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Short Communication

Isolation and Localization of Type IIb Na/Pi Cotransporter in the Developing Rat Lung

Mitsuyoshi Hashimoto,* Dong-Yu Wang,* Takaharu Kamo,* Yue Zhu,*† Toshifumi Tsujiuchi,‡ Yoichi Konishi,‡ Masamitsu Tanaka,* and Haruhiko Sugimura*

From the First Department of Pathology, Hamamatsu University School of Medicine, Hamamatsu, Japan; the Cancer Research Institute,† China Medical University, Shenyang, China; and the Department of Oncological Pathology,‡ Cancer Center, Nara Medical College, Nara, Japan*

Differential display analysis of rat lung at different developmental stages identified a fragment, HG80, which appeared on embryonic day 16.5 and thereafter. A full-length cDNA derived from a cDNA library of newborn rat lung probed with HG80 was the rat counterpart of sodium-dependent phosphate transporter type IIb and was designated rNaPi IIb. *In situ* hybridization showed that rNaPi IIb was expressed in type II alveolar cells, suggesting a role in the synthesis of surfactant in the alveoli. The time-dependent changes in localization of this gene in the developing lung and its possible use as a type II pneumocyte marker are discussed. *(Am J Pathol 2000, 157:21–27)*

The specific temporal and spatial patterns of gene expression during mammalian pulmonary development remain poorly characterized.[1] Several factors including retinoid receptors, homeobox genes, hepatocyte nuclear factors, and thyroid transcription factor have been reported to be involved in bronchogenesis, but the only factors that have been specifically associated with alveolar development are surfactant proteins.[2–8] To identify genes that are specifically expressed in different stages of lung development, we performed a differential display analysis of pulmonary development of the Wistar rat from embryonic day 15. One differentially expressed band yielded a cDNA clone that had substantial homology to the mouse sodium-dependent phosphate transporter (Na/Pi cotransporter) Npt2b.[9] Several cDNAs encoding sodium-phosphate cotransport systems have been identified from several species and have been classified into three groups (types I, II, and III) based on sequence homology.[10] The Na/Pi cotransporter type II has been further subdivided into two groups (type II and type IIb) based on structure, tissue distribution, and pH dependence.[9,11] We report here the isolation of one of these genes that is specifically expressed in the developmental stages of lung formation. Sequence comparison of the complete cDNA sequence identified it as a novel isoform of the rat Na/Pi cotransporter, and we designated it rat Na/Pi cotransporter type IIb (rNaPi IIb). The human Na/Pi cotransporter type IIb (NaPi3b) transcripts were very recently found in relatively high abundance in total RNA prepared from lung tissues, although the localization in this tissue has not yet been determined.[11] We performed *in situ* hybridization to determine the localization of rNaPi IIb in developing lung tissue, and demonstrated restricted localization of expression to type II alveolar cells. We discuss the potential use of rNaPi IIb as a marker to trace heterogeneous pulmonary constituents.

Materials and Methods

Animals and Tissue Preparation

Wistar rats purchased from Nippon SLC Co. (Hamamatsu, Japan) were bred and maintained in the Institute for Experimental Animals at the Hamamatsu University School of Medicine. After overnight mating, the presence of a vaginal plug or sperm-positive vaginal smear in the morning was considered as gestational day 0.5. All pregnant rats were housed in individual cages and allowed food and water *ad libitum* under controlled conditions of temperature (23 ± 2°C) and lighting (12-hour light-dark cycle). The timed pregnant female rats

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Address reprint requests to Haruhiko Sugimura, First Department of Pathology, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu, 431-3192, Japan. E-mail: hsugimur@hama-med.ac.jp.

were sacrificed by exsanguination through the abdominal aorta after deep anesthesia with diethyl ether. Embryos from gestational day 15.5 to 21.5 (term = 22 days) were removed from the mothers, and fetal rat lungs were obtained by microdissection under stereoscopic microscope. Postnatal 1-day-old, 1-week-old, and adult rats were sacrificed by inhalation of diethyl ether, and lungs were rapidly removed from the chest cavity. Fetal and postnatal lung tissues were immediately frozen in liquid nitrogen and stored at −70°C. All of these procedures were performed according to the guidelines for animal experiments in the institute.

RNA Isolation

Total RNA was isolated from the lung of fetal rats (ranging in age from gestational day 15.5 to 21.5 and postnatal 1-day-old, 1-week-old, and 14-week-old rat) using the commercially available ISOGEN kit (Nippongene, Tokyo, Japan). Poly$(A)^+$ RNA was then prepared by using Oligotex-dT 30 Super (Roche, Tokyo, Japan) according to the manufacturer's instructions.

Differential Display

Differential display was performed by using the RNAimage Kit 10 (GenHunter, Brookline, MA) according to the manufacturer's protocol. This kit contains three anchored oligo-dT primers (H-T_{11}A, H-T_{11}C, and H-T_{11}G) and eight arbitrary primers (H-AP73∼80) with 24 possible combinations of upstream and downstream primers. Briefly, 0.2 μg of total RNA, treated with DNase I (MessageClean Kit, GenHunter, Brookline, MA), was reverse transcribed to a first strand complementary DNA (cDNA) using an anchored oligo-dT primer, 5′-AAGCTTTTTTTTTTTG-3′ (H-T_{11}G). Following the reverse transcription, cDNAs were amplified by polymerase chain reaction (PCR) using the same anchored oligo-dT primer (H-T_{11}G) and an arbitrary primer, 5′-AAGCTTCTATTTC-3′ (H-AP80) in the presence of α-[^{35}S]dATP (NEN, Boston, MA). The amplified products were then separated by electrophoresis on a 6% denaturing polyacrylamide gel and visualized by autoradiography.

Characterization of the Differentially Expressed Band

The differentially expressed bands were dissected from the dried gel and eluted in distilled water, followed by boiling for 15 minutes. Reamplified PCR products with the same pair of primers used in the initial PCR reaction were then subcloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced by the dye-deoxy terminator methods with a Thermo Sequenase II dye terminator cycle sequencing kit (Amersham, Buckinghamshire, UK) and an ABI 373A DNA Sequencer (Applied Biosystems, Foster City, CA). Homology search was performed using the BLAST program at the National Center for Biotechnology Information.

Northern Blot Analysis

Northern blot analysis was carried out according to the standard methods.[12] Total RNA (25 μg/lane) from fetal, newborn, and adult rat tissues were fractionated by formaldehyde-1% agarose gel electrophoresis and transferred to a nitrocellulose membrane (Micron Separations Inc., Westborough, MA). RNA was fixed to the membrane by baking at 80°C for 2 hours. The membrane was prehybridized in 50% formamide, 5× saline sodium citrate (SSC), 5× Denhardt's solution, 5.0 mmol/L EDTA, 0.1% sodium dodecyl sulfate (SDS), and 100 μg/ml denatured salmon sperm DNA at 42°C for 4 hours. The cDNA probe isolated from the differential display gel was labeled with [α-^{32}P]dCTP (ICN, Costa Mesa, CA) using Random Primer DNA Labeling Kit version 2.0 (Takara, Osaka, Japan) and hybridized in 50% formamide, 5× SSC, 5× Denhardt's solution, 5.0 mmol/L EDTA, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, and 10% dextran sulfate at 42°C for 14 hours. The membrane was washed twice with 1× SSC, 0.1% SDS at room temperature for 15 minutes and twice with 0.25% SSC, 0.1% SDS at 58°C for 15 minutes and exposed to X-OMAT AR film (Kodak, Rochester, NY) with intensifying screens at −80°C for 48 hours. The membrane was subsequently rehybridized with mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA as an internal control for equivalent RNA loading. The size of the hybridizing RNA was inferred by comparing ribosomal RNAs in an adjacent lane.

cDNA Library Construction and Screening for Full Sequence

A 1-day-old newborn rat lung cDNA library (oligo-dT primed) was constructed in Lambda Zap II vector following the manufacturer's instruction (Stratagene, La Jolla, CA). A total of 300,000 plaque-forming units (pfu) was plated at 30,000 pfu/150-mm agar plate. The plaques were lifted onto Hybond-N+ nylon transfer membranes (Amersham), alkali denatured, and fixed to the membranes by a UV cross-linker (Stratagene). The membranes were screened with the ^{32}P-labeled cDNA probe generated by random prime labeling under high stringency conditions.[12] Positive plaques were picked from plates and subjected to secondary and tertiary screening at lower titers to ensure the purity of the clones. pBluescript SK(−) plasmids containing the positive cDNA were rescued from the Lambda Zap II vector using ExAssist helper phage according to the *in vivo* excision protocol (Stratagene). The cDNAs were sequenced by the dye primer method with a Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham) and a DSQ-2000L DNA sequencer (Shimadzu, Kyoto, Japan).

Reverse Transcription (RT)-PCR

To obtain the full-length cDNA, RT-PCR was performed as follows. Total RNA (4 μg) of adult rat lung was reverse transcribed using a oligo$(dT)_{12-18}$ primer. The upstream

primers, 5′-CCCTGCG(A/GAGGGAGCGCTGA(T/C)C-3′, just outside the predicted initiation codon were designed based on sequence data obtained from mouse and human isoforms of the Na/Pi cotransporter type IIb.[9,11] PCR was performed using the upstream primers paired with a downstream primer, 5′-GATGATTGACGAGGACGTGC-3′ (nucleotide 502–521, in rNaPi IIb), under 94°C denaturation, 55°C annealing, and 72°C extension temperatures for 22 cycles. The predicted product size with these primers was 512 bp. The RT-PCR products were subcloned into the pGEM-T Easy vector (Promega) and sequenced.

In Situ *Hybridization and Tissues*

Sense and antisense complementary RNA (cRNA) probes were synthesized from the linearized pBluescript SK(−) plasmid containing the longest cDNA fragment of rNaPi IIb, designated HG80 28a, obtained from the cDNA library screening by *in vitro* transcription in the presence of digoxigenin (DIG)-labeled UTP (Boehringer Mannheim, Mannheim, Germany) with the use of T7 and T3 RNA polymerase, respectively, following the protocol recommended by the company. The cRNA probes were alkali hydrolyzed to generate probes about 500 bases long. The mouse surfactant protein C (SP-C) probe[13] was a gift from Dr. N. Miura, Second Department of Biochemistry, Hamamatsu University School of Medicine. The SP-C sequence homology between mouse[14] and rat[15] at the cDNA level was 92%. For developmental studies, Wistar rat embryos aged gestational day 15.5 to 18.5, 20.5, and 21.5, postnatal 1-day-old, and adult rat lung tissues were fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (PB) at 4°C overnight and processed under RNase-free conditions for standard paraffin embedding.

In situ hybridization on sections was performed as previously described[16] with minor modifications. Briefly, 4-μm sections were deparaffinized, washed with PB, treated with proteinase K (10 μg/ml) at 37°C for 30 minutes, washed again with PB, fixed again in 4% paraformaldehyde in PB, treated with 0.1% Nonidet P-40 in 2× SSC at 37°C for 30 minutes, treated with 0.2N HCl at room temperature for 20 minutes to inactivate internal alkaline phosphatase, treated with 0.1 mol/L triethanolamine-HCl (TEA) pH 8.0, acetylated with 0.25% acetic acid in 0.1 mol/L TEA, pH 8.0, dehydrated through a graded series of ethanol, and air-dried. The cRNA probes were hybridized to the tissue sections at 50°C for 16 hours in a moisture chamber. After hybridization, sections were washed in 5× SSC briefly, 50% formamide in 2× SSC at 50°C for 30 minutes, and 10 mmol/L Tris pH 7.6, 5 mmol/L EDTA, and 500 mmol/L NaCl (TNE) at 37°C for 15 minutes. Sections were then treated with RNase A (25 μg/ml) at 37°C for 30 minutes and washed in TNE at 37°C for 10 minutes. Next, sections were washed twice in 2× SSC and 0.2× SSC at 50°C for 15 minutes. To further reduce background signals, sections were rinsed in 0.1% Nonidet P-40 in 2× SSC at 37°C for 10 minutes. Finally, sections were dehydrated in a graded ethanol series and air-dried. The hybridization signal was detected using DIG Nucleic Acid Detection Kit (Boehringer Mannheim). Controls included tissue sections that were hybridized with sense cRNA probes and those incubated in hybridization buffer without probes added.

Results

Differential Display

To isolate genes involved in lung development, mRNA expression in the embryonic and postnatal lung was compared by differential display analysis. One band appeared faintly on gestational day 16.5 and increased in expression with development (Figure 1A). This cDNA fragment, designated HG80, was subcloned into the pGEM-T Easy vector for sequencing. The HG80 cDNA was 247 bp long and contained both upstream (H-AP80) and downstream (H-T_{11}G) primer sequences used for amplification. Sequence alignment revealed that HG80 had substantial similarity to the mouse type IIb Na/Pi cotransporter (Npt2b).[9]

Confirmation of Differential Expression in the Developing Lung

We confirmed the differential expression of HG80 in the developing lung by Northern blot analysis (Figure 1B). The ^{32}P-labeled HG80 cDNA probe hybridized to a single mRNA that was faintly expressed in gestational day 17.5 lung tissue but was rapidly augmented after gestational day 18.5.

cDNA Cloning

Screening of a 1-day-old newborn rat lung cDNA library with the HG80 as a probe resulted in the isolation of six individual overlapping cDNA clones, the largest of which, HG80 28a, was about 3.8 kb. Sequence comparison with the Npt2b (GenBank accession no. AF081499) and NaPi3b (GenBank accession no. AF111856) suggested that approximately 100 nucleotides were missing at the 5′ end. To obtain the full-length cDNA sequence, RT-PCR was performed as described in Materials and Methods. Three clones of the RT-PCR products were sequenced. The full-length cDNA sequence contains 3950 nucleotides with an open reading frame of 2088 nucleotides. The deduced amino acid sequence consisted of putative eight hydrophobic transmembrane domains and contained clusters of cysteine residues in the C-terminal region and showed the highest homology with Npt2b. Comparison of the deduced amino acid sequences of the rat with that of mouse Npt2b and human NaPi3b confirmed a high degree of sequence similarity: 93.7 and 80.4%, respectively. Identity with the rat Na/Pi cotransporter type II (NaPi2A, GenBank accession no. L13257) was 65.6% at the amino acid level.[17] Taken together, we assigned this gene as the rat counterpart of Npt2b and NaPi3b, and designated it as rNaPi IIb (GenBank accession no. AF157026).

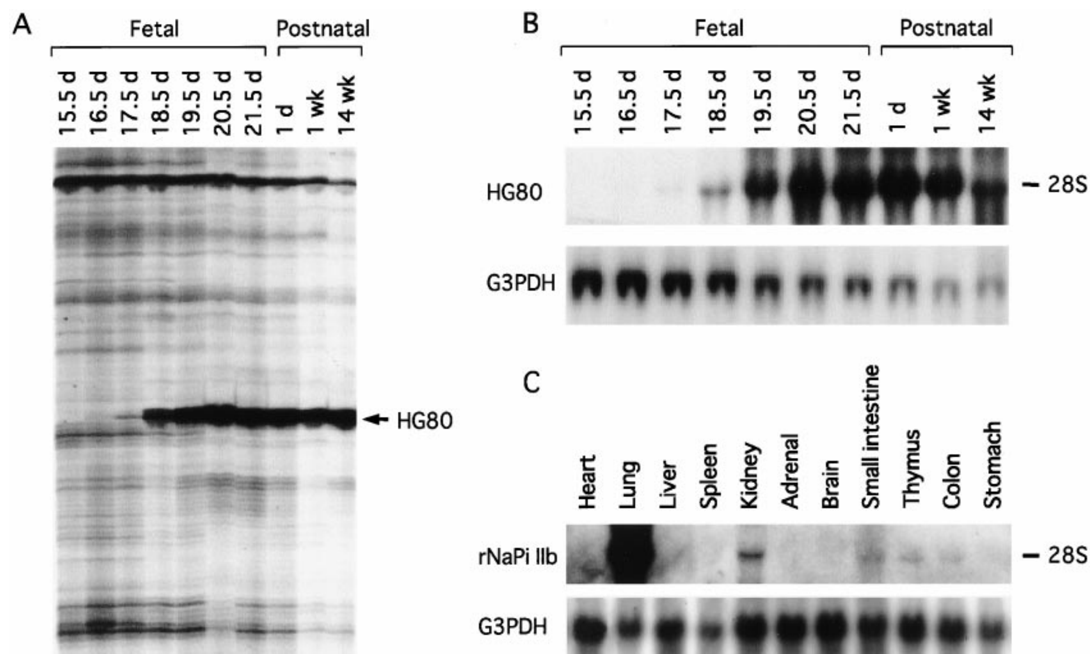

Figure 1. A: Differential display analysis was performed with poly(A)$^+$ RNA isolated at different gestational and postnatal ages from the developing rat lung. A faint signal was first detected on day 16.5 of gestation (**arrow**). Clear expression was observed from gestational day 17.5 onward. **B:** Northern blotting of HG80 expression. Expression of HG80 mRNA in the developing rat lung increased with advancing gestation. This pattern of expression was similar to that of the differential display analysis. A faint signal for HG80 transcripts was first detected on day 17.5 of gestation and a dramatic increase was observed in fetal lungs on days 18.5–19.5 of gestation. Transcript size identified by radiolabeled HG80 cDNA probe was similar in the rat fetal and postnatal lung mRNA. The position of migration of 28S ribosomal RNA was indicated. HG80 cDNA probe was stripped off and same membrane was hybridized with mouse G3PDH cDNA probe and exposed for 48 hours. **C:** Northern blot analysis of rNaPi IIb mRNA expression in various adult rat tissues. A single rNaPi IIb transcript was detected. High level expression was observed in lung, moderate level expression in kidney, and low level expression in small intestine, colon, and thymus.

Northern Blot Analysis

Northern blot analysis was performed to characterize the rNaPi IIb gene expression. Total RNAs were isolated from the brain, heart, lung, stomach, small intestine, colon, liver, spleen, kidney, thymus, and adrenal gland of 14-week-old Wistar rats. Northern blot analysis using the HG80 cDNA probe identified a single 4.0-kb transcript expressed predominantly in the lung (Figure 1C). Signals were also detected in the kidney and, to a lesser degree, in the small intestine, colon, and thymus. No hybridization signals were observed in the brain, heart, stomach, liver, spleen, or adrenal gland.

Expression of rNaPi IIb during Lung Development Analyzed by in Situ *Hybridization*

In situ hybridization was performed to determine the spatial and temporal expression of rNaPi IIb mRNA in the developing rat lung. In mammalian lung development, the following different but partially overlapping stages have been suggested based on morphological criteria[1,18]: the embryonic (gestational day 11.5–14 in the rat), pseudoglandular (gestational day 15–18), canalicular (gestational day 19–20), saccular (gestational day 21–22), and postnatal stages (late saccular stage, alveolar stage, and stage of microvascular maturation). Lung sections on and after the pseudoglandular stage were prepared because, with differential display and Northern blot analysis, rNaPi IIb mRNA expression was first detected on gestational days 16.5 and 17.5, respectively.

In the pseudoglandular stage, the primitive bronchial tree composed of tall columnar epithelial cell lining, which grows into the surrounding mesenchyme, resembles an exocrine gland on light microscopy. During the late pseudoglandular stage, the most prominent structural change is a marked increase in the number of epithelial tubules in the peripheral area of the developing lung.[18] Fetal lungs on gestational day 15.5, corresponding to the early pseudoglandular stage, did not demonstrate any detectable rNaPi IIb message, even after prolonged color development (data not shown). On gestational day 16.5, faint and indistinct hybridization signals were first detected in a minority of epithelial cells at several distal edges of the primitive bronchial tree (data not shown). On the next day (gestational day 17.5), diffuse, weak hybridization signals which appeared to be restricted to the rounded ends of the primitive bronchial tree were detected (data not shown). On gestational day 18.5, rNaPi IIb signals were very strong and distinctly confined to the bulbous ends of the branching bronchial tree (Figure 2, A-C). Clusters of positive cells were sharply demarcated from adjacent negative epithelial cells. No hybridization signals were detected in the more proximal bronchial tubules and mesenchyme during this pseudoglandular stage.

In the canalicular stage, there is an enormous increase in capillarization of the primitive interstitium and close

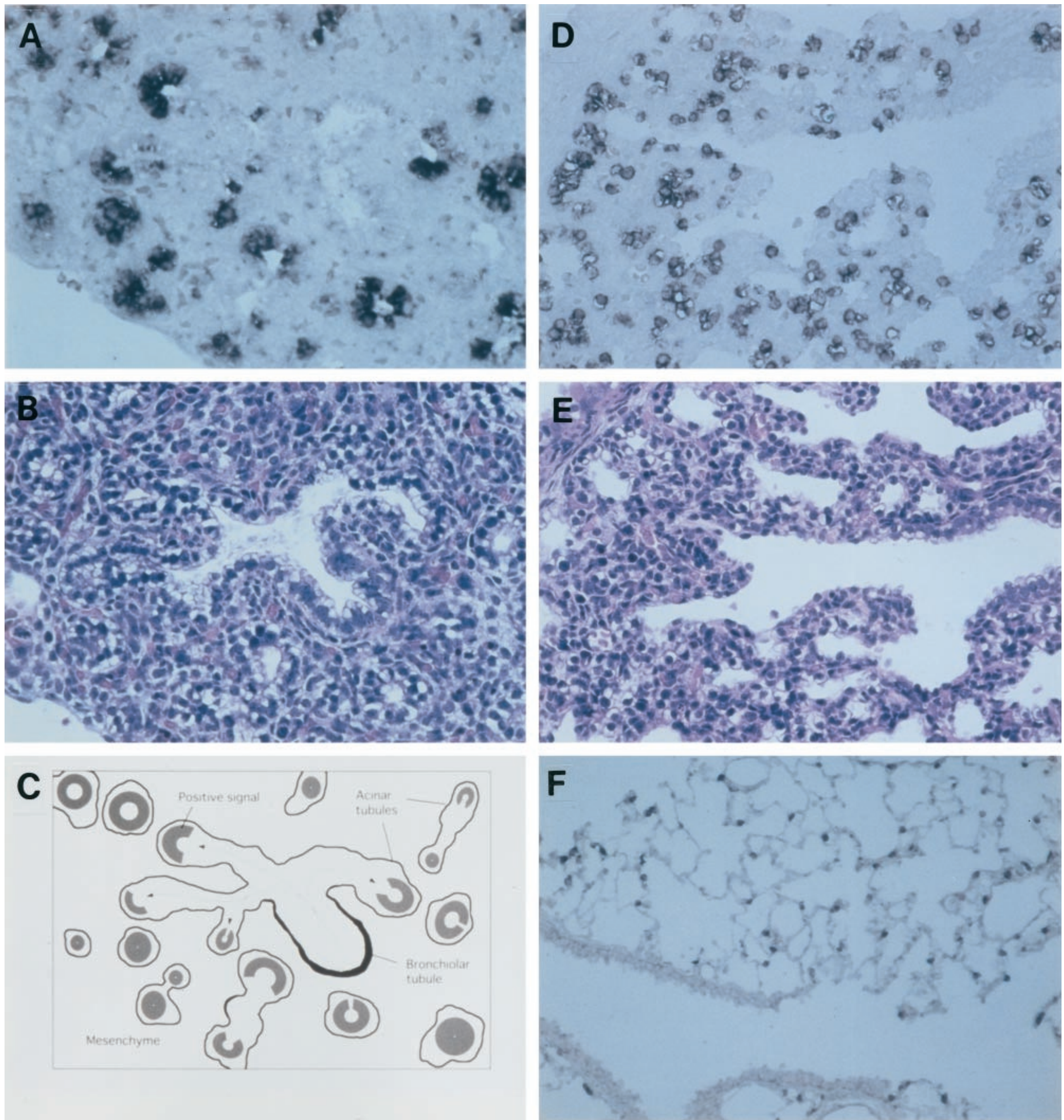

Figure 2. *In situ* hybridization analysis of rNaPi IIb expression in the developing rat lung on gestational day 18.5 (**A**) and 21.5 (**D**), and in the adult rat lung (**F**). Serial sections of **A** and **D** were stained with hematoxylin and eosin (**B** and **E**, respectively). **C:** Schematic diagram of **A**. Original magnifications, ×400 (**A**, **B**, **D**, and **E**) and ×200 (**F**).

apposition between epithelium and capillaries, which contribute to the formation of air-blood barriers.[18] It has been demonstrated that the cuboidal cells of the acinar tubules at the periphery of the primitive bronchial tree correspond to prospective alveolar epithelium[19,20] and in this stage more cuboidal epithelial cells of the acinar tubules and terminal buds are present. These cells are considered to be type II cells because an accumulation of lamellar bodies has been demonstrated in the cytoplasm. Hybridization signals on gestational day 20.5 were found in the cuboidal cells of the acinar tubules and terminal buds of the most distal portion of the primitive respiratory tree (data not shown). The more proximal bronchial epithelial cells, interstitial cells, and vascular endothelial cells were all negative for rNaPi IIb expression.

In the saccular stage, the acinar tubules become further differentiated into primitive alveolar ducts or terminal sac and the lung exhibits an increase in its alveolar appearance with the more expanded terminal sacs,

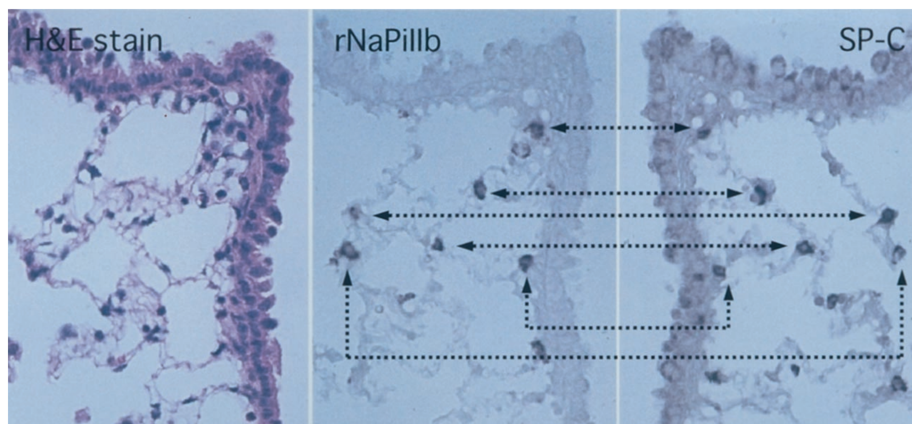

Figure 3. *In situ* hybridization analysis of rNaPi IIb expression in the adult rat lung. Mirror sections were hybridized with antisense DIG-labeled cRNA probes for rNaPi IIb (**center**) and SP-C (**right**). Serial section was stained with hematoxylin and eosin (**left**). Original magnifications, ×400 in all sections.

which are already lined by both mature type II cells and flattened type I cells. The distribution of rNaPi IIb mRNA expression in the early saccular stage (gestational day 21.5) was similar to that of the previous canalicular stage (Figure 2, D and E). On postnatal day 1, corresponding to the late saccular stage, the positive cuboidal cells of the terminal sac were observed to be more sparse (data not shown). No hybridization signals were detected in the flattened cells of the terminal sac, bronchiolar epithelial cells, vascular cells, and interstitial cells in this stage.

In the adult rat lung, hybridization signals were localized to the cuboidal cells of the alveolar epithelium, located in the corners of the alveoli (Figure 2F). No hybridization signals were detected in the flattened cells of the alveolar epithelium, bronchiolar epithelium, and interstitium. These positive cells scattered in the alveolar area were obviously alveolar type II cells, based on their morphological appearance and pattern of distribution.

To confirm this, we performed *in situ* hybridization with antisense rNaPi IIb cRNA probe and SP-C cRNA probe on mirror sections, because it had been shown that SP-C expression was strictly specific to alveolar type II cells of the rat lung at this stage.[15,21] SP-C and rNaPi IIb mRNA were expressed in the same cells (Figure 3). This result confirmed that the rNaPi IIb mRNA was also expressed in type II cells.

In all of the *in situ* hybridization experiments, none of the negative control sections showed positive signals (data not shown).

Discussion

During a search for developmentally regulated genes in alveologenesis, we isolated a cDNA encoding the rat Na/Pi cotransporter, which appears on embryonic day 16.5, corresponding to approximately 8 weeks of gestation in human. No strong sequence similarity was found with the previously reported rat renal type II Na/Pi cotransporter NaPi2A. Based on the sequence similarity to Npt2b and NaPi3b and structural characteristics of cysteine clusters in the amino terminal portion, this gene (rNaPi IIb) is assigned as the rat counterpart of Npt2b and NaPi3b, though a functional study has not yet been done. Tissue distribution by Northern blot analysis shows the rNaPi IIb transcripts in the lung, small intestine, kidney, colon, and thymus. High expression is detected in the lung, with moderate expression seen in the kidney and lesser levels in the small intestine. A similar tissue distribution pattern was described for Npt2b and NaPi3b,[9,11] but the exact cellular localization has not been described. As expected, we have clearly demonstrated by comparison with SP-C gene expression that rNaPi IIb is localized in type II alveolar cells and continues to be expressed in the adult lung.

The functional significance of rNaPi IIb in type II alveolar cells is probably related to surfactant production, because in alveolar type II cells phosphate may be an essential constituent of phospholipids, which are the major components of surfactant. Clerici et al[22] suggested that phosphate availability for surfactant synthesis might be accomplished by a sodium-dependent phosphate uptake, and that the gene we report here performs this function. If rNaPi IIb plays a critical role in collecting inorganic phosphate for making phospholipids, the expression of this gene in alveolar type II cells but not in bronchiolar epithelium, including Clara cells, demonstrated here will further clarify the issue of whether phospholipids and SP-C are synthesized in Clara cells.

There are currently few lineage markers available for type II alveolar cells of the lung. To date, SP-C is the only marker that is specific to type II cells in the lung. rNaPi IIb is a candidate marker, because it is continuously expressed in type II alveolar cells. RT-PCR demonstrated that SP-C mRNA was detected on day 13 of gestation,[23] whereas rNaPi IIb expression seems to appear 3 days later. Thus, rNaPi IIb mRNA can be used as a later marker for studying the spatial and temporal differentiation of alveolar type II cells.

The histological lineage of adenocarcinoma of the lung remains unclear, and rNaPi IIb may be a useful marker to analyze the histopathogenesis of lung cancer. Characterization of human adenocarcinoma by this probe is also expected. We also expect that this gene may be useful for analyses of pathological conditions of alveolar cells, including various types of alveolar cell damage.

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