THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 15, Issue of April 12, pp. 12762–12769, 2002

Printed in U.S.A.

Plunc, a Member of the Secretory Gland Protein Family, Is Up-regulated in Nasal Respiratory Epithelium after Olfactory Bulbectomy*

Received for publication, July 3, 2001, and in revised form, January 25, 2002
Published, JBC Papers in Press, January 30, 2002, DOI 10.1074/jbc.M106208200

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Subtraction suppression hybridization was used with high throughput screening to identify transcripts of genes that are differentially expressed in nasal epithelium following lesioning of the olfactory bulb, termed bulbectomy. We isolated the rat homologue of *plunc*, a murine gene highly expressed in lung and nasopharyngeal regions, by this method. Rat *plunc* encodes a 270-amino acid protein containing a putative signal peptide. *plunc* up-regulation in respiratory epithelium was confirmed by Northern blot and *in situ* hybridization. *plunc* mRNA was expressed in nasal epithelium, heart, lung, thymus, and salivary gland in adult rodent. *plunc* was expressed in nasal epithelium, thymus, and salivary gland during embryogenesis. Antibodies against Plunc detected a 31-kDa protein in lung, heart, and spleen. Rat nasal epithelium displayed robust immunoreactivity that was highly localized to the microvilli layer of respiratory epithelium. The expression of *plunc* was up-regulated after bulbectomy in respiratory epithelium. We also detected secreted plunc in rat and human mucus. Sequence and homology analyses suggest that Plunc is a member of the secretory gland protein family with putative bactericidal/bacteriostatic function. This is the first protein found in respiratory epithelium whose expression is regulated by olfactory neuronal injury and may provide protection against infection subsequent to injury.

The olfactory epithelium, situated within the nasal cavity, is composed of olfactory receptor neurons (ORNs),[1] sustentacular supporting cells, and a mixed population of basal cells, some of which serve as ORN stem/progenitor cells for the generation of new olfactory sensory neurons throughout life (1–3). The initial events of odor detection occur in ORNs, making their survival vital to the survival of the animal (4). Interspersed among olfactory epithelium is respiratory epithelium, consisting of columnar epithelial cells and underlying basal cells. Any contribution of respiratory epithelium in regulating or responding to olfactory epithelial injury/physiological changes is unknown.

ORN axons project to the olfactory bulb (OB), which is their target. ORNs depend on the OB for their survival, and injury to the OB causes ORNs to degenerate, resulting in compromise of the olfactory epithelium (5). The degeneration of ORNs is followed by an increase in proliferation and maturation of basal cells to repopulate the olfactory epithelium. The OB can be lesioned experimentally, termed bulbectomy. Bulbectomy results in the hyper-induction of neurogenesis in the olfactory epithelium ipsilateral to the side of the lesion (6–8). These cellular changes in the damaged olfactory epithelium are accompanied by the changes in expression for many molecules that participate in olfactory neurogenesis (5, 10–12). These molecules may include neurotrophins, cytokines, growth factors, and, potentially, factors that serve a protective role during compromise of the olfactory epithelium.

Subtraction suppression hybridization (SSH) (13), like representational difference analysis (14) and differential display (15), is a PCR-based subtractive cDNA hybridization method used to identify and isolate cDNAs of differentially expressed genes. The method is based on suppression PCR and combines normalization and subtraction in a single procedure. The normalization step equalizes the abundance of cDNAs within the target population, and the subtraction step excludes common sequences between the target and driver populations (13). The high level of enrichment, low background, and normalized abundance of cDNA in the subtracted mixture make this method ideal for rapid cloning of cDNAs from differentially expressed genes. By combining SSH with high throughput differential screening, 625 differentially expressed cDNAs from metastatic adenocarcinoma cell lines have been isolated with a true positive rate of 94%, when subtracted from its non-metastatic counterpart (16).

Using similar approaches in this study, clones of differentially expressed gene fragments were isolated in the adult rat nasal epithelium after olfactory bulbectomy. We used this method to identify a number of differentially expressed genes and have characterized one of these clones in detail. A full-length clone was obtained from a rat olfactory cDNA library. This gene is highly homologous to a recently identified mouse gene, *plunc* (palate, lung, and nasal epithelium clone). Here, we provide data to indicate that Plunc is a secreted protein and

* This work was supported by National Institutes of Health Grants NIDCD DC-2979 and NINDS NS-39657 (to G. V. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ORN, olfactory receptor neuron; OB, olfactory bulb; SSH, subtraction suppression hybridization; PBS, phosphate-buffered saline; OMP, olfactory marker protein; E, embryonic day; BPI, bactericidal permeability-increasing protein; STAT, signal transducers and activators of transcription; OE, olfactory epithelium; EST, expressed sequence tag.

generate insight into one or more of the potential functions of Plunc by investigating its differential expression in our olfactory epithelium damage model as well as sequence analysis.

EXPERIMENTAL PROCEDURES

Experimental Animals and Tissue Preparation—All experimental protocols were approved by The Johns Hopkins University Institutional Animal Care and Use Committee, and all applicable guidelines from the National Institutes of Health Guide for the care and use of laboratory animals were followed. Male adult Sprague-Dawley rats (100 g) were obtained from Harlan (Indianapolis, IN). For protein and RNA preparation, rats were decapitated and the olfactory epithelium was dissected and immediately processed. For immunohistochemistry and *in situ* hybridization, animals were anesthetized with Xylaket and perfused with PBS followed by Bouin's fixative. Tissue was dissected, post-fixed overnight in Bouin's at 4 °C, washed in PBS, placed in 20% sucrose, and embedded in Tissue-Tek. Bouin's fixed rat olfactory tissue was sectioned at 18 μm using an HM500M cryostat (Zeiss, Germany). Sections were affixed to Superfrost plus slides (Fisher Scientific), thaw-mounted for 2 h at room temperature, and stored at −80 °C until use.

Bulbectomy—Adult Sprague-Dawley rats were anesthetized with Xylaket and fixed in a stereotaxic apparatus for surgery. The right olfactory bulb was exposed via a partial dorsal craniotomy and was ablated by suction. Care was taken to avoid damage to the contralateral (left) olfactory bulb. The ablation cavity was filled with Gelfoam to prevent invasion of frontal cortex neurons into this cavity, which could provide an alternative target for regenerating olfactory axons. The skin above the lesion was sutured, and animals were allowed to recover from anesthesia under a heat lamp. Following recovery from anesthesia, rats were returned to the animal colony and maintained on a normal diet until animals were killed at 3 day, 1 week, 2 weeks, or 3 weeks post-bulbectomy.

Subtracted Library Construction by SSH—Total RNA was purified from nasal epithelium tissues by the acid guanidinium thiocyanate phenol/chloroform extraction method (17). Nasal epithelium as isolated contained both olfactory epithelium and respiratory epithelium. Polyadenylated RNA was further isolated using mRNA purification kit (Qiagen). Normal nasal epithelium (driver) and 3-day post-bulbectomized nasal epithelium (tester) cDNA synthesis and SSH were performed using a PCR-select cDNA subtraction kit (CLONTECH) as per the manufacturer's protocol. Briefly, tester and driver cDNA were digested separately with *Rsa*Θ to obtain shorter, blunt-ended molecules. The tester cDNA was divided into two populations, each of which was ligated with different adaptors provided in the kit, whereas the driver cDNA had no adaptors. Tester and driver cDNA were heated to 98 °C for 5 min, and each of the tester cDNA samples was separately hybridized with the driver at 68 °C for 10 h. The two hybridization mixtures were mixed without being denatured again. Additional denatured driver was added, and the mixture was hybridized for 16 h. After two rounds of hybridization as above, templates for PCR amplification were generated from differentially expressed sequences. Using two rounds of suppression PCR, differentially expressed sequences were enriched. The subtracted library cDNA (secondary PCR products) was cloned into pCR 2.1 (original TA cloning kit, Invitrogen) vector. Electromax DH10B cells (Invitrogen) were transformed using a Bio-Rad electroporator (2.5 kV, 25 microfarads, 100 Ω). Bacterial cells were plated on LB agar plates containing 100 μg/ml ampicillin, 100 μM isopropyl-1-thio-β-D-galactopyranoside, and 50 μg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

Dot Blotting and Differential Screening—960 white colonies were picked and inoculated into 10 sterile 96-well plates containing 100 μl of LB media and ampicillin. After overnight incubation, 15 μl of bacterial culture was diluted with dH_2O and lysed by heating the aliquots to 100 °C for 10 min. A portion of each lysate was used as a PCR template. The conditions for PCR amplification of cloned inserts using nested primers were 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 1 min, for 36 cycles. PCR products were denatured by the addition of 0.4 M NaOH/10 mM EDTA, pH 8.2, and heating to 100 °C for 10 min, followed by blotting onto Hybond N^+ (Amersham Biosciences, Inc.) using a dot blotter (Bio-Rad). The membranes were hybridized under 5× SSC, 5× Denhardt's, 1% SDS, and 100 μg/ml denatured salmon sperm DNA, with equivalent amounts of *Rsa*I (a four-base cutter, as used for the construction of the initial subtracted library)-digested ^{32}P-labeled double-stranded cDNA of equal specific activity (2.2×10^6 cpm/ml) derived from driver and tester mRNA, respectively. Filters were washed twice consecutively in 2× SSC/0.1% SDS at 25 °C, 0.2× SSC/0.1% SDS at 42 °C, and 0.1× SSC/0.1% SDS at 68 °C. The membranes were exposed to the PhosphorImager (Molecular Dynamics) overnight. A partial list of the genes up-regulated after bulbectomy included $\alpha 2\mu$ globulin-related protein, clathrin heavy chain, collagen, clusterin, fibronectin, histone (H2A), mitochondrial cytochrome-oxidase *c* subunits, procollagen type I α-2 chain, RBM3, thymosin β-4, transferrin, and tuftelin.

Northern Hybridization—Total RNA from each experimental sample was separated by 1% agarose/2.2 M formaldehyde denaturing gel, which was then transferred to Hybond N^+ nitrocellulose membranes (Amersham Biosciences, Inc.) by capillary action using 10× SSC. RNA was cross-linked to the membranes using a UV cross-linker (Stratagene). Probes were generated by random prime labeling of a 0.35-kb subtracted cDNA fragment (later identified as rat plunc nucleotides 690–1040). Hybridization and washing conditions were the same as described. A commercial multiple tissue blot (CLONTECH number 7762-1) was probed for adult tissue Northern analysis.

Rat Olfactory Epithelium cDNA Library Construction and Screening—An oligo(dT)-primed rat olfactory epithelium cDNA library was constructed in Uni-ZAP XR (Stratagene) by using polyadenylated RNA isolated from normal olfactory epithelium and a ZAP-cDNA synthesis kit (Stratagene). Approximately 1×10^6 plaques from the library were screened under high stringency conditions using the 0.35-kb cDNA probe isolated from the subtracted cDNA fragment as above (rat *plunc* nucleotides 690–1040). Positive clones were sequenced by The Johns Hopkins University DNA Synthesis and Sequencing Facility.

In Situ Hybridization—The protocol was adapted from the Roche Molecular Biochemicals publication Nonradioactive *In situ* Hybridization Application Manual. Embryos sectioned at 12 μm were obtained from Novagen (Madison, WI). Embryo slides were washed twice for 5 min in xylene to remove paraffin and rehydrated through a series of graded ethanol solutions. Sections were washed twice in TBS (50 mM Tris-HCl (pH 7.5), 150 mM NaCl) and treated for 10 min in 0.2 M HCl. Slides were washed twice in TBS and permeabilized with 1 μg/ml RNase-free Proteinase K (Roche Molecular Biochemicals) for 30 min at 37 °C in TBS containing 2 mM $CaCl_2$. Slides were post-fixed in Bouin's for 5 min at room temperature, washed twice with TBS, incubated with 0.1 M triethanolamine buffer, pH 8.0, containing 0.25% (v/v) acetic anhydride (Sigma Chemical Co.) for 10 min, and pre-hybridized at 37 °C for 10 min with 2× SSC containing 50% (v/v) deionized formamide. Sections were hybridized overnight at 55–65 °C in 50–100 μl of hybridization buffer (50% deionized formamide, 10% dextran sulfate, 2× SSC, 0.02% SDS, and 0.01% sheared salmon sperm DNA) containing digoxigenin-labeled RNA. The following day, coverslips were removed by immersion in 2× SSC, and slides were washed twice for 10 min each in 2× SSC and twice in 1× SSC. Single-stranded RNA probe was digested by RNase A (20 μg/ml) buffer (10 mM Tris, pH 7.5, 500 mM NaCl, and 5 mM EDTA) for 30 min at 37 °C and then washed twice for 20 min each in 0.1× SSC at 37 °C.

To visualized bound probe, sections were washed twice in TBS, and blocked with blocking buffer (10% fetal calf serum in TBS) for 15 min at room temperature. The slides were incubated for 1 h in 1:5000 dilution of anti-DIG Fab fragment (Roche Molecular Biochemicals) in blocking buffer. Sections were then washed twice in TBS and incubated for 5 min in AP buffer (0.1 M Tris, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$). The color signal was developed in AP buffer containing 3.375 mg/ml nitro blue tetrazolium (Roche Molecular Biochemicals), 3.5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Roche Molecular Biochemicals), and 0.24 mg/ml levamisole. The color reaction was carried out at 4 °C and then terminated with a dH_2O wash. Coverslips were added to the developed slides with Aquapolymount (Polysciences). Serial sections were hybridized with identical quantities of sense cRNA as a control for specificity. In all cases, no signal was observed using sense cRNAs probes.

Immunohistochemistry—Immunohistochemistry was performed following the Vectastain ELITE protocol (Vector Laboratories). Tissue sections were permeabilized for 1 h in PBS containing 0.1% Triton X-100 and blocked for 1 h in PBS containing 1% bovine serum albumin and 4% normal serum. Section slides were incubated overnight at 4 °C in PBS containing the affinity-purified Plunc antibody at a dilution of 1:50. The next day, slides were rinsed with PBS and incubated with Vectastain biotinylated secondary antibody (1:1000) for 30 min, then incubated in 0.5% H_2O_2 for 10 min at room temperature. Slides were incubated in the BC reagent for 30 min, rinsed in PBS, and developed using 25 mg/100 ml diaminobenzidine (Sigma Chemical Co.) in 50 mM Tris, pH 7.4.

Generation of Antibodies to Plunc Peptide—Rabbit polyclonal antiserum to Plunc was generated against a peptide (CNITAEIVAMKD-NQGR, C for conjugation to KLH) consisting of amino acids 174–188 of the predicted rat Plunc sequence. The resulting immune sera (Zymed

Laboratories Inc.) were affinity-purified against SulfoLink gel (Pierce)-bound peptide as per the manufacturer's instructions.

Western Blot Analysis—Whole adult nasal epithelium and adult tissues were homogenized in radioimmune precipitation buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (v/v) deoxycholic acid, and 0.1% SDS) and incubated on ice for 30 min. The extracts were cleared by centrifugation for 15 min in an Eppendorf microcentrifuge at 13,000 rpm at 4 °C. Supernatants (50 μg of protein per gel lane) were subjected to SDS-PAGE on a 4–15% gradient gel. Protein content was measured using a Bradford assay with bovine serum albumin as a standard. The separated proteins were transferred to nitrocellulose membrane, and the membranes were probed with rabbit anti-Plunc antibody. Horseradish peroxidase-conjugated goat anti-rabbit Ig (Roche Molecular Biochemicals) was used as a secondary antibody at a 1:5000 dilution. Bands were visualized by enhanced chemiluminescence (Amersham Biosciences, Inc.). For mucus collection, anesthetized rats were injected with isoproterenol (30 mg/kg, intraperitoneal) to induce secretions. Secreted nasal mucus and tears were obtained and analyzed. Human tears and mucus were from male lab volunteers (a Caucasian and an Asian).

Bioinformatic Analyses—DNA and protein sequences were analyzed using the Wisconsin Package Version 10.1 (Genetics Computer Group (GCG), Madison, WI). Pair-wise sequence alignments were obtained using the GAP (or BESTFIT) programs of GCG using default parameters. For analysis of the statistical significance of pair-wise alignments, *Z* scores were obtained using the quality score of each alignment, as well as the quality score and standard deviation of the measurement of 100 alignments in which the sequence of one protein is scrambled.

BLASTP 2.1.2 searches were performed on the GenBank™ data base. UniGene was searched using protein names and accession numbers using build #131 (human), build #86 (mouse), build #84 (rat), or build #6 (cow). For phylogenetic analysis, multiple sequence alignments were generated using PileUp with default parameters (gap creation penalty 8, gap extension penalty 2). Alignments were visualized using the software package Phylogeny Analysis Using Parsimony (PAUP version 4.0b6, Sinauer Associates, Sunderland, MA). Heuristic searches were performed to identify the best tree for 11 taxa. 500 bootstrap replicates were performed.

RESULTS

plunc Is Identified as an RNA Transcript That Is Up-regulated after Bulbectomy—Total RNA isolated from normal nasal epithelium and from nasal epithelium of bulbectomized rats was used as the tester and driver for SSH, respectively. Both mRNA populations were reverse-transcribed and subjected to SSH as described previously (13). 960 cloned inserts were amplified by colony PCR, and the PCR products were dot-blotted to a Hybond N^+ membrane. Duplicate filters were hybridized with restriction enzyme cleaved double-stranded cDNA of equal specific activities derived from driver and tester mRNA. The comparison of hybridization signals revealed differentially expressed genes. *plunc* (p for palate, lu for lung, n for nose, and c for clone) was one of the clones identified as up-regulated after bulbectomy.

plunc showed high homology to a mouse gene sequence (Fig. 1) (18). The predicted amino acid sequence of rat *plunc* was aligned with the mouse sequence (278 amino acids; accession NP_035256), and the two proteins share 91% amino acid identity. More recently, a human *plunc* sequence was identified by screening a human EST data base with a full-length mouse *plunc* cDNA sequence (19). Analysis of GenBank™ protein and nucleic acid data bases revealed that rat Plunc shared 73% amino acid identity with this human Plunc protein called Lunx (NP_057667). Based on its identities to rat and mouse Pluncs, human Lunx is orthologous to Plunc. The human *lunx* gene was assigned to chromosome 20. We predicted that our longest rat *plunc* clone (1079 bp) represented a near full-length cDNA sequence, because mouse *plunc* and human *lunx* are 1113 and 1020 bp, respectively, and the mobility of our transcript was ~1.1k bp. The complete rat *plunc* sequence contained an open reading frame of 810 base pairs (270 codons) predicted to encode a protein with a molecular mass 27.8 kDa. There are 50 base pairs of 5′-untranslated sequence, and 219 base pairs of 3′-untranslated sequence.

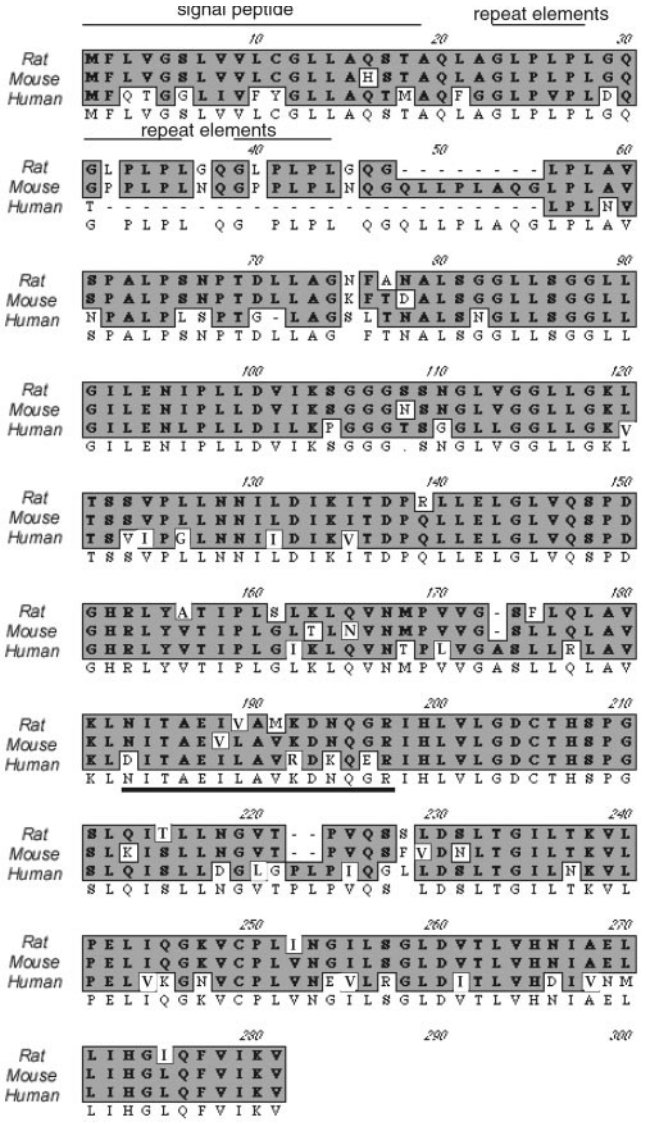

FIG. 1. **Multiple sequence alignment of rat, mouse, and human Pluncs.** The alignment was performed using the PileUp program (see "Experimental Procedures"); final alignment was manual. Positions in which the three residues in rat, mouse, and human Plunc are identical are *shaded gray*. Signal peptide motif and G(L/P)PLPL repeated sequence motifs are indicated by *thin lines*. Peptide sequence used for generation of anti-peptide antibody is indicated by the *thick line*.

To verify that *plunc* is expressed in nasal tissue and differentially expressed following bulbectomy, Northern blot analysis was performed (Fig. 2). When compared with its expression in normal nasal epithelium (Fig. 2*A*, *lane 1*, *control*), rat *plunc* was highly expressed in nasal epithelium ipsilateral to the side of the bulbectomy (Fig. 2*A*, *lane 2*, *Bulbx*). These results were quantified to reveal a 1.8-fold difference using glyceraldehyde-3-phosphate dehydrogenase as a standard. To examine the tissue expression profile of rat *plunc*, we performed RNA hybridization using a commercial tissue blot prepared from adult mouse (Fig. 2*B*). A partial rat *plunc* fragment (nucleotides 690–1040) was used as a probe. Both heart and lung expressed *plunc* (Fig. 2*B*). This observation contrasts with an earlier report that demonstrated that mouse *plunc* was detected only in lung tissue (18). Moreover, the signal in heart was much stronger than that of lung. No signal was detected in brain, spleen, liver, muscle, kidney, or testis.

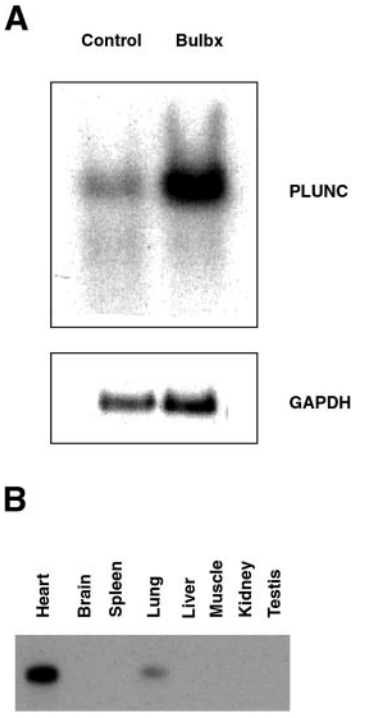

FIG. 2. **Expression of *plunc* in nasal epithelium and adult tissues.** *A*, Northern analysis of *plunc* after bulbectomy. Equal amounts (40 μg) of total RNA were isolated from control (*Control*) and 3-day post-bulbectomy (*Bulbx*) and hybridized with probes to *plunc* or glyceraldehyde-3-phosphate dehydrogenase. *B*, Northern analysis of *plunc* in mouse tissues. A commercially prepared mRNA blot from adult mouse tissues (2 μg mRNA/lane) was used. A single band in each lane for heart and lung.

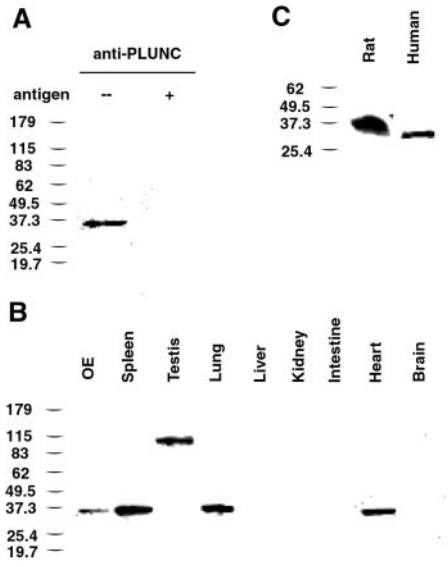

FIG. 3. **Expression of Plunc protein in the tissues.** *A*, generation of antibody against Plunc. Homogenates were prepared from whole adult rat nasal epithelium. One hundred micrograms of protein per lane were electrophoretically separated on a 4–15% SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking, lanes were separated and probed with affinity-purified anti-Plunc antiserum (*lane 1*, 1:500 dilution). Immunoreactivity was blocked when the antibodies were pre-absorbed with a 10-fold excess of the peptide antigen (*lane 2*). *B*, immunoblot analysis of Plunc in the rat adult tissues. *C*, immunoblot analysis in secretory samples. Thirty microliters of mucus from rat and human were examined.

Plunc Is a Secreted Protein Expressed in the Nasal Respiratory Epithelium—Plunc expression in tissues was also examined by Western blotting using affinity-purified antibodies against a Plunc peptide (Fig. 1, *dark line* below amino acids 174–189 represents sequence used as the antigen for anti-peptide antibody preparation). Plunc immunoreactivity was present in lysates prepared from nasal epithelial tissue (Fig. 3*A*). The relative mobility of the single band identified corresponded to a molecular mass of 31 kDa (Fig. 3*A*, *lane 1*). The size of he protein is larger than the estimated size of 27.8 kDa, suggesting there may be post-translational modification of rat Plunc, such as protein glycosylation. When the antibodies were pre-absorbed with the peptide antigen, the immunoreactive band was no longer detected (Fig. 3*A*, *lane 2*).

To examine the patterns of tissue localization of Plunc protein in the adult, we performed immunoblot analysis on various tissue extracts from adult rats. As observed by Northern blot analysis, Plunc was present in lung and heart (Fig. 3*B*). In contrast to the results obtained by Northern blot analysis, Plunc immunoreactivity was abundant in spleen. Plunc was also detected in testis. However, the relative molecular mass of the band in testis was about 100 kDa, a mobility that was quite different from the 31-kDa band detected in other organs. This discrepancy between the results obtained by Western and Northern analyses of testis tissue may be attributed to the ability of the antiserum to recognize a related but not identical protein in testis. *plunc* mRNA was not detected in testis, suggesting that the message of the 100-kDa protein identified in testis may contain distinct mRNA sequences that prohibit its recognition using a cRNA probe to *plunc* by high stringency Northern analysis. Plunc protein was variably detected in kidney. There was no expression in liver, intestine, or brain (Fig. 3*B*). We also examined tissues from adult mice. As in rats, Plunc was robustly expressed in heart and lung (data not shown). However, in contrast to rats, Plunc expression was minimal in mouse spleen (data not shown). Interestingly, a considerable amount of Plunc immunoreactivity was observed in mouse kidney (data not shown). The significance of these findings is addressed in the discussion.

Plunc has a putative signal peptide sequence (amino acids 1–19), implying that it is a secretory molecule. To investigate whether Plunc might be a secretory protein, we induced mucus secretion in adult rats through the administration of isoproterenol and collected the mucus as previously described (20). The collected mucus was subjected to immunoblot analysis (Fig. 3*C*). Plunc was detected in the mucus from the external nares, indicating that Plunc is a secreted protein. We also demonstrated that human mucus contained Plunc. The relative mobility of human Plunc was smaller that that of rat Plunc, corresponding to the estimated size difference between these proteins. Rat Plunc immunoreactivity was visualized on Western blot as a diffuse band, a characteristic not seen when human mucus was examined, implying that rat Plunc may have post-translational modifications that are different from the human Plunc, such as the degree of glycosylation. We also tested tears and saliva from both rat and human subjects, but no significant amount of Plunc immunoreactivity was observed in these preparations (data not shown).

Plunc Is Highly Expressed in Adult Nasal Respiratory Epithelium—To investigate the cellular localization of *plunc*, *in situ* hybridization was performed. Serial coronal sections of adult rat nasal epithelial tissues were prepared and hybridized with a digoxigenin-labeled antisense RNA probe to *plunc* (Fig. 4). Nasal epithelium consists of stretches of respiratory epithelium that are dovetailed with stretches of olfactory epithelium, each with a distinctive cellular architecture (Fig. 4, *diagram*). Representative sections from anterior to posterior were examined (Fig. 4, *A–D*). *plunc* was highly expressed in the anterior and ventral regions of nasal epithelium, where respiratory

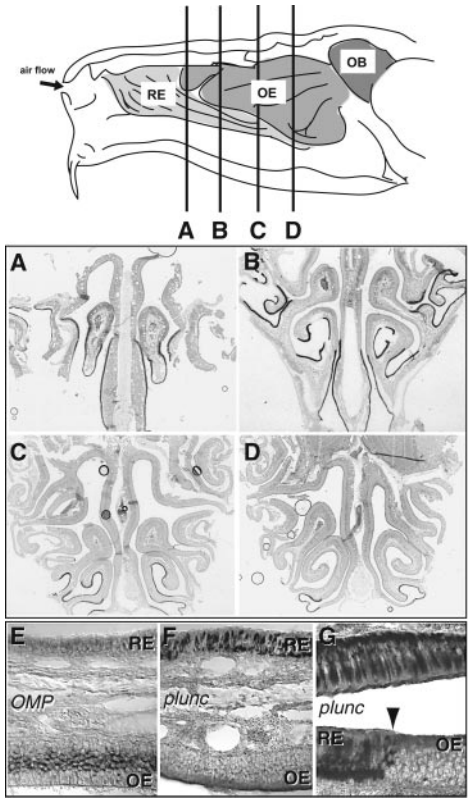

FIG. 4. **Expression of *plunc* mRNA in the nasal epithelium.** Diagram indicates the position across the nasal epithelium at which sections shown in *A–D* were taken. *In situ* hybridization analysis was performed in adult rat coronal sections from anterior to posterior (*A* through *D*) across the nasal epithelium. *plunc* mRNA was expressed exclusively in the lower parts of the nasal epithelium, where regions of respiratory epithelium are located. *E* and *F*, serial sections were used for *in situ* hybridization for OMP and *plunc* to demonstrate that OMP was expressed exclusively in olfactory epithelium (*OE*), whereas *plunc* was exclusively expressed in respiratory epithelium (*RE*). *G*, higher magnification photomicrograph of a section of nasal epithelium showing *in situ* hybridization for *plunc* at a region of transition (*arrowhead*) between respiratory epithelium (*RE*) and olfactory epithelium (*OE*), demonstrating that *plunc* is expressed exclusively in respiratory epithelium.

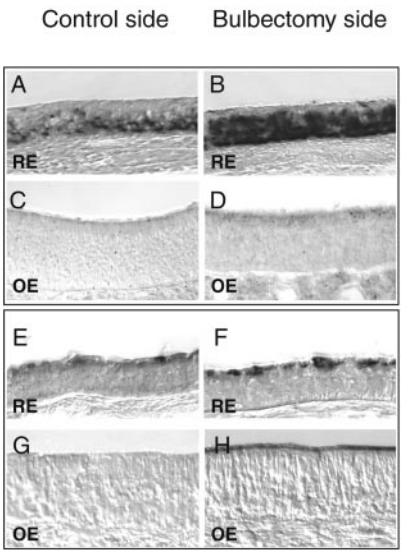

FIG. 5. **Expression of *plunc* mRNA and protein in the nasal epithelium after unilateral bulbectomy.** *A–D*, *in situ* hybridization analysis of *plunc* mRNA expression in the nasal epithelium after bulbectomy. Coronal sections of adult nasal epithelium are displayed. *A* and *C*, control side; *B* and *D*, 3 days post-bulbectomy side. Compared with control respiratory epithelium (*A*, *RE*), *plunc* mRNA was up-regulated ipsilateral to the bulbectomy side in respiratory epithelium (*B*). No signal for *plunc* message was detected in olfactory epithelium (*OE*) on control (*C*) or lesioned (*D*) sides. *E–H*, immunohistochemical analysis of Plunc protein expression in the nasal epithelium after bulbectomy. *E* and *G* represent the control side, whereas *F* and *H* represent the bulbectomized side 3 days after lesioning. Plunc protein expression is detected in control RE (*E*), and is up-regulated on the side ipsilateral to the bulbectomy (*F*). Although Plunc protein is not detected in the OE on the control side (*G*), Plunc protein is detected in the cilial/mucus layer of the OE ipsilateral to the bulbectomy (*H*).

epithelium is located. No signal was observed in regions containing olfactory epithelium. To confirm that *plunc* was expressed exclusively in columnar epithelial cells in the respiratory epithelium, *in situ* hybridization was performed for olfactory marker protein (OMP) or *plunc* on serial sections of nasal epithelium that contained both respiratory and olfactory epithelium (Fig. 4, *E* and *F*). OMP is expressed in mature ORNs and, therefore, identifies regions of olfactory epithelium that contain ORNs. *OMP* was expressed in olfactory epithelium and absent from respiratory epithelium as expected, whereas *plunc* was expressed in the opposite distribution. In addition, *plunc* expression was examined in sections containing transition zones between respiratory and olfactory epithelium (Fig. 4*G*, *arrowhead*). *plunc* expression ceased at these transition zones (Fig. 4*G*, *arrowhead*), confirming its restriction to cells of the respiratory epithelium.

plunc Is Up-regulated in Respiratory Epithelium Post-bulbectomy—plunc was initially identified by screening for genes whose messages were up-regulated after olfactory bulbectomy. The up-regulation of *plunc* message was confirmed by Northern blot analysis using RNA isolated from control and 3-day post-bulbectomy nasal epithelium (Fig. 2). This result indicated that SSH could identify differentially expressed messages and suggested one of two possibilities regarding *plunc* expression post-lesioning of the olfactory bulb. One possibility is that *plunc* expression is restricted to respiratory epithelium in normal animals but is induced in olfactory epithelium post-lesioning. Alternatively, *plunc* is restricted exclusively to respiratory epithelium, and its expression is up-regulated in respiratory epithelium post-lesioning of the olfactory bulb. To examine these possibilities, *in situ* hybridization (Fig. 5, *A–D*) and immunohistochemistry (Fig. 5, *E–H*) for Plunc were performed on nasal tissues isolated from animals that had received unilateral bulbectomies 3 days earlier. Sections containing nasal epithelium ipsilateral to the side of the lesion (*Bulbectomy side*) and contralateral to the side of the lesion (*Control side*) were examined. *plunc* message was expressed in the normal respiratory epithelium of non-lesioned animals (Fig. 4) and was essentially unchanged on the side of the epithelium contralateral to olfactory bulbectomy (Fig. 5*A*). In contrast, the expression of *plunc* increased dramatically in the respiratory epithelium ipsilateral to the bulbectomy (Fig. 5*B*). This up-regulation of *plunc* expression persisted even after 2 weeks post-lesioning (data not shown). As seen in normal olfactory epithelium (Fig. 4), *plunc* expression was not detected in olfactory epithelium on control or bulbectomized sides (Fig. 5, *C* and *D*, respectively).

Immunohistochemical analysis was performed on the same tissues using affinity-purified antibodies directed against a peptide representing amino acids 174–189 of Plunc (Fig. 5, *E–H*). Plunc immunoreactivity was localized to the apical region of the cells of the respiratory epithelium on the control side contralateral to the olfactory bulbectomy (Fig. 5*E*), with a distribution and abundance similar that observed in non-lesioned animals (data not shown). Plunc protein expression was increased in the respiratory epithelium ipsilateral to the lesion (Fig. 5*F*). There was no immunoreactivity observed in the olfactory epithelium on the control side (Fig. 5*G*). Interestingly,

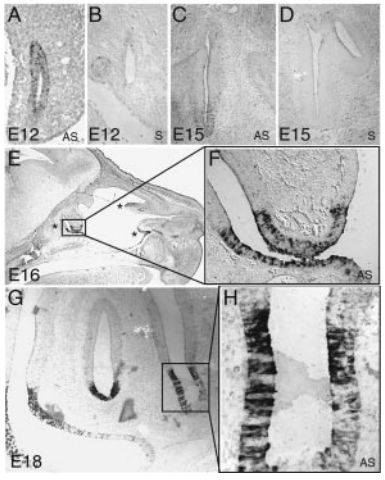

FIG. 6. **Developmental study of *plunc* expression in rat embryonic tissues.** *A–H*, *in situ* hybridization analysis in the nasal epitheliums of embryos. Sense probes were utilized for specific signals of *plunc* (*B* and *D*). High power resolution clearly demonstrated specific expression of *plunc* in the respiratory epithelium (*F* and *H*). *E* with *numbers*, embryonic days. *A* and *AS*, sense and antisense probes utilized, respectively.

faint, but consistent Plunc immunoreactivity was visualized in the ciliary/mucus layer of the olfactory epithelium ipsilateral to the bulbectomy (Fig. 5*H*). These data support our *in situ* hybridization results that showed that OMP and Plunc expression were mutually exclusive. In addition, this is the first report of a change in the expression of a protein in response to injury of the olfactory bulb in respiratory epithelial cells.

plunc Is Expressed during Embryonic Development—To evaluate the onset of *plunc* during development, we examined *plunc* expression by *in situ* hybridization using rat sagittal embryo sections (Fig. 6). *plunc* was first detected at embryonic day (E) 12 in rat (Fig. 6*A*), similar to the expression pattern of mouse *plunc* in the nasal epithelium (18). At this stage, cells expressing *plunc* were distributed throughout the presumptive olfactory epithelium. With increasing embryonic age, *plunc* expression was localized to regions of putative respiratory epithelium (Fig. 6, *C*, *E*, and *G*). This is consistent with its lack of expression in olfactory epithelium in the adult. At higher magnification, *plunc* was expressed in columnar epithelial cells, consistent with respiratory epithelial cells (Fig. 6, *F* and *H*). None of these signals were observed in the sections incubated with sense probe (Fig. 6, *B* and *D*).

plunc was also expressed during embryogenesis in lung tissues. *plunc* mRNA was first detected at E16 (data not shown). Robust expression was localized to segmental bronchi. In addition to lung and nasal tissues, *plunc* message was also observed in thymus and salivary gland (data not shown).

Sequence Analysis of plunc cDNA—Further BLAST searches of the non-redundant GenBank™ data bases revealed that Plunc proteins in rat, mouse, and human are significantly related to eight other proteins (Table I). These are: two putative proteins identified from mouse sequencing efforts (accession numbers BAB26290 and BAB24670); a von Ebner's gland minor salivary gland protein (named in a data base entry); two closely related bovine parotid secretory proteins (bSP30) (21); rat submandibular gland protein A (22); and parotid secretory protein from rat and mouse (23). The statistical significance of the amino acid homologies was confirmed by obtaining *Z* scores for pair-wise comparisons of proteins (see "Experimental Procedures" and Table I). All of the alignments were highly significant ($Z > 3$), except for the alignment of rat parotid secretory protein to putative protein 2 from mouse.

To evaluate the relationships of the rat, mouse, and human Pluncs and to define the relationships of these proteins to other homologues, we performed multiple sequence alignment of the 11 related proteins. As expected from the pairwise sequence alignments, the three Plunc orthologues shared many identical amino acid residues (of 248 multiply aligned positions, 172 were identical). We observed several features of interest. Rat, mouse, and human Plunc are predicted to contain a signal peptide at residues 1–19, based upon the SignalP software package (www.cbs.dtu.dk/services/SignalP/) (24). We identified a motif, G(L/P)PLPL, which occurred three times near the amino terminus of rat and mouse (but not human) Plunc. A search for other proteins sharing this motif using pattern-hit initiated blast (www.ncbi.nlm.nih.gov/blast/) failed to identify other proteins sharing this motif. The multiple sequence alignment revealed only three positions (Cys-196, Cys-238, and Pro-239 of rat Plunc) that are identically conserved among all 11 proteins. However, conservative amino acid substitutions among all the proteins were apparent throughout the multiple sequence alignment and reached 85%. Another interesting domain is bactericidal permeability-increasing protein (BPI) domain.

A search (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) of conserved domain data base with rat Plunc sequence revealed statistically significant relatedness to a family that includes bactericidal permeability-increasing protein (BPI)/lipopolysaccharide-binding protein/cholesteryl ester transfer protein amino-terminal domain (SMART data base accession number BPI1). The expected value is 0.005. This family includes murine von Ebner minor salivary gland protein, strengthening support for membership of Plunc in this group. Other members of the family are secreted from glands, such as a blood fluke developmentally regulated albumen gland gene (accession AAB00448) and several lipid-binding proteins. Additionally, two secreted proteins localized to nasal glands, rat RYA3 and rat RY2G5 (accession numbers CAA43065 and CAA43067, respectively) (25, 26) are members of the BPI family and share limited sequence identity with rat Plunc (26% amino acid identity over a span of 140 residues).

DISCUSSION

Here, we report the molecular cloning, expression profile study, and sequence analysis of a rat *plunc*. Rat *plunc* is highly homologous to mouse and human *plunc*, whose functions are still unclear. Based on the observation that Plunc is present in the nasal mucus and that its electrophoretic mobility in PAGE is higher than the estimated molecular weight, Plunc appears to be a secreted protein with post-translational modification. Rat Plunc was up-regulated in respiratory epithelium ipsilateral to the side of olfactory bulbectomy by both *in situ* hybridization and immunohistochemistry. In addition, Plunc appeared to "coat" the surface of the olfactory epithelium ipsilateral to the lesioned side. Because *in situ* hybridization analyses showed up-regulation after bulbectomy only in the respiratory epithelium, Plunc may be expressed and secreted from the respiratory epithelium and migrate into the olfactory epithelium.

One of our goals was to identify candidate genes involved in the olfactory neurogenesis by isolating genes expressed olfactory epithelium that were up-regulated following bulbectomy. In all clones identified by SSH that had follow-up by Northern blot analysis, it appeared that our screening method indeed identified genes that were up-regulated following bulbectomy. The *plunc* fragment was chosen to investigate further. Unexpectedly, *plunc* was expressed in respiratory epithelium, not in olfactory epithelium. It is unlikely that Plunc is a stimulatory factor for neurogenesis in olfactory neuroepithelium, because

TABLE I

Relationship of rat plunc to other proteins

Rat *plunc* sequence was analyzed by BLAST searching (BLAST 2.1.2, TBLASTN, and PSI-BLAST). Protein abbreviations: SP30, Bos taurus common salivary protein; SMGB1, neonatal submandibular gland proacinar cell protein precursor; VEM, Von Ebner minor salivary gland protein. Organism abbreviations: cow, *Bos taurus*; human, *Homo sapiens*; mouse, *Mus musculus*; rat, *Rattus norvegicus*. Expressed sequence tags (ESTs) are listed from UniGene (see "Experimental Procedures"). Protein pairwise sequence comparisons were performed using the BESTFIT or GAP programs of GCG. Values in boxes indicate the percent amino acid identity between each two proteins, the number of gaps in the alignment (in parentheses), and the *Z* score using 100 random shufflings of a protein sequence in the pairwise alignment.

Protein	Organism	Accession	UniGene	Length (aa)	Mouse plunc	Human lunx	Mouse VEM	Rat SMGB1	Mouse PSP	Rat PSP	Cow bSP30a	Cow bSP30b	Mouse Protein 1	Mouse Protein 2
Plunc	Rat		(none)	270	91% (1) $Z = 119$	73% (4) $Z = 97$	24% (7) $Z = 22$	24% (7) $Z = 12$	32% (13) $Z = 14$	30% (10) $Z = 13$	23% (9) $Z = 14$	21% (7) $Z = 15$	60% (2) $Z = 110$	31% (8) $Z = 30$
Plunc	Mouse	NP_035256	Mm.28430 (259 ESTs)	278	—	74% (5) $Z = 88$	24% (6) $Z = 25$	24% (8) $Z = 9$	33% (14) $Z = 13$	31% (10) $Z = 15$	21% (6) $Z = 15$	23% (7) $Z = 19$	62% (3) $Z = 98$	32% (8) $Z = 37$
Lunx	Human	NP_067667	Hs.211092 (21 ESTs)	258		—	30% (10) $Z = 24$	22% (8) $Z = 8$	31% (12) $Z = 15$	28% (11) $Z = 12$	23% (8) $Z = 16$	22% (9) $Z = 18$	62% (8) $Z = 93$	28% (5) $Z = 26$
VEM	Mouse	NP_061205	Mm.3783 (185 ESTs)	474			—	24% (5) $Z = 14$	25% (9) $Z = 18$	22% (6) $Z = 14$	23% (8) $Z = 23$	24% (10) $Z = 17$	24% (9) $Z = 20$	20% (8) $Z = 7$
SMGB1	Rat	A42337	Rn.9925 (6 ESTs)	206				—	40% (3) $Z = 37$	40% (4) $Z = 42$	27% (7) $Z = 22$	28% (6) $Z = 15$	21% (8) $Z = 0.8$	28% (16) $Z = 3$
Parotid secretory protein	Mouse	NP_032979	Mm.4538 (1524)	235					—	70% (0) $Z = 75$	34% (8) $Z = 39$	31% (8) $Z = 31$	30% (12) $Z = 15$	18% (7) $Z = 4$
Parotid secretory protein	Rat	AAC06334	Rn.9766 (0 ESTs)	235						—	33% (6) $Z = 36$	31% (7) $Z = 32$	29% (11) $Z = 11$	19% (10) $Z = 0.7$
bSP30s	Cow	AAB38282	Bt575 (1 EST)	243							—	83% (2) $Z = 118$	25% (9) $Z = 17$	27% (10) $Z = 17$
bSP30b	Cow	AAB38283	Bt576 (0 ESTs)	240								—	22% (8) $Z = 16$	28% (13) $Z = 16$
Putative protein 1	Mouse	BAB26290		270									—	27% (6) $Z = 38$
Putative protein 2	Mouse	BAB24670		232										—

bPlunc is expressed exclusively in the respiratory epithelium and localized in the apical layer of the respiratory epithelium. One might suspect that Plunc would have difficulty coming in contact with olfactory stem/precursor cells located in basal layer of olfactory epithelium, although Plunc proteins are present in the ciliary layer of the olfactory epithelium after bulbectomy. Instead, *plunc* may be the target gene of cytokines or growth factors that are up-regulated upon olfactory bulbectomy to serve another function in response to injury. This is the first report of a protein expressed in respiratory epithelial cells whose expression is altered by injury to the olfactory epithelium or olfactory bulb.

Based on our data, Plunc is up-regulated after injury to the olfactory epithelium. It is not clear how Plunc is up-regulated in the respiratory epithelium after removal of olfactory bulb, which is the target for ORNs resident in olfactory epithelium. Our result implies that there may be some kind of communication between olfactory and respiratory epithelia. This communication might be caused by local fluctuation of cytokines, growth factors, or other factors after bulbectomy. Our results also demonstrate that Plunc is expressed in epithelial cells in segmental bronchi and in heart. By analogy to the olfactory system, Plunc may function in response to injury or insult in the respiratory epithelium. It is more difficult to hypothesize about the function of Plunc expression in heart. However, it would be very interesting to examine Plunc expression after injury to the heart and lung.

The NCI-H647 adeno-squamous carcinoma cell line and the PG-3 adenocarcinoma cell line are known to express Plunc (19, 27). It is interesting to note that Plunc expression in NCI-H647 is reduced by interferon γ, implying that Plunc is regulated either directly or indirectly by this cytokine. Examination of the 5′ regulatory region of *plunc* to determine whether there is any cytokine-responding element would be interesting. In the mouse 5′ regulatory region, there are, in fact, seven potential binding sites for STATs,[2] which are known to regulate expression by various cytokines, including interferon γ (28–30).

By using peptide antibody to Plunc, we demonstrated that Plunc is secreted in the nasal mucus of rat and human. This finding, together with a conserved domain data base search that showed that Plunc has a bactericidal permeability-increasing protein (BPI) domain, suggest that Plunc might have secretory/antimicrobial functions. BPI is a cationic protein displaying a pivotal role in host defense against Gram-negative bacteria (31, 32). The spleen is known to be a critical organ to clear a Gram-negative bacteremia (33), and asplenia can cause sudden death of children as a result of infections of the respiratory systems (9). Interestingly, *plunc* message is expressed in the upper airways and Plunc protein (but not message) is abundant in rat spleen. These findings suggest that plunc may be a host defense molecule against inhaled Gram-negative bacterial infection.

Very recently, human Plunc (also called Lunx) was proposed as a marker of micrometastasis in non-small cell lung cancer (27). Lunx has been identified by two independent groups (19, 27). It is expressed around the developing palate and thymus, in nasal septum and nasal conchae, as well as in the trachea and main stem bronchi (18, 19). Lunx is found in all the lung carcinoma cell line, and it is differentially expressed in lymph nodes of cancer patients (27). Although Lunx was proposed as a marker of micrometastasis in non-small cell lung carcinoma (27), the nature and function of Plunc are largely unknown. Therefore, it would also be worth determining if there is a neuroproliferative and/or survival role of Plunc.

There is a discrepancy between a previous report describing the expression patterns of mouse/human *plunc* (18, 19) and our rat *plunc* Northern result. Others did not detect mouse *plunc* expression in heart, but, in our study, rat *plunc* was highly expressed in heart by both Northern and Western analyses. Mouse plunc UniGene has a few ESTs derived from heart, so a possible explanation is that, in mouse, *plunc* expression in the heart may not be as high as that in rat.

Thus, Plunc may protect against infection in epithelial tissues post-injury. Variations in the level of Plunc expression could well be correlated with differences in susceptibility to infections. There is precedent for this hypothesis. The relative abundance of a salivary protein, bSP30, is correlated with susceptibility to bloat in cattle herds (21). A similar situation might exist for Plunc and respiratory infections. Alternatively, Plunc may also play a role in the etiology of bloat. The specific tissue expression pattern of Plunc provides invaluable tools to researchers interested in these tissues.

[2] C. Moon, J.-Y. Yoo, and G. V. Ronnett, unpublished observations.

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