode of somatic ectoderm of the roof and floor of Rathke's pouch (Godine *et al.*, 1982; Simmons *et al.*, 1990). In mouse embryos, we observed expression of *neuronatin* in Rathke's pouch floor, tip, and roof at 9.0 days p.c. In addition, around 8.5 days p.c. we observed expression of the *neuronatin* gene in the preoral gut (Seessel's pocket) and the closely associated surface ectoderm that forms the oral placode and the floor of Rathke's pouch. At this timepoint of development the latter two ectodermal tissues could not be distinguished.

In conclusion, the early and late expression of *neuronatin* in the anterior pituitary suggests an important role in the specification of primordial pituitary cells, as well as a role in the differentiation and/or physiological function of the adenohypophysis.

Rhombomere-Specific Expression of neuronatin

The hindbrain contains a series of morphological distinguishable transient bulges around 8.5–10.5 days p.c. (Vaage, 1969). These bulges, called rhombomeres, cooperate in pairs to generate the repeating sequence of cranial branchiomotor nerves (Lumsden and Keynes, 1989). As soon as the rhombomeric boundaries are present, they act as significant lineage restriction barriers (Birgbauer and Fraser, 1994; Fraser *et al.*, 1990; Guthrie and Lumsden, 1991). *Neuronatin* was observed to be expressed during hindbrain development before morphological segmentation. The regions of expression coincide with r3 and r5 and overlap with the expression domains of the transcription factor Krox-20 (Nieto *et al.*, 1991; Wilkinson *et al.*, 1989a) and its target gene, the transcription factor Hox-b2 (Schneider-Manoury *et al.*, 1993; Sham *et al.*, 1993; Wilkinson *et al.*, 1989b) and with the receptor tyrosine kinase *sek* (Gilardi-Hebenstreit *et al.*, 1992; Nieto *et al.*, 1992). The genes of the Hox family are expressed in the spinal cord and have anterior limits of expression at rhombomeric borders in the hindbrain (for review see Krumlauf, 1993). Disruption of the *Hox-a1* and *Hox-a3* genes interfere with normal hindbrain formation (Carpenter *et al.*, 1993; Chisaka and Capecchi, 1991; Chisaka *et al.*, 1992; Dollé *et al.*, 1993; Lufkin *et al.*, 1991; Mark *et al.*, 1993). The disruption of *Krox-20* demonstrated a marked reduction or elimination of r3 and r5. This gene did not appear to be required for the initial delimitation but for maintenance of r3 and r5 (Schneider-Manouri *et al.*, 1993). The spatial and temporal overlap of expression of *Krox-20*, *Hox-b2*, *sek*, and *neuronatin* suggest a possible connection in the gene regulatory cascade and suggest an important role for the *neuronatin* gene in the process of hindbrain development and differentiation.

In conclusion, the neuronatin gene encodes a novel group of putative transmembrane proteins that might participate in signal transduction, cell–cell communication, or cell adhesion processes in the maturation of the nervous system and in the development or differentiation of the hindbrain and adenohypophysis.

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FIG. 6. Localization of *neuronatin* RNA in the head of 10.5-days p.c. mouse embryos. *In situ* hybridization on sections. (A, C, E) Photomicrographs under dark-field illumination. (B, D, F) Photomicrographs of the same series under bright-field illumination. (A, B) Transverse section through the head at the level of the sulcus telediencephalicus. (C, D) Transverse section through the head at the level of the optic stalk. (E, F) Transverse section through the head at the level of the otic vesicle. ba, branchial arch; d, diencephalon; h, hindbrain; o, otic vesicle; pm, preocular muscle mass; r5, rhombomere 5; rp, Rathke's pouch; t, telencephalon; V, trigeminal ganglion; VII–VIII, facioacoustic ganglion complex. Scale bar indicates 20 μm.

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