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Differential Expression of a Proteasomal Subunit during Chick Development

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Removal of cardiac neural crest disrupts normal development of the heart and pharynx. Subtractive hybridization was used to identify differentially expressed messages after neural crest ablation in chick embryos. A 1 kb clone, homologous to *PROS-28*, a 28 kD α subunit of a *Drosophila* proteasome, was differentially expressed in embryos lacking neural crest. An increase of *GPROS-28* expression in the head and pharyngeal arches of stages 12-21 chick embryos without cardiac neural crest accompanied generalized low-level expression throughout experimental and normal embryos. In addition, high levels of *GPROS-28* expression were detected in normal embryos at particular sites and times in development in the limb buds, mesonephros, heart, liver, neural tube, dorsal root ganglia, and lung buds, when the cells in these regions were undergoing intense proliferation. © 1997 Academic Press

Cardiac neural crest cells migrate from the neural tube, through the pharyngeal arches and participate in septation of the outflow tract of the heart. In addition to contributing to normal heart development, the cardiac neural crest cells also support development of the aortic arch arteries and glands derived from the pharyngeal arches (1). Ablation of the cardiac neural crest leads to defects in the heart, great vessels and glands of the neck (2). Although Bockman et al. (3) have shown that the amount of ectomesenchyme in the pharyngeal arches is reduced when the cardiac neural crest cells are removed, the mechanism by which the absence of cardiac neural crest leads to abnormal development is unknown.

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Abdulla et al. (4), showed that several proteins are expressed at stage 14 in sham-operated chick embryos that are not expressed in the pharyngeal arches of neural crest-ablated embryos. Conversely, the neural crest-ablated embryos expressed one protein at stage 14 and several proteins by stage 18 which were not expressed in the sham-operated embryos. We performed a subtractive hybridization of pharyngeal arches of normal and neural crest-ablated chick embryos at stage 14, and found a 1 kb clone, differentially expressed in embryos without cardiac neural crest. The clone was the chick homologue of *PROS-28*, an α subunit of a *Drosophila* 20S proteasome (5) and its developmental expression pattern has been correlated with known sites of proliferation.

METHODS

Fertilized Arbor Acre chick eggs were incubated in forced-draft incubators at 37°C with a relative humidity of 90-100%. The eggs were windowed at stages 8-10 (6), and the embryos stained with neutral red. The cardiac neural crest cells, located at the lateral edges of the neural fold extending from the level of the mid-otic placode to the caudal limit of the third somite, were laser-ablated (experimental embryos). The eggs were then sealed with cellophane tape and re-incubated until the embryos reached the desired stage of development. Sham-operated controls were produced identically with the exception of the ablation.

Control or experimental embryos were removed from the egg, rinsed in saline for RNA extraction. Whole or partially dissected embryos at various stages were collected for Northern analysis. For library construction the area caudal to the first pharyngeal arch extending caudally to the sinus venosus and including the heart, was dissected from the rest of the embryo. All tissue was placed in GIT buffer (4 M guanidine isothiocyanate, 0.025 M sodium acetate, pH 6 and 0.12 M β-mercaptoethanol), frozen and stored in liquid nitrogen until the RNA extraction. Total RNA was prepared from the frozen tissue using RNAzol (Biotecx Laboratories) following the manufacturers instructions. mRNA was purified from the total RNA using an oligo dT cellulose column (7).

An experimental pharyngeal arch cDNA library was made using an oligo dT primer containing a NotI site at the 5′ end for directional cloning. The cDNA was ligated into pGEM5AF+ plasmid (Promega) using BstXI linders with NotI digestion. A control pharyngeal arch

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cDNA library was constructed with EcoRI linkers and ligated into lambda gt10.

The subtraction protocol was based on a procedure described by Travis et al. (8) and has been reported previously (9). The differentially expressed clone was digested with EcoR1 and the two fragments, a 520 bp and a 430 bp fragment, were subcloned into pSPORT (Gibco BRL). Both subclones, p520 and p430, were sequenced by the dideoxy chain termination method (Sequenase 2.0 kit, USB). The sequences were entered into the PC/GENE sequence analysis programs. The GenBank, EMBL, and Swiss-PROT databases were searched with the nucleotide and putative amino acid sequences for homologies to known sequences.

For Northern analysis 20 μg of total RNA was separated using a formaldehyde-agarose gel. The RNA was blotted onto Hybond-N (Amersham) and hybridized with oligo-labeled cDNA or digoxygenin-labeled riboprobe generated from the differentially-expressed clone. The hybridization was carried out at 60°C. Autoradiograms were scanned into an NIH image analysis program. The computer-generated image of the bands was represented in numbers of pixels. For each blot the 18S band with the highest number of pixels was set equal to 1 and the rest of the 18S bands were set equal to a fraction of 1. These numbers were then used to correct the number of pixels determined for each positive band.

Embryos for whole-mount *in situ* analysis were rinsed in PBS and fixed overnight in 4% paraformaldehyde at 4°C. Whole-mount *in situ* hybridization, using a digoxigenin-labeled riboprobe, was done as described by Wilkinson (10). The riboprobe was transcribed from p520 (the plasmid containing the 5′ end of *GPROS-28*)(Promega). All hybridizations were performed overnight at 70°C. After the post-hybridization washes the embryos were digested with 100 mg/ml of RNase A for 20-30 minutes at 37°C. The embryos were then incubated with anti-digoxigenin which was detected by alkaline phosphatase-linked immunohistochemistry. Finally embryos were either embedded in 4% agarose for vibratome sectioning or dehydrated, embedded in paraffin and sectioned at 10-14 μm.

RESULTS

The subtracted cDNA probe recognized 38 clones in a normal stage 14 chick pharyngeal arch library, after primary and secondary screening. Duplicate Southern blots of the EcoR1 restriction digests of several of the positive clones from the secondary screening were probed with single-stranded cDNA from pharyngeal arches of control (Fig. 1A) or experimental stage 14 chick embryos (Fig. 1B). A 1 kb fragment, clone 12, hybridized strongly on a blot probed with single-stranded cDNA from pharyngeal arches of the neural crest-ablated embryos (Fig. 1B). Clone 12 was subcloned and sequenced. The nucleotide sequence and deduced amino acid sequence are shown in Fig. 2.

The 959 bp sequence had an ATG start codon 21 base pairs from the 5′ end, a TGA stop codon at nucleotide 768 and was predicted to have a single open reading frame coding for a 249 amino acid peptide (M_r of 2.8×10^3, pI=9.8) with 68% identity at the nucleotide level and 82% homology at the peptide level to *Drosophila PROS-28.1* (5), an α subunit of a *Drosophila* 20S proteasome (Fig. 2) (11). All of the sequences showing homology to clone 12 are α subunits: the yeast *Pre 6* α subunit (12); the *DD5* α subunit from *Dictyostelium* (Scauer et al., unpublished data); and an α subunit

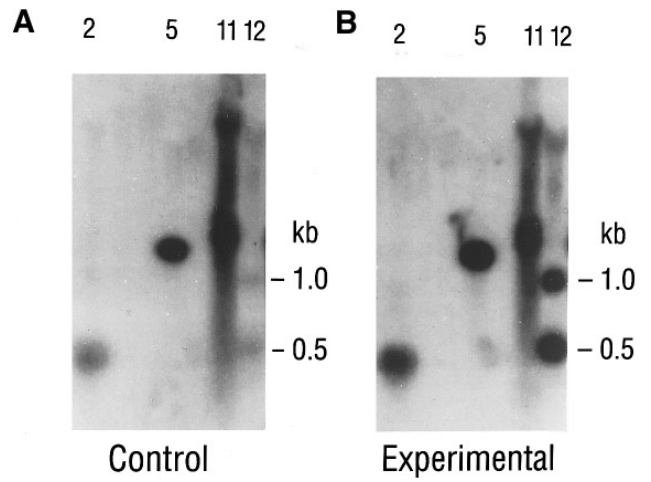

FIG. 1. Results of tertiary screening of the positive clones from the secondary screening of the control stage 14 cDNA library. (A) was hybridized with single-stranded cDNA from control pharyngeal arches. (B) was hybridized with single-stranded cDNA from experimental pharyngeal arches. Clone 12 hybridized more strongly to the experimental cDNA (B) compared to the hybridization seen with the control probe (A). Clone 12 is 1 kb and has an EcoRI site approximately in the middle of the insert. The 0.5 kb band is actually 2 bands of 520 and 430 bp and is the completely digested insert.

from *Arabidopsis thaliana* (13) (Fig. 3). A dendrogram indicates that *GPROS-28* and its nearest neighbor, *DmPROS-28,* are members of the same family while the C3 subunits belong to a closely related but different branch of the family (Fig. 4).

Sites with potential biological significance are shown in Figs. 2 and 3 (14,15,16). The cAMP/cGMP protein phosphorylation site and the TPK site are conserved in several of the α proteasome subunits and provide evidence for the potential biological significance of these sequences (Fig. 3). A potential nuclear targeting signal (NTS) (17; 18; 19), is found at the COOH-terminus.

In all of the stages analyzed, *GPROS-28* hybridized to a single message of 1.1 kb (Fig. 5A). *GPROS-28* expression was increased in the head and pharyngeal region of stage 14, 18 and 21 experimental embryos. By stage 24, *GPROS-28* expression was no longer increased in experimental pharyngeal arches.

Sense and antisense riboprobes were generated from the 5′ end of *GPROS-28* for whole-mount *in situ* hybridization analysis. No hybridization signal was detected in embryos hybridized with the sense riboprobe at any stage (Fig. 6A). *GPROS-28* was expressed in the pharyngeal arches of embryos at all stages tested (Fig. 6B,C,D). In stage 12, when the pharyngeal arches were just beginning to form, the expression pattern of *GPROS-28* (data not shown) was unremarkable. At stage 14 the cranial three pharyngeal arches were formed and the expression of *GPROS-28* was seen in

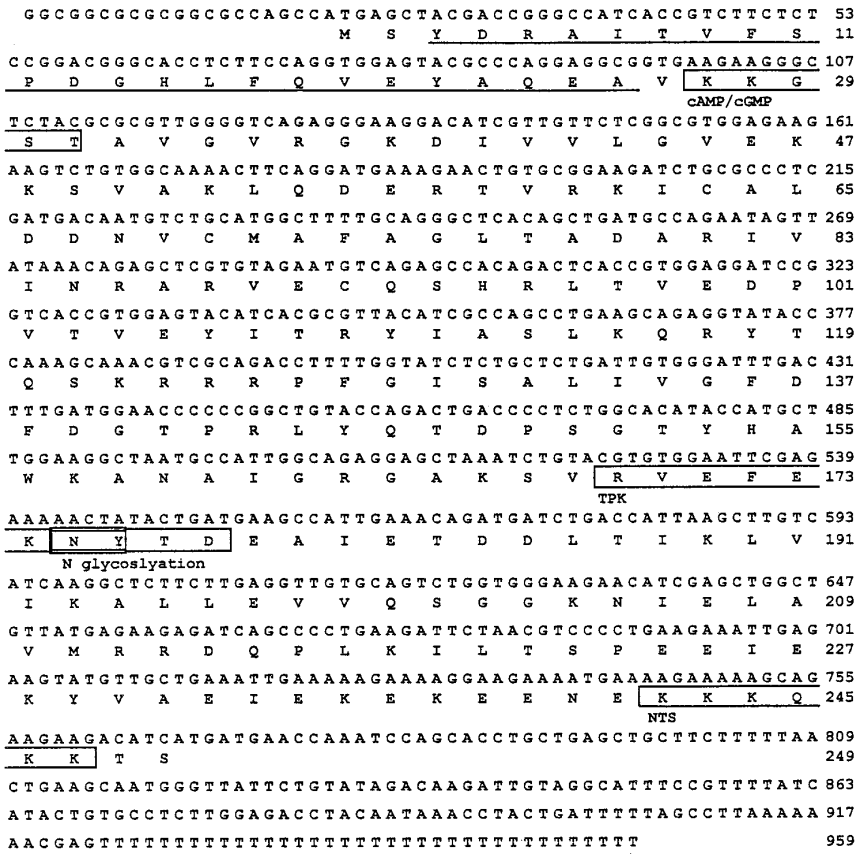

FIG. 2. Nucleotide and deduced amino acid sequence of clone 12 (*GPROS-28*). Underlined amino acids are homologous to the signature pattern of the α type proteasome subunits. The boxed sequences are consensus sequences for cAMP/cGMP-dependent protein kinase, a consensus sequence for phosphorylation by tyrosine protein kinase (TPK), a consensus sequence for N-glycosylation, and a consensus sequence for a nuclear targeting signal (NTS). (GenBank accession number U09226).

the ectomesenchyme surrounding the arch arteries. No expression was detected in the heart at stage 14 (data not shown). By stage 18, the first arch artery had broken down into a capillary network and *GPROS-28* expression appeared to be concentrated in the ventral portion of the arch (Fig. 6D). In addition, the region in which the sixth pharyngeal arch was forming showed intense hybridization (Fig. 6B). In the rest of the pharyngeal arches, the *GPROS-28* signal was detected in the ectomesenchyme surrounding the arch artery (Fig. 6C). By stage 21 the difference in *GPROS-28* expression between the control and experimental embryos was not visible. The ventral portion of the first and second arches where the arch arteries had been remodeled into capillaries strongly expressed *GPROS-28* (Fig. 6D). By stage 24, the expression pattern of *GPROS-28* in the first two pharyngeal arches was less regular and intense, however, *GPROS-28* was still detected around the arch arteries.

GPROS-28 expression was detected in the presumptive ventricles of the heart coincident with the apperance of trabeculations at stage 18, and in the developing lung buds beginning at stage 21 (Fig. 6D,I). The liver exhibited a punctate expression pattern beginning at stage 21 and shown at stage 24 (Fig. 6E and J). The developing kidney also expressed *GPROS-28* with the staining localized to the tubules (6H). In all the stages studied, *GPROS-28* was detected at the rhombomere boundaries in the hindbrain as well as the dorsal edges of the neural tube (Fig. 6K). By stage 24 the expression became concentrated medially within the neural tube. The somites were positive for *GPROS-28* expression, and the expression seemed to increase with age (Fig. 6K). Beginning at stage 18, the dorsal root ganglia showed an increased expression of *GPROS-28* (Fig. 6L). In addition, by stage 24 the cranial ganglia strongly expressed *GPROS-28* (data not shown). *GPROS-28* expression was detected in the condensed mesenchyme of the limb (Fig. 6F). By stage 24, the pattern of *GPROS-28* expression in the core of the limb buds was no longer homogeneous (Fig. 6G). The expression was concentrated in the mesenchyme surrounding the cartilaginous bone precursors but conspicuously

FIG. 3. The amino acid alignment of *GPROS-28* with homologous α subunits. The residues boxed in black are identical to the *GPROS-28* residues. The shaded residues indicate conserved substitutions (Argos, 1987) compared to *GPROS-28*. Boxed areas are consensus sequences common to *GPROS-28* and the other subunits. All of the subunits have the α-type subunit signature, the TPK (tyrosine protein kinase) phosphorylation site, as well as the consensus sequence for phosphorylation by cAMP/cGMP. Only *GPROS-28* and *Dm PROS-28* have a consensus sequence for a NTS (nuclear targeting signal) at the carboxyl terminus.

absent from the differentiating cartilage. Table 1 summarizes the stages and localizations of *GPROS-28* expression.

DISCUSSION

We have isolated and characterized a chick 20S α proteasomal subunit which is up-regulated in the pharyngeal arches of chick embryos destined to have cardiac defects. The proteasome or multicatalytic protease (MCP) is a ubiquitous 20S cylindrical particle composed of 12-25 different subunits with nonlysosomal protease activity and is involved in metabolism of proteins and RNA. Expression of the proteasome subunits is tissue specific and under developmental control (20). Proposed functions include a general role in nonlysosomal protein turnover (21), ATP-ubiquitin-dependent protein degradation (22; 23; 24), posttranslational modification of newly synthesized proteins (25; 26; 27), and generation of peptides presented by the MHC-class I molecules (28). Proteasomes also play an important role in cell proliferation (29; 30; 31). Several reports have shown that the distribution of the proteasome complex changes in invertebrates during oogensis (32), embryonic development (33; 34), and differentiation (35), and that the embryonically expressed proteasome subunits are significantly different from those expressed in adult tissue (20); however this is the first report of such changes in a vertebrate embryo.

The 20S proteasome subunits are highly conserved from archaebacteria to humans (36; 37): the 20S proteasome of *Thermoplasma acidophilum*, is considered the ancestral or Urproteasome (37). This proteasome consists of only two subunits, α and β. All other 20S proteasome subunits can be aligned with these α or β subunits. The 20S proteasome complex consists of a stack of four rings forming a hollow cylinder with 7 α subunits forming each of two outer rings and 7 β subunits forming each of two inner rings (38; 39). A narrow channel controls access to the inner compartments. The β subunits contain the proteolytically active site while the α subunits have targeting and regulatory functions. The distinguishing characteristic of the α subunit is a highly conserved region at the N-terminus distinct from the β subunits. *GPROS-28* possesses the characteristic α-type sequences at the N-terminus and therefore is an α subunit. The presence of a nuclear localization signal indicates that *GPROS-28* may be located in

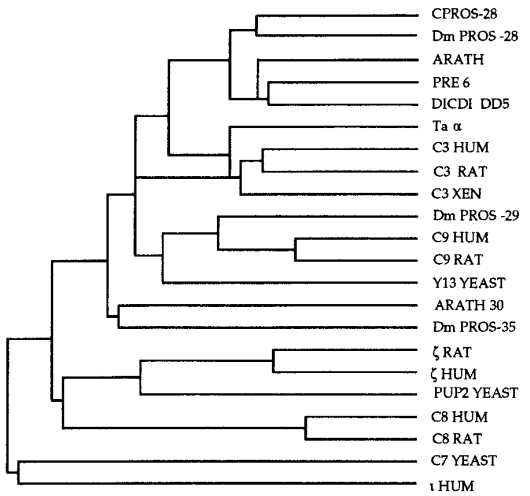

FIG. 4. Dendrogram showing the homologies between *GPROS-28* and other α subunits. *Dm PROS-28: Drosophila* 28 kD subunit (5); *ARATH:* from *Arabidopsis thaliana* (43); *DICDI DD5:* from *Dictyostelium* (Scauer *et al.*, unpublished data); *PRE 6:* yeast subunit (12); *Ta α:* from *Thermoplasma acidophilum* (37); *C3 HUM:* human C3 subunit (44); *C3 RAT* subunit (45); *C3 XEN: Xenopus laevis C3* subunit (46); *Dm PROS-29:* 29 kD *Drosophila* subunit (5); *C9 HUM:* human C9 subunit (44); *C9 RAT* subunit (47, 48); *Y13 YEAST* subunit (49); *ARATH 30: Arabidopsis thaliana* subunit (50); *Dm PROS-35:* 35 kD *Drosophila* subunit (51); *ζRAT:* rat zeta subunit (44); *ζHUM:* human zeta subunit (53); *PUP2* YEAST subunit (53); *C8 HUM:* human C8 subunit (44); *C8 RAT* subunit (45); *C7 YEAST* subunit (49); *ι HUM:* the human iota subunit (52).

the nucleus suggesting a role in degradation of nuclear proteins.

Because of its expression pattern in the experimental pharyngeal region and the sites of upregulated expression in normal embryos, the increased expression of *GPROS-28* is likely to reflect increased proliferation of cells attempting to reconstitute the missing cardiac neural crest cells. Kirby (40) showed that nodose placode cells migrate into the pharyngeal arches beginning at stage 14 and contribute ectomesenchyme to compensate for the missing cardiac neural crest cells. This would be a relatively small population of cells and expression of a single message by this population would be expected to be a minor component of the messages in the region. In yeast, short-lived regulatory factors of the cell cycle, such as Fos, p53, Mos, and cyclins are degraded by the proteasome-ubiquitin pathway (41), and expression is down-regulated as is proliferation as the cells differentiate (42). In the neural crest-ablated chick, differentiation of the displaced nodose placode cells in the pharyngeal region begins between stages 21 and 24 which corresponds to the time when *GPROS-28* expression returns to normal levels. Proteasomal subunits in the *Drosophila* embryo also undergo changes in localization and accumulation in proliferating cells (34). In an oncogene-transformed granulosa cell line, immunohistochemistry studies have shown increased 20S proteasome protein expression in proliferating cells. However, when the cells were stimulated to differentiate, the 20S proteasome protein levels decreased (30) supporting a role for 20S proteasomes in proliferation but not in differentiation. Abdulla et al. (4) also noted a return to normal protein patterns by stage 21, which suggests that the major period of reconstitution of the missing neural crest is between stage 14 when the nodose placode cells first migrate and stage 21 when differentiation of the pharyngeal arches begins.

Our results with *GPROS-28* support previous data in that proliferation is accompanied by the appearance of the message in many tissues during normal development (summarized in Table 1). Many of the areas (limb buds, lung buds, liver, kidney as well as the pharyngeal arches) with increased *GPROS-28* expression are areas of intense cell proliferation. As cells differentiated in these regions *GPROS-28* expression was decreased. In the limb buds, for example, the apical ectodermal ridge

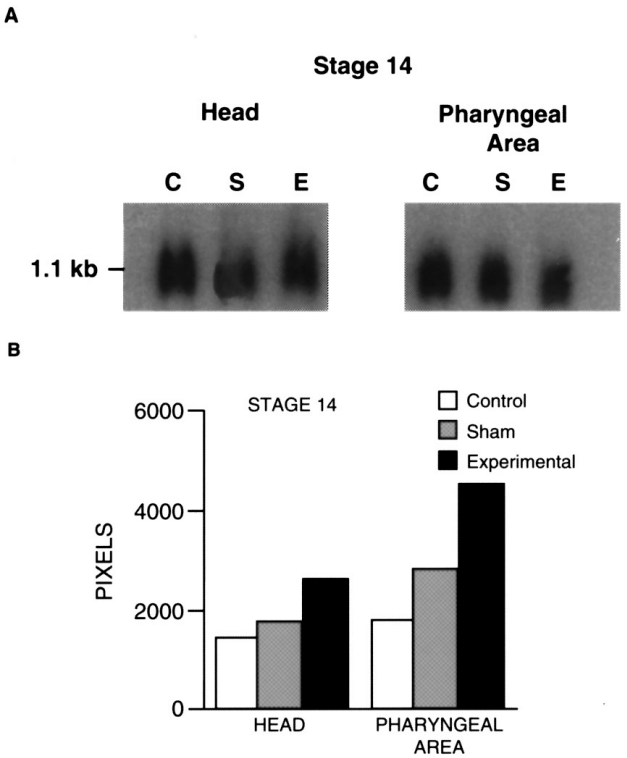

FIG. 5. (A) Northern blot of the head/heart and tail of a stage 14 control (C), control, and experimental (E) embryos. The blots were probed with the 530 bp fragment from the 5′ end of *GPROS-28.* (B) Bar graphs of the corrected values for the levels of *GPROS-28* mRNA expression in stages 14 control, sham-operated, and experimental embryos. Each bar represents the number of pixels generated by a computer image of the autoradiography bands from the Northern blots and corrected for loading errors. *GPROS-28* expression was increased in the pharyngeal area of stage 14 experimental embryos.

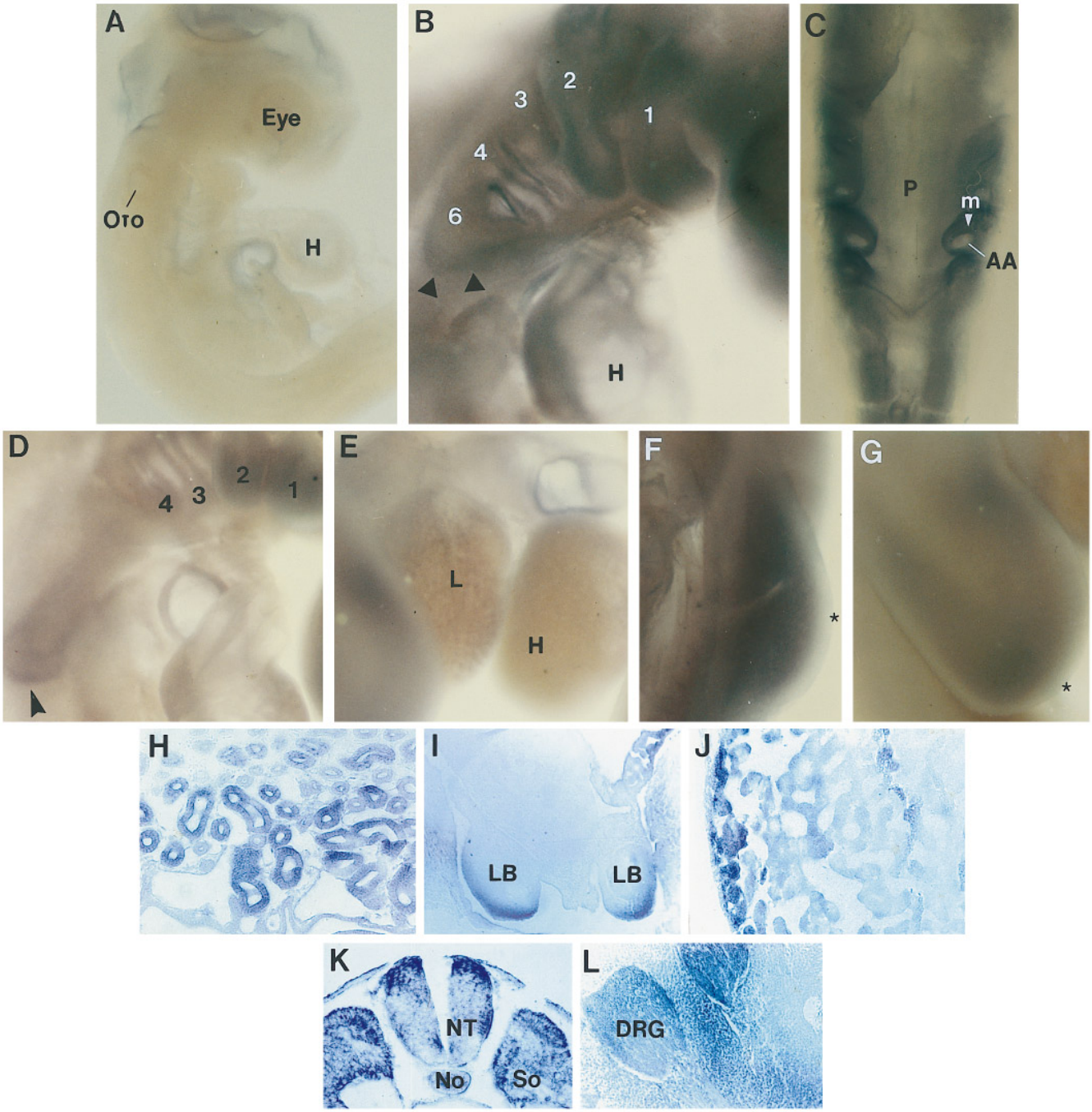

FIG. 6. Whole-mount embryos (A-G) and paraffin sections (H-L). (A) Sense probe for *GPROS-28* in stage 18 control embryos. (B) Stage 21 control embryo. Pharyngeal arches 1, 2, 3, 4, and 6 are labeled. The aortic arch artery in arch 1 has regressed. Intense expression of *GPROS-28* is still detected in the ventral portion of the first and second pharyngeal arches. Arrowheads indicate intense expression in the caudal portion of the arch 6 area. (C) A coronal section through the pharyngeal arches of a stage 18 embryo. *GPROS-28* expression is in the ectomesenchyme (m) surrounding the arch artery (aa). (D) Expression of *GPROS-28* in the lung bud (arrow) of a stage 21 embryo. (E) Punctate expression pattern of *GPROS-28* in the liver (l) and heart (h) of a stage 24 embryo. (F) Stage 18 limb bud showing *GPROS-28* expression throughout the condensed mesenchyme. By stage 24, *GPROS-28* expression is limited to the mesenchyme surrounding the cartilaginous bone precursors. (G) Neither the apical ridge (*) nor the surrounding ectoderm expresses *GPROS-28* in stages 18-24. Sagittal section through the mesonephros (H) showing *GPROS-28* expression in the developing nephrons. Cross sections through (I) lungs (LB) and (J) liver of stage 24 embryos. (K) Cross section of stage 14 neural tube (NT) and somites (So) showing expression in the epithelial somite and dorsal neural tube with no expression in the notochord (No). (L) Sagittal section of stage 21 embryo showing continued expression of *GPROS-28* in the dorsal root ganglia (DRG) and sclerotome.

TABLE 1

Summary of *CPROS-28* Expression

	St 12	St 14	St 18	St 21	St 24
Somites	+	+	+	+	+
Rhombomeres	+	+	+	+	+
Neural tube/spinal cord		+	+	+	+
Pharyngeal arches		+	+	+	+
Limb buds			+	+	+
Dorsal root ganglia			+	+	+
Heart			+	+	+
Lung				+	+
Liver				+	+
Mesonephros					+

induces intense proliferation in the lateral plate mesoderm to form a dense mesenchymal core that is intensely *GPROS-28* positive. By stage 24, *GPROS-28* expression was decreased in the center of the limb bud where cartilage was differentiating. Taken altogether, there is compelling evidence that *GPROS-28* expression is a developmentally regulated proteasomal subunit associated with degradation of factors involved in cell cycle regulation.

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