Quantitation and Localization of ENaC Subunit Expression in Fetal, Newborn, and Adult Mouse Lung

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The newborn lung is cleared of fetal liquid by active Na^+ transport. The heterotrimeric (α, β, γ) epithelial Na^+ channel, ENaC, mediates this process. To understand the role of individual ENaC subunits in Na^+ transport during development, we quantified murine ENaC (mENaC) subunit messenger RNA (mRNA) expression levels of fetal, neonatal, and adult mouse lung by Northern blot analysis and studied regional expression by *in situ* hybridization. αmENaC and γmENaC mRNA expression increased sharply in late fetal gestation and reached near-adult levels by Day 1 of postnatal life. βmENaC expression increased more gradually through late fetal and early postnatal life and increased progressively until adulthood. *In situ* hybridization studies showed similar localization patterns of αmENaC and γmENaC subunit expression in fetal and postnatal lung. γmENaC and αmENaC subunits were initially localized to fetal lung bud tubules and by late gestation both subunits were expressed in all regions (acinar and bronchiolar) of the distal lung epithelium. βmENaC was detected from 16 d gestation onward and was expressed most intensely in small airways. There was little expression of βmENaC in the alveolar region. In postnatal lung all three subunits were expressed intensely in small airways. In adult lung, αmENaC and γmENaC were expressed in a pattern consistent with an alveolar type II (ATII) cell distribution. The timing of quantitative changes in mENaC subunit expression is consistent with a role of Na^+ transport in liquid clearance of the perinatal lung. Intense expression of mENaC subunits in medium and small airway epithelium and in ATII cells suggests that these regions are a primary location for liquid absorption in the perinatal and postnatal murine lung. **Talbot, C. L., D. G. Bosworth, E. L. Briley, D. A. Fenstermacher, R. C. Boucher, S. E. Gabriel, and P. M. Barker. 1999. Quantitation and localization of ENaC subunit expression in fetal, newborn, and adult mouse lung. Am. J. Respir. Cell Mol. Biol. 20:398–406.**

The net flux of liquid into or out of the lung lumen results from a balance of active liquid secretory and absorptive forces. During fetal life, liquid is secreted by the epithelium lining the acini and small airways of the developing lung. Liquid secretion results from the active transport of Cl^- from the interstitium to the lumen. Around the time of birth, this secreted liquid must be cleared rapidly from the future air spaces to allow adaptation to independent respiration (1). It has long been proposed that Na^+ absorption through amiloride-sensitive channels is the driving force for the rapid clearance of fetal-lung liquid from the lung at birth (2). The recent cloning of the amiloride-sensitive epithelial Na^+ channel, ENaC (3), has greatly advanced our understanding of Na^+ transport in the lung. Definitive evidence that ENaC plays a critical role in perinatal lung liquid clearance came from recent studies of mice that were deficient in the α-subunit of ENaC (4). Affected mice were unable to clear liquid from their lungs at birth and died within the first 2 d of life with lung water content that was not different from fetal values.

The precise function of the three individual subunits of ENaC—α, β, and γ—in perinatal and postnatal lung liquid balance has not yet been established. Oocyte expression studies suggest that all three subunits are required for optimal function of channel, and that only the α subunit appears to have intrinsic ion-transporting capacity (3, 5). To explore further the role of individual ENaC subunits in liquid absorption during fetal and postnatal life, we mapped regional murine ENaC (mENaC) subunit messenger RNA (mRNA) expression by *in situ* hybridization and quantified changes in subunit expression by Northern blot analysis. mENaC mRNA subunit expression was stud-

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Abbreviations: alveolar type II, ATII; base pairs, bp; amiloride-sensitive epithelial Na^+ channel, ENaC; hematoxylin and eosin, H&E; murine ENaC, mENaC; messenger RNA, mRNA; saline sodium citrate, SSC.

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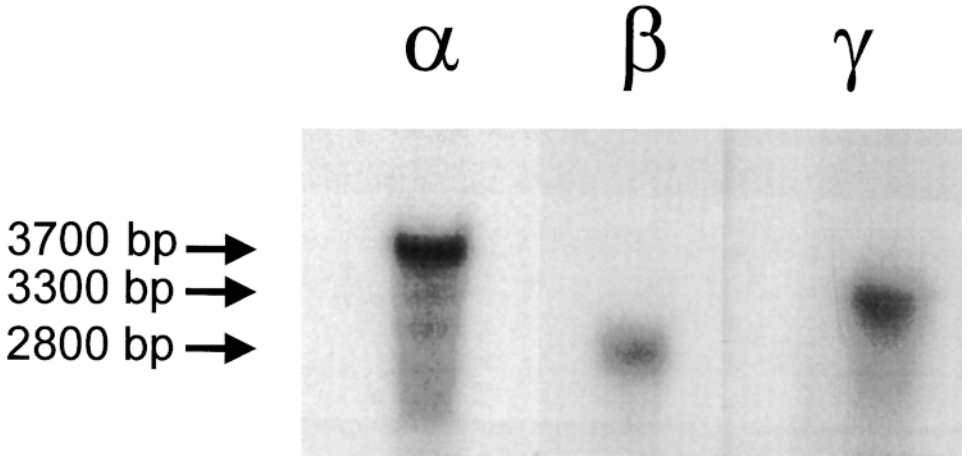

Figure 1. Northern blot analysis of ENaC subunits. Northern blot of adult lung mENaC mRNA showing the relative intensity of expression and size of each subunit. Length of subunits: αmENaC, 3,700 bp; βmENaC, 2,800 bp; γmENaC, 3,300 bp.

ied from Day 14 through Day 19 of murine fetal lung development (pseudoglandular and cannilicular phases), in the early postnatal period (terminal sac development) and in adult mouse lung.

Materials and Methods

Animals

Timed pregnant C57/B16 mice were obtained from Jackson Laboratories (Bar Harbor, ME). After maternal CO_2 inhalation, fetuses were dissected free from the uterus and lungs were dissected free from heart and great vessels. For Northern analysis, fetal lungs were pooled from 15-d (four litters, 24 fetuses), 17-d (three litters, 22 fetuses), and 19-d (two litters, six fetuses) mice to obtain sufficient RNA for the analysis. For postnatal time points, four mouse lungs were used for Day 1 analysis, and two lungs or lung segments were used for the remaining time points. For *in situ* studies, lungs from three fetuses from each gestation (14 to 19 d) and postnatal (1 wk and adult) time point were studied.

Preparation of Mouse-Specific ENaC Probes by Reverse Transcriptase

Total RNA was isolated from mouse lung and flash-frozen in liquid N_2. Reverse transcription of murine lung RNA was primed with a cocktail of three oligonucleotides (specific for each subunit) derived from the 3′ end of the rat sequence specific for each subunit (6). The complementary DNA from these reactions was used to amplify specific fragments, via polymerase chain reaction (PCR), of 636 base pairs (bp) (αmENaC), 429 bp (βmENaC), and 678 bp (γmENaC). These fragments were subcloned into the PCR II vector using the Stratagene TA cloning kit (Stratagene, La Jolla, CA). The α fragment has been sequenced and is about 98% homologous to rat αENaC; however, the sequences for the β- and γmENaC fragments have not been confirmed by sequential analysis.

Northern Blot Hybridization

Whole lungs were excised from fetal (15 to 19 d) and postnatal (1 d, 1 wk, 4 wk, and adult [> 8 wk]) mice and dissociated in guanidine isothiocyanate using a Polytron

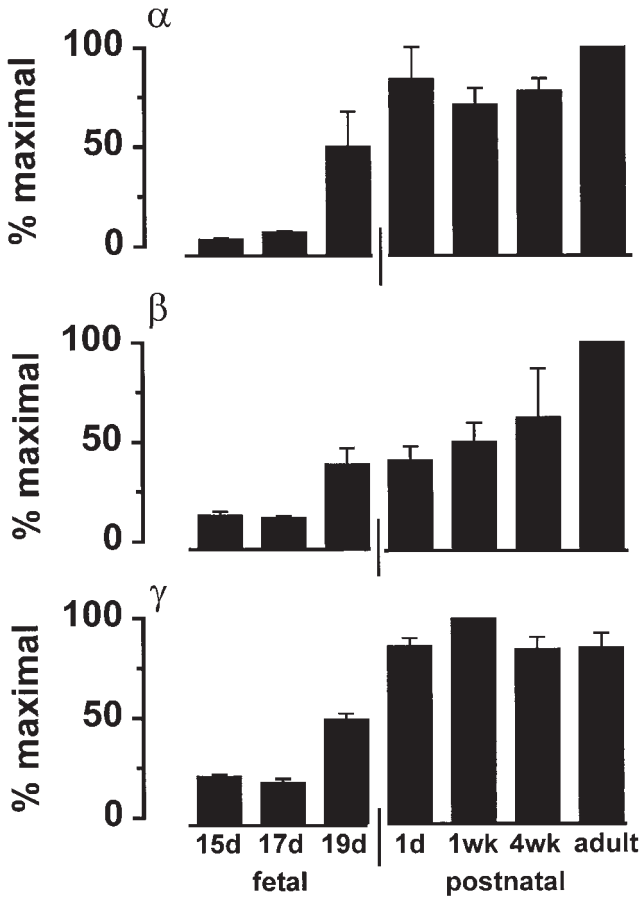

Figure 2. Relative intensity of α-, β-, and γmENaC mRNA for whole lung at different fetal gestations and postnatal ages. mRNA expression intensity is relative to maximum expression for each subunit (maximum = 100%). Maximum expression occurred on Day 1 for αmENaC, at 1 mo for βmENaC, and at 1 wk for γmENaC. Values are means ± standard deviation of pooled samples for each fetal gestation and postnatal age (two pooled samples each for α and γ, and three pooled samples for β).

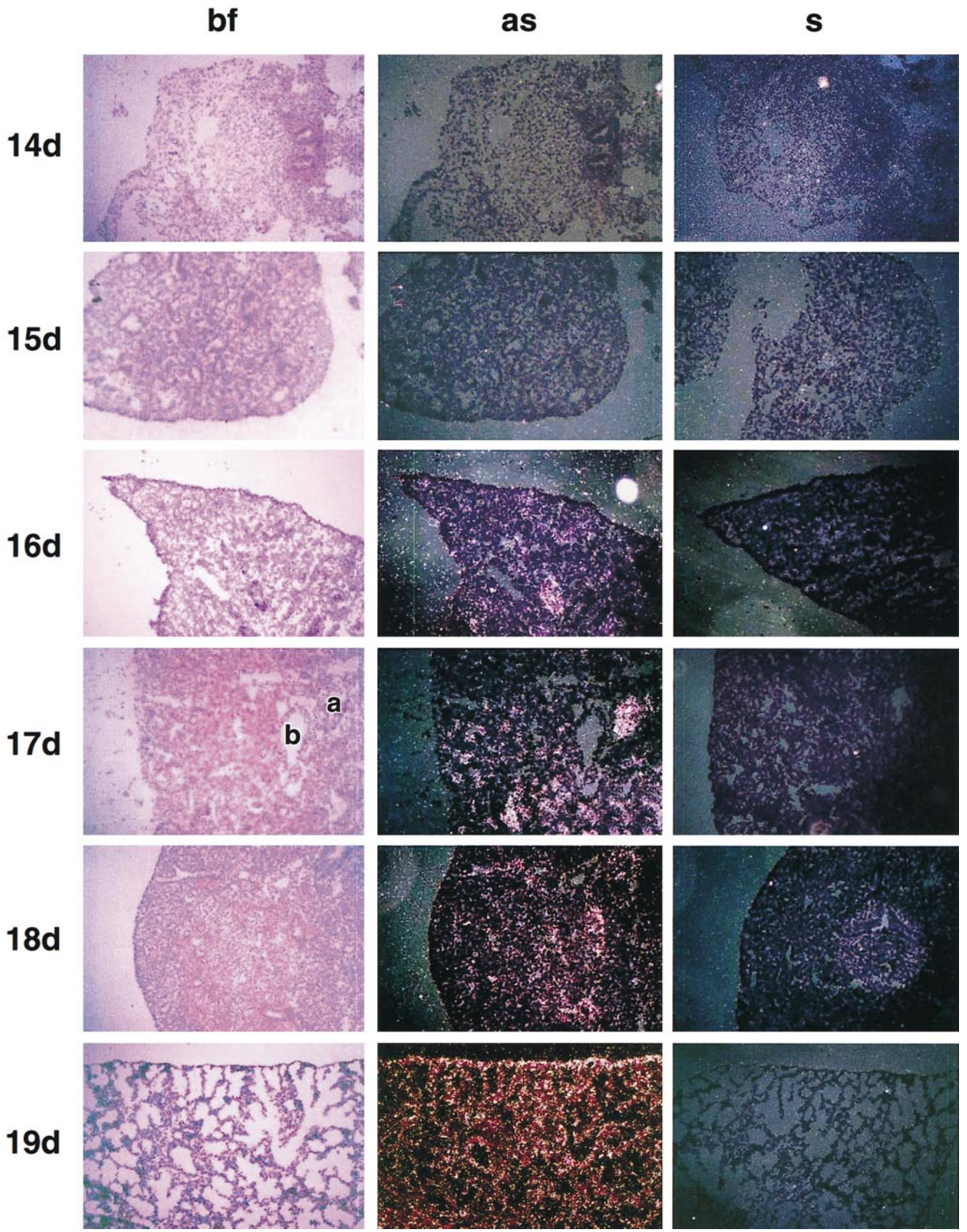

Figure 3. In situ hybridization study of αmENaC mRNA expression in fetal lung. Brightfield (H&E) image is shown on the *left*. Darkfield images of frozen sections exposed to ^{35}S-labeled antisense and sense mRNA probes are shown for each time point. a, small airway; b, blood vessel. Magnification: ×40.

PT1200 (Brinkmann Instruments Inc., Westbury, NY). RNA was purified from mouse lung as described previously (6). Approximately 10 μg/lane of total RNA was fractionated by electrophoresis on a 1.2% agarose–formaldehyde gel. Total RNA was transferred to a Duralon nylon membrane (Stratagene) by capillary blotting according to manufacturer's protocols. The membrane was ultraviolet-crosslinked and stored at −20°C until hybridization. Membranes were prehybridized at 42°C for 1 h in a 20-mM $NaPO_4$ buffered solution containing 50% deionized formamide, 5× saline sodium citrate (SSC), 1× Denhardt's, 1% glycine, and 250 μg/ml sonicated salmon-sperm DNA (Sigma Chemical Co., St. Louis, MO). The membranes were then hybridized with ^{32}P-labeled probes generated from purified DNA fragments, corresponding to either α-, β-, or γmENACs, using the Prime-a-Gene Labeling System (Promega, Madison, WI). Hybridizations were performed in 50% formamide, 5× SSC, 1× Denhardt's, 10% dextran sulfate, and 100 μg/ml salmon-sperm DNA at 42°C for 18 h. The membrane was washed at a final stringency of 0.1× SSC/0.1% sodium dodecyl sulfate at 65°C and exposed to Kodak BioMax X-ray film (Eastman Kodak, Rochester, NY) with intensifying screens. Developmental expression levels of mENAC RNA were quantified by densitometry (Molecular Dynamics, Sunnyvale, CA). mENAC RNA levels were normalized to the 28S ribosomal band (stained by methylene blue) to control for variance in loading and transfer of Northern blots, as described previously (7). The length of mENaC RNA was calculated by plotting the log (length [bp]) of 28S and 18S ribosomal bands versus the migrating distance (cm) of the ribosomal bands. Migrating distances of mENaC subunits were determined from a standard curve.

In Situ Analysis for the ENaC Subunits in Fetal Mouse Lung

Frozen sections (8 μm) were mounted on slides and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h. After fixation, slides were rinsed twice in PBS, dehydrated, air-dried, and stored at −20°C until use. *In situ* hybridization was performed by standard methods, as described previously (8). Briefly, prehybridization consisted of proteinase K digestion, then acetylation. Serial sections were hybridized overnight at 54°C in a hybridization buffer containing 10^6 counts/min^{-1} of either antisense or sense ^{35}S-uridine triphosphate–labeled RNA probes (α636, β429, γ678) synthesized by *in vitro* transcription with T7 polymerase (according to manufacturer's instructions). After hybridization, slides were washed in 4× SSC at room temperature and subjected to the following sequential protocol: ribonuclease A digestion (20 mg/ml) for 30 min at 37°C, 2× SSC/1 mM dithiothreitol (DTT) at room temperature, a high-stringency wash of 0.5× SSC/1 mM DTT at 58°C (3 × 15 min), followed by ethanol dehydration. Dried slides were dipped in Kodak NTB2 photoemulsion and stored at 4°C until developed. Slides were developed at intervals from 3 to 11 d, counterstained with hematoxylin and eosin (H&E), and photographed using brightfield and darkfield microscopy at ×40 magnification (Nikon Microphot-SA microscope).

Results

Whole-Lung mENaC Subunit mRNA Expression: Northern Blot Analysis

Northern Blot analysis of mRNA isolated from adult rat whole-lung preparations (Figure 1) showed intense expression of αmENaC (1.0 densitometric units) and lesser expression of βmENaC (0.26 units) and γmENaC (0.40 units). The length for each mENaC subunit was determined: αmENaC = 3,700 bp, βmENaC = 2,800 bp, γmENaC = 3,300 bp. Relative subunit expression during development and postnatal life was expressed as a percentage of the maximal expression for each subunit (Figure 2). Low mRNA levels of all three mENaC subunits were detected at 15 d. All three subunits showed a significant increase in expression late in gestation (i.e., Days 17 to 19). α- and γmENaC expression levels reached near-adult levels by the first day of postnatal life. Postnatal βmENaC levels increased more gradually, progressively increasing through to adult life.

Regional mENaC Subunit mRNA Expression in Fetal Lung: *In Situ* Hybridization

αmENaC expression. αmENaC mRNA expression was not detected in developing lung before 16 d gestation (Figure 3, Table 1). At 16 d, expression was observed in the central bronchi only. At 17 d, αmENaC signal was seen in central bronchi with fainter signal in distal airways. At 18 d, signal was seen throughout the airways and developing acinar structures with stronger, more diffuse signal observed in 19-d lung.

βmENaC expression. βmENaC mRNA expression was not detected in 14- or 15-d lung (Figure 4, Table 1). Signal appeared at 16 d in the central lobar airway and, more faintly, in the developing peripheral airways. In 17-d lung, strong signal was seen in lobar and small airways, but not in the developing acini of the peripheral lung. Figure 4 shows an abrupt discontinuation of signal at the distal end of the small airways of 17-d lung. At Days 18 and 19, βmENaC mRNA signal is observed primarily in the lobar

TABLE 1
Ontogeny of mENaC subunit expression by in situ *hybridization*

	Fetal						Postnatal	
	14 d	15 d	16 d	17 d	18 d	19 d	1 wk	Adult
αmENaC								
Small airway	−	−	+	++	++	++	+++	+++
Acinus	NA	NA	NA	−	+	++	++	+++*
βmENaC								
Small airway	−	−	++	+++	++	++	+++	+++
Acinus	NA	NA	NA	−	+/−	+	++	+/−
γmENaC								
Small airway	−	+++	+++	+++	+++	+++	+++	+++
Acinus	NA	NA	NA	++	+++	+++	+++*	+++*

NA = not applicable because the acinus has not developed at these gestations.

*Patchy distribution in a pattern suggestive of ATII localization. Note that comparisons of hybridization intensity cannot be made between subunits because intensity of signal depends on efficiency of probe hybridization.

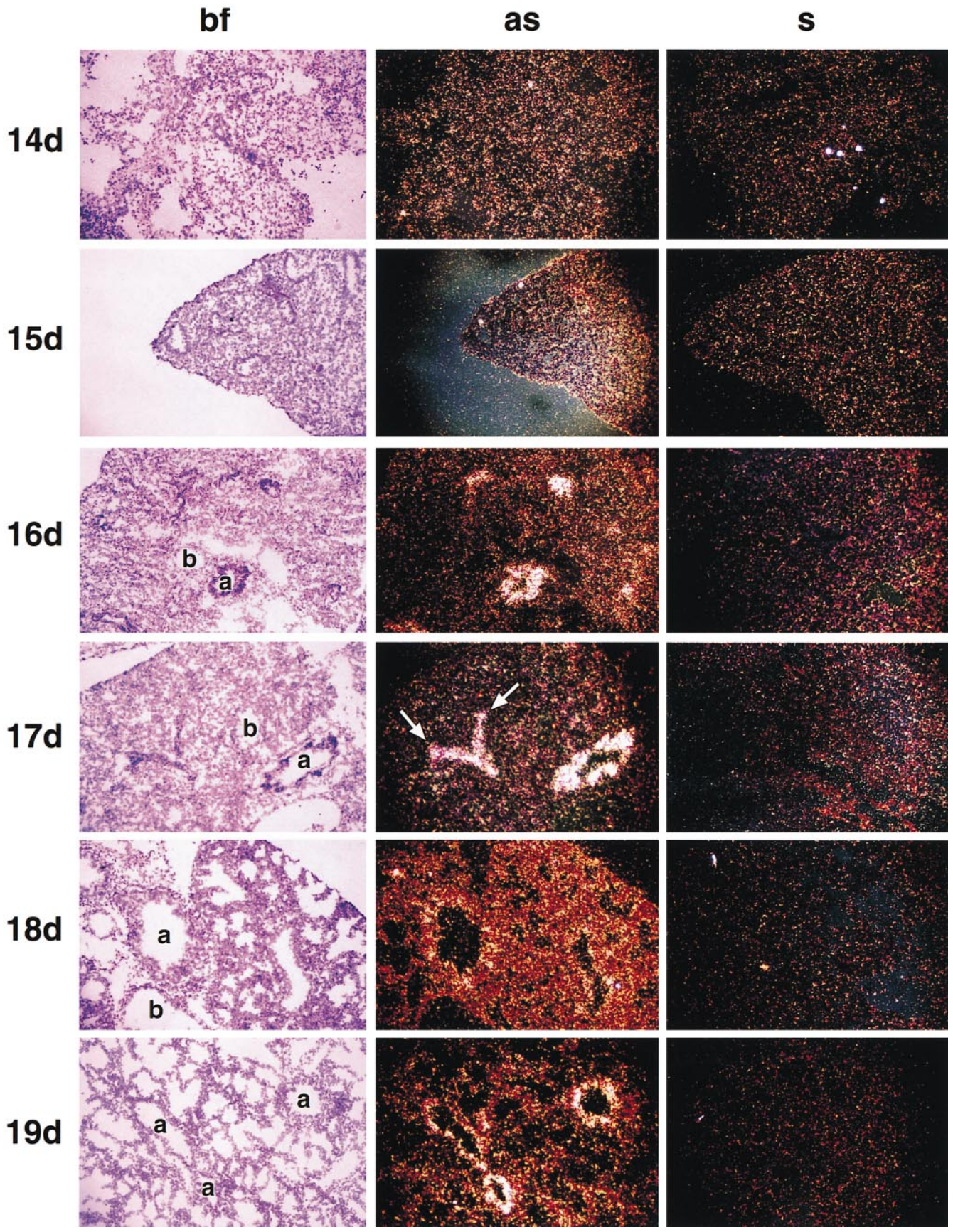

Figure 4. In situ hybridization study of βmENaC mRNA expression in fetal lung. Brightfield (H&E) image is shown on the *left*. Darkfield images of frozen sections exposed to ^{35}S-labeled antisense and sense mRNA probes are shown for each time point. a, small airway; b, blood vessel. *Arrow* shows the abrupt discontinuation of βmENaC signal in the small airway of 17-d fetal lung. Magnification: ×40.

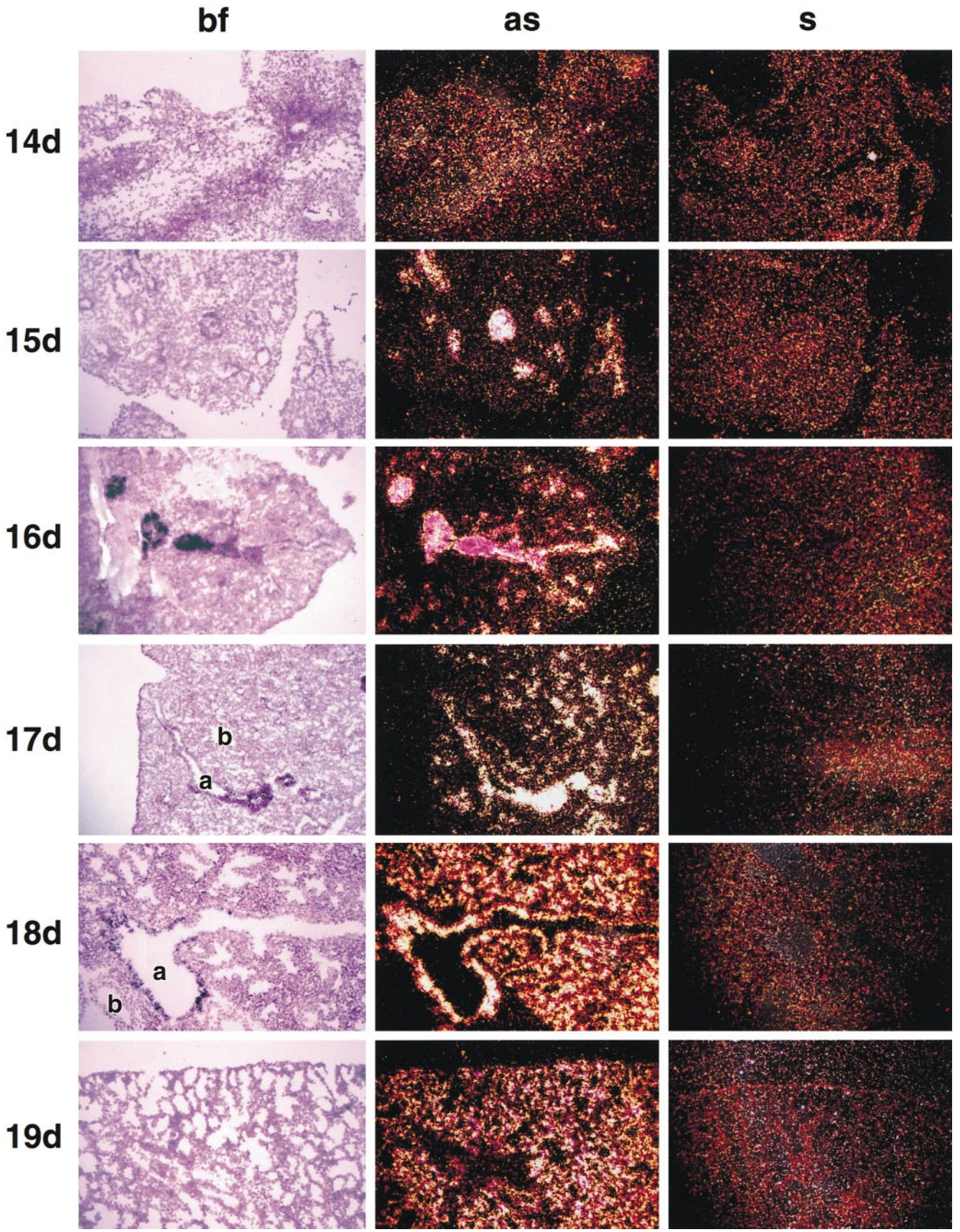

Figure 5. In situ hybridization study of γmENaC mRNA expression in fetal lung. Brightfield (H&E) image is shown on the *left*. Darkfield images of frozen sections exposed to ^{35}S-labeled antisense and sense mRNA probes are shown for each time point. a, small airway; b, blood vessel. Magnification: ×40.

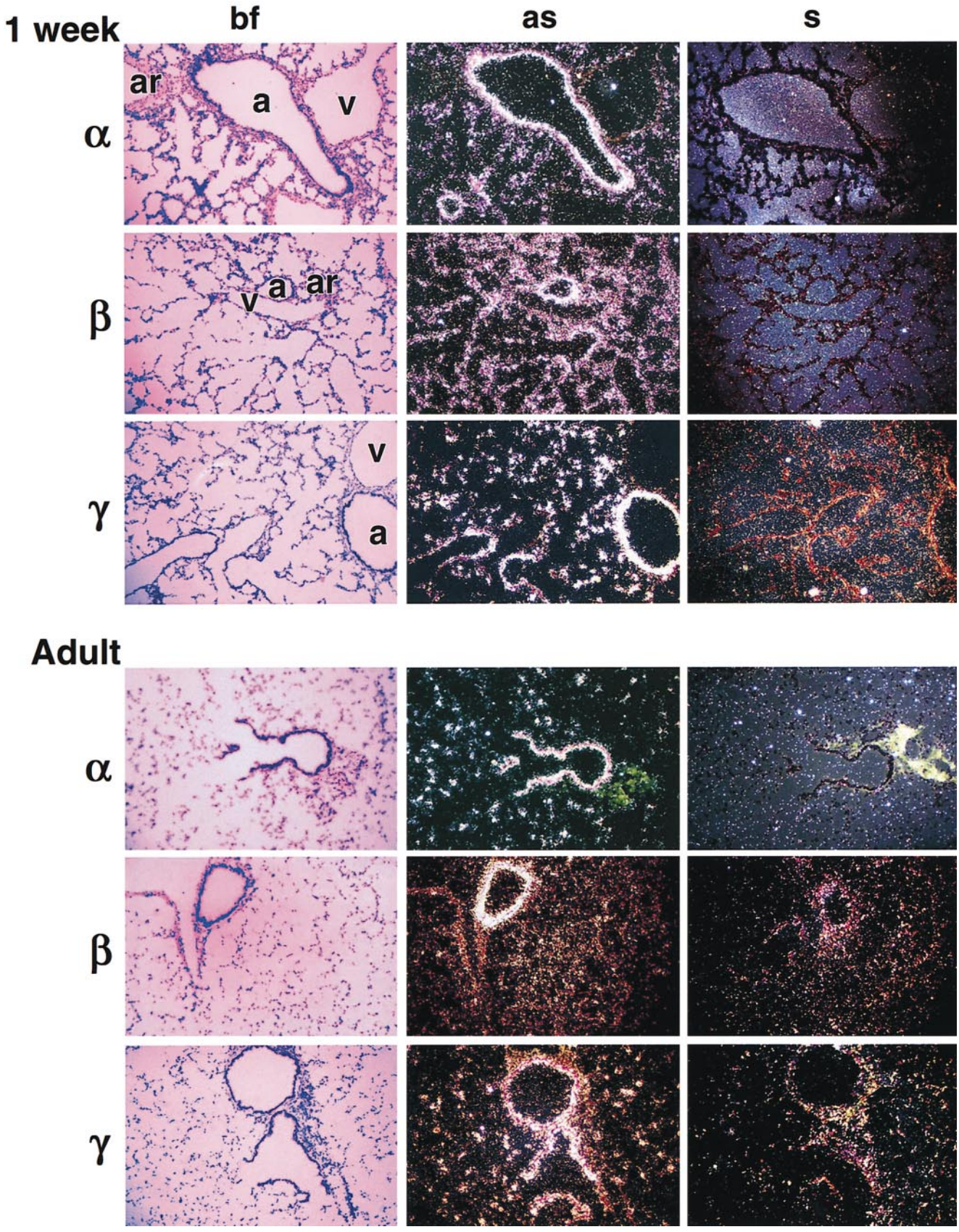

Figure 6. In situ hybridization study of α-, β-, and γmENaC mRNA expression in 1-wk and adult distal lung. Brightfield (H&E) image is shown on the *left*. Darkfield images of frozen sections exposed to ^{35}S-labeled antisense and sense mRNA probes are shown for each time point. a, airway; ar, arteriole; v, vein. Magnification: ×40.

airways, and only faintly in the small airways and developing acinar structures.

γmENaC expression. γmENaC mRNA expression was observed from 15 d (Figure 5, Table 1). mRNA was detected initially in lobar airways and primitive distal tubules of 15-d lungs. In 16-d lung, γmENaC mRNA expression was observed to track out to the most distal branches of the airway and the primitive acinar unit. In 17-d lung, all visible epithelium in the distal bronchial tree and developing acini was outlined by γmENaC mRNA signal. The γmENaC signal in the distal airways and developing alveoli was more intense with advancing fetal gestation.

For all three subunits there was no expression of ENaC in surrounding blood vessels (Figures 3 through 5).

Regional mENaC Subunit mRNA Expression in Postnatal Lung: *In Situ* Hybridization

At 1 wk of postnatal age and in adult lung, intense mRNA expression of all three subunits was observed in medium and small airways (Figure 6, Table 1). In 1-wk lung, α and β subunits were expressed in the alveolar region but with less intensity than in airway epithelium. By contrast, similar intensity of γmENaC subunit mRNA expression was seen in alveolar and small airway regions of 1-wk lung. In the adult lung, the patchy distribution of α and γ subunit expression in the distal lung suggests that these subunits are localized to alveolar type II (ATII) cells. Expression of βmENaC subunit mRNA in the alveolar region of adult postnatal lung was weaker than that observed for αmENaC and γmENaC.

Discussion

At this point the role of individual ENaC subunits in amiloride-sensitive Na^+ transport is not known. The changes in regional localization and whole-lung quantitation of mENaC subunits in the late fetal and perinatal periods can be viewed in the context of known changes in liquid and ionic flow that occur in the lung at this time.

There is now strong evidence that the switch from liquid secretion in the fetal lung to net liquid absorption in the perinatal and postnatal lung is mediated by activation of the amiloride-sensitive Na^+ channel ENaC (2, 4, 9). The pattern of changes in mENaC subunit expression from fetal to postnatal life as detected by Northern blot analysis in this study is similar to that reported for rat whole lung (10). αENaC, βENaC, and γENaC subunit expression increase exponentially before birth in both species. However, we did not observe the pronounced postnatal decline and secondary increase in αmENaC and γmENaC expression levels described for rat lung. We also saw a more progressive increase in βmENaC subunit expression through fetal and postnatal life than is seen in rat, with most of the increase in βmENaC subunit expression occurring after birth, between 4 and 8 wk of postnatal life. In human lung, αENaC mRNA levels also increase sharply between fetal and postnatal life (11), consistent with our observations in mouse lung.

The changes in ENaC subunit expression are associated with no change in fetal rat lung liquid volume (12) and, at most, a modest slowing of basal secretion rate in fetal sheep (13, 14). However, the capacity for amiloride-sensitive liquid absorption in late-gestation fetal sheep lung can be revealed by β-adrenergic stimulation (13). This cyclic adenosine monophosphate (cAMP)–sensitive Na^+ transport capacity is developmentally regulated and is very likely to be linked to an increase in ENaC subunit expression, as was observed in the present study. The speed with which this switch from secretion to absorption occurs (minutes) is likely to be too rapid for new ENaC synthesis. These observations suggest that ENaC is preassembled in fetal lung but remains inactive until phosphorylated by a rise in cAMP associated with the perinatal surge in fetal epinephrine.

The significance of the variation in timing and localization of the three mENaC subunits as detected by *in situ* hybridization studies is difficult to interpret without better understanding of the interactions between ENaC subunits and the stochiometry of the subunits in channel assembly. In the oocyte expression system, β- and γENaC subunits show no Na^+ currents when expressed alone or together (3, 5). αENaC has a trivial intrinsic Na^+ transport capacity when expressed alone, but Na^+ transport is augmented when this subunit is coexpressed with β- or γENaC subunits. However, Na^+ currents are an order of magnitude greater when all three subunits are coexpressed in this system.

The coexpression of αENaC and γENaC subunits in fetal and postnatal lung, particularly in the alveolar region, may modulate liquid flow across the epithelium. The presence of only two of the three subunits may account for the modest effects of liquid flow of these mENaC subunits in late-gestation fetal lung. These subunits appear to be expressed diffusely in fetal lung, whereas expression is patchy in adult lung. The uniform morphology of late-gestation acinar epithelium makes it difficult to determine whether or not mENaC subunit expression is cell-specific. However, high-power examination of the fetal lung exposed to αmENaC or γmENaC antisense shows that silver grains are localized to only about 50% of the cells (data not shown). It is possible that these mENaC subunits may be localized to primitive ATII cells, which are more abundant in fetal than in postnatal rodent lung (15). The coexpression of all three subunits at high levels of expression in the small airways suggests that this is the site of maximal liquid absorption in both perinatal and postnatal lung. The perinatal changes and tissue localization of mENaC subunits closely parallels that of Na/K adenosine triphosphatase subunits (16, 17), suggesting a mechanism for coordinated absorption of liquid in specific regions of the perinatal and adult lung.

The distribution of mRNA expression in the postnatal lung is similar to that reported for rat and human species. In rat lung, all three subunits are intensely expressed in the epithelia of small and medium-sized airways (18). In addition, αENaC is expressed in the alveolar region with a punctate distribution that is very similar to surfactant protein C mRNA localization, suggesting that αENAC mRNA is localized in this region to ATII cells (19). Similar localization of all three ENaC subunits to epithelium of human bronchial and nasal airway was described by Burch and coworkers (6). However, expression of γENaC subunit in

human lung was less intense than that observed in mouse lung.

We showed previously that exposure of the epithelium to oxygen decreases liquid production by the fetal distal lung epithelium, suggesting that the switch from relative hypoxia during fetal life to normoxia at birth is another trigger that initiates liquid absorption at birth (12). Amiloride-sensitive short-circuit current and ENaC mRNA expression (all three subunits) increases after transfer of fetal rat lung distal epithelial cells from low (fetal) to normoxic (postnatal) culture conditions (20). Our observations suggest that most of the ENaC subunits are in place and ready for the rapid clearance of lung liquid that occurs in the first few hours of life. The effect of the switch in ambient O_2 on subunit expression is probably limited because only modest increases in αmENaC and γmENaC expression are seen after birth. Patch-clamp studies suggest that some of the effect of O_2 may result from a more direct effect on ENaC channel function (21).

The combination of Northern blot analysis of whole lung and *in situ* hybridization studies has allowed a more detailed interpretation of the complex maturational and regional changes in Na^+ transport that occur in the mammalian lung, and provides a framework for the study of factors that regulate ENaC subunits and Na^+ transport in fetal, perinatal, and postnatal life. Our data suggest that ENaC regulates liquid absorption by quantitative and regional changes in subunit expression, and by selective association of individual ENaC subunits in different regions of the lung.

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